

# Novel host immune evasion strategy of the endoparasitoid *Drino inconspicuides*

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## Abstract

The tachinid fly *Drino inconspicuides* (Diptera: Tachinidae) is an ovularviparous endoparasitoid whose larvae develop in the host haemocoel and avoids the host immune system. In this study, we investigated the immune evasion mechanisms of this species during infestation in the host *Mythimna separata* (Lepidoptera: Noctuidae). We discovered a unique ‘cloak’ that surrounded *D. inconspicuides* larvae that penetrated into the host and determined through genomic polymerase chain reaction analysis that this structure originated from the host rather than the tachinid. The ‘cloak’ contained both haemocytes and fat body cells from the host, with the haemocytes assembling around the larvae first and the fat body cells then covering the haemocyte layer, following which the two mixed. Living *D. inconspicuides* larvae that were wrapped in the ‘cloak’ were not melanized whereas encapsulated dead larvae were melanized, suggesting that this structure contributes to the avoidance of host immune reactions.

**Keywords:** tachinid fly, *Drino inconspicuides*, *Mythimna separata*, cloak, cell lineage

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## Introduction

Tachinid flies (Diptera: Tachinidae) are natural enemies of many lepidopteran insects (Stireman & Singer, 2003; Dindo, 2011). These koinobiont parasitoids allow their hosts to continue to feed and develop after being parasitized and develop to the late-larval stage by feeding on the host tissues (Stireman *et al.*, 2006). The tachinid fly *Drino inconspicuides* (Baranov) is a gregarious and ovularviparous endoparasitoid that parasitizes a wide range of lepidopteran larvae (Baranov, 1934; Shima, 1999). *D. inconspicuides* larvae hatch within 1 min after oviposition on the body surface of the host, penetrate the host body cavity, attach themselves to the cuticle and create a cap-like respiration organ called a funnel (part of the

sheath; Dindo, 2011). The early larvae remain attached with the funnel until they reach the 3rd larval instar, when they begin to wander freely inside the host haemocoel, eventually killing the host and leaving the remains to undergo pupation.

Insects that are attacked by tachinid flies possess an immune system that can eliminate invading foreign organisms (Lackie, 1988). One cellular immune reaction against invading parasitoid eggs and larvae is encapsulation with haemocytes (Lavine & Strand, 2002), which involves granular cells (an adherent haemocyte cell type) attaching to and forming a single layer around the target, following which plasmatocytes (another adherent haemocyte cell type) aggregate to form a multi-layered capsule; the granular cells attaching to the outermost layer of plasmatocytes undergo apoptosis (Pech & Strand, 2000). Therefore, parasitoids that penetrate into the host haemocoel will come face to face with this immune reaction.

Some tachinid flies escape the host immune response by migrating out of the haemocoel. For example, larvae of *Compsilura concinnata* move into the space between the midgut cells and the periplasmic membrane immediately after

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hatching in the host haemocoel (Ichiki & Shima, 2003), while *Blepharipa sericariae* larvae enter the host by ingestion and migrate from the gut to the ganglion, passing through the haemocoel (Hirose, 2005). Moreover, hatched *Zenillia libatrix* larvae move into the abdominal muscles or silk glands of host lepidopteran insects (Dowden, 1934). However, *D. inconspicuides* larvae spend the entire larval period (approximately 8 days at 25°C) developing inside the host haemocoel (Kalyebi & Nakamura, 2006), forcing them to deal with this immune response in a different way.

