Age- and Density-Dependent Prophylaxis in the Migratory, Cannibalistic Mormon Cricket Anabrus simplex (Orthoptera: Tettigoniidae)

ROBERT B. SRYGLEY

USDA-Agricultural Research Service, Northern Plains Agricultural Research Laboratory, 1500 N. Central Avenue, Sidney, MT 59270

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ABSTRACT  As a result of the increased potential for disease transmission, insects are predicted to show an increased constitutive immunity when crowded. Cannibalistic aggressive interactions further increase the risk of wounding and pathogen transmission in crowds. Nymphal Mormon crickets Anabrus simplex Haldeman were collected in Montana and reared in the laboratory either solitarily or at densities similar to that experienced by Mormon crickets in migratory bands. As teneral adults, solitarily-reared Mormon crickets tended to have greater phenoloxidase activity than those reared in groups. Sampling enzyme activity a second time when the adults were nearing reproductive maturity, group-reared Mormon crickets had elevated levels of prophenoloxidase and encapsulated foreign objects faster than solitarily-reared insects. Rearing density did not have a significant effect on either the darkness of the cuticle or antibacterial activity. This is the first report of age-related responses of adult insect immunity to crowding.

KEY WORDS  cannibalism, disease, katydid, lysozyme, melanism

The density-dependent prophylaxis hypothesis is a predicted elevation of the baseline immunity of an organism as a response to crowding (Reeson et al. 1998, Wilson and Reeson 1998, Wilson et al. 2002, 2003). It is based on theoretical and empirical evidence that pathogens are more likely to be transmitted among conspecifics in crowded environments. As a result of organisms evolving to minimize the chance of infection and maximize survivorship, they should respond to their perception of crowding by increasing their constitutive immunity (Møller and Erritzøe 1996). Implicit is that there is a cost to constitutive immunity that prevents maintenance of this elevated response when the organism finds itself in uncrowded conditions (Sadd and Siva-Jothy 2006).

Crowding also brings opportunities for cannibalistic attacks (Bazazi et al. 2008). For the attacker, cannibalism increases the risk of pathogen transmission above and beyond that because of crowding alone (Pfennig et al. 1998). For potential victims, defense of breaches in the exoskeleton from invasion by pathogens and the rapid closure of wounds may be important to minimize the risk of additional attacks.


There are several reasons why organisms may not respond to crowding with elevated levels of immunity. For one, hosts may be more susceptible to infection at higher densities because of nutritional or physiological stress (Steinhaus 1958, Lindsey et al. 2009). For example, Srygley et al. (2009) found that Mormon crickets, Anabrus simplex Haldeman (Orthoptera: Tettigoniidae), had reduced phenoloxidase activity when their diets were deficient in protein. For another, allocation of resources to locomotion could limit allocation to immunity. In some insects, antibacterial activity can decrease with locomotor activity because the lipid transport protein apolipoprotein III, which also has antibacterial activity, is shuttling lipid fuel to muscles (Adamo et al. 2008, Srygley and Lorch 2011).

Many insects respond to crowding by increasing locomotor and migratory activities (Sword 2005). Locusts, for example, exhibit a suite of life history traits that change between crowded and solitary rearing environments. Proximity to conspecifics results in a developmental regulatory switch that results in darkened, warningly-colored cuticle, swarming behavior, and migration. In Mormon crickets, proximity to conspecifics also results in aggregation behavior and migration, although unlike locusts, these responses to crowding are not the result of a phase-polyphenism (Sword 2005).
Some Mormon cricket populations are composed of consistently solitary individuals that move a few tens of meters or less in a lifetime (Lorch and Gwynne 2000, Lorch et al. 2005), whereas other populations have members that tend to aggregate into dense bands moving up to 2,000 m daily. The migratory Mormon crickets also are characterized by darkened exoskeletons relative to the solitary ones. Bailey et al. (2008) showed that the migratory populations have greater antibacterial lysozyme activity and encapsulate foreign objects faster relative to members of the solitary populations. These differences among populations might be physiological responses to crowding or more deeply rooted in historical differences between the solitary and migratory populations (Bailey et al. 2005, 2007). The purpose of this paper is to investigate whether, within a single population, the immune systems of migratory Mormon crickets respond to local crowding consistently with density-dependent prophylaxis.

