Coping with crowds: Density-dependent disease resistance in desert locusts

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Parasite transmission generally exhibits some form of positive density dependence. Thus, as population density increases, so too does the *per capita* risk of becoming infected. Under such circumstances, natural selection should favor individuals that use cues associated with population density to determine the optimal allocation of resources to disease resistance mechanisms. As a consequence, individuals experiencing crowded conditions are predicted to be more resistant to parasites and pathogens than those experiencing low-density conditions. This phenomenon (termed ''density-dependent prophylaxis'') [Wilson, K. & Reeson, A. F. (1998) *Ecol. Entomol.* 23, 100–101] is predicted to be particularly prevalent in outbreak pest species and in species exhibiting density-dependent phase polyphenism, such as the desert locust, *Schistocerca gregaria*. Here we show that, as predicted, desert locusts reared under crowded conditions are significantly more resistant than solitary locusts to the entomopathogenic fungus, *Metarhizium anisopliae* var. *acridum*, a key natural disease of acridids and an important agent in locust and grasshopper biocontrol. Moreover, enhanced pathogen resistance in crowded locusts is associated with elevated antimicrobial activity, but not with any difference in thermal preferences or behavioral fever response. These results have implications for understanding the development and biocontrol of locust plagues.

It is generally assumed that when animals are crowded, they get "stressed" and so become more susceptible to disease (1, 2). However, it has recently been suggested that, counter to conventional wisdom, we should expect natural selection to favor individuals that invest relatively more in prophylactic disease resistance mechanisms as population density increases and, as a result, susceptibility to disease should decline under crowded conditions (3). Susceptibility declines because, although investment in disease resistance mechanisms is generally costly (4–7), these costs can be reduced or deferred by tailoring levels of investment in disease resistance mechanisms to match the perceived risk of exposure to disease. Because most pathogens apparently exhibit density-dependent transmission (8–10), the *per capita* risk of an individual becoming exposed to infection will generally increase with increasing host population density. Thus, at low population densities, resources that would otherwise be directed into reducing susceptibility to disease can be reallocated to other functions, such as growth or reproduction.

The desert locust *Schistocerca gregaria* (Orthoptera: Acrididae) is the archetypal phase-polyphenic species (11–13). At low population densities, it develops into the green and cryptic *solitaria* phase, whereas at high densities it becomes the conspicuous, yellow-and-black *gregaria* phase (Fig. 1*a*). Several proximate cues trigger this phase transformation, but the most important of these seems to be tactile stimulation (particularly of the hind femur) (14). Many studies have examined the contrasting selection pressures faced by the two phases (11–13, 15), but their relative risk of exposure to entomopathogens has been largely ignored. In the field, populations of desert locusts have been found harboring a range of different parasites and pathogens, including parasitoids, nematodes, viruses, protozoa, bacteria and fungi (including *Metarhizium anisopliae* var. *acridum*) (16, 17), most of which are believed to exhibit some form of density-dependent transmission (8–10). Thus, the *per capita* risk of infection will tend to increase with increasing population density and we can expect natural selection to lead to the evolution of density-dependent prophylaxis (3). Although several studies have provided empirical support for density-dependent prophylaxis, this support is almost exclusively restricted to studies of noctuid caterpillars (3, 18). If density-dependent prophylaxis is a universal phenomenon, then it should apply across a range of insect taxa and, in particular, to phase-polyphenic species like *S. gregaria,* which are known to exhibit phenotypic plasticity with respect the levels of crowding (3).

Materials and Methods

Resistance to an Entomopathogenic Fungus. To test the hypothesis that crowding induces higher levels of investment in disease-resistance mechanisms, we compared relative susceptibility to the entomopathogenic fungus *M. anisopliae* var. *acridum* in recently molted adult *solitaria* and *gregaria* phase locusts. During the nymphal stages, all locusts were reared either in solitary or crowded culture according to the methods described by Roessingh *et al.* (19). The bioassay used adult locusts approximately one or two days after fledging and followed a standard procedure, as outlined in Prior *et al.* (20). In brief, 41 *solitaria* and 45 *gregaria* locusts were inoculated *topically* with 2 μl of an oil suspension of *M. anispoliae* var. *acridum* (isolate IMI 330189) with a micro applicator. This method of application is noninvasive (the suspension is placed on the insect's cuticle beneath the dorsal pronotal shield), and studies have shown that the blank oil formulation has negligible effects on mortality and behavior in the laboratory or field (21–24). Spore concentration in the suspension was adjusted to give 1×10^4 spores per insect, and an equivalent number of *solitaria* and *gregaria* locusts were left untreated as controls. Locusts were maintained in individual containers under a fluctuating 12:12 h temperature regime of 20–35°C, and a 12 h light/12 h dark cycle. Locusts were fed and assessed for mortality due to infection daily. Survival rates were compared by using Cox's Proportional Hazards Model (25, 26).

