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Immediate protein dietary effects on movement and the generalised immunocompetence of migrating Mormon crickets *Anabrus simplex* (Orthoptera: Tettigoniidae)

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Abstract. 1. Mormon crickets form large migratory bands that march over rangeland in the western United States seeking salt and protein. Immune defence is particularly relevant to survival in migratory bands, but little is known about the role of nutrition in insect immunocompetence. We hypothesised that immune defences are compromised in these migratory bands due to nutrient limitations.

2. In a migratory band in Utah, we investigated whether access to a protein relative to a carbohydrate diet would immediately reduce migratory activity, as had been shown for Mormon crickets in a previous study in Idaho, and whether the protein diet would enhance immune defence responses.

3. Radio-tracking Mormon crickets in the field, we found that locomotor activity was significantly and positively associated with body mass. Body mass-adjusted locomotor activity declined marginally following access to a protein diet, whereas spontaneous phenoloxidase (PO) activity was enhanced by the same diet. The encapsulation response and lysozyme-like activity were directly proportional to body mass, but unaffected by the dietary treatments in the short term. Within 6 h of feeding on protein or carbohydrates, Mormon crickets exhibited measurable effects on the immune system.

4. We conclude that nutrition impacts immune function in migrating insects in the field. Spontaneous PO activity may be limited by dietary deficiency in a protein-seeking band of Mormon crickets.

Key words. Biological control, encapsulation, immunity, katydid, lysozyme, migration, nutrition, phenoloxidase.

Introduction

Migration is often associated with a scarcity of local nutrients or other resources. Scarcity may result from changes in temperature, humidity, or other abiotic factors affecting primary productivity, and scarcity may result from inter- or intraspecific competition, such as local crowding. As a result of local scarcities, some or all members of the population move to sites where resources may be more abundant. However, moving long distances is energetically demanding. If changes in primary productivity can be predicted, then animals may prepare for their migratory behaviour by depositing fat before food becomes scarce. In many instances, changes in primary productivity occur without warning and animals find themselves poorly nourished (Mattson & Haack, 1987). Animals in nutrient-poor habitats may redirect resources from other life-history traits to fuel migration.

The flightless Mormon cricket (*Anabrus simplex* Haldeman, Orthoptera: Tettigoniidae) forms dense bands of late-instar nymphs and adults in late spring and early summer. Mormon

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crickets march across rangeland in the western United States in search of nourishment. Solitary Mormon crickets (Bailey *et al*., 2005; Lorch *et al*., 2005) are omnivorous, consuming principally forbs (33–60% of total dry weight of diet), insects (11–37%), and fungi (3–24%; Ueckert & Hansen, 1970). Migrating Mormon crickets are often deficient in proteins and salt, choosing to ingest these over carbohydrates when experimentally placed in their path (Simpson *et al*., 2006). They also cannibalise dead, wounded, and possibly even healthy band members.

In a previous study, food supplementation had immediate effects on the locomotor behaviour of individual Mormon crickets (Simpson *et al*., 2006). Captive Mormon crickets which were fed protein demonstrated less locomotor activity relative to those fed carbohydrates. Once satiated with protein in the short term, captive Mormon crickets subsequently consumed a greater proportion of carbohydrates. Carbohydrates are used to fuel migration, whereas protein is an important component of eggs and spermatophores (Gwynne, 1984).

Despite the known importance of protein, studies of dietary effects on insect immunity are rare (Schmid-Hempel, 2005). Encapsulation, phenoloxidase (PO) activity, and lysozyme activity are all enzyme-based immune responses to foreign invasion or wounding (Siva-Jothy *et al*., 2005) that may be affected by protein deficiency. *Spodoptera* moth caterpillars fed protein-rich diets had enhanced immunity to a viral pathogen relative to those fed on carbohydrate-rich diets (Lee *et al*., 2006). Dietary protein limited PO activity with direct consequences to antibacterial activity, and *Spodoptera* caterpillars increased their protein intake in response to infection while ingesting similar levels of carbohydrates (Povey *et al*., 2009). The ability of *Parasemia* moths to encapsulate foreign objects was dependent on the amount of antioxidants in their diets (Ojala *et al*., 2005). *Tenebrio* beetles fed apple for 1 week had enhanced PO activity relative to starved beetles (Rantala *et al*., 2003a). Food limitation during immature stages of the cricket *Gryllus campestris* reduced lysozyme activity in the adult stage, but did not have an effect on PO activity (Jacot *et al*., 2005).