Materials and Methods

Fifth-instar Mormon crickets were collected at Lodgegrass Montana on 28 May 2009, placed in bug dorms and brought to the installation in Sidney, MT. The next morning in the laboratory, we set up four groups of 12 nymphs (six of each sex) in four metal screen cages (33 cm square by 46 cm high). Feces passed through the screened bottom to a tray that was cleaned daily. In these cages, the density was \( \approx 14.6 \) Mormon crickets per square meter of cage surface, a density very similar to that of a band of Mormon crickets for which the density was measured in 2009 (R.B.S., unpublished data).

On the same day, 24 Mormon crickets (12 of each sex) were placed in clear plastic, 0.95-liter cups. Eight cups were turned upside down on a 0.13-m\(^2\) tray and each cup had a paper sleeve placed around it to prevent neighbors from viewing one another. Cups were screened at the top, and trays were cleaned daily.

Mormon crickets in both treatments were rear ed in designated insect rearing quarters at 30°C during the day and ambient nights with fluorescent lights on local photoperiod. They were fed fresh Romaine lettuce daily and given ample sunflower and mixed bird seeds, wheat bran, and tropical fish flakes. Molting and survivorship were followed daily. The Mormon crickets developed through two additional nymphal instars before reaching their adult molts, which occurred between 12 and 17 June. Adult Mormon crickets were uniquely marked with Bic quick-dry correction fluid. By 16 June, only 1–2 Mormon crickets had died in each group cage, probably as a result of cannibalization, whereas only one solitary female had died from a poor molt. Survival was not significantly different between rearing treatments (\( \chi^2 \) test statistic = 1.51, \( P = 0.219 \)).

By 25 June, 2–5 Mormon crickets had died in each group cage, and significantly more of the group-reared Mormon crickets had died than the solitary-reared ones (\( \chi^2 \) test statistic = 6.27, \( P = 0.012 \)). Mormon crickets encountered by others during molting are particularly prone to being cannibalized (Simpson et al. 2006, R.B.S., unpublished data). A last-instar nymph for which the final molt was delayed relative to the others was cannibalized (R.B.S., unpublished data), and three adults lost all or part of a hind leg on the day that they molted.

Hemolymph was drawn on the same adults when both young and older. On 16 June, eight group-reared Mormon crickets and eight solitary ones of each sex were selected to assay immunity of young adults between 0 and 4 d posteclosion. We measured body mass of each cricket to the nearest mg with an Ohaus microbalance (model AV53, Ohaus Corp., Parsippany, NJ), and then punctured the arthrodial membrane at the base of the hindleg of each insect with a 26-gauge hypodermic needle so that it exuded hemolymph. Puncturing again if necessary, 15 \( \mu l \) of hemolymph were collected into a capillary tube. The hemolymph was diluted 1:50 with phosphate buffered saline (PBS) solution to be used in assays of spontaneous phenoloxidase (PO) and total PO activity and total hemolymph protein. Total PO activity is equal to spontaneous PO plus prophenoloxidase (proPO) activity. Because it is primarily the latter, we call total PO activity proPO in this paper. We immediately froze the hemolymph samples at –20°C. The Mormon crickets were returned to their respective group cages or solitary cups.

On 25 June, the same Mormon crickets that had been bled as young adults were selected except two group-reared ones that had died. They were now between 9 and 13 d posteclosion and nearing reproductive maturity as indicated by two males and two females in the group cages mating the previous day. We measured body mass and extracted 15-\( \mu l \) hemolymph, which was handled in the same way as for the younger adults. We collected an additional 10-\( \mu l \) hemolymph into a separate capillary tube and diluted it 1:4 with PBS. This second sample was kept on ice for assaying lysozyme activity later the same day. Mormon crickets were returned to their respective group cages or solitary cups.

