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Avoidance of the host immune response by a generalist parasitoid, *Compsilura concinnata* Meigen

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Abstract. 1. Ecological interactions between parasitoids and their hosts are extremely strong as parasitoid offspring rely entirely on an individual host to complete development. The ability of a parasitoid to use a host is influenced directly by the degree to which the parasitoid can overcome host defences and grow within the host.

2. Hymenopteran parasitoids have evolved different host-specific strategies to defeat the host immune system, such as the use of venom, endosymbiont virus, or mimicking the host tissue. Dipteran parasitoids from the Tachinidae family do not use these subterfuges and rely mainly on avoiding the host immune system by hiding in specific tissues.

3. Little is known of the effect of this strategy on the host immune system, the absorption of nutrients by the parasitoid larvae, or the implications for parasitoid host range.

4. In this study, the impact of a polyphagous tachinid parasitoid *Compsilura concinnata* Meigen on a pest lepidopteran *Trichoplusia ni* Hübner are assessed. Phenoloxidase levels and haemolymph proteins were measured in parasitised *T. ni* as a function of host immune response.

5. Haemolymph phenoloxidase in the host did not vary with parasitisation but was triggered when a piece of monofilament was implanted in the haemocoel. Haemolymph proteins were depleted in heavily parasitised *T. ni*.

6. These results indicate that *C. concinnata* has a strategy that avoids the host immune system, and accesses the necessary nutrients for larval growth. This strategy could explain the success of this tachinid and its wide host range.

Key words. *Compsilura concinnata*, haemolymph protein, host range, immune system, intra-host competition, parasitoid, phenoloxidase, Tachinidae, *Trichoplusia ni*.

Introduction

Parasitoids have been widely used as biological control agents and have been introduced extensively to control pest species. Ecological interactions between parasitoids and their host are very intimate since the parasitoid offspring relies solely on one host individual to develop to maturity. The main challenges for parasitoids are to find hosts as ovipositing adults, to survive the host immune system, and to absorb nutrients from the host as larvae (Vinson, 1993). The capacity of a parasitoid to use a host is therefore dictated by these criteria.

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The host immune defence against parasitoid larvae is mainly based on encapsulation (Chapman, 1974; reviewed by Strand & Pech, 1995). Encapsulation can be due to melanisation or to a cellular response whereby haemocytes form multiple layers around the organism; this capsule can also be further melanised. In both cases, it is thought that the parasitoid is asphyxiated, but may also die from toxic compounds produced during the immune reaction (Strand & Pech, 1995). Phenoloxidase (PO), an oxidoreductase enzyme, is known to be important in the immune system of insects by catalysing key steps during melanisation and encapsulation of foreign bodies in the haemocoel (Gillespie *et al.*, 1997). PO levels have been shown to alter in response to parasitisation (Hartzer *et al.*, 2005), and to affect rates of encapsulation of foreign bodies (Cotter & Wilson, 2002). It is also involved in wound

healing and sclerotisation (Chapman, 1974; Nation, 2002; Mucklow *et al.*, 2004).

Some parasitoids have evolved specific strategies to evade, suppress, and avoid the host immune system responses (Vinson, 1993; Schmidt *et al.*, 2001). Endoparasitoids can evade the immune system by having a similar body surface to the host tissue thereby ensuring the host immune system does not recognise the parasitoid as a foreign body (Vinson, 1993). Some hymenopteran parasitoids suppress the host immune system by injecting an endosymbiont virus during the oviposition process that reduces the ability of the host immune system to respond to invasion (Whitfield, 1994) and also affects PO activation (Shelby & Webb, 1999; Bae & Kim, 2004). These mechanisms are very specific to the host and are thought to be under constant selection pressure (Godfray, 1994).

Dipteran parasitoids are not known to have viruses to suppress the host immune systems, and therefore they must rely on other strategies (Feener & Brown, 1997). Many avoid the host immune system by remaining in locations where the immune system has limited access (glands, nerve ganglia, muscular tissue), or by forming a respiratory funnel when encapsulated. These strategies are believed to contribute to the wider host range found in dipteran parasitoids as compared to hymenopterans (Belshaw, 1994; Feener & Brown, 1997; Stireman *et al.*, 2006). Parasitoids are known to feed on haemolymph nutrients (Baker & Fabrick, 2000; Nakamatsu & Tanaka, 2004), a process that may be inhibited by hiding in specific tissues, which in turn may slow their growth.

