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Does immunity vary with population density in wild populations of Mormon crickets?

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ABSTRACT

Background: Parasite transmission rate often increases with population density, and selection is expected to favour individuals that differentially allocate immune resources according to future population density (density-dependent prophylaxis). Laboratory studies uphold these predictions, but field studies sometimes contradict them.

Question: Do wild populations of Mormon crickets show density-dependent prophylaxis?

Organisms: Cryptically coloured low-density, and darkly melanized high-density, populations of Mormon crickets (*Anabrus simplex*).

Methods: Over 2 years, we assessed two measures of immunity, encapsulation ability and lysozyme activity, in populations with known genetic relationships.

Results: Individuals from consistently high-density populations showed greater immune responses. Increases in each immune parameter were positively associated with cuticular melanization. Despite the broad correspondence between high population density and increased immunity, immune variation in wild Mormon crickets appears to be predominated by population-level effects, as opposed to the short-term flexibility in immunity expected under a conventional interpretation of density-dependent prophylaxis.

Keywords: *Anabrus simplex*, density-dependent prophylaxis, ecological immunology, encapsulation, lysozyme activity.

INTRODUCTION

Population density can be highly variable in natural settings, and risk of infection by parasites likely increases with density (Steinhaus, 1958; Anderson and May, 1979). Immune defence against such infections can be costly (Sheldon and Verhulst, 1996), and selection may favour individuals that optimize their allocation of resources to immunity according to future population density (Wilson and Reeson, 1998; Wilson *et al.*, 2002). This phenomenon is known as density-dependent prophylaxis (Wilson and Reeson, 1998), and it has been studied in a wide range of taxa, including birds, mammals, and insects (Saino *et al.*, 2000; Hagen *et al.*, 2006; Møller *et al.*, 2006).

Insect species displaying density-dependent phase polyphenisms are expected to be particularly disposed to density-dependent prophylaxis (Wilson and Reeson, 1998; Wilson *et al.*, 2002).

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Phase polyphenisms are two or more intraspecific phenotypic forms mediated by environmental factors, such as rearing density or climate (Kennedy, 1956; Simpson *et al.*, 2001). In many Lepidoptera and Orthoptera, phase changes correspond to phenotypes that differ markedly in patterns of cuticular melanization, with high-density forms typically being darker (Rhoades, 1985). Cuticular melanization and density-dependent prophylaxis may be functionally linked in insects, because individuals with darker cuticles have been found to be more resistant to infection than those with lighter ones (Reeson *et al.*, 1998; Barnes and Siva-Jothy, 2000; Wilson *et al.*, 2001; Cotter *et al.*, 2004; Armitage and Siva-Jothy, 2005; but see Robb *et al.*, 2003; Hagen *et al.*, 2006). The classic example of a phase polyphenic insect is the desert locust, *Schistocerca gregaria*, which can exist in a cryptic green low-density form and a yellow and black high-density form (Uvarov, 1966; Applebaum and Heifetz, 1999). In line with the theoretical expectations of density-dependent prophylaxis, *S. gregaria* individuals reared at high density in the laboratory showed increased immune responses and greater resistance to fungal infection (Wilson *et al.*, 2002).

Although density-dependent prophylaxis has been studied extensively under controlled laboratory conditions, few studies have examined this phenomenon in the wild (Hagen *et al.*, 2006). Mormon crickets (*Anabrus simplex*, Haldeman) undergo periodic high-density outbreaks throughout the western United States (Gurney, 1939), but a low-density form also occurs primarily on the eastern slope of the Rocky Mountains (Gwynne, 1984; Bailey *et al.*, 2005). The two forms differ across a broad suite of behavioural and morphological traits (Bailey *et al.*, 2007a). Individuals in low-density populations are small, sedentary, and cryptically coloured in shades ranging from green to brown. In contrast, individuals in high-density bands have darkly melanized cuticles and are conspicuously black to reddish-black in colour. These populations are often up to a thousand times denser than low-density populations (Gwynne, 1984), and they undergo concerted mass migrations at rates approaching 2 km per day (Cowan, 1929; Lorch *et al.*, 2005). The differences in the natural history and population dynamics of Mormon crickets make them ideally suited for a study of density-dependent prophylaxis in the wild, because individuals in the considerably more crowded outbreak populations are expected to allocate more resources towards immunity because of the increased risk of parasite transmission.

