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Social and Personal Immunity of Parents and Larvae in the Burying Beetle, Nicrophorus orbicollis

For the degree of Master of Science

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Head of the Department Graduate Program Date

SOCIAL AND PERSONAL IMMUNITY OF PARENTS AND LARVAE IN THE BURYING BEETLE, NICROPHORUS ORBICOLLIS

A Thesis

Submitted to the Faculty

of

Purdue University

by

Adam John Riley

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of

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

ABSTRACT

Riley, Adam John. M.S., Purdue University, August 2014. Social and Personal Immunity of Parents and Larvae in the Burying Beetle, *Nicrophorus orbicollis*. Professor: J. Curtis Creighton.

The burying beetle *Nicrophorus orbicollis*, through biparental care, raise their young on small vertebrate carrion which exposes them to microbial parasites and competitors. These interactions have led to elaborate strategies to combat microorganisms. Through the application of anal and oral secretions adults are able to preserve a carcass from which larvae feed, constituting a social immune response. Evidence suggests that larvae also contribute to this social immunity through their own secretions. Social immunity was tested through exposing larvae to an isolated food source with an experimentally elevated microbial signal, dead *Micrococcus luteus*. Larvae maintained higher lysozyme-like activity (LLA) in their oral secretions in response to the microbial signal. However, if personal immunity was compromised LLA was not maintained. Larvae and parents were tested for response to increased competition of feeding on fresh or aged carcasses with greater levels of decay. Larvae were not shown to alter LLA of their oral secretions but female parents did have a negative relationship of LLA in their oral secretions and in relation to brood size. Larvae had a negative relationship of phenoloxidase activity in their oral secretions in relation to brood size. This suggests influence of individual larval

investment to social immunity affecting both parents and sibling investment. These findings show that oral secretions in *N. orbicollis* larvae, like adults, has adaptive antimicrobial activity which acts as a social immune response for defending a carrion food source and is sacrificed for personal immunity.

CHAPTER 1: INTRODUCTION

Microbial parasites can have a major impact on the fitness of their host. Hosts in response, have evolved elaborate defense strategies (Adamo 2004; Moret & Schmid-Hempel 2000; Simmons 2011). Upon exposure to microbes in vertebrates, humoral and cellular immune responses are initiated constituting a personal immune response. Among invertebrates these defenses are often initiated by hemocytes and include phagocytosis, encapsulation and melanization of microbes, promoted by phenoloxidase (PO), as well as the production of antimicrobial peptides and lysozyme (Flatt *et al.* 2008; Siva-Jothy *et al.* 2005; Zuk & Stoehr 2002). Exposure of social organisms to parasites has led to the evolution of behaviors that are beneficial to both the individual and the collective group in combating parasites thus constituting a social immune response (Cotter & Kilner 2010a). Social immunity has been observed in behaviors such as allogrooming and trophallaxis among termite and ant colonies, which correlates positively with increased survival of immune-challenged individuals (Hamilton *et al.* 2011; Scharf *et al.* 2012; Traniello *et al.* 2002).

For many social animals, the family structure relies on parents providing care to multiple young. For developing young, parasites often pose a threat making immune defense important (Vogelweith *et al.* 2013). Parents often invest immune defenses to

counter these parasites, thus benefiting offspring through a social immune response (Cotter & Kilner 2010b). This can include responding to immune challenge by incorporating antimicrobial peptides into the eggs of offspring as observed in female mealworm beetles (*Tenebiro molitor*) (Zanchi *et al.* 2012), or direct passage of immune factors to embryos as seen in the zebrafish (*Danio rerio*) (Wang *et al.* 2009). Social immune responses can also come at costs. Female burying beetles (*Nicrophorus vespilloides*) produce fewer offspring over a lifetime when their investment in social immunity is up regulated (Cotter *et al.* 2010).

The threat of microbial competition with organisms in a social group can also lead to offspring investment in social immunity, although there are few documented cases. For example European paper wasp larvae (*Polistes dominulus*) produce antimicrobial saliva to protect the food stored in their gut throughout pupation benefiting themselves and their parent, which actively consume the secretions (Turillazzi *et al.* 2004). How offspring social immune responses affect their fitness or what potential trade-offs exist between personal and social immunity in the young is not well understood. In this paper, I evaluate these questions in the burying beetle, *Nicrophorus orbicollis*.