Knowledge of nutritional effects on immune defence is particularly lacking in field populations (Boggs, 2009), and even more so in migrating animals (Weber & Stilianakis, 2007). Here we investigate the immediate effects of protein and carbohydrate diets on migratory activity and immunocompetence of Mormon crickets in marching bands in northeast Utah.

Materials and methods

Study site and the Mormon cricket band

This study was conducted on 16–17 June 2007 on a band of Mormon crickets at the Head of Rye Grass (40°47′52″N, 109°8′57″W; 2220 m elevation) in Daggett County, Utah approximately 50 km northeast of Vernal, UT. The band comprised pre-reproductive adults. Mormon crickets mature and begin to mate and oviposit approximately 2 weeks after moulting to adults. In the experimental band, signs of mating and oviposition appeared a few days after the experiment. Following methods of Simpson *et al*. (2006), we conducted standard intake trials on the band and confirmed that the Mormon crickets fed most avidly from the high-protein diet. The valley floor is dominated by sagebrush (*Artemisia tridentata*) with scattered juniper tree groves (*Juniperus*)–typical vegetation for high Great Basin desert.

Diet manipulation, radio-tracking, and haemolymph collection

In total, 25 male and 25 female Mormon cricket adults were captured from the middle of the band over the course of 2 days. They were held separately in 1 litre clear-plastic containers. Half of the crickets captured each day were fed a 42% protein diet consisting of a 3:1:1 mix of casein, peptone, and albumen; the others were fed a 42% carbohydrate diet consisting of equal parts of sucrose and dextrin. Both diets contained 54% cellulose and 1.8% Wesson's salt mixture and 2.2% vitamins, linoleic acid and cholesterol. Shaded insects were allowed to feed from this diet for 1 h at mid day.

Recording sex, treatment, and the unique radio frequency that identified each insect, a 0.4 g radio-transmitter (Biotrack Ltd, Wareham, U.K.) was glued to the pronotum of each cricket. The added mass did not affect locomotion in the laboratory (Lorch *et al*., 2005). Using a Trimble GPS datalogger to record location and time for each cricket, we released the insects back into the band between 14.00 and 14.30 hours, with each cricket initially separated by at least 1 m on a linear transect perpendicular to the general direction of band movement at that time. After approximately 4 h, tagged crickets were located with hand-held radio receivers (Lorch & Gwynne, 2000) between 18.00 and 19.00 hours (MDT). The location and time were recorded, and the cricket was placed in a 50 ml Corning plastic centrifuge tube. Velocity ($mm\,s^{-1}$) was calculated as the straight-line distance between release and recapture over time. It is important to note that in contrast to previous tracking studies of Mormon cricket migratory bands (Lorch & Gwynne, 2000; Lorch *et al*. 2005; Sword *et al*., 2008), the band was not as dense (G. Sword and P. Lorch, pers. obs.). Consistent with theoretical and lab studies of band movement at intermediate densities (Buhl *et al*., 2006), directional consistency tended to vary relative to straight line paths that had been measured in other locations and other years. The straight-line distances measured over the relatively short 4 h tracking interval do not reflect any underlying directional changes and may underestimate the Mormon crickets' velocities.

Within 1 h of capture, we punctured the arthrodial membrane at the base of the hindleg of each insect with a 26 gauge hypodermic needle so that it exuded haemolymph. Puncturing again if necessary, 20 μl of haemolymph were collected into a capillary tube. To be used in assays of PO activity and total haemolymph protein, the haemolymph was diluted 1:50 with phosphate-buffered saline (PBS) solution and placed on ice. To be used to assay lysozyme activity, an additional capillary tube with at least 4 μl of haemolymph was wrapped in parafilm

and stored on ice. Crickets were returned to their centrifuge tubes and given water. Returning to our field laboratory in Vernal 90 min later, we froze the haemolymph samples at −18 °C in a field-portable Engel freezer (model MT45F-U1). The following morning between 09.00 and 11.00 hours, we measured body mass of each cricket to the nearest milligram with an Ohaus field-portable microbalance (model AV53), and femur length was measured with calipers. We inserted two quartz glass rods (National Scientific Co., Quakertown, PA, U.S.A., 1 mm diameter × 2 mm) dorsally between the first and second abdominal segments. The crickets were given water and tropical fish flakes. Twenty-four hours later (±8 min), we froze the crickets to halt their encapsulation of the rods.