Fixed genetic differences arising from differential selection pressures during separate evolutionary histories can also influence variation in immunity between wild populations (Dupas and Boscaro, 1999). In Mormon crickets, recent work has revealed a deep east–west mitochondrial DNA (mtDNA) division that broadly corresponds to low- and high-density forms respectively, and they appear to have experienced separate phylogeographic histories during Pleistocene glacial cycles (Bailey *et al.*, 2005, 2007b). However, the two forms do not perfectly correspond with the genetic division: a single population of crickets from the predominantly high-density western clade resembles both behaviourally and morphologically low-density crickets from the eastern clade (Bailey *et al.*, 2005, 2007b).

The imperfect correspondence between mtDNA clades and phenotypes afforded us the opportunity to test the density-dependent prophylaxis hypothesis in the wild. Do crickets from low-density populations invest less in immunity regardless of which mtDNA clade they belong to, or is immune variation more readily explained by differences between genetic clades? To address this question, we measured the immune responses of three high-density Mormon cricket bands and three low-density populations over 2 years. One of the low-density populations, named 'Little Brush Creek', was likely composed of the F_3 offpsring of a high-density band and clusters in the western mtDNA clade with other high-density bands. Little Brush Creek was of particular interest, because comparing it with

high-density bands from the west might indicate the extent to which plastic density-dependent effects versus population-level genetic differences influence immune investment.

Two common ways to measure immune responses in insects include assessing an individual's ability to encapsulate and melanize a foreign implant (Rantala and Kortet, 2003; Rantala *et al.*, 2003; Fedorka *et al.*, 2004; Zuk *et al.*, 2004), and quantifying antimicrobial lysozyme-like activity in the haemolymph (Adamo, 2004; Fedorka *et al.*, 2004; Shoemaker *et al.*, 2006). During encapsulation and melanization, foreign invaders such as parasitoid eggs or nematodes are surrounded by haemocytes that die and melanize, thereby asphyxiating the target (Gillespie *et al.*, 1997). Phenoloxidase is an intermediary in the melanization pathway, which catalyses the formation of melanin and results in a characteristic darkly pigmented capsule in many insects (Cerenius and Söderhall, 2004). There is some support for the idea that phenoloxidase itself is important in immune defence (e.g. Adamo, 2004; Leclerc *et al.*, 2006). However, the darkness of encapsulated and melanized implants has been found in previous studies to consistently reflect the number of haemocytes used during an immune challenge, and is a widespread technique for assaying insect immunity (Baer *et al.*, 2006). Lysozymes are a critical component of the humoral insect immune system, degrading peptidoglycans in invading Gram-positive bacterial cell walls (Schneider, 1985). Given the potential fitness benefits of short-term density-dependent prophylaxis, we predicted that encapsulation ability and lysozyme-like activity would be greater in individuals from high-density populations, and lower in individuals from low-density populations, regardless of which mtDNA clade they belonged to. We also assessed macroparasite loads in the first year, predicting that high-density individuals would harbour more macroparasites because of the increased chances of transmission in crowded conditions.

MATERIALS AND METHODS

Cricket collection and housing

Individuals were collected in early July 2006 and early July 2007 from populations in Colorado, Utah, and Nevada. Mormon crickets have a highly discontinuous distribution, and outbreaks of high-density crickets can be unpredictable, so we sampled over 2 years to obtain data from an adequate number of populations. High-density bands were sampled from sagebrush flats near (1) Dinosaur, Colorado, (2) Mountain City, Nevada, and (3) Big Bend, Nevada. Low-density populations were sampled from two montaine meadows in the Rocky Mountains near Kelly Flats and Indian Meadows, both in Colorado. We also collected males from a third low-density population near Little Brush Creek in north-eastern Utah. The Little Brush Creek site has been studied previously, and prior to 2004 it was inhabited by a low-density population of Mormon crickets that was morphologically similar to the smaller, cryptically coloured crickets found on the eastern slope of the Rockies (Bailey *et al.*, 2005, 2007b). Late in the summer of 2004, however, a high-density band of Mormon crickets migrated through Little Brush Creek (N. Bailey, personal observation). In the following years, the resulting generations of crickets at Little Brush Creek have more closely resembled the large, melanized individuals characteristic of high-density bands, strongly suggesting that the band that passed through in 2004 displaced or hybridized with the previous population. The individuals collected for this study were most likely the F_3 offspring of the high-density band. However, the population density of crickets in Little Brush Creek was comparable to that of low-density populations from the eastern slope of

the Rockies, and we were only able to sample males because females, which do not sing, were extremely difficult to locate in the thick sagebrush habitat at the site. To our knowledge, Little Brush Creek is the only consistently low-density population clustering within the western mtDNA clade.

