Nutrient deprivation affects immune response in laboratory and wild populations of the burying beetle, *Nicrophorus orbicollis*

**Introduction**

Organisms in the wild are likely to undergo periods of food shortage due to unsuitable abiotic factors or the presence of competitors, parasites, and predators (Kennish, 1997; Kells *et al*., 1999; Robb and Forbes, 2006; McCue, 2010; Clinchy *et al*., 2013). These periods of restricted food intake are likely to occur in nearly all locations, but vary temporally within specific ecosystems (Dolecal and Long, 2013). Species that feed upon a wide variety of materials may encounter less stringent nutrient deprivation than those restricted to specific resources (Boyer, 2003). Organisms that primarily consume physically or temporally rare items, though, are likely to have food shortages compounded by the general sparseness of, and intense competition over, any of these resources that do become accessible (Debouzie *et al*., 2002; Reitz and Trumble, 2002).

All physiological processes, such as cellular growth, reproduction, or immune system function, constantly require energy and nutrients to maintain proper function (Sheldon and Verhulst, 1996; Siva-Jothy *et al*., 2005; Cotter *et al*., 2011; Ardia *et al*., 2012). The optimal resource allocation model predicts that to maximize fitness, energy should be distributed to physiological processes in different amounts based upon an organism's current condition, along with the type and intensity of external stress encountered (McNamara and Buchanan, 2005). As food availability directly influences the amount of energy and nutrients an organism can partition among physiological processes, periods of food shortage are predicted to cause a reduction of energy allocation in one or more processes, and thus potentially reduce their function. Food deprivation is known to impart physiological and fitness costs in organisms (Valtonen *et al*., 2010; Campero *et al*., 2008; Hoang, 2001), and may affect the immune system by limiting the degree of response an organism can generate during an immune challenge (Moret and Schmid-Hempel, 2000; Siva-Jothy and Thompson, 2002).

Invertebrate and vertebrate immune systems are known to differ, with invertebrates lacking the adaptive immunity and antibody-based defenses of vertebrates (Strand 2008). The invertebrate immune system features innate components based upon antimicrobial peptides, enzymes, and their intermediaries in the hemolymph, as well as hemocyte-based phagocytotic and encapsulation responses towards non-self entities (Gillespie *et al*., 1997; Strand, 2008; González-Santoyo and Córdoba-Aguila, 2012). The interaction of these components is essential, as hemocyte activity is regulated by many peptides and enzymes, and during the immune response, hemocytes produce a number of molecules and intermediaries, such as antimicrobial peptides, quinones, superoxide, and nitric oxide (Strand, 2008; González-Santoyo and Córdoba-Aguilar, 2012). Phenoloxidase is an important enzyme in insect immunity, as its production and activities in the hemocoel produce intermediaries with cytotoxic properties, and is also deeply involved with melanin formation during the process of parasite and foreign object encapsulation (González-Santoyo and Córdoba-Aguila, 2012). As the invertebrate immune system has fewer overall components and shares analogous innate responses compared to the vertebrate system, it has become attractive in the study of evolutionary, ecological, and applied questions (Medzhitov *et al*., 1997; Lord, 2005). Despite having fewer components relative to vertebrates, invertebrate immune systems still require nontrivial amounts of energy to be maintained, indicating that periods of restricted food intake are likely to have deleterious effects on immune function and other physiological processes (Siva-Jothy and Thompson 2002; Bashir-Tanoli and Tinsley, 2014; Adamo et al., 2016).

Burying beetles (*Nicrophorus* sp.) are an effective invertebrate model for examining a variety of evolutionary questions, including the sex differences in the effects of dietary restriction on immune responses. In the wild, these beetles subsist on nutrient-rich, rare, ephemeral carrion, which are also sought by many other species, likely causing these resources to be contested and often unavailable (Scott *et al*., 1987; Scott, 1998). Appropriately responding to food shortage at the physiological level is therefore likely to be a relevant condition that the genus has encountered throughout evolutionary history. *Nicrophorus* females develop ovaries and eggs slowly after eclosion (Scott and Traniello, 1987; Trumbo and Robinson, 2004), while males produce and disperse attractant pheromones after reaching sexual maturity (Eggert and Müller JK, 1989). This may be expected to lead to sex-based differences in resource conflicts among various physiological processes. The beetles also provide substantial biparental care, with adults expending significant time and effort to build a brood chamber and process a vertebrate carcass for rearing larvae, as well as supplying their young with food via regurgitation of consumed carrion, potentially leading to an additional source of nutrient deprivation (Eggert and Müller, 1997; Scott, 1998; Trumbo and Rauter, 2014).

There have been a small number of studies involving *Nicrophorus* species regarding individual immune responses at varying life history stages (Steiger *et al*., 2011; Steiger *et al*., 2012; Reavey *et al*., 2014). Breeding *Nicrophorus orbicollis* did not undergo a reduction in phenoloxidase amount or encapsulation response (Steiger *et al*., 2011), although a subsequent study reported that in competitive scenarios over carrion for reproduction, non-dominant *N. orbicollis* did have a lessened immune response (Steiger *et al*., 2012). In contrast to *N. orbicollis*, breeding *N. vespilloides* were found to have reduced phenoloxidase levels relative to non-breeding controls (Reavey *et al*., 2014). None of these studies, however, have examined the impacts of food shortage, a commonly experienced form of nonreproductive stress (McCue, 2010), on *Nicrophorus* beetles' immune system function.

