Research Article

Is Sexual Dimorphism in the Immune Response of *Gryllodes sigillatus* Related to the Quality of Diet?

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Whereas some authors have proposed that sexual dimorphism in the immune response is fixed, others pose that it is dynamic and depends on diet. The aim of the present study was to explore the second hypothesis. Immunocompetence differences between females and males can be linked to resource availability. We tested this idea by providing a low or high quality diet to two groups of *Gryllodes sigillatus* during their developmental period. Then, at the adult phase half of each group was challenged with LPS from *Serratia marcescens*. The size, phenol oxidase (PO), and lytic activity were compared between groups according to diet, sex, and immune challenge. Results show that diet quality favor size in both sexes. However, the overall immune response did not seem to be significantly different based on diet, but instead on sex. Females showed greater phenol oxidase levels than males, but the opposite was found with lytic activity. Perhaps in *G. sigillatus* the differences in the pathogens commonly confronted by each sex in the distinct habitat of each could explain the differences on PO and lytic activity.

1. Introduction

The understanding of sexual dimorphism (SD) is one of the central challenges in evolutionary biology. SD includes differences in behavior, morphology, and physiology, but perhaps why males are less resistant to parasites and pathogens than females is one of the most studied questions regarding this topic [1–3]. The underlying physiological mechanism of this dimorphic resistance could be that females are more immunocompetent than males, implying SD in the immune response. Hence, immunocompetence is defined as the magnitude and effectiveness of immune response [4].

To understand why females are more immunocompetent than males can be explained by two hypotheses: sexual selection [2, 5] and the immunocompetence handicap hypothesis [6]. The sexual selection hypothesis suggests that males in most species invest in their sexual traits at the expense of survival, meaning that fewer resources are invested in the immune response [2]. For example, males in *Panorpa vulgaris* provide nuptial gifts to attract a female but have the lower values of phagocytic and lysozyme activity [7]. The second hypothesis known as the immunocompetence handicap considers that females invest in the immune response to favor longevity, which is positively correlated with fecundity [8]. However, there are no studies that have tested whether female longevity and egg production are positively correlated with the immune response [8]. The problem with the sexual selection and the immunocompetence handicap hypotheses is that there are some cases in which males are more immunocompetent than females and others in which no differences have been found between sexes [8]. For example, in the tree weta (*Hemideina crassidens*) there was no significant relationship between immune response and weapon or testes size in males or fecundity in females [9]. In addition, in *Teleogryllus commodus* males showed stronger immune response than females, but in *T. oceanicus* no sex differences were found [10]. In general, in invertebrates, females seem not to be more immunocompetent or resistant...
sex [11]. Thus, the question is why males are in some cases more immunocompetent and in other cases females.

A third hypothesis suggests that, rather than being fixed, the immune response is plastic and limited by resource availability [12]. McKeen and Nunney [12] found that with abundant food, *D. melanogaster* males and females experienced no differences in immunity and females produced a large number of eggs, but immunity was reduced for both males and females when food provision was low and sexual activity was intense. It has been reported that nutritional status is related to immune response [13, 14], but this particular hypothesis suggests a physiological plasticity regarding immunity which depends on sex and food availability [10, 12]. Although this hypothesis could explain differences between females and males on immunocompetence, most researchers have paid little attention to this hypothesis.

Crickets have been a good model system to study SD in immune response. For example, in *Gryllus texensis* fed *ad libitum*, phenol oxidase activity and haemocyte number varied according to the sex and age of experimental subjects [15]. In the prereproductive period of these animals, male and female immunocompetence was similar. However, when males displayed reproductive behavior, phenol oxidase (PO) activity declined compared to females and younger males [15]. On the other hand, with the onset of sexual behavior in females, PO activity increased relative to female nymphs and prereproductive adult females [15]. In another cricket (*T. oceanicus*) Zak et al. [10] showed that under normal food conditions no sexual differences were found, but under starving conditions males had a stronger immune response (encapsulation response) than females.