Immunity assays

Adults were temporarily housed and transported in large, mesh-enclosed containers, and they were fed daily with fresh romaine lettuce or apples and dry Purina rabbit chow. Crickets were individually transferred to mesh-enclosed, 540-ml plastic containers before the immunity assays and were fed daily. The containers were kept at room temperature (~25°C) on a 14 h/10 h light/dark cycle for the duration of the experiment.

All of the assays were performed in the laboratory in the first year of the study. However, in the second year we were concerned about continued destructive sampling from sensitive low-density populations, so all immunity assays were performed non-destructively in the field to maintain appropriate sample sizes. In both years, the assays were performed under identical conditions to minimize the chances that any immune variation observed was a result of methodological differences. Similarly, all haemolymph samples were frozen for 2 weeks before the assessment of enzymatic activity, to avoid the possibility that variation in storage time might cause differential degradation of lysozymes across years. Measures of lysozyme-like activity are highly repeatable across runs using the technique described below, even after samples have been thawed and refrozen (N. Bailey, unpublished data). Equal numbers of low- and high-density samples were run during each assay, so variation between runs was unlikely to have contributed to differences between groups.

In the first year, 3 μl of haemolymph was withdrawn from each adult 2 days after arrival in the laboratory by making a small puncture in the abdomen between the second and third abdominal sternites and extracting with a sterile pipette. The haemolymph was immediately placed in 40 μl of 1X PBS (11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl, pH = 7.4, Fisher) and kept on ice until it was stored shortly thereafter at −20°C. In the second year, the haemolymph extraction was performed in the field 2 days after collection following the same procedure. The haemolymph-PBS mixture was kept frozen at −20°C on dry ice until its arrival in the laboratory where we stored it at −80°C. Following the protocol of Fedorka *et al.* (2004), we determined encapsulation ability by challenging each Mormon cricket with an abraded, 3-mm nylon implant inserted into the perforation left after haemolymph extraction. Implanted Mormon crickets were kept individually in plastic containers and provided with lettuce or apples and rabbit chow. Arthropods vary in the amount of time it takes to initiate an encapsulation response (Ryder, 2007), and results from preliminary trials indicated that 48 h was a necessary and sufficient amount of time for Mormon crickets to melanize nylon implants. In the first year, we froze the crickets after 48 h and dissected out the implants. In the second year, the implants were modified to include a knot at one end, allowing for their removal 48 h later. The knots were cut off before analysis. The darkest side of each implant was photographed using a RL Color Spot digital camera mounted on a stereomicroscope under 3.2× magnification. We quantified the extent of encapsulation and melanization of implants using macros in the program NIH Image (v. 1.62). The program calculates a mean value for the grey-scale darkness of each implant, ranging from lightest at 0 to darkest at 256. The aperture of the stereomicroscope and the intensity of light trans-

mitted through the mounted tips from below the stage plate were kept constant across all photographs.

Lysozyme activity is important in Mormon crickets, since they, like other orthopterans, lack specific defences that act against invading bacterial pathogens (Hoffmann *et al.*, 1996). Following the protocols of Fedorka *et al.* (2004) and Adamo (2004), we assayed lysozyme-like activity turbidimetrically by adding 10 μl of PBS-bound haemolymph to a solution (0.35 $mg \cdot ml^{-1}$) of the bacteria *Micrococcus luteus* (*lysodeikticus*) (ICN Biomedicals cat. no. 159972), and measuring the change in absorbance at 490 nm over a period of 120 min during the linear phase of the reaction using a Bio-Rad 550 microplate reader.

Dissections

Dissections were performed exclusively in the first year. Frozen adults were dissected to check for the presence of macroparasites, including gregarines and nematodes. A longitudinal incision was made on the ventral side of the body from the base of the abdomen to the anterior edge of the thorax. The body cavity was searched for macroparasites, and the digestive tract was removed and bisected to determine the presence of gut parasites. During dissections, pronotum length was measured (± 0.01 mm) using digital calipers.