Salt (1968) postulated that such tachinid flies survive the host immune response by moulding the host haemocytes that are engaging in encapsulation into a funnel to avoid suffocation. However, asphyxia is not the only cause of death for encapsulated organisms. For example, Nappi *et al.* (1995) suggested that toxic reactive oxygen species (ROS) produced by the host also kill parasitoids, and the production of various types of ROS has been associated with melanization (Dubovskii *et al.*, 2010; Satyavathi *et al.*, 2014). According to Dudzic *et al.* (2015), two prophenoloxidases (PPOs) contribute to the melanization of the capsules that are formed around parasitoids: PPO2, which is produced by crystal cells (exhibiting similar characteristics to lepidopteran oenositoids; Ribeiro & Brehélin, 2006); and PPO3, which is produced by lamellocytes (exhibiting similar characteristics to lepidopteran plasmatocytes; Ribeiro & Brehélin, 2006). Therefore, since the sheath that was reported by Salt (1968) only covers the posterior end of the larva, leaving the other end open, it seems unlikely that it plays a vital role in protecting tachinid larvae from the host immune reactions although it may contribute to protect the larvae partially. In this study, we investigated whether other defence mechanisms exist in *D. inconspicuides* larvae using *Mythimna separata* (Lepidoptera: Noctuidae) as a host.

## Materials and methods

### Insects

The colony of *D. inconspicuides* originated from parasitized larvae of the fall webworm *Hyphantria cunea* (Drury) (Lepidoptera: Erebididae), which had been collected in Tsukuba, Japan (36°03'N, 140°05'), in September and October 2010. *D. inconspicuides* was reared by using a method of Kalyebi & Nakamura (2006). The adult flies were supplied with sugar cubes and water and were maintained at 20°C. Mated *D. inconspicuides* females were provided with 4-day-old final (6th) instar larvae of *M. separata* as a host. Following oviposition, the host larvae were reared on an artificial diet, Silkmate (Nihon Nosanko), at 25°C under a 16 h light:8 h dark photoperiod.

### Dissection of parasitized *M. separata* larvae

The parasitized *M. separata* larvae were dissected at 2, 3, 4, 24, 48, 52 and 72 h after the invasion and the parasitizing larvae were observed under a stereoscopic microscope, MZ12 (Leica). The surrounding materials and the sheath were peeled from the excised larvae using forceps and were used for genetic analyses. The surrounding materials at 3, 4, 24 and 48 h after the invasion were also surgically separated into the inner and outer layers.

### 4',6-diamidino-2-phenylindole (DAPI) staining

The material surrounding the tachinid larvae at 24 h after the invasion was stained for 5 min with DAPI (Life Technologies), which binds to nuclear DNA, and was observed under a phase-contrast microscope, DM IRB (Leica).

### Cloning of the ribosomal internal transcribed spacer (ITS) region

Genomic DNAs were extracted from *D. inconspicuides* and *M. separata* larvae with the Wizard<sup>®</sup> Genomic DNA Purification Kit (Promega). A primer set for amplifying the ITS region was designed based on the conserved ITS sequences described by Ji *et al.* (2003): 5'-TACACACC GCCCGTCGCTACTA-3' (CAS18sF1 primer) and 5'-ATGT GCGTTCRAAATGTCGATGTTCA-3' (CAS5p8sB1d primer). The polymerase chain reaction (PCR) was performed using GoTaq<sup>®</sup> DNA polymerase (Promega) under the following conditions: 94°C for 5 min followed by 35 cycles at 94°C for 15 s, 60°C for 15 s and 72°C for 15 s. The PCR products that were amplified from the genomic DNAs of *D. inconspicuides* and *M. separata* were subcloned into the pGEM<sup>®</sup>-T Easy Vector (Promega) and sequenced with an ABI 3130 Genetic Analyzer (Applied Biosystems). Based on this ITS sequence information, the following specific primer sets were used to amplify the ITS regions for *D. inconspicuides* and *M. separata*: 5'-AGAAGTAAGACATTCGCAACA-3' (forward primer for *D. inconspicuides*), 5'-TGTCGCAGTAGTCGATCTAG-3' (forward primer for *M. separata*), and 5'-TGTGTCCTGCAG TTCACAC-3' (reverse primer for both species).

### Amplification of genomic DNA by PCR

Genomic DNAs from the 'cloak' and the sheath were amplified with the specific primer sets for the ITS regions of *D. inconspicuides* and *M. separata*. The PCR was performed using Paq5000 DNA polymerase (Agilent) with approximately 2.5 ng of each genomic DNA sample. The PCR programme consisted of initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 30 s.