For immunity assays, we followed the protocols in Srygley et al. (2009). Samples of thawed hemolymph diluted in PBS were centrifuged and activated with 10-mM dopamine solution to measure spontaneous PO activity. The plate was loaded into a temperature-controlled Biotek microplate reader (25°C), and absorbance at 492 nm was read between 5 and 15 min. If sample absorbance was linearly related with time, we calculated mean V (change in absorbance min\(^{-1}\)). One unit PO activity per ml hemolymph is defined as the amount of enzyme resulting in a 0.001 increase in absorbance.

To measure proPO activity, we adapted the protocol of Goldsworthy et al. (2002). We dissolved 1-\( \mu g \) alpha-chymotrypsin from bovine pancreas (Sigma) in 1-ml PBS, combined an equal volume of this solution with a 10-mM dopamine solution to be used in assays of spontaneous phenoloxidase (PO) and total PO activity and total hemolymph protein. Total PO activity is equal to spontaneous PO plus prophenoloxidase (proPO) activity. Because it is primarily the latter, we call total PO activity proPO in this paper. We immediately froze the hemolymph samples at –20°C. The Mormon crickets were returned to their respective group cages or solitary cups.
between 5 and 15 min to measure proPO activity in units ml$^{-1}$ hemolymph. We measured total hemolymph protein in mg protein ml$^{-1}$ hemolymph with a Total Protein Kit, Micro (Sigma) compared with a serial dilution of the human albumin standard.

to measure lysozyme-like antibacterial activity, a turbidimetric method was used. Thawed and PBS-diluted hemolymph (1:50) was added to a well with suspended gram-positive bacteria cells Micrococcus lysodeikticus (Worthington). Clearing of the well was compared with a serial dilution of egg-white lysozyme (Sigma) added to the bacteria suspension. The plate was loaded into a temperature-controlled Biotek microplate reader (25°C), and absorbance at 450 nm read between 10 and 30 min. If the sample absorbance was linearly related with time, we calculated mean V. When sample activity fell below 6.5 μg ml$^{-1}$, the sample was excluded because the standards showed that the data were unreliable when sample activity was this weak.

We measured total hemolymph protein in mg protein ml$^{-1}$ hemolymph with a Total Protein Kit, Micro (Sigma) compared with a serial dilution of the human albumin standard.

To measure encapsulation, we inserted, on 29 June, two quartz glass rods (National Scientific Co., 1 mm diameter × 2 mm) dorsally between the first and second abdominal segments into the same Mormon crickets that had been bled previously, except for two males and two females that had died or disappeared from the group cages after the second blood draw. The crickets were given water and tropical fish flakes and kept isolated in 0.95-liter cups in the insect rearing quarters. Twenty-four hours later (±8 min), we froze the crickets to halt their encapsulation of the rods. To measure encapsulation response, rods were dissected from the Mormon crickets, dried, and weighed. No rods were found in one group female. Weight of the cleaned rod was subtracted.

To measure cuticular darkness, we scanned a dorso-lateral view of the abdominal segments with an Epson Perfection 4990 Pro scanner (Seiko Epson Corp., Long Beach, CA). The abdomen was selected because it is more mottled and hence likely to vary more than the pronotum. Using Adobe Photoshop CS2 (version 9.0, Adobe Systems Inc., San Jose, CA), we excluded all but one side of the abdomen from the second to eighth abdominal segments between the dorsal midline and landmarks on the lateral side where the harder dorsal cuticle met the softer ventral one. This image was converted to 256 shades of gray and the median shade of each insect was measured with Photoshop.

We analyzed data from the two age classes (younger and older adults) separately. When we analyzed immunity measures to see how they were affected by sex, rearing density, and body mass from the day of the hemolymph draw, not all immunity assays were conducted on all insects, and so we ran analysis of covariance (ANCOVA) on each measure of immunity separately. Spontaneous PO activity of younger and older adults, total protein of older adults, and proPO of older adults were log$10$ transformed to normalize these dependent variables. In building our models, body mass was the covariate and sex and rearing density were independent factors with all interactions evaluated. To build the models, variance attributed to interactions that were not significant (alpha = 0.05) was pooled with error.

Changes in spontaneous PO and proPO activity as adults aged were analyzed with Wilcoxon rank sums test.