Analysis

We used general linear models to examine the effects of form, sex, and year on encapsulation ability and lysozyme activity. All two- and three-way interaction terms were included. Mormon crickets from high-density populations are considerably larger than those from low-density populations (Bailey *et al.*, 2007a), so we tested for a relationship between the immune parameters and pronotum length, and included the latter as a covariate in the general linear models. All data included in the general linear models were normally distributed, and Type III sums of squares were used. Individuals from both high-density populations sampled in the second year were combined to provide a more conservative analysis; however, excluding either of these populations had no qualitative effect on the results. We excluded Little Brush Creek samples from the general linear models reported here because a more instructive approach to analysing these samples was to conduct pairwise comparisons between Little Brush Creek males and low-density and high-density crickets from the same year. Mann-Whitney tests were used for pairwise comparisons examining encapsulation ability, because encapsulation data from Little Brush Creek were non-normal and could not be transformed. Results from separate general linear models including Little Brush Creek males did not differ from those reported below (the non-normality of Little Brush Creek encapsulation data is expected to have a negligible impact on the qualitative outcome of the latter general linear models). Statistical analyses were performed using Minitab v. 12.21 and SAS v. 9.1.

RESULTS

High-density individuals encapsulated and melanized nylon implants more strongly (Table 1, Fig. 1a) than low-density individuals. Encapsulation scores were higher overall in year 2 than in year 1, but a non-significant form × year interaction term indicated that the

Table 1. Results from two separate general linear models with encapsulation or lysozyme activity as the response variable, form, sex, and year as fixed factors, and pronotum length as a covariate

	d.f.	*F*	*P*
Encapsulation			
Form	1	7.44	0.007
Sex	1	1.44	0.232
Year	1	40.61	<0.001
Pronotum length	1	0.05	0.820
Form × sex	1	0.00	0.980
Form × year	1	0.05	0.821
Sex × year	1	1.32	0.252
Form × sex × year	1	3.84	0.051
Error	197		
Lysozyme activity			
Form	1	37.91	<0.001
Sex	1	0.00	0.947
Year	1	106.21	<0.001
Pronotum length	1	1.05	0.306
Form × sex	1	0.28	0.598
Form × year	1	50.35	<0.001
Sex × year	1	0.66	0.418
Form × sex × year	1	1.18	0.279
Error	234		

Note: $r^2 = 0.403$ and $P < 0.001$ for the encapsulation model; $r^2 = 0.599$ and $P < 0.001$ for the lysozyme model.

strength and direction of the variation between forms did not differ by year (Table 1, Fig. 1a). Similarly, high-density individuals showed greater lysozyme activity in both years, although the difference between forms was greater in year 1 than it was in year 2 (Table 1, Fig. 1b). Immune responses of the males from Little Brush Creek were indistinguishable from those of high-density individuals in the same year (encapsulation: Mann-Whitney $W = 1087.0$, $P = 0.845$, $n = 100$; lysozyme activity: $t_{35} = 0.27$, $P = 0.790$), but were significantly stronger than those of low-density individuals in the same year (encapsulation: Mann-Whitney $W = 1833.0$, $P < 0.001$, $n = 77$; lysozyme activity: $t_{41} = -3.33$, $P = 0.002$) (Figs. 1a and b). Despite the fact that pronotum length was non-significant as a covariate in the general linear models, encapsulation ability increased significantly with pronotum length (Spearman rank correlation: $r_s = 0.461$, $P < 0.001$, $n = 228$). There was no association between pronotum length and lysozyme activity, however (Spearman rank correlation: $r_s = 0.039$, $P = 0.529$, $n = 243$). We did not observe differences between the sexes in encapsulation ability or lysozyme activity (Table 1). Despite thorough searching, we also did not detect macroparasites in any of the dissected individuals.