Burying beetles (Silphidae: *Nicrophorus*) breed on small vertebrate carcasses, the sole source of food for the larvae and adults (Pukowski 1933). They provide biparental care to their young, which include defending, burying, and preserving the carcass (Milne & Milne 1976). Carcass preservation involves removing the fur or feathers, forming it into a ball, and spreading antimicrobial oral and anal secretions on the surface (Hoback *et al.* 2004). These secretions show increased antimicrobial activity during parental care

(Cotter & Kilner 2010b; Steiger *et al.* 2011), and their application increases survival of the larvae; demonstrating a social immune function (Arce *et al.* 2012). Production of these secretions decreases fitness (Cotter *et al.* 2010) and shows a trade-off with personal immunity when elicited through bacterial challenge in *N. vespilloides* (Cotter *et al.* 2013). Recently work demonstrates that *N. vespilloides* larvae also provide a social immune response in the form of secretions (Arce *et al.* 2013). Thus, burying beetles are an ideal organism for examining investment in personal and social immunity in parents and offspring.

The aim of this study is to investigate personal and social immune responses of larval burying beetles (*N. orbicollis*) in response to increased microbial competition on a carcass. I predict that developing on an aged carcass will elicit a social and personal immune response through increased LLA and PO activity of oral secretions and hemolymph. Parental influence is expected to play a role in the immune response of larvae. I predict larvae respond to a personal and social immune challenge in parental absence through increased LLA and PO activity of oral secretions and hemolymph. Specifically in this paper, I (i) determine adaptive social immunity measured through LLA and PO activity in the oral secretions produced by the larvae, (ii) evaluate personal immune response in larval hemolymph, and (iii) compare the immune response of parents and larvae.

CHAPTER 2: MATERIALS AND METHODS

2.1 Burying Beetle Natural History

Burying beetles use chemoreceptors on their antennae to find small vertebrate carcasses to feed on and use for reproduction (Kalinová *et al.* 2009). If multiple beetles arrive at a carcass they will fight until the largest male and female claim possession (Bartlett & Ashworth 1988; Müller *et al.* 1990). The pair then buries the carcass, removes fur or feathers, forms it into a ball, and applies antimicrobial oral and anal secretions (Eggert & Müller 1997; Hoback *et al.* 2005). After about five days larvae begin to arrive at the carcass to feed at an opening made by the parents. The parents will provide regurgitated carcass tissue to the young for the first couple days and then they rely on self-feeding until dispersal into the surrounding soil (Pukowski 1933). Newly eclosed adult beetles emerge from the soil after approximately three weeks. The young become sexually mature approximately three weeks after eclosion (Trumbo 2009).

2.2 *Nicrophorus orbicollis* Population

The beetle population used for experiments was derived from beetles captured with baited pitfall traps in Big Falls, Wisconsin (fig. 1) in May-June of 2012 and 2013. All *N. orbicollis* collected were housed in individual plastic containers (15 x 11 x 7 cm)

in an environmental chamber at 21°C with 14:10 h light:dark cycle and fed chicken liver twice weekly. These beetles were then used to establish the laboratory population for experiments. They were bred by placing a male and female with a fresh mouse carcass in plastic containers (18 x 15 x 10 cm) two-thirds full with top soil. The males were removed when larvae first appeared on the carcass, and females were removed when larvae dispersed from the carcass. Larvae were left undisturbed until eclosion and then maintained in individual plastic containers until used for the experiments.

Figure 1. Beetles for experiments were derived from wild-caught population collected in Big Falls, Wisconsin (latitude 44.6165° N, longitude -89.0161° W) indicated with an (X) on the map.

2.3 Experiment 1: Larval Immune Challenge

Twenty separate broods were each prepared by placing a virgin male and non-sibling female beetle in a plastic container (18 x 15 x 10 cm) two-thirds full with topsoil and a freshly thawed mouse carcass. When larvae were four days old, four randomly selected larvae were removed from each brood and their oral secretions collected. Each larva was then placed in one of two groups. In the first group, two larvae were placed

individually on 0.5 g of chicken liver dipped in tripticase soy broth (TSB) containing autoclaved (dead) *Micrococcus luteus.* This treatment was designed to examine the immune response of the larvae to a bacterial signal. As a control, two larvae were placed on 0.5 g of chicken liver dipped in TSB only. Within each treatment group, one larva was pierced with a needle dipped in a solution of dead *M. luteus* to impose an immune challenge. Oral secretions and hemolymph were collected after two hours. Three larvae died over the treatment period so no samples were collected from those individuals creating the following sample sizes: liver without *M. luteus,* non-immune compromised (n = 18) and immune compromised (n = 19); liver with *M. luteus*, non-immune compromised (n = 20) and immune compromised (n = 20). All samples were diluted 1:6 in PBS solution (pH 7.2), and then maintained at -80^{o} C until analysis.