Immunocompetence assays

To measure PO activity, we modified the protocol of Wilson *et al*. (2001). Samples of thawed haemolymph diluted in PBS were centrifuged and activated with 10 mM dopamine solution. The plate was loaded into a temperature-controlled Biotek microplate reader (25 °C), and absorbance at 492 nm read between 5 and 15 min. If sample absorbance was linearly related with time, we calculated mean V (change in absorbance min^{-1}). For 80% of the females but only one male (4%), further dilution of the haemolymph was necessary to yield a linear relationship between the absorbance and time. One unit PO activity per millilitre haemolymph is defined as the amount of enzyme resulting in a 0.001 increase in absorbance.

To measure encapsulation response, rods were dissected from the Mormon crickets, dried, and weighed. The weight of the cleaned rod was subtracted, and the encapsulation mass was normally distributed following square-root transformation.

To measure lysozyme-like antibacterial activity, a turbidimetric method was used, following the protocol of Azambuja *et al*. (1991). Thawed and PBS-diluted haemolymph was added to a well with suspended gram-negative bacteria cells *Micrococcus lysodeikticus* (Worthington). Clearing of the well was compared to a serial dilution of egg-white lysozyme (Sigma-Aldrich, St. Louis, MO, U.S.A.) 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 $\mu g\,ml^{-1}$, the sample was excluded, because the standards showed that the data were unreliable when samples were this weak.

We measured total haemolymph protein in milligram protein per millilitre haemolymph with a Total Protein Kit, Micro (Sigma-Aldrich) compared to a serial dilution of the human albumin standard.

Statistical analyses

We conducted an analysis of covariance using JMP 6.0.2 (SAS Inc.) with square-root transformed velocity (root velocity) as the dependent variable, body mass and femur length as covariates, and sex and diet as independent factors. All interactions were evaluated in building the model. We used forward stepwise regression analysis (alpha = 0.15 to enter), adding each feature with the lowest P-value. We report the model for which the Akaike Information Criterion (AIC) was minimised (Draper & Smith, 1998). Not all immunity assays were conducted on all insects, and so we ran analysis of covariance (ANCOVA) on each measure of immunity separately. PO activity and total protein were $\log_{10}$ transformed, and encapsulation mass was square-root transformed to normalise these dependent variables. In building our models, body mass was the covariate and sex and diet were independent factors with all interactions evaluated. We used forward stepwise regression as with root velocity above, reporting the model that minimises the AIC.

Results

Adult body mass is directly proportional to femur length (r = 0.38), but it probably also conveys information about nutrition and age between moulting to the adult stadium and reproductive maturity. Females had greater variance in body mass than males (Bartlett test: $F_{1,40} = 5.33$, $P = 0.02$), but on average, they were not significantly heavier than males (Welch ANOVA: $P = 0.093$). In contrast to body mass, femur length is fixed after the final moult, and relates directly to structural size. Females had significantly longer femurs than males ($n = 25$ for each sex; means ±SD: females 22.0 ± 0.7 mm; males 20.3 ± 0.6 mm; ANOVA $F_{1,48} = 80.97$, $P < 0.0001$).

Velocities ranged from 0.5 to 11.6 $mm\,s^{-1}$ (1.8–41.8 $m\,h^{-1}$). The best model for root velocity included the effects of body mass and diet, selected in that order (Fig. 1a; ANCOVA: $R^2 = 0.334$, $F_{2,47} = 11.8$, $P < 0.0001$). Velocity increased with body mass ($F_{1,47} = 22.0$, $P < 0.0001$), and Mormon crickets fed protein were slower than those fed carbohydrates ($F_{1,47} = 3.1$, $P = 0.085$).

The best model for $\log_{10}$ PO activity included the effects of sex and diet, selected in that order (Fig. 2; ANCOVA: $R^2 = 0.494$, $F_{2,45} = 22.0$, $P < 0.0001$). Females had significantly greater PO activity than males ($F_{1,45} = 40.2$, $P < 0.0001$; adjusted log-transformed means: females = 3.00 units ml^{-1} haemolymph, males = 2.45 units ml^{-1}), and insects fed a protein diet had significantly greater PO activity than those fed carbohydrates ($F_{1,45} = 4.9$, $P = 0.032$; adjusted log-transformed means: protein = 2.82 units ml^{-1}, carbohydrate = 2.63 units ml^{-1}).