To our knowledge, no study has ever assessed the parasitoid larval feeding activity or the response of the host immune system to parasitoids hiding in a protected location. The goal of the present study was therefore to assess the immune reaction and the haemolymph protein levels of *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae), when parasitised by a tachinid, *Compsilura concinnata* Meigen.

Compsilura concinnata was repeatedly introduced to North America from Europe between 1906 and 1986 (reviewed by Boettner *et al.*, 2000). This parasitoid has shown an impressive adaptation to a wide range of hosts; in Europe it is widely distributed and uses about 60 hosts (Culver, 1919), while in North America its host range exceeds 160 species (Arnaud, 1978; Strazanac *et al.*, 2001). Populations of some of the North American hosts have been shown to be negatively influenced by this new parasitoid (Boettner *et al.*, 2000; Kellogg *et al.*, 2003).

Compsilura concinnata larviposits directly into the haemolymph of immature stages of its host using a sickle-shape structure formed by the seventh segment of its abdomen. First-instar parasitoid larvae penetrate the peritrophic membrane of the host and attach to tracheoles with special anal hooks. The larvae remain between the peritrophic membrane and the midgut wall, until the host prepupal stage, when they enter the body cavity and grow rapidly. Third instar parasitoid larvae exit the host shortly after prepupation of the host and form a puparium (Culver, 1919; Bourchier, 1991; Ichiki & Shima, 2003).

To determine if larviposition by *C. concinnata* induces increased activity of the host immune system, the PO activity in the haemolymph of parasitised *T. ni* was assessed. *Compsilura concinnata* larvae migrate immediately after larviposition to the midgut and peritrophic membrane and only remain in contact with the host haemolymph, and therefore immune system, for a maximum of 1 h (Bourchier, 1991; Ichiki & Shima, 2003). For this reason it was hypothesised that several hours after parasitisation PO levels would not be detectably elevated. Alternatively it was predicted that if a foreign body such as a parasitoid remained in the haemolymph, an elevated PO response would be triggered. Secondly, protein levels in parasitised host haemolymph can indicate nutrient consumption by immature parasitoids. It was hypothesised that since *C. concinnata* larvae remain at the first instar while between the peritrophic membrane and the midgut (Ichiki & Shima, 2003), not enough protein would be consumed by the non-growing larvae for the haemolymph protein levels to be affected by parasitisation.

Materials and methods

Insect rearing

Trichoplusia ni. Cabbage loopers were reared at a temperature of 25 °C and a LD 16:8 h photoperiod. Groups of 15 *T. ni* larvae were reared in 175-ml Styrofoam cups containing 20 ml of wheat germ based artificial diet (Ignoffo, 1963). Pupae were removed from their cocoons and soaked in a 0.6% sodium hypochlorite solution to disinfect them against potential viral or bacterial contamination. Adults were kept in cylindrical wire mesh cages in groups of 200, and were fed 10% sucrose solution. Paper towels were put on the outside of the cages to provide oviposition sites and eggs on the paper towels were harvested regularly. These were rinsed with a 0.2% bleach solution, air dried, placed in a plastic bag and stored at 9 °C until use.

Compsilura concinnata. The colony of *C. concinnata* originated from parasitised *T. ni* larvae collected from a broccoli field in Ladner, British Columbia in 2004. Adults *C. concinnata* were kept in 0.25 m^3 screened cages and were provided with water and 10% sugar solution. Groups of 15 fourth-instar *T. ni* larvae in cups containing artificial diet were provided daily for larviposition. Retrieved cups were kept at 25 °C until parasitoid puparia were found, at which stage they were added to parasitoid cages. Puparia mortality levels were constantly lower than 5%.

Phenoloxidase assay

Exposure to parasitoid. For this experiment, *T. ni* larvae that had been in contact with *C. concinnata* and were potentially parasitised ($n = 58$ randomly chosen from the rearing colony) and control larvae that had not been in contact with the parasitoid ($n = 20$) were assessed. To quantify parasitisation, *T. ni* that had been in contact with the flies were dissected after the assay, and the number of parasitoids found per host was recorded. Non-parasitised, parasitised, superparasitised, and control larvae were compared, forming the four treatments of the experiment.