2.4 Experiment 2: Parental and Larval Investment on Carcass

Two treatments were established with both consisting of a 21-28 day old, virgin male and non-sibling female beetle placed in a plastic container (18 x 15 x 10 cm) two-thirds full with topsoil. Beetles used were selected randomly with genetic line, mass and pronotum size recorded. The control treatment pairs (n = 20) were given a freshly thawed 30 g (± 1 g) mouse carcass and the experimental treatment pairs (n = 20) were given a 30 g (± 1 g) mouse carcass that had aged at room temperature under a fume hood for seven days. This allowed for an abundance of microbial growth, which does have costs to fitness (Rozen *et al.* 2008). Each pair was observed daily, and the male was removed when larvae first appeared. On the fourth day of larvae being present on the carcass, the

female was weighed and then hemolymph, anal and oral secretions were collected. On the same day, oral secretions were collected from five randomly selected larvae, which were then returned to the carcass. At the same time, a hemolymph sample was taken from one randomly selected larva, which was then removed from the brood. All samples were diluted 1:6 in PBS solution (pH 7.2), and then stored at -80^{o} C until analysis. Brood size was also determined at this time.

2.5 Phenoloxidase Testing

The fluid samples collected were all tested for the presence of PO activity. For the PO assay 2 µL of sample was added to 100 µL of LPS solution (Sigma-Aldrich L3129) followed by 100 µL of 5 mM L-Dopa (3,4-Dihydroxy-L-phenyl-alanine from Sigma-Aldrich D9628). L-Dopa acts as a substrate for measuring PO (Cotter *et al.* 2010). Samples were then incubated in a BioTek Synergy 2 microplate reader at 30^{o} C and measurements of absorbance taken at 490 nm every minute for an hour. PO activity was expressed as the max change in absorbance of light over the hour.

2.6 Lysozyme-Like Activity

Lysozyme-like activity (LLA) for each fluid sample was measured using a zone of clearance assay. Agar plates containing lyophilized *M. luteus* were prepared with 1 µL of sample loaded into wells (1.5 mm diameter). For each plate a control of 1 µL of 1% hen egg white lysozyme (Sigma-Aldrich L6876) was added to one well. The plates were

then incubated for 48 h at 27^{o} C. Once removed zone of clearance was measured using Image J software (http://rsweb.nih.gov/ij/index.html).

2.7 Statistical Analysis

To determine how presence of bacteria on the carcass and an immune compromise affect concentrations of immune molecules in oral secretions and hemolymph of larvae I used a mixed model analysis of variance (ANOVA) for Experiment 1. A separate analysis was run for each of three response variables as follows: oral PO, oral LLA, and hemolymph PO. For oral secretions that could be taken without killing the larvae, I used the difference between pre and post-treatment values as the response variable. This controls for individual variation in baseline values. Both oral PO and oral LLA fit the assumptions of the parametric model without the need for transformations. For hemolymph measures, taking samples resulted in immune compromise, so only post-treatment samples were taken. Hemolymph LLA was not analyzed because only a few individuals had non-zero readings. Hemolymph PO was transformed using a natural logarithm to meet the assumptions of a parametric model. Bacterial presence (experimental treatment of TSB with dead *M. luteus* or control of TSB alone), and immune compromise (compromised versus control) were included as fixed effects, and their interaction in the model. Brood ID was considered a random effect to account for the relatedness among individuals within a brood. The MIXED procedure in SAS (SAS 9.3 SAS Insitute, Cary, North Carolina, USA) was used for all three analyses.

To determine how age of the carcass, source of the secretions and brood size affect concentration of immune molecules in female parents a mixed model analysis of covariance (ANCOVA) was used for Experiment 2. A separate analysis was run for each of two types of immune molecules as response variables: LLA and PO. Values of LLA fit the assumptions of the parametric model without the need for transformations. Values of PO were square-root transformed to meet the assumptions of a parametric model. Treatment of carcass (fresh versus aged) and source of secretion (oral, anal, or hemolymph) were included as fixed effects, and their interaction in the model. Brood size was included as a covariate. In preliminary analyses the interactions of brood size with two fixed effects and female size were included as additional covariates, but they were not significant in any analyses and were removed for final analysis. Brood ID (consistent with female ID in this case) was considered a random effect to account for repeated sampling of the immune molecules from the same female. The MIXED procedure in SAS (SAS 9.3 Institute, Cary, North Carolina, USA) was used for both analyses.

