Endoparasitoids have the ability to evade the cellular immune responses of a host and to create an environment suitable for survival of their progeny within a host. Generally, the host immune system is suppressed by endoparasitoids. However, polyembryonic endoparasitoids appear to invade their hosts using molecular mimicry rather than immune system suppression. It is not known how the host immune system is modified by polyembryonic endoparasitoids. Using haemocyte counts and measurement of cellular immune responses, we evaluated modification of the host immune system after separate infestations by a polyembryonic parasitoid (Copidosoma floridanum) and another parasitoid (Glyptapanteles pallipes) and by both together (multi-parasitism). We found that the polyembryonic parasitoid maintains and enhances the host immune system, whereas the other parasitoid strongly suppresses the immune system. Multi-parasitization analysis revealed that C. floridanum cancelled the immune suppression by G. pallipes and strengthened the host immunity. This enhancement was much stronger with male than with female C. floridanum.

1. Introduction

Endoparasitoids develop in a host haemocoel by consuming haemolymph and later the internal organs, and eventually kill the host [1]. Various physiological interactions and regulations exist between the parasitoid and the host. The most important challenge for parasitoids is to circumvent the host cellular immune response, which will lead to haemocytic encapsulation [2,3]. Many parasitoids are known to suppress host immunity [4]; for example, some parasitoids in the Braconidae and Ichneumonidae suppress it by maternal secretions of polydnaviruses, venom and ovarian proteins [2,5,6]. However, such suppression is likely to enhance the host’s susceptibility to fungal, bacterial and viral infections [7], resulting in an immunological dilemma for the parasitoids.

The polyembryonic parasitoid Copidosoma floridanum appears to have solved this dilemma in a unique way. This species exhibits clonal reproduction in which one egg yields several thousand reproductive larvae and about a hundred soldier larvae, all of which evade the host’s immune responses by molecular mimicry [8-10]. Both the embryo and larva of this species are surrounded by an extraembryonic syncytium (membrane) that shields these life stages from the host cellular immune response [11], thereby circumventing the need for immunological suppression of the parasitized host.

Another important observation on host immunity comes from studies of multi-parasitism [1]. When a single host is parasitized by two different parasitoids, one parasitoid species emerges victorious following competition between them. Previous studies have shown that C. floridanum usually kills and eliminates the larvae of its competitor, Glyptapanteles pallipes (Braconidae) by attacking them with soldier larvae and a unique toxic humoral factor [12]. There are
distinctive differences between male and female broods of C. floridanum in the numbers, behaviours and competitive abilities of the soldiers [13,14]. In addition, in a mixed brood where one male egg and one female egg are laid on a host egg, many male offspring are killed by female soldiers, but surviving males typically mate with most of the female reproductives [14]. These differences in the parasitoid sexes imply that their control of the host immune system is also different. There are many reports of the differences in immune responses between the host sexes [15]; but virtually none on the differences between the parasitoid sexes.

We hypothesized that C. floridanum maintains the host immune system and that this immunological control is qualitatively different between males and females of this parasitoid. We therefore compared the parasitization of a host by the males and/or females of C. floridanum and a braconid (G. pallipes) that is known to suppress host immunity and multi-parasitism. In insects, the cellular immune defence is determined by the quantity and quality of the haemocytes [7]. Thus, we examined the host immune system in terms of haemocyte counts and immunological responses to abiotic and biotic foreign objects. To our knowledge, this is the first study that investigates both the effects on the host immune system and sex-based differences in those effects in a polyembryonic parasitoid that employs molecular mimicry.