This study examined the effects of dietary restriction on constitutive immunity and mounting an immune response between sexes in the burying beetle, *Nicrophorus orbicollis.* Constitutive immunity serves as the primary defense against invasion, using a mixture of humoral components such as enzymes, enzyme intermediaries, and hemocytes to destroy or phagocytize and encapsulate parasites (Gillespie *et al*., 1997). We predicted that as beetles became increasingly underfed, immune responses would correspondingly drop.

Food shortages are thought to exact similar physiological costs upon the sexes, though they may be expressed or presented in different ways. Organisms may reallocate nutrients or synthesize biological compounds in varying amounts based upon their sex, reproductive strategy, and developmental stage in order to maximize their chances of survival or reproductive success during periods of food deprivation (Hoyenga and Hoyenga, 1981; Brace *et al*., 2015; Wegmann *et al*., 2015). Sex-based differences are also predicted for immune system function, dependent in part on the reproductive strategy of the species (Zuk and Stoehr, 2002; McKean and Nunney, 2005). If the species' reproductive strategy relies upon strong competition between individuals for mates, or low parental investment from one member of the breeding pair, immune function is predicted to differ between the sexes (Zuk and Stoehr, 2002). The individual with higher residual reproductive value is expected to invest more in immune system maintenance or development, as longevity directly improves their fitness. Alternatively, if male and female investment in reproduction and offspring are similar, it has been found that differences between immune responses are minimized (Zuk and McKean, 1996). As *Nicrophorus orbicollis* beetles mate freely, except in the presence of carrion (Eggert and Sakaluk, 1995), and both females and males potentially share significant parental investment in the creation and maintenance of a brood chamber for the larvae (Fetherston *et al*., 1990), we predicted that male and female immunity in *N. orbicollis* would not be significantly different, as suggested by theory (Zuk and Stoehr, 2002) and results from other species (Zuk and McKean, 1996).

Numerous studies have investigated immune responses of organisms under various types of stress in laboratory settings (Koella and Sorensen 2002; Siva-Jothy and Thompson 2002; McKean and Nunney 2005), but few have used field-related data to compare and discuss relevance between experimentally-based findings and those collected in wild settings (Rolff and Siva-Jothy, 2004). In this report, we also include immune response data from field-caught beetles in order to assess the biological importance and germaneness of our laboratory-based results.

**Materials and Methods**

*Experimental Beetles*

Experimental *Nicrophorus orbicollis* beetles were reared from a colony that was founded by beetles caught at T.L. Davis Prairie, Nebraska, USA. The colony was initially started with mite and nematode-free *N. tomentosus* acting as surrogate parents to *N. orbicollis* larvae, so that the resulting offspring would not foster these commensalists and mutualists (Wilson and Knollenberg, 1987; Richter, 1993). At the time of this study, the beetles had been maintained in an insectary at the University of Nebraska Omaha for 15 generations. Adults were identified to sex after eclosion and placed into individual plastic containers (15 cm x 10 cm x 5 cm), filled with approximately 2 cm of moist peat. These boxes were held in the insectary at 22.0 ± 0.2 °C with 15 hours of light and 9 hours of dark per day. One month was then allowed for beetles to reach sexual maturity, during which time they were fed, twice per week, 1/8 teaspoon of canned cat food (Science Diet ®, Hill's Pet Nutrition Inc, Topeka, KS, USA).

*General Experimental Design*

To determine the effect of sex on immune response, when beetles had reached sexual maturity, their body mass, elytra length and pronotum width were measured. Afterwards, the beetles were randomly assigned to one of three treatment groups for seven days. In the first treatment group, individuals were fed 1/8 teaspoon of canned cat food per day. The second treatment group consisted of individuals that were fed 1/8 teaspoon of canned cat food twice during the seven day period. The third treatment group received no food for the seven day period, but had access to water *ad libitum* via water-filled glass vials containing small cotton rolls. All beetles were housed in the same location of the insectary, and only disturbed when food was added to their container or water replenished.

At the end of the seven day period, beetles were weighed again and subsequently had hemolymph drawn for use in the phenoloxidase and protein assay, or were subjected to a simulated encapsulation immune challenge via the insertion of a small nylon monofilament. By quantifying phenoloxidase and encapsulation response, we examined aspects of what are considered humoral and cellular innate immunity, although this distinction is somewhat arbitrary (Strand, 2008). In order to avoid possible confounding effects, beetles from all treatment groups were handled for approximately equal amounts of time, and all handling and assays were conducted at approximately the same time of day.

*Hemolymph Collection*

Hemolymph was extracted from beetles using a 25-gauge syringe tip that was sterilized in 70% ethanol to make a small puncture at the suture above the prosternum, which was also lightly wiped with 70% ethanol. This procedure allowed us to draw between 2-5 μl of hemolymph into a disposable microcapillary tube. Using established protocols (Cotter and Wilson, 2002), before use in phenoloxidase and protein assays, samples were immediately forced into individual 1.5 mL Eppendorf tubes, diluted with a ratio of 1 μl hemolymph to 50 μl of ice-cold phosphate-buffered saline (PBS, pH 7.4), vortexed, and then frozen at -20 °C for one hour to rupture hemocytes.