To determine how parental immune response interacts with larval immune responses a mixed model analysis of covariance (ANCOVA) was used for Experiment 2. A separate analysis was run for each type (LLA or PO) and source (oral or hemolymph) of immune molecule derived from larvae, resulting in four separate analyses with response variables as follows: larval oral LLA, larval oral PO, larval hemolymph LLA, and larval hemolymph PO. Values of response variables fit the assumptions of the parametric model without the need for transformations. For larval oral LLA and hemlymph LLA treatment of carcass (fresh versus aged) was included as a fixed effect,

and female parent oral, anal, and hemolymph LLA and brood size as covariates. Interactions between the fixed effect and covariates in the model were also included. For larval oral PO and hemolymph PO treatment of carcass (fresh versus aged) was included as a fixed effect, and female parent oral, anal, and hemolymph PO and brood size as covariates. Interactions between the fixed effect and covariates were included in the model. The MIXED procedure in SAS (SAS 9.3 Institute, Cary, North Carolina, USA) was used for both analyses.

CHAPTER 3: RESULTS

3.1 Experiment 1: Personal vs. Social Immunity in Larvae

There was a significant difference in the change in LLA in oral secretions of larvae between experimentally increased bacterial load treatments and control treatments (table 1). Larvae in treatments with increased bacterial loads showed a smaller decline in oral LLA compared to controls (fig 2a). The immune compromise treatment had a significant effect on the change in oral LLA in larvae (table 1). Immune compromised larvae exhibited a greater decline in oral LLA compared to controls (fig 2a). There was no significant interaction between bacterial load and immune compromise treatments (table 1). The change in PO activity of oral secretions over the treatment period showed a significant difference with immune compromise (table 1). Immune compromised larvae had less of a decline in PO activity compared to controls (fig 2b). There was no significant difference between bacterial load treatments or interaction effect between the bacterial load and immune compromise treatments (table 1).

LLA in the hemolymph was only present in two individual at low levels so no statistical analysis was performed. There was a marginally significant difference in the PO activity of hemolymph of larvae between experimentally increased bacterial load treatments and control treatments (table 1). Larvae in treatments with increased bacterial

loads had increased concentration of hemolymph PO activity compared to controls (fig 3). There was no significant difference with immune compromise or interaction effect between the bacterial load and immune compromise treatments (table 1).

Table 1. ANOVA from Experiment 1 for the effects of bacterial load (liver without *M. luteus* or with *M.* luteus) and immune compromise (with or without) on measures of larval social immunity (oral LLA and oral PO activity) and personal immunity (hemolymph PO activity). Bold values are statistically significant.

Response Variable	**Effect**	**Df**	**F-Value**	**p-value**
Oral LLA				
	Bacterial load	54.6	8.60	**0.0049**
	Immune compromise	54.7	4.51	**0.0382**
	Bacterial load*Immune compromise	54.7	1.83	0.1816
Oral PO activity				
	Bacterial load	54.5	0.01	0.9153
	Immune compromise	54.6	6.25	**0.0154**
	Bacterial load*Immune compromise	54.6	0.62	0.4344
Hemolymph PO activity				
	Bacterial load	54	3.83	0.0556
	Immune compromise	54.1	0.22	0.6416
	Bacterial load*Immune compromise	54.1	2.15	0.1486