2. Material and methods

In this study, the host used was Ctenoplusia agnata (Lepidoptera, Noctuidae) and the parasitoids were males and females of the polyembryonic parasitoids C. floridanum (Encyrtidae) and its competitor G. pallipes (Braconidae) (see the electronic supplementary material for experimental conditions). To evaluate host haemolymph, we recorded the total haemocyte counts (THCs) and the differential haemocyte counts (DHCs; granulocytes, plasmatocytes, oenocytoids, spherulocytes and prohaemocytes [16]). Haemocyte responses to foreign objects were assessed at the sixth instar host stage by polystyrene microbeads (abiotic object) and fluorescein-labelled zymosan (Saccharomyces cerevisiae; biotic object). The responses against microbeads were classified into four types: no reaction, attachment, phagocytosis and nodule formation.

For statistical analysis, a generalized linear model with Poisson or quasi-Poisson distribution was used for both haemocyte counts and responses to foreign objects; when significant, we used the Steel–Dwass test to assess post hoc statistical significance (see the electronic supplementary material).

3. Results

(a) Host haemocyte counts

THC varied (figure 1) depending on the parasitoid ($p = 3.02 \times 10^{-16}$; generalized linear model with quasi-Poisson distribution; see the electronic supplementary material, table S1 for individual statistics). Copidosoma floridanum alone exhibited no effect on THC, whereas G. pallipes with or without C. floridanum
Table 2. Cellular immune response against microbeads of haemocytes of *C. agnata* parasitized by *C. floridanum* (male or female), *G. pallipes* alone, and multi-parasitized by *C. floridanum* and *G. pallipes*. Cf, *C. floridanum*; Gp, *G. pallipes*.

<table>
<thead>
<tr>
<th>hosts</th>
<th>n</th>
<th>no reaction (%)</th>
<th>attachment (%)</th>
<th>phagocytosis (%)</th>
<th>nodule formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-parasitized</td>
<td>15</td>
<td>16.7 ± 9.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.1 ± 8.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>27.7 ± 9.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.9 ± 10.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>parasitized by:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cf&lt;sup&gt;+&lt;/sup&gt; alone</td>
<td>15</td>
<td>25.7 ± 11.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.2 ± 7.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.9 ± 9.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>10.2 ± 6.5&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cf&lt;sup&gt;+&lt;/sup&gt; Gp</td>
<td>15</td>
<td>15.5 ± 9.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.8 ± 4.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>31.0 ± 6.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.8 ± 9.2&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>multi-parasitized by:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cf&lt;sup&gt;+&lt;/sup&gt; + Gp</td>
<td>15</td>
<td>46.0 ± 25.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.4 ± 8.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>19.4 ± 17.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>8.2 ± 7.0&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td>6.32 × 10&lt;sup&gt;−09&lt;/sup&gt;</td>
<td>0.00107</td>
<td>0.00126</td>
<td>1.25 × 10&lt;sup&gt;−05&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Numbers in columns followed by the different letters (a–c) indicate significant difference between treatments (*p* < 0.05, generalized linear model with quasi-Poisson distribution).

reduced THC significantly. The level of reduction in THC was lowered significantly by male (*p* = 0.00003), but not by female (*p* = 0.64584) *C. floridanum*.

DHC showed that granulocytes and plasmatocytes comprised more than 70% of the total haemocytes, with oenocytoids and spherulocytes comprising about 10% each, and prohaemocytes about 2% (table 1). The effects of parasitism on DHC also varied (*p* < 0.05; generalized linear model with Poisson distribution; table 1; electronic supplementary material, tables S2–S6). The percentage of granulocytes was increased by *G. pallipes* alone, but not by *C. floridanum* with or without *G. pallipes*. The percentage of plasmatocytes was also increased by *G. pallipes* alone, whereas no statistically significant change was detected with male or female *C. floridanum* alone. The magnitude by which *G. pallipes* increased the percentage of plasmatocytes was greatly reduced when the host was multi-parasitized by male *C. floridanum*. The percentage of oenocytoids was lowered by *G. pallipes* in the absence of competition, whereas this effect was cancelled when *C. floridanum* was also present. The percentage of spherulocytes was lowered by *G. pallipes* with/without *C. floridanum*.