Here, the interaction between sex, diet, and immune challenge was tested using *Gryllodes sigillatus* as model system. The advantage of using this system is that males live longer than females [16]. Hence, according to the (a) immunocompetence handicap hypothesis, a better immune response is expected in males than in females; (b) the sexual selection hypothesis, males should have a lower immune response than females; and (c) plastic sexual dimorphism dependent on diet, the better feed sex should have the better immune response. Given that more than one immune response parameter should be employed to evaluate immunocompetence [17, 18], we measured two immune markers of the innate immune response of these insects—PO and lytic activity—both of which have previously been recorded in crickets [4, 17, 18] including *G. sigillatus* [19]. During melanogenesis PO catalyzes the hydroxylation of tyrosine to L-dihydroxy phenylalanine (L-DOPA) to produce the melanin precursor, indolequinone, to combat parasites and pathogens [20]. Quinoid compounds are potent catalysts of the production of reactive oxygen molecules (ROS) [20, 21], and the oxidation of dihydroxy phenylalanine (L-DOPA) can generate superoxide anion and hydrogen peroxide, which are toxic molecules to parasites [20–22]. An increase of lytic activity has also been implicated in resistance to a bacterial challenge [23]. To activate the immune response we used lipopolysaccharides of *S. marcescens*, a highly pathogenic bacterium for insects that has been shown to activate PO and lytic activity in crickets [4] including *G. sigillatus* [24].

2. Materials and Methods

2.1. Species. *G. sigillatus* is known to be sexually dimorphic and is denominated the decorated cricket. This species was originally described from specimens collected in Sri Lanka [25]. At present it is well established in tropical urban ecosystems around the world [26, 27]. Females are flightless and larger than males. Male wing expanse only covers the thorax area, and therefore these wings are smaller than those of other cricket species. At night in the field, females are phonetically attracted to males and they oviposit soon after mating, and the eggs hatch approximately 30 days after copulation [28]. Nutritional stress has a negative impact on female and male body size as well as on time and stability of offspring development [29, 30]. In males, body size is related to mating success. Thorax width and right hind leg length are negatively correlated with song pulse, and shorter pulse is preferred by the females [31].

2.2. Cricket Collection and Rearing. We established a colony from last instar nymphs collected in Zapotitlán Salinas, Puebla, Mexico (latitude: 18°16′59″N; longitude: 97°26′51″W; 1480 m above sea level). The collecting site is xerophytic shrub land with an annual precipitation of 412 mm. Animals were maintained in environmental conditions at 29 ± 1°C with L/D periods of 12 h. They were provided *ad libitum* with water vials and a Purina chicken diet (Start & Grow). The colony was regularly supplied with new individuals to avoid inbreeding. Second instar nymphs were removed from the colony and placed individually in 55 mL transparent plastic jars. The nymphs were randomly assigned to a high or a low quality diet. The high quality diet (protein diet, H) consisted of 90% rabbit food and 10% bran, and the low quality diet (L) 10% rabbit brand food and 90% bran. When crickets became adults we measured (with a digital caliper; Mitutoyo Corp., Tokyo, Japan) thorax width and right femur III length for both sexes.

2.3. Immune Challenge. Animals were divided according to diet (low and high quality) and sex and afterwards subdivided into two groups. One of them was challenged (experimental group) and the other unchallenged (control group). The challenge was administered inside the thorax of each cricket [4] by using a 10 μL syringe (Hamilton) to inoculate 2 μL of phosphate buffer saline (PBS, Sigma, pH 7.4) with 10 μg of lipopolysaccharides (LPS) from *S. marcescens* (Sigma). The control animals received only an injection of 2 μL PBS. Both challenged and control groups were housed individually in plastic, transparent containers (10 cm²) for one hour.

A three-way factorial design was established (sex, diet, and challenge). The three factors have two levels that were crossed to yield 8 different treatment combinations: males with a low quality diet that were challenged (MLC; *n* = 76) or unchallenged (MLU; *n* = 65), males with a high quality diet that were challenged (MHC; *n* = 40) or unchallenged (MHU; *n* = 46), females with a low quality diet that were challenged (FLC; *n* = 92) or unchallenged (FLU; *n* = 69), and females with a high quality diet that were challenged (FHC;
Table 1: Effect of sex, diet, challenge, and their interactions on the amount PO in haemolymph.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>238130</td>
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<tr>
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<tr>
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<td>0.142</td>
<td>0.70</td>
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<tr>
<td>Sex * diet</td>
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<td>3444</td>
<td>0.166</td>
<td>0.68</td>
</tr>
<tr>
<td>Sex * challenge</td>
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<td>1</td>
<td>38</td>
<td>0.002</td>
<td>0.97</td>
</tr>
<tr>
<td>Diet * challenge</td>
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<td>0.031</td>
<td>0.86</td>
</tr>
<tr>
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<td>47194</td>
<td>2.275</td>
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</tr>
<tr>
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<td>10247094</td>
<td>494</td>
<td>20743</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SS: sum of squares; DF: degrees of freedom; MS: mean square; F: test value; P: significance.