*Phenoloxidase Activity Assay*

Phenoloxidase acts as a catalyst for the conversion of L-3,4-dihydroxyphenylalanine (L-DOPA) to dopachrome, which can be measured spectrophotometrically. Hemolymph samples that had been frozen for one hour were thawed, vortexed briefly, and 60 μl of sample was pipetted into a spectrophotometer cuvette that contained 440 μl of ice-cold phosphate-buffered saline. 500 μl of L-DOPA (4 mg/ml of H2O) was then added to this mixture, and the resultant solution was thoroughly mixed using a micropipette. The subsequent reaction was allowed to proceed for fifteen minutes at 25 °C in a spectrophotometer (Beckman Coulter DU530, USA), with readings being taken at an absorbance of 490 nm. Phenoloxidase activity was measured, using the specrophotometer's software, as the rate of substrate conversion (absorption/minute) during the linear phase of the reaction, which is known to correlate with the concentration of phenoloxidase in the sample (Wilson *et al*., 2001, Haine *et al*., 2008). Each sample was analyzed in duplicate for assurance of repeatability (F1, 144 = 32.88, P < 0.0001, R=0.941), and an average of the result was taken to arrive at a final phenoloxidase activity value for each beetle.

*Protein Assay*

The total amount of protein in each hemolymph sample was measured by combining 40 μl of sample with 960 μl of Bio-Rad protein assay dye in a spectrophotometer cuvette, thoroughly mixing the two with a micropipette. This compound was allowed to react for ten minutes, before measuring absorbance at 520nm with a spectrophotometer (Beckman Coulter DU530, USA). Protein levels were derived using a calibrated standard curve with fixed concentrations of bovine serum albumin (Sigma): 0.216 mg/mL, 0.432 mg/mL, 0.648 mg/mL, and 0.864 mg/mL. This procedure was conducted twice for each sample in order to assess its repeatability (F1, 126 = 12.67, P < 0.0001, R=0.854).

*Encapsulation and Melanization Assay*

To simulate a novel challenge of the immune system via an encapsulation response, a nylon monofilament was inserted into beetles that did not have hemolymph previously drawn. A clear nylon fishing line (0.15 mm diameter) was roughened with fine-grain sandpaper, tied into knots, and cut into 2 mm pieces, each piece having a knot in its center. A disposable 27-gauge syringe tip needle was used to create a small hole in the pleural membrane between the second and third abdominal sternites. Fine-tipped surgical forceps, sterilized in 70% ethanol between each use, were used to insert a piece of the nylon filament fully into the beetle's abdominal cavity. Beetles were then placed into clean, empty individual plastic containers (5.5 cm diameter, 4 cm tall) and allowed to move freely for two hours. Each container held a moist paper towel, which allowed beetles to hide and avoid further stress, as well as desiccation. After two hours had elapsed, beetles were frozen at -20 °C until the filaments were dissected out.

Filaments were removed from thawed beetles using a dissecting microscope and surgical forceps, after which the filament was gently and quickly dipped in water to remove any clumps of tissue, and stored in individually-labeled 1.5 mL Eppendorf tubes at -20 °C until they were analyzed. During analysis, filaments were placed on a depression microscope slide and covered with a glass slip, the slide placed atop a piece of black plastic that would later serve as the control gray value. Each filament was photographed from the front and the back using a microscope with attached camera (Olympus SZX12 Dissecting Microscope, 20 megapixel ImagingPlanet camera). Photographed images were analyzed using freely available ImageJ software (http://rsb.info.nih.gov/ij/). This was accomplished by using the program to select the knot in each filament, along with an additional 0.2 mm on each side, then analyzing the selected area for its average gray value. Each selection was performed twice to assess the repeatability of this process (F1, 77 = 1956.49, P < 0.0001, R=0.999). Darkness scores ranged from 0 (completely black) to 215 (near-white). The final encapsulation rating for each beetle was calculated as the difference between the average gray value of the control and the average of the front and back photographs of each filament, respectively.

*Wild-Caught Beetles*

Wild beetles were captured from a forested area within the University of Nebraska Omaha's TL Davis conservation area, and at a private forest in northern Omaha, Washington County, Nebraska, USA. The TL Davis and Washington County sites are referred to as field sites one (FS1) and two (FS2), respectively. At each site, beetles were caught using elevated Japanese beetle traps that contained 1 oz plastic cups with small holes, which were filled with decomposing ground beef to attract *Nicrohorus* beetles. The traps were half-filled with moist peat for beetles to hide in and avoid desiccation. Traps were placed in transects, approximately ten meters apart, and were checked on alternate days. Wild-caught beetles were brought back to the lab the day they were collected. Once in the laboratory, the beetles had their mass recorded, elytra length measured, and were identified to sex. They were then individually placed in plastic containers (15 cm x 10 cm x 5 cm) filled with moist peat and allowed to acclimate to insectary conditions for 24 hours. After resting, wild-caught beetles were weighed again and randomly assigned to be subjected to a phenoloxidase and protein assay or simulated immune challenge with a nylon monofilament.