### Transplantation experiments

Polystyrene beads (Φ600 μm; Polysciences) or intact *D. inconspicuides* larvae that had been excised from the host 24 h after the invasion and killed by freezing were transplanted into the abdominal leg of ice-anaesthetized 6th instar larvae of *M. separata*, following which the leg was immediately tied with thread to prevent haemolymph leakage. The host larvae were then dissected at 2 h and 24 h after transplantation, the transplanted objects were excised, and the capsules around them were separated for genetic analysis.

### Cloning of a gene encoding aldehyde dehydrogenase (aldh) in *M. separata*

To find a candidate marker gene for distinguishing fat body cells from haemocytes, suppression subtractive hybridization (SSH) was performed using the GeneFishing DEG Kit (Seegene) following the manufacturer's instructions. Briefly, RNAs were extracted from the fat body and haemocytes of 6th instar larvae of *M. separata* with TRIzol reagent (Invitrogen) and adaptor-linked cDNAs were synthesized. PCR was then conducted using Taq<sup>™</sup> HS DNA polymerase

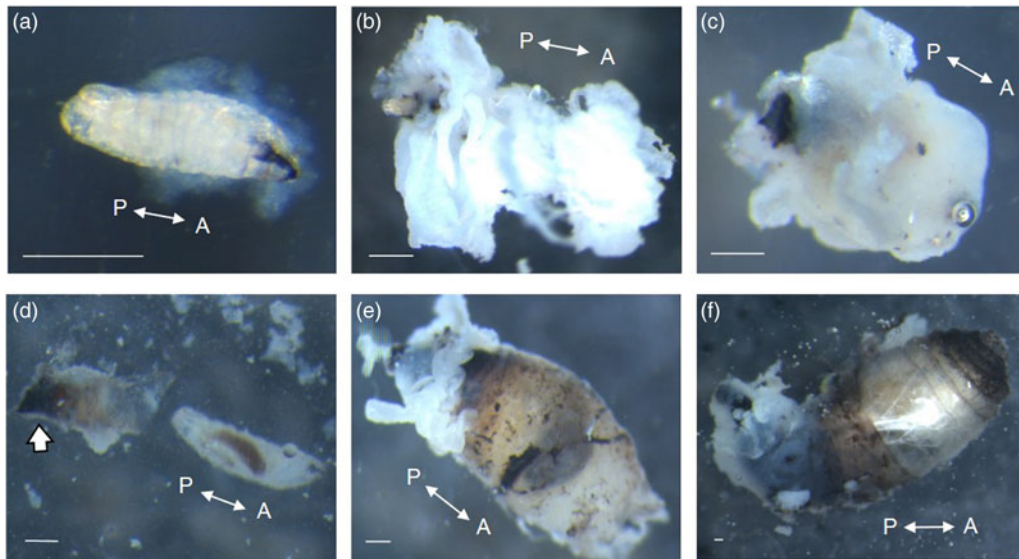


Fig. 1. Parasitizing *Drino inconspicuides* larvae excised from *Mythimna separata* host larvae at (a) 2 h, (b) 3 h, (c) 24 h, (e) 52 h and (f) 72 h after the invasion. (d) Photograph of the sheath (left) and larva (right) inside the surrounding structure at 24 h after the invasion. Scale bars = 0.25 mm. Double-headed arrows indicate the anterior-posterior (A/P) axes of the larvae. The arrow in (d) indicates the funnel.

(Takara) with a primer set that contained the adaptor sequence. The PCR products that were amplified from only the cDNA of the fat body were subcloned into the pGEM-T Easy Vector and sequenced.