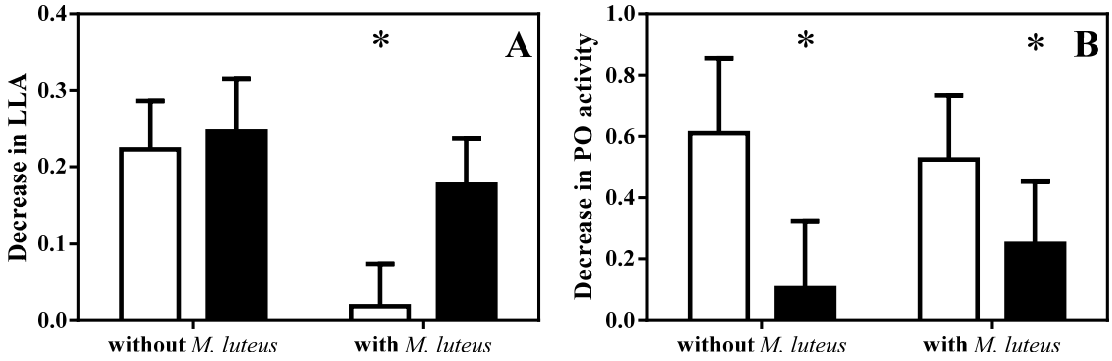

Figure 2. Mean (+SE) decrease in social immune response of larval oral secretion measures of LLA (A) and PO activity (B) between bacterial load treatments (liver without *M. luteus* or with *M.* luteus) and non-immune compromised (white bar) or immune compromised (black bar) treatments. Results of statistical analysis are reported in table 1. Statistically significant differences ($P < 0.05$) are denoted by asterisk.

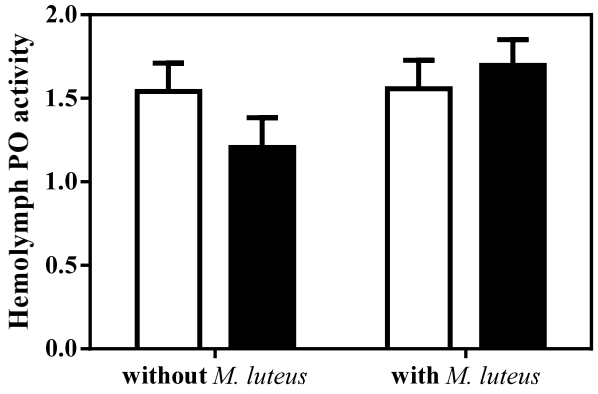

Figure 3. Mean (+SE) PO activity in larval hemolymph between bacterial load treatments (liver without *M. luteus* or with *M.* luteus) and non-immune compromised (white bar) or immune compromised (black bar) treatments. Results of statistical analysis are reported in table 1.

3.2 Experiment 2: Carcass Quality

I determined the effects of carcass age, source of the secretions and brood size on concentration of immune molecules in female parents. There is no difference in female LLA in relation to treatment of fresh versus aged carcasses (table 2). There is a significant difference in female PO activity in relation to treatment (table 2) with greater

PO activity in the aged (LSM 3.793, ±SE 0.183) compared to fresh (LSM 3.221, ±SE 0.182). Female PO activity had a significant interaction effect between treatment and source (table 2). There is a marginally significant increase in female PO activity of hemolymph ($F_{1, 36} = 3.40$, $P = 0.0734$; fig. 4) from the aged compared to fresh treatment but no significant relationships for oral ($F_{1, 36} = 0.46$, $P = 0.5033$; fig. 4) or anal ($F_{1, 36} = 0.17$, $P = 0.6806$; fig. 4) secretions. LLA and PO activity of female hemolymph, oral and anal secretions differ significantly based on the source (table 2). LLA is greater in the oral secretions (LSM 1.563, ±SE 0.056) followed by anal secretions (LSM 0.914, ±SE 0.056), and hemolymph (LSM 0.050, ±SE 0.056). PO activity is greater in the hemolymph (LSM 7.224, ±SE 0.211) followed by oral (LSM 1.750, ±SE 0.211), and anal secretions (LSM 1.545, ±SE 0.211). There is a significant negative relationship between female LLA and brood size (table 2). Female LLA has a marginally significant interaction between brood size and source (table 2). Brood size has a significant negative relationship with female oral secretions ($F_{1, 36} = 5.18$, $P = 0.0218$; fig 5) but no significant relationship with female anal secretions ($F_{1, 36} = 5.18$, $P = 0.1902$) and hemolymph ($F_{1, 36} = 5.18$, $P = 0.4288$). There is no difference in female PO activity based on brood size (table 2).

I determined how parental immune response, age of carcass and brood size interact with larval immune responses of LLA and PO activity in their oral secretions and hemolymph. I found no significant relationship of larval oral LLA with respect to carcass treatment, female factors, or brood size (table 3). The PO activity in the larval oral secretions did have a positive relationship with female anal secretion and hemolymph PO

activity with an interaction effect between anal secretions PO activity and treatment (table 3). There was a significant negative relationship between brood size and larval oral PO activity (table 3; fig 6). The LLA of larval hemolymph had a positive relationship with female hemolymph LLA and brood size (table 3). There was a significant interaction effect of treatment and female hemolymph LLA with LLA in larval hemolymph (table 3). There was a marginally significant interaction effect of treatment and brood size with LLA in larval hemolymph (table 3). No significant relationships were found among factors with PO activity in larval hemolymph (table 3).