Figure 2. Phagocytic activity of haemocytes (granulocytes and plasmatocytes) to fluorescin-labelled zymosan (*S. cerevisiae*). Data are presented as mean ± s.d. (arc-sin-detransformed data). Means with different letters indicate significant difference between treatments (*p* < 0.05, generalized linear model with quasi-Poisson distribution). Host conditions: non, non-parasitized (control); Cf<sup>+</sup>, parasitized by male *C. floridanum*; Cf<sup>+</sup>, parasitized by female *C. floridanum*; Gp, parasitized by *G. pallipes*; Cf<sup>+</sup> + Gp, multi-parasitized with male *C. floridanum* and *G. pallipes*. Numbers in parentheses are sample sizes.

(b) Host immunological responses

The immunological responses to the injected microbeads in unparasitized hosts (control) included no reaction (16.7%), attachment (35.1%), phagocytosis (27.7%) and nodule formation (19.9%). *Glyptapanteles pallipes* greatly increased the frequency of no reaction (54.1% with *G. pallipes* alone, 43.9 and 46.0% with male and female *C. floridanum*, respectively) and decreased the frequency of phagocytosis (14.3% alone, 15.3 and 19.4% with male and female *C. floridanum*, respectively) and nodule formation (9.6% alone, 4.5 and 8.2% with male and female *C. floridanum*, respectively), whereas *C. floridanum* subjects were not significantly different from the control (e.g. no reaction: male: 25.7%; female: 15.5%; *p* > 0.05, generalized linear model with quasi-Poisson distribution; table 2; electronic supplementary material, tables S7–S10).

The *in vitro* assessment of phagocytosis against zymosan showed that the percentage of phagocytosis was decreased significantly by *G. pallipes* alone or with female *C. floridanum* but not by *G. pallipes* with male *C. floridanum*, or by male or female *C. floridanum* alone (*p* = 5.59 × 10<sup>−05</sup>; generalized linear model with quasi-Poisson distribution; figure 2; electronic supplementary material, tables S11 and S12).

4. Discussion

To our knowledge, this is the first study assessing not only the enhancement of host immune systems by parasitoids, but also of the parasitoid sex differences in these effects. As with other known parasitoids [7,17,18], *G. pallipes* was found to suppress the host immune system (*G. pallipes* infection alone in figures 1 and 2, and table 2). By contrast, such suppression was not detected with *C. floridanum* alone, regardless of sex. Multi-parasitization experiments further revealed that *C. floridanum* actively enhanced host immunity.
This enhancement was much stronger in males than in females (see multi-parasitism in figure 1).

The differences between THC and DHC may be important. In G. pallipes, the percentages of granulocytes and plasmocytes, which are responsible for cellular defence, were increased, but THC was reduced by almost one half, resulting in a large reduction in absolute numbers (figure 1 and table 1). With multi-parasitism, THC of male C. floridanum was higher than that of females (figure 1) but DHC was not significantly different (table 2). The toxic humoral factor produced by C. floridanum may be responsible for this sex difference in THC [12]. Our results may imply that male C. floridanum suppress the polydnavirus infection, which is used by many bracoonid parasitoids to suppress host immunity [19,20]. Gene expression and/or protein translation of polydnaviruses may be blocked by multi-parasitizing male C. floridanum. More detailed studies on the comparisons between THC and DHC, and their underlying mechanisms are needed.

The stronger enhancement of host immunity by male C. floridanum may be a compensation for their weaker competitive abilities. If male C. floridanum invest more in the enhancement of host immunity, the parasite increases the reproductive success by increasing the host resistance against pathogens and competitors. In mixed broods, female soldiers kill many male reproductives without reducing male success because only a few male reproductives are needed to inseminate all the female reproductives present in the host [14]. However, if male soldiers were more competitive, then they would be more likely to kill female sibling reproductives, resulting in a reduction in both inclusive fitness and the number of female mates. Therefore, fitness is enhanced if female eggs invest more in soldiers, while male eggs invest more in host immunity enhancements [21].

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