#### Reverse transcription PCR (RT-PCR)

Total RNAs were extracted from the 'cloak', the capsules around the transplanted objects, and the host fat body and haemocytes with TRIzol reagent (Invitrogen), and 100 ng of each RNA were converted to cDNA with PrimeScript™ RTase (Takara) in a total volume of 20 µl. cDNA synthesis was confirmed using the housekeeping gene *rp49* (submitted as *RpL32*, accession number: AB669190; Yamaguchi *et al.*, 2012). *aldh* was used as the target gene that was specifically expressed in the host fat body and was amplified using the following primer set: 5'-GCTGGTGTCATCACC AACGA-3' and 5'-CTCCAAGTATTGTGTGATACCTTCCT-3'. The *M. separata* C-type lectin gene *epl* (Ishihara *et al.*, 2017) was used as the target gene that was specifically expressed in the host haemocytes and was amplified using the following primer set: 5'-ACGATCAGGCTGGTGAATGAT-3' and 5'-GATACAGA ATTCTGCGGTTGCAT-3'. PCR was performed using Paq5000 DNA polymerase (Agilent) with 0.8 µl of the cDNA synthesis mix as a template. The PCR programme consisted of initial denaturation at 94°C for 5 min followed by 31 (*aldh*) or 25 (*epl*) cycles at 94°C for 40 s, 55 °C for 40 s and 72°C for 40 s.

#### Sudan black B staining

The fat body and inner and outer layers of the 'cloak' at 3 h and 24 h after the invasion were fixed with 50% MeOH for 15 min and stained with Sudan black B (Muto Pure Chemical Co., Ltd) at 37°C for 15 min. The specimens were then washed with 50% MeOH and observed under a stereoscopic microscope (Leica).

## Results

### Characteristics of *D. inconspicuides* larvae in the host haemocoel

Invading *D. inconspicuides* larvae remained near the penetration point throughout the observation period. At 2 h after penetration, the larvae were surrounded by a cloudy material (fig. 1a), following which a cottony mass gradually coagulated around them (fig. 1b). The size of this cottony mass increased over 24 h to completely conceal the moulted 2nd instar larvae (fig. 1c). We named the distinctive cloudy and cottony mass the 'cloak'. Beneath the 'cloak', there was a brownish sheath with a blackened funnel surrounding the surface of the larvae (fig. 1d). The larvae inside the sheath were not melanized. The larvae moulted into 3rd instar larvae after approximately 52 h, by which time no further thickening of the 'cloak' occurred. Consequently, the 'cloak' no longer surrounded the entire larvae, with only a small portion of debris being observed on the sheath (fig. 1e), although the sheath still completely surrounded the larvae. Approximately 72 h after the invasion, the larvae increased in size so that their anterior half protruded from the sheath (fig. 1f).

### DNA analysis of the 'cloak'

The DAPI-stained 'cloak' removed at 24 h after invasion emitted a multitude of blue fluorescent signals (fig. 2a), suggesting that it consisted of cell aggregates. To identify the origins of the 'cloak', we obtained the ITS sequences of both the parasitoid *D. inconspicuides* (accession number: LC361445) and the host *M. separata* (accession number: LC361446). Only the host primer set amplified the specific DNA products from the 'cloak', with no DNA products being amplified by the *D. inconspicuides* primer set (fig. 2b), suggesting that the 'cloak' was composed of *M. separata* cells. The sheath also contained only host cells.

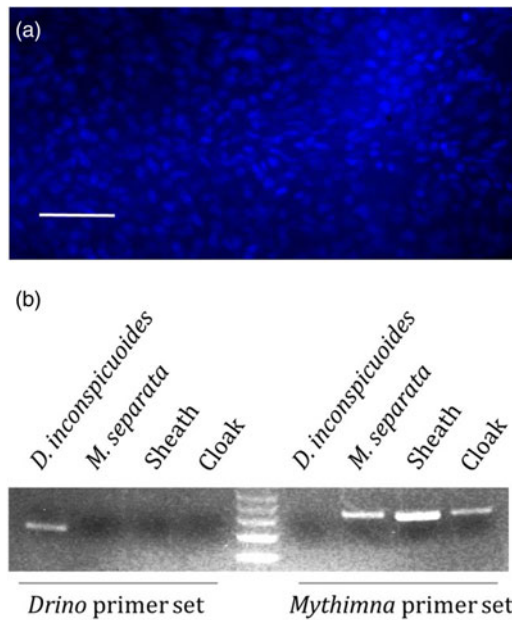


Fig. 2. Genomic analysis of the 'cloak'. (a) 'Cloak' stained with 4',6-diamidino-2-phenylindole (DAPI); scale bar = 0.05 mm. (b) Genomic polymerase chain reaction (PCR) with specific primer sets for the parasitoid *Drino inconspicuooides* and the host *Mythimna separata*.