In terms of sex, females had less lytic activity (0.0066 ± 0.0003) than males (0.0082 ± 0.0004, Figure 3(b)).

$n = 68$) or unchallenged (FHU; $n = 56$). In each of these groups we recorded size, haemolymph protein content, and phenol oxidase and lytic activity.

2.4. Haemolymph. One hour after bacterial challenge (or injection of PBS only), the haemolymph was obtained by perfusion. A syringe (10 µL, Hamilton) was used to inject 5 µL of PBS in each animal thorax, and then a puncture in the coxa allowed the emanation of a drop of haemolymph. Five µL of haemolymph were collected with a micropipette (Rainin, 10 µL) and deposited in a precooled vial of 1.5 mL (Eppendorf) that contained protease inhibitors (Sigma) and 80 µL of PBS (see [32]). Tubes were stored at −70°C and analyzed the next day.

2.5. Phenol Oxidase. To estimate PO activity in each sample, an aliquot with 40 µL of haemolymph/PBS was deposited in a microplate template of 96 round-bottomed wells (Corning) that contained 60 µL of PBS and 100 µL of L-DOPA diluted in PBS (4 mg/mL). PO activity was determined indirectly by oxidation of 3,4-Dihydroxy-L-phenylalanine (L-DOPA, Sigma). As blanks, only 50 µL of PBS and 50 µL of L-DOPA (4 mg/mL) were used. The slope of the curve was calculated by using the optical density at 490 nm with an ELISA reader (Microplate Reader Series, BMGLABTECH). Optical density readings were taken every 5 minutes during one hour at 27°C.

2.6. Lytic Activity. To estimate the lytic activity, 9 mg of Micrococcus luteus (Sigma) were dissolved in 25 mL of Phosphate buffer solution (0.1 M, Sigma). An aliquot (200 µL) of the M. luteus diluted in phosphate buffer solution was mixed with 30 µL of each sample. Only 0.1 M phosphate buffer solution (230 µL) were used as control. All samples were added to each well of a microplate template of 96 round-bottomed wells. Samples were read every 5 minute during ten minutes at 540 nm with an ELISA reader (Microplate Reader Series, BMGLABTECH) and the slope of the curve was calculated for comparison between groups.

3. Results

3.1. Size (Femur Length). Size was affected by sex ($F_{1,508} = 10.69; P < 0.0001$) and diet ($F_{1,508} = 21.09; P < 0.0001$) but the interaction size * diet was not significant ($F_{1,508} = 0.4; P = 0.52$). Females (9.68 ± 0.04; $n = 284$) were larger than males (9.05 ± 0.04; $n = 226$). However, females (9.79 ± 0.05; $n = 160$) and males (9.53 ± 0.06; $n = 141$) reared on high quality diet were larger than females (9.1 ± 0.06; $n = 124$; Fisher LSD $P = 0.002$) and males (8.83 ± 0.07; $n = 85$; Fisher LSD $P = 0.0004$) reared on low quality diet, respectively (Figure 1).

3.2. Phenol Oxidase. In a three-way analysis of variance the results did not indicate significant difference in PO activity between treatments (Table 1; $F_{6−480} = 1.31; P = 0.24$). Only sex explained the variation in the PO activity. Females had more PO (0.0078 ± 0.0008) than males (0.0053 ± 0.0006, Figure 2).

3.3. Lytic Activity. The results showed a significant difference in lytic activity between treatments ($F_{6−480} = 3.45; P < 0.0001$). Sex and challenge explained the variation in the amount of lytic activity. The immune challenge increased lytic activity (0.0077 ± 0.0003) compared to controls (0.0071 ± 0.0004, Figure 3(a)). In terms of sex, females had less lytic activity (0.0066 ± 0.0003) than males (0.0082 ± 0.0004, Figure 3(b)).
4. Discussion

Regarding sexual dimorphism (SD), some authors suggest that it is fixed, males being less immunocompetent than females [1, 5, 6], while others pose that it is dynamic and depends upon diet [10, 12]. Comparing *G. sigillatus* fed a high and low quality diet in the present study, it was found that a better diet resulted in larger crickets but did not affect the immune response (evidenced by the unchanged PO and lytic activity). On the other hand, females showed greater phenol oxidase levels than males, but the opposite was found with lytic activity.