Behavioral Fever. Resistance of locusts and grasshoppers to disease is strongly affected by temperature and the thermoregulatory behavior of the infected host (27–30). Studies have shown that *gregaria* desert locusts enhance their resistance to *M. anisopliae* var. *acridum* by initiating a behavioral fever upon infection, actively raising body temperatures above the normal set point, inhibiting pathogen development (28). To determine whether *solitaria* locusts also demonstrate the capacity for

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Abbreviation: PO, phenoloxidase.

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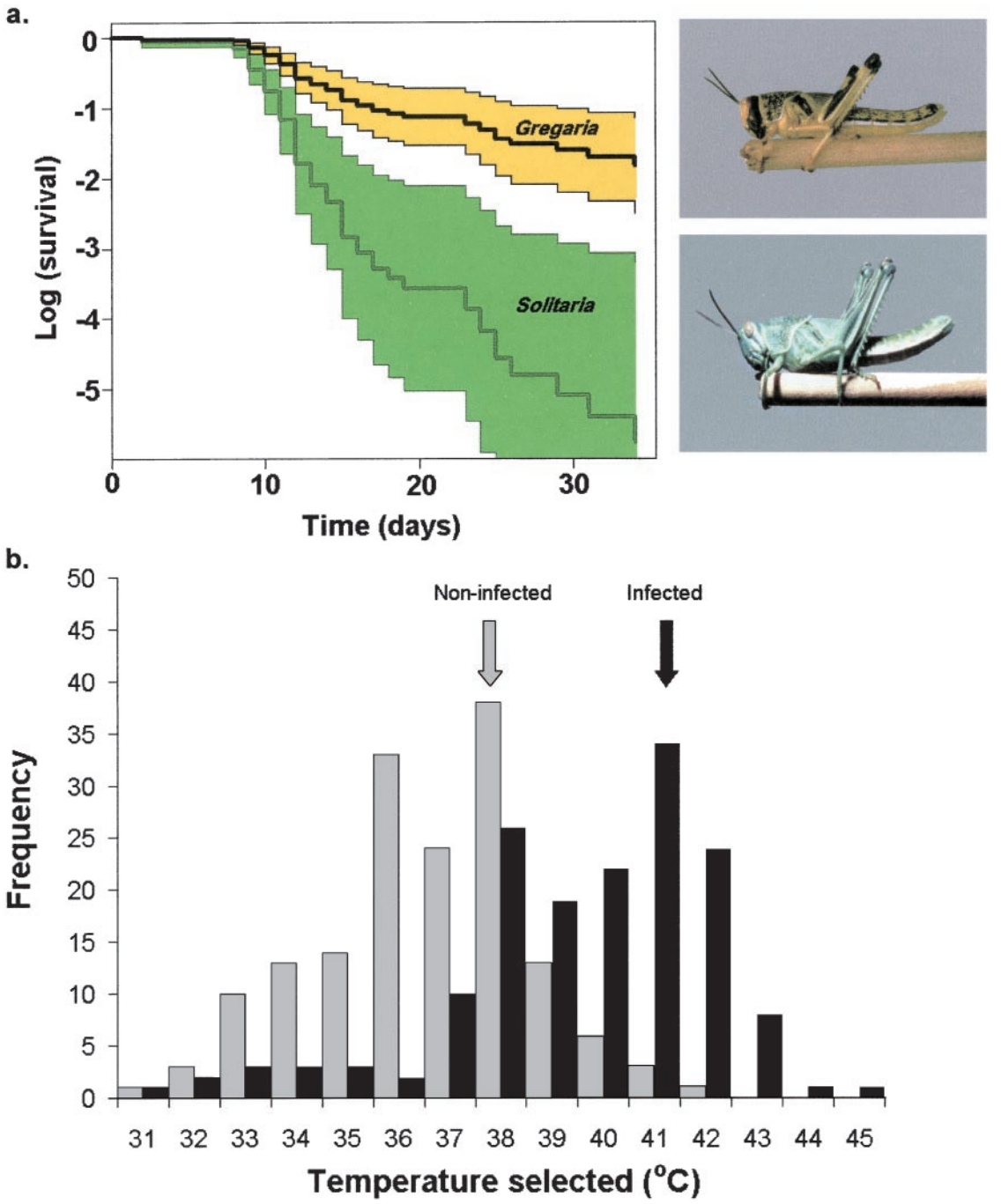