#### Comparison with encapsulation capsules formed by the host

Since large foreign objects are usually encapsulated with host haemocytes, we considered it possible that the 'cloak' was a by-product of the cellular immune reactions. However, we observed lipid-like droplets within the cells of the cottony mass, leading us to hypothesize that production of the 'cloak' involved fat body cells. To verify this hypothesis, we identified the cell lineage of the 'cloak' and compared this with that of the encapsulation capsules.

The transplanted immune targets, micro-polystyrene beads and freeze-killed *D. inconspicuooides* larvae, became encapsulated with transparent capsules within 2 h (fig. 3a, b), at which time melanin deposition was visible on the surface of the dead larvae. The encapsulation capsules were completed by 24 h after insertion of the materials (fig. 3c, d).

We confirmed that *epl* (Ishihara *et al.*, 2017) was specifically expressed in the haemocytes and not in the fat body cells. Therefore, we used this as the host haemocyte-specific marker gene. Use of the SSH technique identified a gene encoding an aldehyde dehydrogenase (*aldh*; accession number: LC361447) as a candidate fat body-specific marker gene. Since ALDH is known to detoxify acetaldehydes, which are generated through lipid metabolism (Chen *et al.*, 2010), we used this gene as the fat body-specific marker gene. RT-PCR analysis using the primer sets for the haemocyte- and fat body-specific marker genes showed that only the *epl* gene fragment was detected from the cDNAs of the encapsulation capsules surrounding both the micro-beads and freeze-killed *D. inconspicuooides* larvae (fig. 3e). By contrast, both the *epl* and *aldh* gene fragments were amplified from the cDNA of the 'cloak', indicating that it originated from both the haemocytes and fat body.