**Results**

**Table 1.** Two-way analysis of variance for immunity assays of younger and older adults modelled with probabilities for sex, rearing density (Trt), and their interaction (Int.) where $P < 0.05$

<table>
<thead>
<tr>
<th>Adult age class</th>
<th>Assay</th>
<th>F</th>
<th>d.f.</th>
<th>$P$</th>
<th>Sex</th>
<th>Trt</th>
<th>Int.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Younger</td>
<td>Log PO</td>
<td>0.92</td>
<td>2.15</td>
<td>0.92</td>
<td>0.23</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>Younger</td>
<td>ProPO</td>
<td>3.05</td>
<td>2.29</td>
<td>0.063</td>
<td>0.40</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>Younger</td>
<td>Protein</td>
<td>2.57</td>
<td>3.28</td>
<td>0.054</td>
<td>0.64</td>
<td>0.087</td>
<td>0.030</td>
</tr>
<tr>
<td>Older</td>
<td>Log PO</td>
<td>1.75</td>
<td>2.26</td>
<td>0.19</td>
<td>0.10</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Older</td>
<td>Log proPO</td>
<td>7.45</td>
<td>2.27</td>
<td>0.003*</td>
<td>0.17</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Older</td>
<td>Lysozyme</td>
<td>1.27</td>
<td>2.10</td>
<td>0.447</td>
<td>0.50</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Older</td>
<td>Log protein</td>
<td>7.60</td>
<td>2.27</td>
<td>0.002*</td>
<td>0.002</td>
<td>0.065</td>
<td></td>
</tr>
<tr>
<td>Older</td>
<td>Log encapsulation</td>
<td>4.24</td>
<td>2.22</td>
<td>0.025*</td>
<td>0.18</td>
<td>0.021</td>
<td></td>
</tr>
</tbody>
</table>

*Indicates significant models ($P < 0.05$).

Did body mass differ between rearing treatments? In the younger adults, body mass did not differ significantly between the sexes or rearing environments ($F = 0.96, P = 0.40$). In the older adults, body mass did not differ significantly between group and solitary reared adults ($P = 0.67$), although females were significantly heavier than males (3.75 relative to 3.10 g, on average, $P = 0.004$). This result indicates that Mormon crickets in different treatments had similar access to food. Furthermore in ANCOVAs for assays of immunity and circulating protein, body mass was not a significant factor in any model. Thus the covariance was pooled with error, and I present the results from simpler two-way analysis of variance (ANOVA)5s with sex and rearing treatment as independent factors.

In young adults, solitary individuals had significantly greater concentrations of proPO than group-reared ones, but the general model was not significant ($P = 0.063$, Table 1). A significant interaction was observed between sex and treatment for total circulating protein, but again the general model was not significant ($P = 0.054$, Table 1).

In contrast to when they were younger, older adults had significantly greater proPO titers when reared at higher densities ($P = 0.001$, Fig. 1a). Mean PO did not differ significantly between treatments (Table 1), but...
individuals reared in groups increased their PO levels more as they aged than those reared solitarily (Wilcoxon test, $z_{H11005} = 2.57$, $n_{H11005} = 16$, $P_{H11005} = 0.010$, Fig. 2a). The change in proPO levels with age also was much greater in group-reared relative to solitarily-reared Mormon crickets (Wilcoxon $z_{H11005} = 3.43$, $n_{H11005} = 30$, $P_{H11005} = 0.0006$, Fig. 2b). Controlling PO and proPO levels for changes in total protein as the insects aged did not qualitatively affect the differences observed between rearing treatments (Wilcoxon tests for protein-adjusted PO and proPO: $P_{H11005} = 0.010$ and $P_{H11005} = 0.012$, respectively).

Group-reared Mormon crickets encapsulated a foreign object with more mass than solitary adults ($P_{H11005} = 0.021$, Fig. 1b). Indeed, group-reared individuals had adhered on average 30% more mass on the introduced rods than those in solitarily-reared Mormon crickets. However, encapsulation mass was not significantly related to circulating spontaneous PO or proPO titers ($P_{H11005} = 0.67$ and $P_{H11005} = 0.30$, respectively).