PO activity was measured based on the methods described in Wilson *et al.* (2001). At the fifth instar (i.e. 2 days after being parasitised), caterpillars were bled by immobilising them on a piece of parafilm and removing one of the prolegs. Blood pearling on the parafilm was collected with a pipette and 10 μl were put into a 500-μl micro-centrifuge tube containing 240 μl of ice-cold Dulbecco's phosphate buffer saline (DPBS, 0.2 M, pH 7.2, Sigma-Aldrich, St Louis, Missouri). Samples were vortexed and then frozen at −20 °C for 24 to 48 h to disrupt haemocyte membranes. To measure the PO activity, three replicates of 50 μl of each sample were put in a 96-well microtitre plate with 100 μl of 15 mM dopamine hydrochloride (Sigma-Aldrich). Absorbance was measured at 492 nm every 30 s whereas an endpoint absorbance reading at 595 nm was used to determine protein concentration. All absorbance readings were performed with a Spectramax 190 microplate reader (Molecular Devices Corporation, Sunnyvale, California). Proteins were measured using methods developed by Bradford (1976), in three replicates of 5 μl for each sample using bovine serum albumin (BSA) as a protein standard (Protein Assay Kit #2, BIORAD Laboratories, Hercules, California). Plate-to-plate variation was eliminated with the inclusion of a BSA dilution series in each microtitre plate. The specific activity of PO per haemolymph sample is expressed as PO units per mg protein, where one PO unit is the amount of enzyme needed to increase absorbance by 0.001 min^{-1}.

Mock parasitisation. In this experiment, fourth-instar *T. ni* that were pricked with a small surface sterilised pin were compared to a group of *T. ni* that had a 1.5 mm long by 0.022 mm diameter monofilament line inserted at a shallow angle in the lateral, medial quadrant, and to a control group of unmanipulated larvae. Five microlitres of haemolymph were collected 24 or 48 h after the mock parasitisation event and put in 100 μl of ice-cold DPBS. PO activity was measured as for the previous experiment.

Statistical analysis

PO activity and protein concentration in haemolymph for *T. ni* which were parasitised, superparasitised, not parasitised and not exposed to the parasitoid (control) were compared using a one-way ANOVA in JMPIN 4.0.3 (SAS Institute Inc., 2000). PO activity was natural log transformed and protein concentration was square-root transformed to approximate normal distribution. In the exposure to parasitoid experiment, the four treatments were not all represented in all of the microplates. To test for a possible difference between microplates, each treatment was analysed separately with microplate as a factor determining PO and protein. For all four treatments the microplate effect was non-significant and was not included in subsequent analyses (PO: parasitised: $F_{3,18} = 2.282$, $P = 0.121$; superparasitised: $F_{3,10} = 0.485$, $P = 0.705$; not parasitised: $F_{2,24} = 2.513$, $P = 0.104$; control: $F_{2,19} = 0.352$, $P = 0.709$; protein: parasitised: $F_{3,18} = 2.100$, $P = 0.143$; superparasitised: $F_{3,10} = 1.458$, $P = 0.317$; not parasitised: $F_{2,24} = 0.070$, $P = 0.933$; RC: $F_{2,19} = 0.992$, $P = 0.391$). To compare the effect of the treatments on PO activity in the mock parasitisation experiment, a two-way ANOVA was used with treatment and time at sampling as factors.

Results

Dissection of *T. ni* that were presented to *C. concinnata* colony showed that parasitisation was frequent. Of the 58 larvae that were dissected, 52% were parasitised. Superparasitism was common in *T. ni* with up to five *C. concinnata* larvae per host (Table 1). The total number of parasitisation events was 59, and 11 hosts were superparasitised. All fly larvae were found between the peritrophic membrane and the midgut and were first instars, except for one second-instar larva in a superparasitised host.

Exposure to parasitoid

PO was not influenced by parasitisation in *T. ni*. PO activity in the haemolymph was not significantly different when comparing unparasitised controls, non-parasitised but exposed larvae, singly parasitised and superparasitised *T. ni* larvae ($F_{3,74} = 0.604$, $P = 0.614$). Protein quantity in the haemolymph was significantly lower in superparasitised hosts compared to control and non-parasitised *T. ni*, but singly parasitised hosts were not significantly different from the other categories of *T. ni* ($F_{3,74} = 3.398$, $P = 0.022$) (Fig. 1).