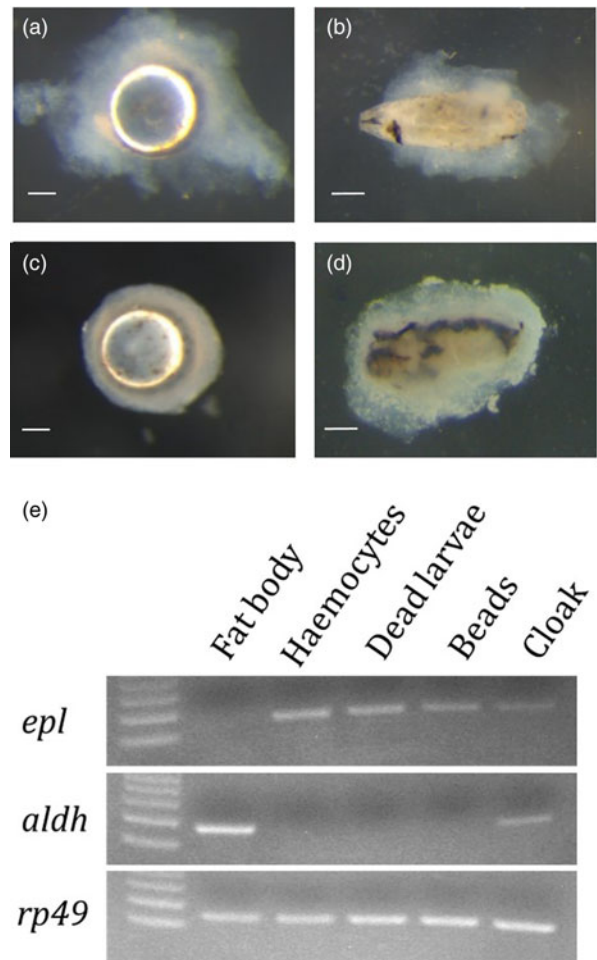


Fig. 3. Cell lineages of the encapsulation capsules that formed around transplanted objects and the 'cloak'. (a–d) Encapsulation of the micro-polystyrene beads (a and c) and dead *Drino inconspicuooides* larvae (b and d) at 2 h and 24 h, respectively, after artificial transplantation into *Mythimna separata* larvae; scale bars = 0.25 mm. (e) Expression of haemocyte-specific (*epl*) and fat body-specific (*aldh*) genes in the capsules around the transplanted objects and the 'cloak' at 24 h after the invasion. *rp49* gene is used as a control for cDNA synthesis.

#### Sequential changes in the components of the 'cloak'

RT-PCR analysis showed that the cloudy material and the inner layer of the 'cloak' expressed mainly the *epl* gene, whereas the outer layer expressed only the *aldh* gene at 3 h and 4 h after the invasion (fig. 4a). However, both genes were expressed in all layers after 24 h, suggesting that both the haemocyte and fat body cell lineages were mixed in the 'cloak'. The staining of each layer with Sudan black B, which binds lipids, confirmed that those layers that expressed *aldh* (i.e. the outer layer at 3 h and both the inner and outer layers at 24 h after the invasion) included lipid-containing fat body cells (fig. 4b). Together, these findings indicate that haemocytes assembled around the larvae first and then the fat body cells covered the haemocyte layer, following which the two lineages progressively converged.

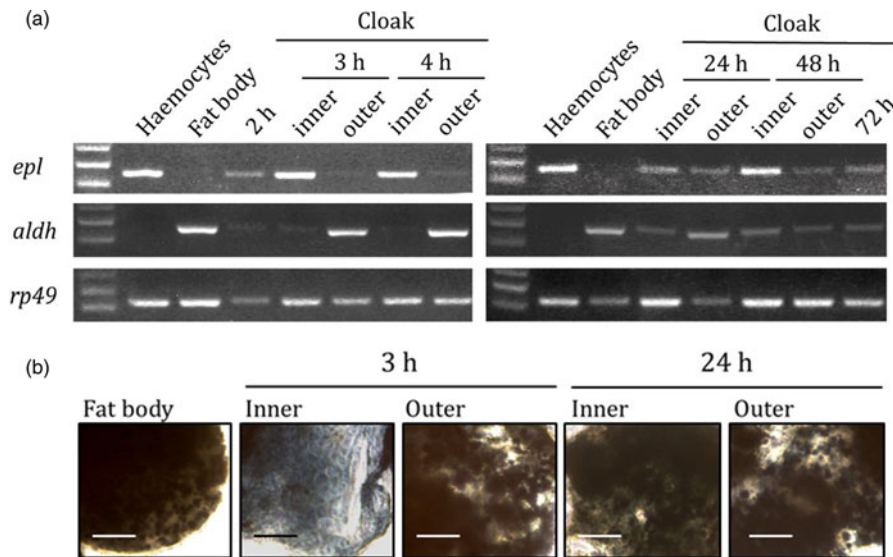


Fig. 4. Sequential changes in the cell lineage of the 'cloak'. (a) Expression of haemocyte-specific (*epl*) and fat body-specific (*aldH*) genes in the 'cloak'. *rp49* gene is used as a control for cDNA synthesis. (b) Sudan black B staining of the fat body (control), and the inner and outer layers of the 'cloak' at 3 h and 24 h after the invasion. Scale bars = 0.25 mm.

## Discussion

In this study, we discovered a unique structure that formed around *D. inconspicuides* larvae several hours after the invasion, which we termed the 'cloak', and demonstrated that this structure was constructed from the host cells (fig. 2). A similar structure that is produced by the host tissue has been observed in the endoparasitoid *Stichotrema dallatorreanum* (Strepsiptera: Myrmecolacidae), the larvae of which camouflage themselves in a host-derived epidermal 'bag' by manipulating the host epidermal tissue (Kathirithamby *et al.*, 2003), enabling them to avoid recognition and thus attack by the host immune system.