Fig. 1. Response of desert locusts to infection by the fungus *M. anisopliae* var. *acridum*. (*a*) Log-survival curves for *solitaria* and *gregaria* phase locusts infected with the fungus. The two bold lines show the fitted values from the Cox's Proportional Hazard Model and the narrow lines and shading represent the 95% confidence intervals [calculated by using the S-Plus statistical package (62)]. The model includes mean body weight as a covariant in the model (see main text for details). None of the 84 locusts in the control group succumbed to fungal infection during the assessment period, compared with >90% positive mycosis for the treated insects. Infected *gregaria* locusts survived significantly longer than *solitaria* locusts ($P < 0.0001$). (*b*) Frequency distribution of temperatures selected on a thermal gradient by control (gray bars) and infected (black bars) *solitaria* adults. Infected *solitaria* locusts exhibited behavioral fever ($P < 0.001$).

behavioral fever (or whether this response, in line with increased in risk of infection, is restricted to the high-density phase), we placed treated and control *solitaria* locusts from the fungal bioassay on thermal gradients and determined their preferred body temperatures. The method followed that reported by Blanford and Thomas (28) whereby batches of six locusts were introduced into the center of 1-m aluminum "runways," which had one end heated to create a temperature gradient of 20–50°C. One gradient was used for control locusts and one for treated. After 20 min, the ambient temperature at the position of each locust was recorded by using a fine thermocouple placed as close to the vertical midpoint of the insects' thorax as possible. Repeat measurements from stationary locusts were taken every 15 min for 1.5 h. The whole process was then repeated with different locusts during a 5-day period commencing from the seventh day after inoculation. Data were then pooled to give an overall

frequency distribution of selected temperatures for the observation period.

Immune Function Parameters. To determine whether any phase difference in pathogen resistance is reflected in different levels of investment in prophylactic immune defense, we assayed four aspects of immune function in a further batch of newly molted, adult *solitaria* and *gregaria* phase locusts. To perform this assay, we took hemolymph samples from individual locusts and measured phenoloxidase activity (31), encapsulation response (32), total hemocyte count (33), and antibacterial activity (measured by using an inhibition-zone assay) (34, 35). All four of these assays are used routinely to assay insect immune function (35–38).

Enzymes of the prophenoloxidase cascade are involved in cellular encapsulation, humoral encapsulation, and nodule formation and have been implicated in resistance to a range of different parasites and pathogens, including viruses, protozoa, and nematodes (31, 39, 40). The methods used to determine phenoloxidase (PO) activity followed those of Wilson *et al.* (41). In brief, 8 μl of hemolymph was added to 400 μl of ice-cold PBS (pH 7.4) (42) and mixed in a plastic Eppendorf tube. The sample was frozen at -20°C to disrupt hemocyte membranes, and PO activity in the defrosted sample was assayed spectrophotometrically with L-dopa as a substrate (43). This assay involved adding 100 μl of 10 mM L-dopa to the buffered hemolymph and incubating triplicate samples of the mixture on a temperature-controlled VERSAmax tunable microplate reader (Molecular Devices) for 20 min at 25°C. The absorbance was then read at 492 nm during the linear phase of the reaction. With use of 10 μl of the hemolymph/PBS mixture, the amount of protein in the sample was also assayed with a Bio-Rad protein assay kit (calibrated by using a standard curve created on the same microtiter plate with a BSA standard). Phenoloxidase activity is expressed as PO units per milligram of protein, where one unit is the amount of enzyme required to increase the absorbance by 0.001 per minute.