Group-rearing did not result in significantly darker individuals. When comparing abdominal coloration between the two blood draws, Mormon crickets became significantly darker as they aged (Student’s $t$-test statistic = 3.36, d.f. = 28, $P = 0.0023$), but there were no significant differences between the sexes or treatments in the amount of darkening (2-way ANOVA: $F_{H11005} = 0.34$, d.f. = 2,26, $P = 0.58$).

**Discussion**

There was a strong effect of adult age on the immune response of Mormon crickets to crowding. Younger adults between 0 and 4 d posteclosion did not respond by increasing their immune activity as expected. If anything, the solitarily-reared adults tended to have greater proPO activity than those reared in groups at this early age. Over the 9 d of adult life that followed the first sampling, group-reared Mormon crickets increased their PO and proPO concentrations more than the solitarily-reared adults. Older group-reared adults also encapsulated foreign bodies more quickly but did not have greater antibacterial activity. Crowded Mormon crickets have more opportunities for cannibalism. Thus, observed density-dependent prophylaxis in older adults might result from recognition of conspecifics through external contact or ingestion, and potential or realized pathogen transmission via these two routes (Pfennig et al. 1998, Barnes and Siva-Jothy 2000). Studies rarely sample insects more than once in a lifetime, and so to the best of my knowledge, this is the first report of age-related responses of adult insect immunity to crowding.

PO also occurs in an inactive zymogen called prophenoloxidase (proPO), which is assayed along with spontaneously-active PO in our measure of total PO activity. On average, circulating proPO was an order of magnitude more concentrated than PO. However there was no relationship between the amount of active PO and that of proPO in the hemolymph ($r = 0.05$). Srygley and Lorch (2011) found a similar lack of association between circulating PO and proPO in Mormon crickets migrating in Nevada. Circulating PO titers increased with infection of Mormon crickets by the entomopathogenic fungus *Beauveria bassiana* and were associated with attempted clearing of *B. bassiana*.
blastospores and hyphae from the hemolymph (Srygley and Jaronski 2011).

Surprisingly, neither PO nor proPO titers were associated with encapsulation. The first stage of encapsulation of a glass rod is recognition of the foreign object by hemocytes and hemocyte adhesion to the object (Gillespie et al. 1997). The PO cascade is an important part of encapsulation, particularly in the latter stages, because melanization causes the cell mass to harden around the foreign body by cross-linkages. Thus, encapsulation involves both cellular and humoral responses to invasion. In field studies of Mormon crickets, the mass adhering to the rod over 24 h increased with the concentration of spontaneously-active PO (Srygley et al. 2009, Srygley and Lorch 2011). Whereas both the PO and proPO titers are measures of constitutive immunity, the encapsulation response is an induced measure. This difference between background and induced levels of immunity may be one reason why neither enzyme assay was correlated with the rate of encapsulation.

The captive subjects were exposed to a more stable environment than Mormon crickets are likely to have available in the field. From the fifth nymphal instar onwards, they were reared in the laboratory, fed and watered daily, and probably not nutritionally stressed as evident by the lack of difference in adult mass between those reared in groups and those reared solitarily. In contrast, migrating Mormon crickets are likely to be nutritionally stressed. In some bands, Mormon crickets have a dietary need for proteins (Simpon et al. 2006, Srygley et al. 2009), whereas those in other bands lack sufficient carbohydrates (Srygley and Lorch 2011). In addition, those lacking protein have lower spontaneous PO titers, whereas those lacking carbohydrates have lower antibacterial activity. As a result of these nutritional deficiencies, migrating Mormon crickets in the field may not exhibit the density-dependent prophylaxis seen in captive subjects (Steinhaus 1958, Lindsey et al. 2009).

In addition, the captive subjects were reared without access to radiation sources that would permit elevation of body temperature above ambient. Some orthopterans increase their body temperatures when infected (Boorstein and Ewald 1987, Adamo 1998), and immunity assays. I also thank two anonymous reviewers for their comments on an earlier version of the manuscript.

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