Mock parasitisation

Despite the invasiveness of the implant surgery, larvae implanted with a piece of monofilament line recovered and developed normally. *Trichoplusia ni* from the implant treatment that were not used in the experiment pupated and survived to adults. PO activity was significantly influenced by the different treatments ($F_{2,182} = 6.163$, $P = 0.003$). PO activity was higher for *T. ni* that had a piece of monofilament implanted in their body cavity, but pricked and naive larvae were not significantly different (Fig. 2). Time at sampling had a significant effect on PO; levels were higher after 24 h than after 48 h ($F_{1,182} = 9.335$, $P = 0.003$). PO was higher 24 h (mean = 7.657, SE = 0.134) after the mock parasitisation event than at 48 h (mean = 7.093, SE = 0.127). There were no interactions between treatment and the time at sampling ($F_{2,182} = 0.007$, $P = 0.994$).

Table 1. Number of *Compsilura concinnata* found per dissected host and number of parasitisation events.

Number of flies per host	Number of hosts
0	28
1	19
2	1
3	4
4	4
5	2
Total	58

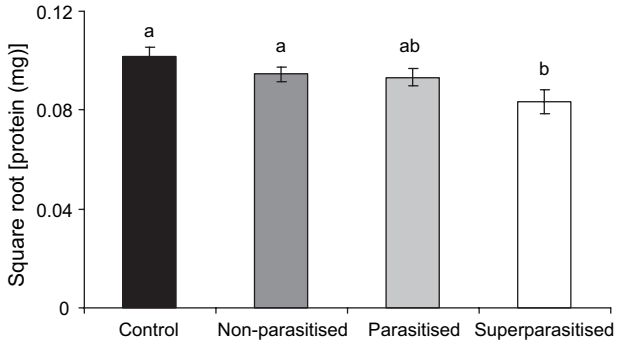

Fig. 1. Protein quantity in haemolymph for control, non-parasitised, singly parasitised, and superparasitised fifth-instar *Trichoplusia ni*. Treatments with the same letter are not significantly different.

Discussion

Parasitoids have evolved various approaches to overcome host immune responses by avoiding or suppressing the host immune system. Dipteran parasitoid larvae use two major strategies to avoid the host immune system. First they can keep constant contact with air by forming a respiratory funnel and thus avoiding oxygen depletion caused by encapsulation by attaching themselves to host tracheoles. Second, they can move to specific tissues where the immune system does not have access, such as glands, nerve ganglia, or muscles (Feener & Brown, 1997). In *T. ni*, *C. concinnata* adopts the second strategy by remaining between the peritrophic membrane and the midgut for most of the host's larval development. It also uses this in *Lymantria dispar* (Culver, 1919; Bourchier, 1991) and *Bombyx mori* (Ichiki & Shima, 2003). It is possible that the polyphagy found in this species is facilitated by the avoidance of the immune system (Bourchier, 1991), which is supported by the failure of PO levels to respond to parasitisation as compared to the significant PO response that occurred when a piece of monofilament was inserted into the haemocoel. By remaining in between the peritrophic membrane and the midgut, *C. concinnata* larvae are not triggering the encapsulation or melanisation processes that normally take place when foreign bodies enter the haemocoel (Cotter *et al.*, 2004).

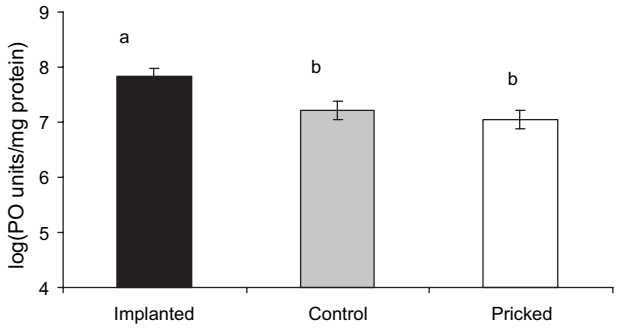

Fig. 2. Phenoloxidase activity in haemolymph of *Trichoplusia ni* implanted with monofilament, pricked with a pin and untreated control. Treatments with the same letter are not significantly different.