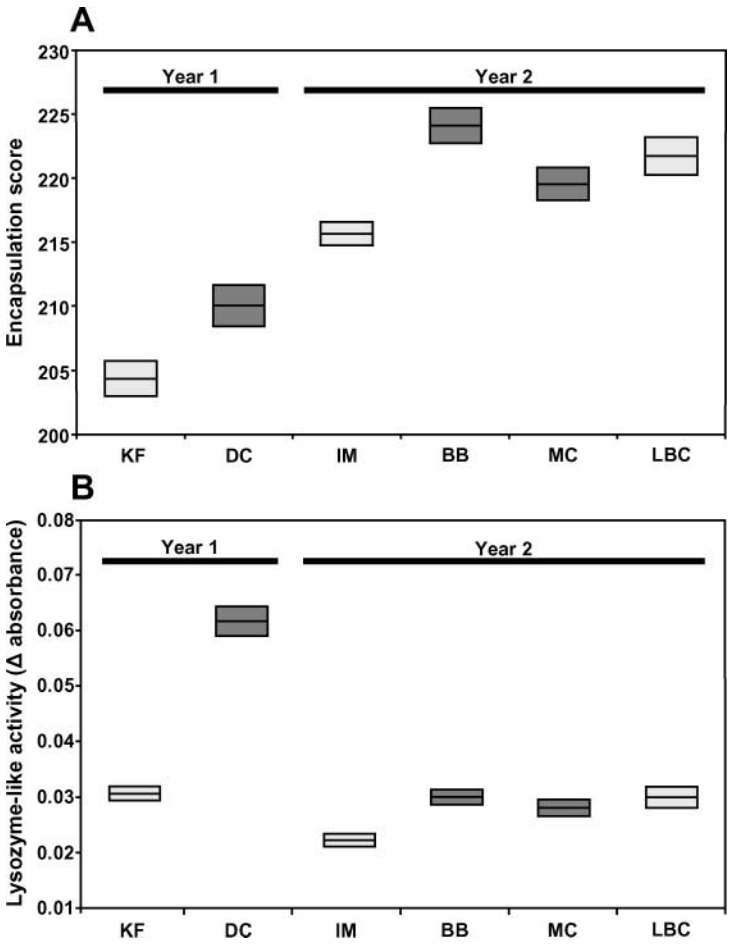

Fig. 1. Immune responses of high-density (dark grey) and low-density (light grey) Mormon crickets, separated by year. (A) Encapsulation ability, measured by assessing implant darkness score, and (B) lysozyme activity. Horizontal lines indicate mean responses and the boxes represent one standard error. KF = Kelly Flats; DC = Dinosaur, Colorado; IM = Indian Meadows; BB = Big Bend; MC = Mountain City; LBC = Little Brush Creek.

DISCUSSION

The pattern of immune variation in wild populations of Mormon crickets broadly followed the predictions of density-dependent prophylaxis, but differences in immunity appeared to be predominated by population-level effects as opposed to short-term density-dependent phenotypic plasticity. Although many other environmental and biotic factors could influence immunity in the wild, such as condition, reproductive status, seasonal variation, and resource availability (Zuk and Stoehr, 2002), individuals from high-density Mormon cricket populations had stronger encapsulation abilities and higher lysozyme activity than those from historically low-density populations, which is consistent with the idea that they invest more in immunity. The direction of this effect was consistent across the 2 years for both

immune assays, although the strength of immune responses varied by year. Work in several bird species has demonstrated that temporal variation in environmental conditions can influence immunity, and it is likely that climatic variability across the 2 years of our study affected the strength, but not the direction, of immune differences in Mormon cricket populations (Gonzalez *et al.* 1999, Christe *et al.* 2001).

The ultimate cause of the persistence of high- and low-density Mormon cricket forms is still unclear (Sword, 2005; Bailey *et al.*, 2007a), but the density differences between them satisfy the criteria for a situation where density-dependent prophylaxis might be favoured. However, the results from Little Brush Creek males present an important test of the extent to which immunity might be constrained by long-term evolutionary history. Unlike the other low-density populations belonging to the eastern mtDNA clade, Little Brush Creek males are the likely descendants of a high-density band in the western mtDNA clade that invaded the field site 3 years before the present study. Little Brush Creek males were behaviourally solitary at the time of the study and population density was very low. Morphologically, however, they closely resembled the larger, darkly melanized crickets found in high-density bands, and their immune responses were indistinguishable from those of their high-density congeners in the western mtDNA clade. Increased immunity in both Little Brush Creek and the high-density populations in the western mtDNA clade is consistent with several scenarios. First, the variation in immunity we have documented in Mormon crickets may not be as facultative as the behaviours that are associated with the different forms. Behavioural changes can occur rapidly within a single generation in locusts and other species that display true phase transitions, whereas physiological changes can be influenced by conditions experienced in previous generations, leading to cumulative and enduring physiological and morphological transitions across generations (Applebaum and Heifetz, 1999; Simpson and Miller, 2007). Trans-generational immune priming has recently been demonstrated in other insects (Sadd *et al.*, 2005; Moret, 2006), and the immune responses of Little Brush Creek males may reflect continuing maternal effects due to their recent derivation from a high-density band, in which case the data presented here provide a useful baseline for future investigations.