The 'cloak' observed in this study contained not only haemocytes but also fat body cells (figs 3 and 4). Namba *et al.* (2009) reported that the fat body is involved in the formation of the cuticular cyst that *Autographa nigrisigna* (Lepidoptera: Noctuidae) creates around parasitizing eggs of the endoparasitoid *Camponotus chlorideae* (Hymenoptera: Ichneumonidae), causing them to be discarded with the old cuticle at ecdysis. Therefore, we considered it possible that the fat body-containing 'cloak' observed in the present study was also specifically generated around *D. inconspicuides* larvae as part of the host immune reactions. However, we found that the encapsulation capsules that formed around freeze-killed larvae and micro-polystyrene beads were composed of only haemocytes (fig. 3e), suggesting that formation of the fat body-containing 'cloak' does not specifically depend on the surface cuticle structure of *D. inconspicuides* larvae but rather factors secreted by the living larva. It has previously been shown that the tachinid fly *Exorista larvarum* possesses large salivary glands (Gardenghi & Mellini, 1995; Michalková *et al.*, 2009) that can secrete massive amounts of enzymes to extraintestinally digest the host tissue. Moreover, Hayakawa (1986) reported that the tachinid fly *Blepharipa sericariae* secretes a factor that inhibits lipid transport in the host. Therefore, it is possible that living *D. inconspicuides* larvae secrete factors

that regulate the assembly of the host fat body to form the 'cloak'. We have not yet determined whether the haemocytes in the 'cloak' were accumulated through some action of the parasitoid larvae or as a result of the host immune reactions. However, since the cloudy material is formed within 2 h of invasion, which is similar to the encapsulation capsule that surrounded dead tachinid larvae, this material may assemble as a result of the host immune reaction.

We found that dead larvae were melanized after transplantation (fig. 3b, d) whereas living larvae that were surrounded with the 'cloak' were not melanized (fig. 1d). In *Drosophila* species, PPO3 is specifically produced by haemocytes that engage in encapsulation and contributes to melanization around parasitoid eggs during encapsulation (Dudzic *et al.*, 2015). However, we currently have no evidence that PPOs activation is suppressed by the parasitoid larvae. Since normal encapsulation capsules contain only haemocytes and the composition of the 'cloak' was similar to that of fat body-contaminated encapsulation capsules, it is possible that the fat body physically interferes with the normal encapsulation process leading to suppression of the activities of PPOs. Alternatively, the secretion of chemicals from the larvae or the fat body cells within the 'cloak' may suppress the activation of PPOs.

Salt (1968) suggested that the sheath that forms around intruding larvae (fig. 1d, left) is made from the host haemocytes but he did not confirm this through genetic analyses. Here, we confirmed through genomic PCR that both the sheath and the 'cloak' are composed of only host cells. However, since the removal of the entire 'cloak' to prepare pure sheath material is difficult, we cannot exclude the possibility that there may have been some contamination of the sheath material by the 'cloak'. In addition, since tachinid larvae moult twice within 72 h of invasion, it is likely that the cast-off cuticles are also incorporated into the sheath.

Once the *D. inconspicuides* larvae became 3rd instar larvae, the 'cloak' no longer surrounded the entire larval body

(fig. 1f). Therefore, it is likely that some other defence mechanism protects the larvae after this time. Alternatively, it is possible that they no longer require any particular means of defence because the host immune system has been sufficiently weakened by the consumption of immune tissues such as the fat body and haemocytes.

When females of endoparasitoid wasps deposit their eggs into the host, they also inject immune-suppressive factors such as polydnviruses to allow their offspring to develop inside the host haemocoel (Moreau & Asgari, 2015). However, parasitizing *D. inconspicuoides* larvae require the urgent activation of mechanisms that protect them from the host immune reactions in the absence of materials from their parents. Therefore, it may be that host-derived substances are used to form a barrier. Further studies that focus on the molecular mechanisms that are involved in the formation of the 'cloak' and the suppression of melanization will help further our understanding of the unique parasitization strategies of tachinid flies.

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