Body mass was the only variable selected to explain variation in square-root transformed encapsulation. The encapsulation response increased with body mass (Fig. 3a; $n = 11$ carbohydrate-fed 'C' females, $n = 9$ protein-fed 'P' females, $n = 11$ C males, $n = 11$ P males; $R^2 = 0.431$, $F_{1,40} = 30.3$, $P < 0.0001$). Inclusion of the clean rod mass as a covariate for encapsulation mass did not change the variables selected nor did it change the best model qualitatively ($R^2 = 0.441$, $F_{2,39} = 15.4$, $P < 0.0001$). Controlling for body mass, root encapsulation mass was significantly and positively correlated with log PO activity (Fig. 3b; $n = 40$, partial r = 0.447,

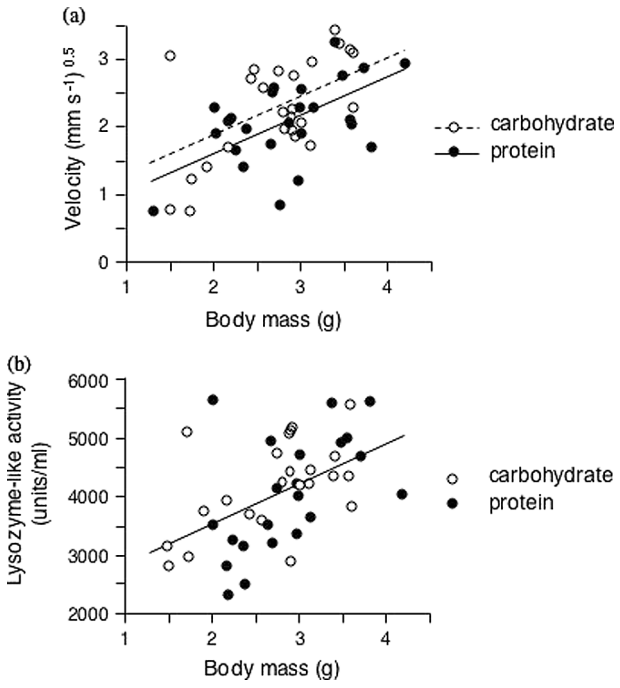

Fig. 1. Relative to those fed a carbohydrate diet, migrating Mormon crickets fed a protein diet reduced their (a) migratory velocity following adjustment for body mass, but did not show a difference in (b) lysozyme-like activity.

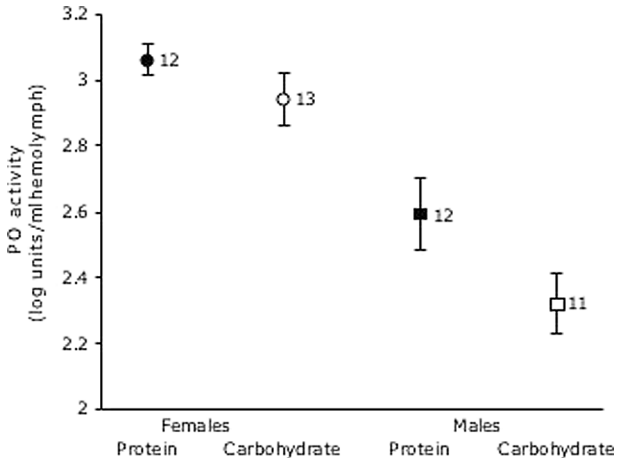

Fig. 2. For the same migrating Mormon crickets as those in Fig. 1a, females had greater phenoloxidase (PO) activity than males and a protein diet enhanced PO activity relative to a carbohydrate diet in both sexes (numbers: sample sizes; error bars: SE of the means).

$P = 0.004$). In contrast to the effect of diet on PO activity, there was not a significant effect of diet on the encapsulation response.

Body mass was the only variable selected to explain variation in lysozyme-like activity, with lysozyme-like activity increasing significantly with body mass (Fig. 1b; n = 12 C females, $n = 12$ P females, $n = 11$ C males, $n = 11$ P males; $R^2 = 0.244$, $F_{1,44} = 14.2$, $P = 0.0005$).