Alternatively, the differences in immune measurements we have documented may not be facultative, and instead might reflect genetic differences fixed during separate evolutionary histories. Population-level differences in immunity are evident from our analysis. Geographic and genotypic variation in immunity has been documented in other species (Dupas and Boscaro, 1999; Barbosa *et al.*, 2007), and complex traits such as immune responses are governed by genetic effects, environmental effects, and the interaction between the two. Regardless of the ultimate cause of the differences found in this study, Mormon crickets display an immunological pattern that is consistent with the central idea of density-dependent prophylaxis: individuals from populations that are likely to experience high-density conditions appear to invest more in immunity. However, the immunological similarities between Little Brush Creek and other high-density populations in the west suggest that immunity in Mormon crickets may be subject to constraints imposed by long-term effects such as continuing maternal effects or separate evolutionary histories, as opposed to short-term responses to density cues as would be expected under a conventional interpretation of the density-dependent prophylaxis hypothesis.

Few studies of density-dependent prophylaxis have been conducted in the wild, and those that have been provide conflicting results. For example, one prediction of density-dependent prophylaxis is that it should be particularly prevalent in species showing density-dependent phase polyphenisms (Wilson and Reeson, 1998; Wilson *et al.*, 2001). Since the high-density forms of such

species are typically darker in colour, it follows that cuticular melanization may be functionally linked to immunity. This hypothesis has been supported in most laboratory studies (Barnes and Siva-Jothy, 2000; Wilson *et al.*, 2001; Armitage and Siva-Jothy, 2005; but see Goulson and Cory, 1995), but is not corroborated by studies in nature. In a study of colour polymorphic stone wetas (*Hemideina maori*) collected from the wild, Robb *et al.* (2003) reported an association between colour morph and melanotic encapsulation response, but in the opposite direction than expected. Darker individuals had lower encapsulation abilities and haemocyte counts (Robb *et al.*, 2003). Similarly, in pygmy grasshoppers (*Tetrix undulata*), darker individuals had lower encapsulation responses and were more heavily parasitized (Civantos *et al.*, 2005). The colour differences within *H. maori* and *T. undulata*, however, are genetically determined polymorphisms, unlike the differences in cuticular melanism in species displaying true phase polyphenisms. It is currently unknown whether the colour differences in Mormon crickets arise from genetic polymorphisms or are facultative responses to environmental conditions. Nevertheless, the strong correspondence between immunity and cuticular melanization in our study suggests a link between cuticular melanism and immunity in wild populations of Mormon crickets.

We found no macroparasites in any of the dissected Mormon crickets. There are two opposing forces that control parasitization rates in natural settings: parasite prevalence and host immunity. Anderson and May (1978) found that the rate of parasite transmission is greatest in high-density populations. Host populations are expected to respond to this increased likelihood of exposure to parasites by showing greater resistance to infection (Reeson *et al.*, 1998). Thus, whether or not one finds a greater number of macroparasites in high-density populations depends on the dynamic between the increased rate of parasite transmission and the greater resistance to infection predicted for hosts (Reeson *et al.*, 2000). Parasites known to attack Mormon crickets are mainly hymenopteran wasps and microsporidians (Sorenson and Jeppson, 1940; MacVean, 1987; Gwynne, 2001). It is noteworthy that high-density Mormon crickets have rarely been found to harbour significant parasite loads, a fact that has thwarted efforts to control them biologically (Cowan and McCampbell, 1929; MacVean, 1987). Baker and Capinera (1997) also noted that nematodes do not normally parasitize grasshoppers and locusts. Future investigations might therefore benefit from assessing differences in fungal or microsporidian parasitization rates between the two forms of Mormon crickets. Additionally, the lysozyme and encapsulation assays we used here are not exact indicators of the ability of an organism to resist infection (Adamo, 2004), and future efforts should be made to assess disease resistance using infectious agents that Mormon crickets would naturally encounter in the wild.

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