The encapsulation response occurs when a metazoan parasite (such as a nematode or parasitoid) invades the host hemocoel. Hemocytes are attracted to the foreign object and surround it, forming a capsule which then melanizes (because of the action of PO enzymes) so killing the parasite, probably because of the cytotoxic effects of compounds associated with melanin production (44). As described (32, 37, 45–47), we assayed the encapsulation response by measuring the relative area of cells surrounding an encapsulated novel antigen (a small piece of nylon, $\approx$3 mm long, inserted into the hemocoel between the second and third tergites). Twenty-four hours later, the nylon was carefully dissected out of the locust and stored in 70% ethanol. The pieces of nylon were subsequently rehydrated, mounted on glass slides, and photographed with a Polaroid digital camera. The encapsulation response was quantified by determining capsule area (the area of cells covering the nylon implant) with IMAGE PRO-PLUS image-analysis software. This value was then corrected for the length of nylon implant by using the residuals from the linear regression of capsule area against implant length.

Hemocytes are involved in phagocytosis, nodule formation, and cellular encapsulation, and we can expect the efficiency of each of these processes to be increased by elevated hemocyte densities (48). Across *Drosophila* species, relative resistance to the parasitoid *Asobara tabida* is positively correlated with total hemocyte count (33), and in *Drosophila melanogaster* genetic lines selected for parasitoid resistance have significantly higher hemocyte counts than conspecifics from control lines (38). Total hemocyte count was determined by adding 15 μl of hemolymph to both counting chambers of a hemocytometer, and counting the number of hemocytes at a magnification of $\times$400 under phase-contrast illumination. Each chamber was counted once and the average taken to give a single estimate for each locust.

Resistance to microbial parasites is due, in part, to the activity of a number of antimicrobial proteins (humoral factors), including cecropins, attacins, defencins, and lysozymes (34). Lytic activity against *Micrococcus lysodeikticus* was determined by using a lytic zone assay. Agar plates containing 10 ml of 1% agar with 5 mg/ml freeze-dried *M. lysodeikticus* were prepared as described by Kurtz *et al.* (49). Holes (2-mm diameter) were punched in the agar and filled with 70% ethanol saturated with phenylthiourea; phenylthiourea inhibits melanization of the hemolymph. After the ethanol had evaporated, 1 μl of hemolymph was placed in each well, two replicates per sample. The plates were incubated at 33°C for 24 h, after which they were digitally photographed and the diameter of the clear zones calculated by using IMAGE PRO PLUS software. Standard curves were obtained by using a serial dilution of hen egg white lysozyme. The concentration of "hen egg white lysozyme equivalents" was then calculated.

Results

Resistance to an Entomopathogenic Fungus. After accounting for a sex difference in fungus-induced mortality, phase had a highly significant effect on mortality rate (likelihood ratio tests: phase, $\chi^2_1 = 10.66$; $P = 0.0011$; full model, $\chi^2_2 = 21.4$, $P < 0.0001$, $r^2 = 0.22$), with the average daily mortality risk for *solitaria* locusts being 1.47 times greater than that for *gregaria* locusts (95% confidence interval, 1.17–1.85). This difference was apparent despite the fact that, on average, *solitaria* locusts were significantly heavier than *gregaria* locusts (2.33 $\pm$ 0.63 g vs. 1.90 $\pm$ 0.51 g, respectively; $t = 2.339$, df = 38, $P = 0.024$) and resistance to entomopathogens generally increases with body weight (50). If average body weight is included in the survival analysis as a covariant, the effect of phase on fungus-induced mortality increases (likelihood ratio tests: phase, $\chi^2_1 = 17.67$, $P < 0.0001$; full model, $\chi^2_2 = 20.9$, $P < 0.0001$, $r^2 = 0.37$; Fig. 1*a*) and the relative daily mortality risk for the *solitaria* phase increases to 1.76 times that of the *gregaria* phase (95% confidence interval: 1.35–2.31). Locusts in the control group exhibited no fungus-induced mortality, and no significant effect of phase on survival rate (likelihood ratio tests: phase: $\chi^2_1 = 1.14$, $P = 0.286$; full model, including weight, $\chi^2_2 = 1.24$, $P = 0.538$, $r^2 = 0.02$).

Behavioral Fever. In line with equivalent work on *gregaria* locusts (28), the preferred (modal) body temperatures of uninfected *solitaria* was 38°C (Fig. 1*b*). In addition, a significant difference occurred in the temperature distribution between treated and control insects (Mann–Whitney U test = 12318, $P < 0.001$), with infected *solitaria* locusts selecting a modal temperature (41°C) that was 3°C higher than that of control insects, consistent with the fever response reported for *gregaria* locusts (28).