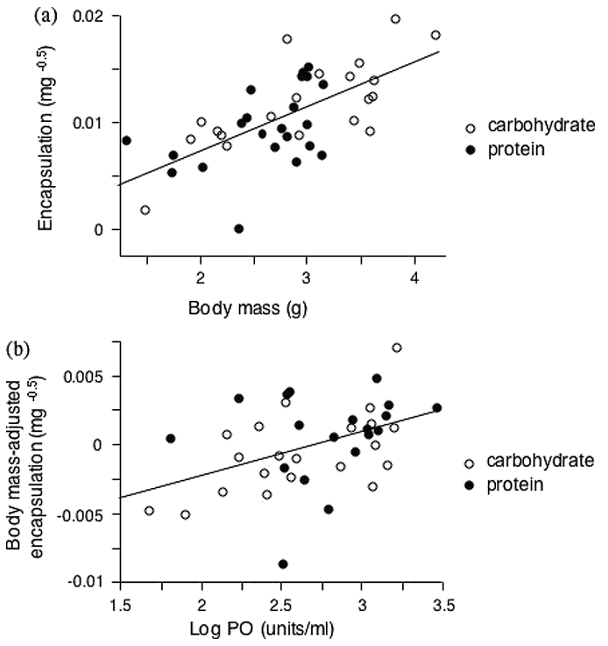

Fig. 3. The encapsulation of glass rods *in vivo* increased with (a) body mass and (b) phenoloxidase (PO) activity but did not vary significantly with diet.

The best model for $\log_{10}$ total protein included the interaction of sex and body mass, and that of diet and body mass, selected in that order ($n = 13$ C females, $n = 12$ P females, $n = 11$ C males, $n = 13$ P males; ANCOVA: $R^2 = 0.704$, $F_{5,43} = 20.4$, $P < 0.0001$). Log total protein was directly proportional to body mass ($F_{1,43} = 75.9$, $P < 0.0001$), and males had significantly greater circulating protein than females ($F_{1,43} = 21.1$, $P < 0.0001$; adjusted log-transformed means: females = 1.41 mg ml^{-1} haemolymph, males = 1.61 mg ml^{-1}). Insects fed a protein diet had significantly greater circulating protein than those fed carbohydrates ($F_{1,43} = 8.0$, $P = 0.007$; adjusted log-transformed means: protein = 1.57 mg ml^{-1}, carbohydrate = 1.45 mg ml^{-1}).

Discussion

Body mass had major effects on locomotion and immunocompetence in migrating Mormon crickets. Migratory velocity, total circulating proteins, encapsulation response, and lysozyme-like activity all increased with body mass. Whether this reflects differences among individuals in nutritional state (with smaller insects being less well fed) or age during the adult stadium is not known. The lack of a significant effect of femur length on velocity indicates that heavier insects were not simply larger structurally. Since we do not know the age of the migrating adults (other than that they were in the first 2 weeks of their adult lives), we can only suggest that body mass is an excellent predictor of the insect's migratory performance and immune defence as reflected by squared increases in

velocity and encapsulation response, and logarithmic increases in lysozyme-like activity.

Diet influenced migratory activity in the short term with a protein diet reducing locomotion. Although the effect of diet on locomotor activity was marginally significant, our results were consistent with those on a migratory band in Idaho (Simpson *et al*., 2006). In Idaho, individually housed insects showed a 50% reduction in locomotor activity when kept with a high-protein, low-carbohydrate diet relative to Mormon crickets maintained with a low-protein, high-carbohydrate diet. Protein satiation for 4 h also reduced cannibalism. The effect of a high-protein diet on locomotion was less in Utah where individuals were released back into the band rather than being housed solitarily. The difference in dietary effects may be related to increased stimulation from band-mates to move independent of nutritional state in the Utah band. In the laboratory, locomotion of individuals fed a high-quality diet *ad libitum* was shown to be induced simply by the immediate presence of conspecifics (Sword, 2005).

Access to a protein diet increased total haemolymph protein concentrations. Ingested protein is digested into amino acids in the gut and absorbed into the haemolymph. In *Locusta migratoria* which were fed a 28% protein diet, amino acids are absorbed from the gut immediately after the onset of feeding (Abisgold & Simpson, 1987). Concentrations of haemolymph peptides and proteins peak 45 min later, indicating that the 42% protein diets fed to the Mormon crickets could raise total haemolymph protein concentrations within the 6 h time frame given here.