*Statistics*

All statistical analyses were performed using SAS (SAS Institute Inc. Cary, NC, USA). The effect of starvation regime, sex, and their interactions on phenoloxidase activity, protein levels, and melanization were analyzed with a general linear model including the main effects of starvation regime, sex, and their interaction. Protein was used as a covariate during the phenoloxidase activity analysis. To verify that equal start conditions existed among the treatments in regard to beetle age, body mass, elytra length, and body condition, the effects of starvation regime, sex, and their interactions on these control variables were tested using a general linear model including starvation regime, sex, and their interaction. To achieve normality before statistical analysis, beetle mass, elytra length, average grey value, and phenoloxidase activity were normalized by log-transformation, while protein was normalized by square root transformation. Body condition was calculated as the residual of the regression of the beetle’s mass on the beetle’s elytra.

**Results**

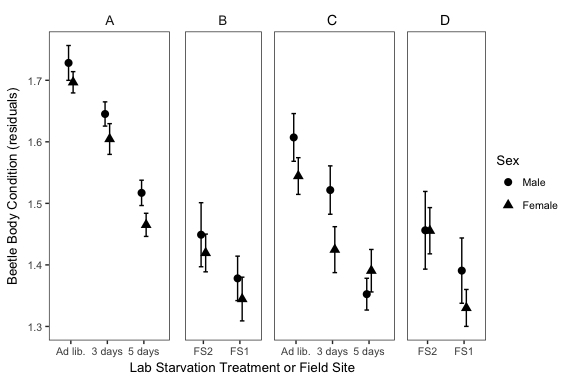
*Laboratory Experiments*

At the beginning of the experiment, the beetles of the three starvation treatments did not differ in age (*F*2, 220=0.64, *P*=0.5302), initial body condition (*F*2, 221=2.24, *P*=0.1089), or elytra length (*F*2, 221=0.22, *P*=0.8027). Similarly, there were no significant interactions found between sex, treatment, or assay group in the analyses for age, initial body condition, and elytra length.

The starvation regime had a significant effect on the post-experimental body condition of the beetles (*n*=79, *F*2, 221=60.98, *P*<0.0001, see also Table 1). Beetles that experienced longer durations of starvation were associated with lower body condition (Fig. 1). Males generally displayed higher post-treatment body condition than females (Table 1, Fig. 1). No interactions among sex, treatment, and the assay groups yielded significant results (Table 1).

**Table 1:** The results of a general linear model analysis of the effect of starvation treatment on post-experimental body condition in beetles

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Source | DF | SS | MS | *F* | *P* |
| Starvation treatment | 2 | 1.6253 | 0.8126 | 60.98 | < 0.0001 |
| Sex | 1 | 0.0859 | 0.0859 | 6.45 | 0.0118 |
| Experiment type | 1 | 0.9485 | 0.9485 | 71.17 | < 0.0001 |
| Sex x Starvation treatment | 2 | 0.0335 | 0.0167 | 1.26 | 0.2859 |
| Sex x Experiment type | 1 | 0.0000 | 0.0000 | 0.00 | 0.9783 |
| Starv. Trtmnt x Exp. Type | 2 | 0.0087 | 0.0043 | 0.33 | 0.7214 |
| Sex x Trt x Exp. Type | 2 | 0.0537 | 0.0265 | 1.99 | 0.1390 |
| Error | 221 | 2.9453 | 0.0133 |  |  |
| Total | 232 | 5.9180 |  |  |  |

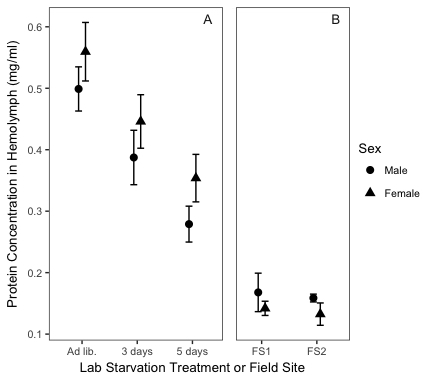
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**Figure 1.** Body condition of laboratory-raised and field-caught beetles, differentiated by assay group (A, B: laboratory and field-caught beetles, respectively, used for phenoloxidase assay; C, D: laboratory and field-caught beetles, respectively, used in melanization assay), starvation treatment, field site (FS1: TL Davis Site; FS2: Washington Co. Site), and sex.

The duration of starvation had a significant effect on beetles’ hemolymph-based protein concentration (*n*=52, *F*2, 150=15.38, *P*<0.0001; see also Table 2). Longer durations without food were associated with progressively lower concentrations of protein (Fig. 2). There was not a significant difference in protein concentration between beetles' sex, and there was no significant interaction between sex and starvation treatment.

**Table 2:** The results of a general linear model analysis of the effect of starvation treatment on protein concentration in hemolymph for laboratory-reared beetles

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Source | DF | SS | MS | *F* | *P* |
| Starvation treatment | 2 | 0.8339 | 0.4169 | 15.38 | < 0.0001 |
| Sex | 1 | 0.1039 | 0.1039 | 3.83 | 0.0521 |
| Sex x Starvation treatment | 2 | 0.0045 | 0.0022 | 0.08 | 0.9203 |
| Error | 150 | 4.0678 | 0.0271 |  |  |
| Total | 155 | 5.0108 |  |  |  |

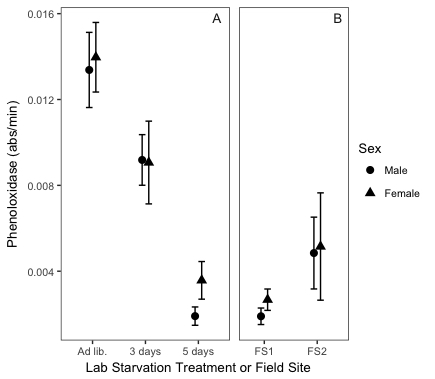


**Figure 2.** Protein concentrations of males and females from (A) three different lab starvation treatment groups and (B) two field sites.