Immune Function Parameters. *Gregaria* phase locusts exhibited significantly greater antibacterial activity than locusts in the *solitaria* phase ($P < 0.001$; Table 1). *Gregaria* phase locusts also had marginally higher total hemocyte counts, but the difference was statistically nonsignificant ($0.05 < P < 0.1$; Table 1). No significant differences occurred between the phases with respect to PO activity or encapsulation response ($P > 0.8$; Table 1).

Discussion

These results show that the two phases of the desert locust differ in their resistance to an entomopathogenic fungus. Given the demonstrated importance of temperature and thermoregulation in mediating resistance to disease in locusts and grasshoppers (27–30, 51, 52), it is interesting that both phases show equivalent preferred temperatures and capacity to fever. This result suggests that differences between the two phases result from some

Table 1. Effect of phase on four measures of immune functions in desert locusts

Immune parameter	*Gregaria*	*Solitaria*	Effect of phase
PO activity, log PO units/mg protein	1.509 ± 0.040	1.524 ± 0.035	$F_{1,109} = 0.034$; $P = 0.853$
Encapsulation response, capsule area in arbitrary units	0.011 ± 0.021	0.003 ± 0.018	$F_{1,84} = 0.007$; $P = 0.933$
Total hemocyte count, log mean number/ml	5.821 ± 0.036	5.812 ± 0.035	$F_{1,109} = 3.393$; $P = 0.068$*
Antibacterial activity, log μg of lyzozyme equiv/ml hemolymph	2.182 ± 0.045	1.969 ± 0.038	$F_{1,97} = 12.343$; $P < 0.001$**

All means are adjusted for any differences between the sexes and any effects of locust body weight, and, for the encapsulation response, also for variation in the length of the nylon implant. *, $0.05 < P < 0.1$; **, $P < 0.001$.

other aspect of resistance, such as differences in the physical attributes of their cuticles, differences in immune factors within the cuticle, or differences in the antifungal properties of their hemolymph. Although we did not investigate hemolymph antifungal activity directly, we did observe a marginally nonsignificant increase in the density of hemocytes in the hemolymph of *gregaria* locusts, and a highly significant increase in hemolymph antibacterial activity, which shows that at least some components of the *gregaria* locusts' immune system are up-regulated under crowded conditions.

Although density-dependent phase polyphenism is typified by the desert locust, it has evolved many times in other insect taxa, including Lepidoptera, beetles, phasmids, and aphids. Differential exposure to entomopathogens may have been an important selection pressure during the evolution of phase polyphenism (3, 36, 53), and several studies have examined density-dependent prophylaxis in phase polyphenic species. However, with the exception of a recent study on the beetle *Tenebrio molitor* (18), all previous studies had been restricted to the larvae of phase-polyphenic Lepidoptera (36, 53–56). Thus, the present study is important in highlighting the generality of the density-dependent prophylaxis phenomenon; a failure to detect a positive effect of crowding on disease resistance in the archetypal example of phase polyphenism, the desert locust, would have severely undermined the credibility of the hypothesis.

Future studies have many issues to address. First, we need to determine the precise mechanisms underlying the observed phase differences in fungal resistance and establish whether the phase difference is caused by the physical properties of the cuticle or immunological differences in the hemolymph or elsewhere. Second, we need to establish whether enhanced fungal resistance in *gregaria* locusts is matched by enhanced resistance to other types of parasite and, in particular, whether the observed difference in hemolymph antibacterial activity is reflected in differences in resistance to entomopathogenic bacteria. Third, we need to determine how quickly these mechanisms can be stimulated in response to changes in crowding [given that behavioral phase change occurs within a matter of hours of simulated crowding (14)], and the relative importance of longer-lasting phase differences, such as those mediated by maternal and paternal effects (57, 58). We also need to examine the consequences of phase differences in pathogen resistance for locust population dynamics and for the development of locust plagues; simple mathematical models suggest that density-dependent investment in disease resistance could contribute to the unstable dynamics of phase polyphenic insects (59). Finally, substantial interest now exists in reducing reliance on chemical insecticides for locust and grasshopper control and adopting more environmentally sustainable technologies (60). To this end, biopesticides based on isolates of *M. anispoliae* var. *acridum* have shown considerable potential and have been tested extensively throughout Africa, Australia, and parts of Europe and Central and South America (60, 61). What implications the results of the current study have for these new biopesticides remains to be seen, but they do suggest the possibility of improving efficacy and economics of biocontrol through the development of preventive spray strategies targeting potentially more susceptible, low-density populations.

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