In some species, such as *Spodoptera littoralis* [33] and *Anabrus simplex* [18], the amount of protein is related to the immune response. However, in these reports only one immune response marker was evaluated. Other reports indicate that diet protein increases some but not all immune markers. For example, food limitation during immature stages of *G. campestris* reduces lysozyme activity in the adult stage but does not have any effect on PO activity [34]. In *A. simplex* the access to a high-protein diet increases PO activity but not the encapsulation response or lytic activity [18]. These studies reveal that the plasticity of the immune response is related to the diet protein content. However, in the current study SD in the immune response of *G. sigillatus* is not affected by food protein and, together, all these results suggest a complex relationship between immune response and food quality.

The immunocompetence handicap hypothesis and the sexual selection hypothesis suggest that the immune response is fixed. The former states that, if female fecundity and longevity are positively correlated, an overall immune response should be better and longevity higher in females than in males [6]. The latter, taking into account that both sexual traits and immunity are costly, asserts that males should invest their resources in sexual traits at the expense of longevity (e.g., the immune response; [1, 5]). In *G. sigillatus*, males survival is longer than females [16], so, according to the former hypothesis it was expected to have more elevated values of immune response in males than in females, but according to the latter hypothesis the opposite was expected. Hence, our results do not support either of these scenarios because some but not all immune parameters were increased in females and decreased in males. Similarly, SD in *P. zoe* was found to be dependent on the particular immune marker: the black winged male morph showed lower levels of nitric oxide but higher levels of PO than that of females [35]. The insect immune response is highly dynamic and it varies depending on different physiological contexts as well as its interaction with different parasites [36] and a single measurement of immunity should not be interpreted as a demonstration of overall resistance [4, 17, 37, 38].

The immunocompetence handicap hypothesis and the sexual selection hypothesis explain the evolution of SD from the host point of view. However, a third scenario is the role of parasites in sexual differences [39, 40]. For example, female monarch butterflies (*Danaus plexippus*) had greater haemocyte counts than males in the absence of infection with *Ophryocystis elektroscirrha*, but the opposite was found in the presence of infection [41]. It is the parasite and pathogen hypothesis that allows us to explain the higher levels of PO in *G. sigillatus* females and the greater lytic activity of males. Whereas PO is responsible for combating macroparasites, such as nematodes and parasitoids [42–44], lytic activity is related to resistance to bacteria (including *S. marcescens*). Thus, it is possible that females of *G. sigillatus* are more exposed to macroparasites than males and that males are more frequently attacked by bacteria. In line with this, *G. sigillatus* is attacked by the nematode *Mehdinema alii* [45]. This nematode is innocuous to females but infective for males [45]. The proposals as to why females are more resistant than males are that nematodes could require sex specific nutrients or that the anatomy of females could impede the dauer's entrance to the intestine [45]. However, an alternative hypothesis is that females better control nematodes growing by producing more PO than males. Finally, apart from the idea that females have greater resistance against this nematode than males because it exists in their habitat, it is also possible that females produce more PO than males because this molecule is also used to produce eggs [46, 47]. Hence, if parasites play a key role in SD, males should be more resistant than females against bacteria because we found that male lytic activity was activated against LPS of *S. marcescens*. This should certainly be an interesting topic for future research.
because the sexual dimorphism could not solely be related to sexual selection and longevity but how sexes invest their resources on immune defense, condition, and reproduction at the same time as they confront parasites with different virulence or different food quality, or reproductive history [40].

In conclusion, the current results do not support our hypothesis that diet should affect SD in the immune response. Since neither immune marker recorded was affected by diet, the costliness of production of either does not seem to be based on resource availability in Gryllus sigillatus. The fact that females showed more PO activity than males but less lytic activity has two implications. Firstly, it supports the idea that more than one immune marker should be recorded in studies of immunocompetence [36]. Secondly, it suggests that future studies should explore the possible relation between SD in the immune response and the parasites and pathogens that differentially affect each sex.

Conflict of Interests
The authors declare no financial relation with the trademarks mentioned in this paper as well as no conflict of interests.

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