The hemolymph-based conversion of phenoloxidase to dopachrome was significantly affected by the starvation treatment (n=52, *F*2, 149=32.05, *P*<0.0001, see also Table 3), with longer durations of starvation being associated with lower conversion rates of phenoloxidase (Fig. 3). There was no significant difference between the sexes, nor was there a significant interaction between sex and starvation treatment (Table 3).

**Table 3:** The results of a general linear model analysis of the effect of starvation treatment on phenoloxidase in laboratory-reared beetles

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Source | DF | SS | MS | *F* | *P* |
| Starvation treatment | 2 | 77.7619 | 38.8809 | 32.05 | < 0.0001 |
| Sex | 1 | 0.2709 | 0.2709 | 0.22 | 0.6372 |
| Protein | 1 | 6.6319 | 6.6319 | 5.47 | 0.0207 |
| Sex x Starvation treatment | 2 | 5.0773 | 2.5386 | 2.09 | 0.1270 |
| Error | 149 | 180.7530 | 1.2131 |  |  |
| Total | 155 | 307.6736 |  |  |  |

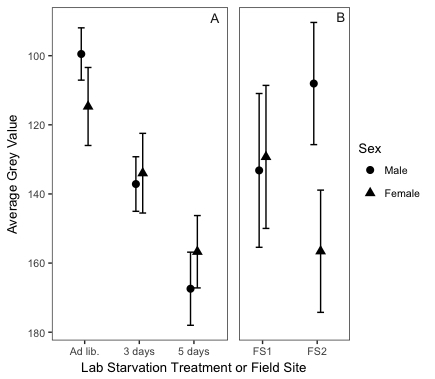
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**Figure 3.** Phenoloxidase absorbance rates of males and females from (A) three different laboratory treatment groups and (B) two field sites.

The starvation treatment employed had a significant effect on the ability of the beetles to melanize a nylon filament (*n*=27; *F*2, 71=2.56, *P*<0.0001, see also Table 4). Longer durations of starvation were associated with a filament that, upon analysis, yielded a higher average gray value (Fig. 4), which indicated less melanization. There was no significant difference between the beetles' sex, nor was there a significant interaction between terms (Table 4).

**Table 4:** The results of a general linear model analysis of the effect of starvation treatment on encapsulation (melanization) of a nylon monofilament in laboratory-reared beetles

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Source | DF | SS | MS | *F* | *P* |
| Starvation treatment | 2 | 2.5612 | 1.2806 | 16.31 | < 0.0001 |
| Sex | 1 | 0.0005 | 0.0005 | 0.01 | 0.9329 |
| Sex x Starvation treatment | 2 | 0.1453 | 0.0726 | 0.93 | 0.4011 |
| Error | 71 | 5.5753 | 0.0785 |  |  |
| Total | 76 | 8.3304 |  |  |  |

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**Figure 4.** Melanization levels of males and females from (A) three different laboratory treatment groups and (B) two field sites. Lower average grey values indicate higher amounts of melanization.

*Field Experiments*

The two populations of wild-caught beetles exhibited a significant difference in elytra length between the sexes (*F*1, 54=7.92, *P*<0.01), with females generally possessing longer elytra than males. There was not, however, a difference in elytra length between the two field sites that hosted these populations (*F*1, 54=1.81, *P*=.1847). There was no significant interaction between sex, field site, or assay group in the analyses for elytra length. Beetle populations at the two field sites had significant differences in body condition (*F*1, 54=7.59, *P*<0.01), with those at the Washington County site (FS2) having better body condition than those at the TL Davis site (FS1). Body condition was not found to differ between the sexes at each field site (*F*1, 54=0.87, *P=*0.3545), nor were there significant interactions between sex, field site, or assay group. There were no significant differences between field populations or the sex of beetles with regards to phenoloxidase conversion to dopachrome, protein concentration in the hemolymph, or melanization response, nor were there significant interactions found between sex, field site, or assay group in these analyses.