Access to a protein diet also increased PO activity, suggesting that migrating Mormon crickets are deficient in PO activity as a result of their nutritional deficiency. To the best of our knowledge, this is the first report of a direct effect of diet on immune function in naturally migrating organisms. Immune defence requires energy and results in the breakdown and loss of proteins (Lochmiller & Deerenberg, 2000). Thus, protein deficiency in the Mormon crickets' diets could reasonably result in a deficiency in PO activity. Deficiency in PO activity results in vulnerability to wounding (as caused by predators or cannibals), parasitism, and bacterial and fungal invasion–all agents that regulate populations. In a laboratory study of *Spodoptera*, dietary protein had a greater effect on immunocompetence than carbohydrates, and following viral attack, the caterpillars increased intake of protein to compensate protein costs of resistance (Lee *et al*., 2006). Although the encapsulation response of the Mormon crickets was associated with PO activity, it was not significantly affected by diet. However, prior blood letting for the enzyme assays may have introduced variability to our subsequent measure of the encapsulation response, obscuring any effect of diet.

What is surprising is the rapidity of the increase in PO activity after only 1 h feeding and a few hours migrating. PO also occurs in an inactive zymogen called prophenoloxidase (proPO), which is an order of magnitude more concentrated than PO in the migratory locust *Locusta migratoria*, for example (Mullen & Goldsworthy, 2003). It is possible that the protein diet does not upregulate PO-gene transcription, but causes the release of proPO from the gut and proteolytic conversion of circulating proPO to spontaneously active PO. proPO is activated by a serine protease cascade with additional proPO activating factors, which themselves are zymogens activated by microbial products and protein components of the proPO system. With additional proteinase inhibitors, the proPO cascade is thought to be tightly regulated to avoid excessive or premature activation (Cerenius & Söderhäll, 2004). We are presently developing an assay to measure proPO in Mormon crickets.

Females and males did not differ significantly in body mass, but females had, on average, 3.5 times the PO activity of males. It has often been observed that females have greater immunological activity than males of the same species. PO activity in *Gryllus texensis* crickets declined with age of males, whereas it peaked at the age of reproduction in females (Adamo *et al*., 2001). Proximate and ultimate explanations have been proposed for this pattern. Ultimately, female investment in their offspring is correlated with lifespan, and thus extension of lifespan through investment in immunity to disease will directly affect lifetime reproductive success more so than for males (Bateman's Principle; Rolff, 2002). This may, however, be a plastic response to the availability of food and mates (McKean & Nunney, 2005). Migratory Mormon crickets reverse sex roles relative to low-density populations, and males select larger females to which they contribute their protein-rich nuptial gifts during mating (Gwynne, 1981; 1984; Gwynne & Simmons, 1990). Males donate 20–30% of their body mass in nuptial gifts and may be expected to be more protein limited than females in band forming populations. Thus, males may suppress immune activity as a result of increased sexual activity in migratory populations relative to solitary ones (see also, McKean & Nunney, 2001). Proximately, insect juvenile hormone (JH) is secreted with mating activity. JH is antagonistic to PO activity in tenebrionid beetles (Rolff & Siva-Jothy, 2002), but it enhances male attractiveness (Rantala *et al*., 2003b). Whether males augment JH at the expense of PO in migratory Mormon crickets is not known. It should also be noted that in this band of pre-reproductive adults, males encapsulated foreign objects with significantly less mass than females (one-tailed Student's $t = 1.89$, d.f. $= 40$, $P = 0.0328$), but this sexual difference was not significant after controlling for body mass.

One likely cost of band formation is disease transmission, and the limited PO activity of protein-starved individuals is likely to enhance this. A benefit in associating with a band is found in the reduced risk of predation (Sword *et al*., 2005), but the risk of cannibalism by protein-deficient band members may also cause insects within the group to avoid contact with other band members (Simpson *et al*., 2006). Cannibalistic acts have a cost in disease transmission by ingestion, but they may also provide beneficial nutrients to enhance immune functions.

Here we have shown that a major component of the insect's defence against wounding and invasion is limited by the nutrient deficiency that characterises members of Mormon cricket bands. Migrating Mormon crickets quickly enhanced spontaneous PO activity following protein ingestion. In contrast to PO, either lysozyme-like activity was not protein limited or the Mormon crickets were unable to up-regulate lysozyme-like activity in the short term.

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