It is also a possibility that *C. concinnata* has a mechanism to suppress PO activity in the host, as shown in some hymenopteran parasitoids (Shelby & Webb, 1999; Bae & Kim, 2004; Nappi *et al.*, 2004; Hartzer *et al.*, 2005) and other organisms such as nematodes (Brivio *et al.*, 2002). This is unlikely since no such mechanism has ever been found in a tachinid species (Feener & Brown, 1997). Furthermore, the lack of an ovipositor in tachinids and more specifically in *C. concinnata*, which uses a piercing structure made of the seventh segment of the abdomen (Culver, 1919), prevent the injection of substances such as viruses that would counteract the host immune system (reviewed by Stireman *et al.*, 2006).

PO is used in wound healing (Chapman, 1974; Nation, 2002; Mucklow *et al.*, 2004), and has been positively correlated with encapsulation rates (Cotter & Wilson, 2002). However, despite the cuticle being pierced, PO was not triggered by fly parasitisation or the pin piercing treatment. This is possibly due to the fact that piercing the cuticle is less invasive than the insertion of a foreign body in the haemocoel and therefore would not stimulate increased PO levels sufficiently to be detected at the time of the assays. Furthermore, it is possible that 48 h after piercing the cuticle, the PO activity had already resumed. PO is an immune response reaction that occurs quickly following injury. Since oxidisation of phenols into quinones generates toxic by-products that are also harmful to the insect (Söderhäll & Cerenius, 1998; Schmid-Hempel, 2005), the PO cascade is tightly regulated and therefore PO activity in the haemolymph must decline over time. This is supported by the PO levels being higher 24 h after the mock parasitisation than 48 h later.

By remaining between the peritrophic membrane and the midgut, *C. concinnata* likely avoids *T. ni*'s immune system, but at the same time is not in direct contact with nutritional resources. Since growth of the parasitoid is reduced at the host larval stage (Culver, 1919; Ichiki & Shima, 2003), it was hypothesised that the location of the parasitoid may limit the parasitoid's nutritional requirements. In this study, one *C. concinnata* larva was found at the second instar when the host was dissected, indicating that some nutrient absorption had occurred. Also, protein quantity in the haemolymph was lower in superparasitised hosts than in non-parasitised hosts. This is in agreement with Nakamatsu and Tanaka (2004) who showed that proteins and other haemolymph nutrients were depleted faster in heavily parasitised hosts. This also indicates potential competition for nutrients between the parasitoid larvae in superparasitised hosts. To allow the absorption of nutrients from the digestion process, the peritrophic membrane is permeable (Chapman, 1974). In the case of *C. concinnata*, nutrients may be consumed as they pass through the peritrophic membrane and the midgut wall, before entering the haemolymph. The concentration of protein in the haemolymph would therefore be reduced due to lower rates of replacement from the midgut.

Parasitoids can be quite specific in their host range. Host range is thought to be determined mostly by host ecology and taxonomy. Parasitoids can be specialised to one species or one group of insects since they share similar physiology and/or defence mechanisms. Parasitoids can also target hosts having a similar ecology, such as the same food plants (Godfray, 1994). Dipteran parasitoids tend to have wider host ranges than

hymenopteran parasitoids, and this may be due to strategies used to avoid host immune systems (Belshaw, 1994; Feener & Brown, 1997; Stireman *et al.*, 2006). This is supported by the narrower host range found in tachinids that remain freely in the host haemocoel compared to tachinids that evade encapsulation by forming respiratory tunnels (Belshaw, 1994). *Compsilura concinnata* is highly polyphagous and can use hosts in different insect orders, mainly Lepidoptera, but also Hymenoptera and Coleoptera hosts (Webber, 1926; Arnaud, 1978). These orders differ in many ways but share many anatomical similarities. This study shows that by remaining between the peritrophic membrane and the midgut, *C. concinnata* avoids activation of the PO cascade while still accessing nutrients. This strategy may reduce the need for a strong adaptation to the host. Introduced parasitoids with a wide host range are more likely to have detrimental impacts on native fauna (Louda *et al.*, 2003; Pearson & Callaway, 2003; Louda & Stiling, 2004). Understanding strategies used by parasitoids to overcome host immune responses may help to predict host range and thereby reduce the potential non-target effects of introduced biological control agents.

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