**Table 5. Summary of immune parameter results in lab-reared and wild *Nicrophorus orbicollis* beetles**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Fed *Ad libitum* (Nt=79) | Fed Every 2 Days (Nt=73) | Not Fed (Nt=81) | TLD 2011 (Nt=35) | WA. Co. 2011 (Nt=28) | d.f. | F | P |
| Elytra (mm) | 10.95±0.96 | 10.81±0.96 | 10.89±1.02 | 11.831±0.646 | 11.764±1.271 | 2, 221 | 0.22 | 0.8027 |
| Age (days) | 61.46±20.84 | 59.96±24.65 | 62.29±18.16 | *NA* | *NA* | 2, 221 | 0.64 | 0.5302 |
| Body Condition | 1.67±0.14 | 1.58±0.14 | 1.45±0.12 | 1.364±0.117 | 1.440±0.108 | 2, 221 | 16.06 | <0.0001 |
| Protein (mg/ml) | 0.53±0.22 (Na=52) | 0.41±0.22 (Na=50) | 0.31±0.18 (Na=54) | 0.150±0.032 (Na=19) | 0.151±0.054 (Na=17) | 2, 150 | 15.38 | <0.0001 |
| PO (abs/min) | 0.014±0.009 (Na=52) | 0.009±0.008 (Na=50) | 0.003±0.004 (Na=54) | 0.005±0.006 (Na=19) | 0.002±0.001 (Na=17) | 2, 150 | 46.09 | <0.0001 |
| Melanization Response (AGV) | 106.82±34.99 (Na=27) | 135.63±32.26 (Na=23) | 162.27±38.35 (Na=27) | 132.32±54.42 (Na=16) | 131.06±47.74 (Na=11) | 2, 149 | 32.05 | <0.0001 |

Multivariate analysis, followed by univariate post hoc tests. Values are means ± s.d. Field-caught beetle data not included in statistical analyses. Nt =Total sample size for the treatment or field site; Na =Sample size for the assay. AGV: average gray value, lower values indicate higher melanization.

**Discussion**

In this study, we investigated the influence of starvation on immunity in male and female burying beetles, *N. orbicollis*, and compared our laboratory data to that gathered from field-caught beetles to ascertain the relevance of our findings. We found clear impacts of starvation on individual immunity, with beetles that had experienced several days of reduced food intake having lower concentrations of protein and phenloxidase in their hemolymph, as well as decreased ability to encapsulate foreign objects. Male beetles maintained better body condition than females, but there were no significant differences in immune parameters between the two sexes. Field-caught beetles of both sexes had very low levels of protein and phenoloxidase, comparable to severely starved laboratory beetles, while their encapsulation rates, however, generally corresponded to moderately starved laboratory beetles. This suggests that our laboratory studies set ecologically appropriate periods of starvation for the beetles, as well as that organisms that feed on rare or ephemeral resources are likely to experience below optimal constitutive immunity between encounters of food resources.

*Body Condition*

Male and female *N. orbicollis* maintained similar body conditions while receiving adequate nutrition, but diverged when starvation occurred. Starved male *N. orbicollis* generally maintained their body condition at higher levels than females. This may be due to physiological differences between the sexes in *Nicrophorus* species. After eclosion, female burying beetles begin to develop ovaries and eggs (Scott and Traniello 1987, Trumbo et al. 1995), but only develop them completely after they encounter carrion suitable for reproduction (Scott and Traniello 1987, Trumbo et al. 1995). This may lead to continual reproductive expenses for females before the initiation of an actual breeding attempt (Trumbo et al. 1995). As the beetles' primary food source, carrion, is generally nutrient dense and rich in energy (Barton et al. 2013), it is likely that it adequately supports the physiological needs of either sex when available with some regularity. However, the additional costs of ovarian and oocyte development that females must withstand may not be fully apparent until periods of food shortage. Eggs are generally considered more energetically expensive to produce than sperm, and gametic-related biomass production is typically higher in females (Hayward and Gillooly 2011), which may also influence males' ability to more easily maintain body condition during periods of starvation, relative to females. This interpretation is supported by the observation that starved female burying beetles take longer to lay eggs than well-fed females after finding a carcass for reproduction (Trumbo and Xhihani 2015).

As carrion that is suitable for reproductive purposes is considered a rare and unpredictable resource (Scott 1998), it would not benefit females to leave their ovaries completely undeveloped after eclosion until an opportunity for reproduction occurred. Similarly, it would be disadvantageous to cease biological maintenance of their ovaries and oocytes if females experienced one or more periods of starvation before finding reproductively suitable carrion. This is because such behavior could result in a critical delay between discovering carrion, laying eggs, and having active larvae on a carcass, during which time competitors may find the corpse and displace the original beetle or beetles (Trumbo 1990, Wilson and Fudge 1984). Females instead appear to use available or stored energy to maintain their premature reproductive organs, but do not invest in complete development until a suitable opportunity for reproduction arises (Trumbo and Xhihani 2015, Trumbo et al. 1995).

Differences in responses and strategies to stressors, such as starvation, between sexes are well documented: females across many invertebrates and vertebrates have reduced body condition after periods of food limitation relative to males (Matsura 1981, Martin et al. 2007, Aggarwal 2014, Angelier et al. 2015). Restricted access to food triggers trade-offs between many different life history or physiological traits, including growth, immunity, and reproduction. This is particularly true when either of the sexes has higher developmental or maintenance costs (Dubiec et al. 2006).

*Protein Concentration in Hemolymph*

Starvation led to decreased protein concentrations in the hemolymph of male and female burying beetles, which was not unexpected. Burying beetles normally consume protein-rich foods, but in our experiments, two treatment groups experienced varying intensities of nutrient deprivation. As a result, these beetles received little to no new amounts of amino acids with which to maintain physiological processes or anatomical characters, including immunity. Amino acid-based antimicrobial compounds, such as cytotoxic or cell-lysing enzymes, represent a highly conserved innate immune response from invertebrates to vertebrates (Bulet, Stöcklin, and Menin 2004), and there is a strong correlation between protein consumption and the level of these antimicrobial substances within the body (Lochmiller and Deerenberg 2000). This indicates that production and maintenance of amino acid-based immune parameters may typically be limited more by nutritional intake and life history trade-offs than strict upper bounds imposed by evolutionary pressures. In insects, increased protein intake is known to positively influence body condition and immune function by adding to the available amount of nitrogen for investment in such physiological processes (Lee, Simpson, and Wilson 2008). Protein levels in the hemolymph may serve as an indicator of available enzymes that have bactericidal activity (Cotter et al. 2008), and higher concentrations of protein in hemolymph have been identified as allowing individuals in different taxa to survive immune challenges better than those with lower protein concentrations (Povey et al. 2009, Adamo 2004). Therefore, the steady decline in our experimental beetles' hemolymph protein concentration as food deprivation increased indicates that their immunity was likely compromised in one or more ways, which is consistent with other insect studies examining immunity and food stress (Adamo et al. 2016).

*Phenoloxidase in Hemolymph*

Similar to protein concentrations in hemolymph, phenoloxidase levels were reduced when beetles experienced nutrient limitation. Phenoloxidase and its intermediaries are considered important components in the constitutive immune and repair systems of insects (Cotter and Wilson 2002), but have been found to change with physiological status (Srygley et al. 2009, Adamo 2016), indicating that they may be involved in trade-offs or pleiotropy. The production and use of phenoloxidase during immune responses has been implicated in several physiological trade-offs, such as altered development rates (Cotter et al 2008), reduced fecundity or offspring quality (Stahlschmidt et al. 2013, Moret and Schmid-Hempel 2001), self-harm (Sadd and Siva-Jothy 2006), and reduced longevity (Schwarzenbach and Ward 2006). Pleiotropy has caused indirect selection on melanin-derived immunity, such as phenoloxidase, in the cricket *Allonemobius socius*, because there is direct thermal selection on melanin-based cuticle color in this species (Fedorka et al. 2013). As phenoloxidase production is partly dependent on the amount of amino acids and other nutrients consumed during feeding, it is not surprising that there were decreased levels of this enzyme in starved *Nicrophorus orbicollis* beetles. Studies utilizing different insect groups have found similar phenoloxidase-reducing responses to starvation in coleopteran, odonate, and lepidopteran groups (Siva-Jothy and Thompson 2002, Rantala et al. 2003, DeBlock and Stoks 2008, Adamo 2016), though another experiment noted an inverse response in a lepidopteran (Yang, Ruuhola, and Rantala 2007). This suggests the possibility that responses to starvation may not be consistent across insect orders due to evolved differences in responding to stress at the immune system level.

*Encapsulation*

Adequate protein intake in insects is known to positively influence melanin production by allowing for the maintenance of a nitrogen pool that is suitable to be drawn from (Lee, Simpson, and Wilson 2008). Therefore, the reduced encapsulation and melanization of the nylon monofilament in the treatment groups undergoing starvation was not unexpected. Encapsulation and melaninization are associated with a robust immune response, as hemocytes must be recruited to the location of the pathogen, surround it, and potentially be involved with a series of oxidation reactions that culminate in melanin production (González-Santoyo and Córdoba-Aguila, 2012). Therefore, if there is a decrease in the degree of melanization response an insect is able to generate, this should correspond to a decrease in amounts of available phenoloxidase and protein as well, which was observed in this study. Decreases in melanization response have been reported in other insect species when undergoing nutrient deprivation, such as *Anopheles stephensi* (Koella and Sorensen 2002) and *Manduca sexta* (Diamond and Kingslover 2011). Others, however, have found starvation lead to no significant differences from controls in melanization responses for *Tenebrio molitor* (Siva-Jothy and Thompson 2002, Rantala et al. 2003), indicating that insect responses to starvation can be diverse at the immune system level (Adamo 2016).

*Laboratory Beetles Versus Field-caught Beetles*

This study included a field research component in order to establish a basic understanding of the general nutritional conditions that *Nicrophorus* beetles experience in natural settings, and to verify that our laboratory beetles were subjected to biologically relevant levels of nutrient deprivation. Beetles from surveyed field populations had body condition levels most similar to laboratory beetles undergoing moderate to total starvation: no field-caught beetles were in a condition equivalent to laboratory beetles fed *ab-libitum*. This provides new evidence that carrion is a rare, ephemeral, and contested resource for these beetles, as previously suggested (Wilson and Fudge 1984, Trumbo 1990, Scott 1998, Smith 2001). Protein concentrations in the hemolymph of field-caught *N. orbicollis* were at or below levels of laboratory beetles that were undergoing total starvation, further suggesting that *Nicrophorus* beetles in field settings experience periods of time where protein-rich carrion availability is very low. Starvation frequency and duration are known to vary widely among organisms in the wild, resulting in many different adaptations and responses to periods of less-than-optimal feeding (McCue 2010). Reduced protein concentrations are a common response across many different organisms experiencing extended starvation. Blood protein levels in wild populations of vertebrates can be low, relative to amply fed individuals, due to reduced availability or quality of food (Sinclair et al. 1982; Torbit et al. 1985; Seewagen et al. 2016), which can lead to starvation, catabolism, and gluconeogenesis in extreme cases (McCue 2010). Wild populations of invertebrates are known to experience periods of less than adequate food intake as well, resulting in decreased protein content in hemolymph (Gu et al. 1996; Yang et al. 2007). Although exceptions are known to exist (McCue 2010), organisms that are experiencing starvation have reduced circulating protein levels unless catabolism occurs, which may break down previously stored proteins for energy.

Field-caught *N. orbicollis* beetles primarily exhibited similar phenoloxidase levels as experimental beetles experiencing total starvation, and had significantly different levels from laboratory beetles that were regularly fed. This finding adds further evidence that wild beetles exist during periods of time where carrion, which is nutrient-dense, is highly limited. Phenoloxidase is produced from a variety of amino acid and enzyme precursors, which are continually required in sufficient amounts for its synthesis, both before and during an immune event (Cerenius and Söderhäll 2004; González-Santoyo and Córdoba-Aguila 2012). Multiple adaptations to suboptimal feeding have arisen due to the wide variation in starvation frequency among organisms in the wild (McCue 2010). It is not unexpected, then, that levels of constitutive immune defenses, such as phenoloxidase, have been found to vary between species. Invertebrates as diverse as damselflies, crickets, oysters, and moths (Rolf and Siva-Jothy 2004; Butt et al. 2007; Srygley et al. 2009; Myers et al. 2011) have been found to exhibit lowered phenoloxidase responses when experiencing dietary deficiencies or reduced fat reservers. An alternative moth species, however, exhibited enhanced phenoloxidase response during starvation (Adamo 2016). The variations in phenoloxidase response to starvation suggest that different species have developed a wide range of immune system adaptations and reconfigurations to stressors, such as starvation (Adamo 2016, Adamo 2017). Our findings provide additional support that food stress in invertebrates can lead to lower phenoloxidase levels as part of a more comprehensive reconfiguration of immune responses to maximize an organism’s survival.

The melanization response of beetles from field sites was significantly greater than expected, relative to the protein and phenoloxidase results. Males and females generally had melanization responses similar to experimental beetles that were in the treatment groups being fed twice per week and *ad libitum*. A single population of females at one field site did not follow this pattern, having instead a melanization response similar to experimental females who had been starved during the treatment period. This unexpectedly higher level of melanization, relative to the other immune parameters tested, may potentially be attributable to the beetles’ reproductive status. Steiger et al. 2011 showed that *N. orbicollis* maintains an elevated melanization response during, and for some time after, a reproductive attempt. The researchers explained this by suggesting that *N. orbicollis* maintains robust encapsulation and melanization responses during periods of stress, such as reproduction, due to the possibility of continued fierce competition with others for the carcass. These competitive bouts may result in the loss of appendages and require melanization to seal any wounds. The threat of competition and injury to parent beetles remains elevated until the carcass has been mostly consumed (Trumbo 2006), such that it is advantageous for parent beetles to maintain a robust melanization and encapsulation response during the majority of the time the larvae are feeding from the carcass. The beetles captured from our field sites may have recently emerged from reproductive bout, which could explain their heightened melanization response, relative to starved laboratory beetles. Conditions may also have differed somewhat between our two field sites, such that females from the location with lower melanization (field site 2) were still reproducing within a brood cavity, so that we largely captured females that were not recently involved in reproduction. During reproduction, males may remain on the carcass for shorter lengths of time than females (Scott 1998), which may explain why males captured at this site (field site 2) were found to have still-elevated melanization responses, similar to both sexes of *N. orbicollis* at the alternate field site.

This study demonstrates the continued importance and relevance of comparing results from laboratory-based work to those obtained in field settings to arrive at meaningful conclusions regarding what wild-type organisms likely experience. While we assume that our work is generally representative of what wild *N. orbicollis* beetles experience, future efforts can enhance this knowledge by collecting more samples throughout the entire period the beetles are active, as well as across multiple years, in order to arrive at a more comprehensive understanding of *Nicrophorus* feeding habits and immune status over time. While it is not possible to trap all beetles at a field site for sampling, repeated attempts will yield more data points, which over time guarantee that beetles of varying body conditions and starvation status will be obtained. We could not take into account the reproductive status, phoretic mite or nematode load that field-caught beetles were carrying, as to do so would have stressed them far beyond the degree that our laboratory beetles were. Additional studies need to qualify and quantify actual bacteria and parasites associated with *Nicrophorus* species on carcasses in the wild, in order to gain a more accurate understanding of these threats, and their degree of exposure, experienced by these beetles in non-laboratory settings.

*Conclusion*

Our study shows that as *N. orbicollis* beetles experience longer durations of starvation during non-reproductive periods, both sexes alter the functioning of their immune system by reducing constitutive and inducible immune responses. These shifts indicate an evolutionarily selected reprioritization suited for survival on rare, ephemeral food. Comparison of our laboratory-based results with field-caught beetles indicates that these insects do encounter periods of nutrient deprivation in their natural environment, which affects their body condition and immune responses. When combined, the data from our research demonstrates that organisms in the wild specializing on rare and ephemeral resources have less than optimal immune system function. This study also shows that our experimental starvation treatments were within the range of conditions experienced by these beetles in the wild. Our work suggests that laboratory studies benefit from the use of related field research to discern patterns and implications that might be unrecognized in the laboratory, and also lends support to the theory that insect responses to starvation are quite diverse at the immune system level (Adamo 2016).

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