Table 2. ANCOVA from Experiment 2 for the effects of carcass treatment (fresh or aged), source (hemolymph, oral or anal secretions), brood size (measured on fourth day of larvae present) and their interactions on LLA and PO activity of females. Bold values are statistically significant.

Response Variable	**Effect**	**Df**	**F-Value**	**p-value**
Female LLA				
	Treatment	109	0.00	0.9983
	Source	45.7	49.07	**<0.0001**
	Brood size	89.6	4.45	**0.0377**
	Treatment*Source	45.7	0.72	0.4914
	Brood size*Treatment	108	0.23	0.6361
	Brood size*Source	45.9	3.05	0.0524
Female PO activity				
	Treatment	98.7	4.90	**0.0292**
	Source	46.1	21.02	**<0.0001**
	Brood size	65.9	0.00	.9871
	Treatment*Source	46.1	5.16	**0.0075**
	Brood size*Treatment	93.9	2.31	0.1321
	Brood size*Source	46.1	1.04	0.3568

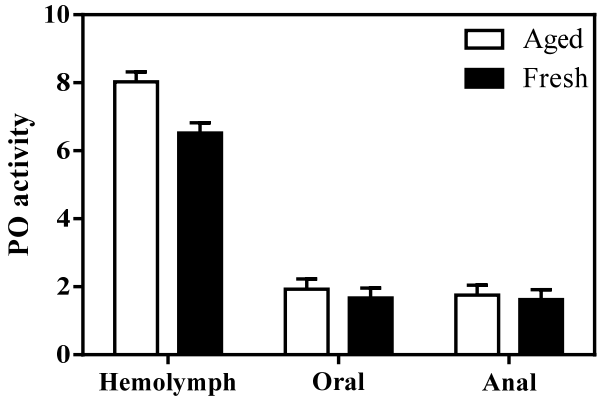

Figure 4. LSM (+SE) for female PO activity in hemolymph, oral and anal secretions from experimental (aged) and control (fresh) treatments. Results of statistical analysis are reported in table 2.

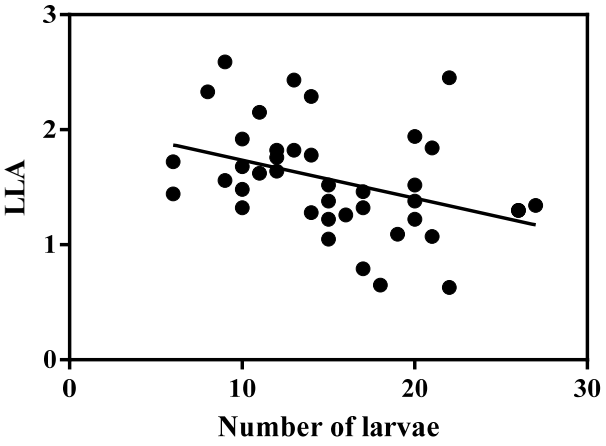

Figure 5. Effect of brood size on LLA of female oral secretions ($F_{1, 36} = 5.18$, $P = 0.0218$).

Table 3. ANCOVA from Experiment 2 for the effects of carcass treatment (aged or fresh) and brood size on larval and female LLA and PO activity from hemolymph, oral and anal secretions and their interaction. Bold values are statistically significant.

Response Variable	Effect	Df	F-Value	p-value
Larval oral LLA				
	Treatment	30	0.52	0.4746
	Female oral LLA	30	1.63	0.2117
	Female anal LLA	30	1.70	0.2028
	Female hemolymph LLA	30	0.55	0.4628
	Brood size	30	0.06	0.8133
	Female oral LLA*Treatment	30	0.01	0.9382
	Brood size*Treatment	30	0.39	0.5353
	Female anal LLA*Treatment	30	1.24	0.2737
	Female hemolymph LLA*Treatment	30	0.28	0.6037
Larval oral PO activity				
	Treatment	30	0.05	0.8257
	Female oral PO activity	30	0.40	0.5342
	Female anal PO activity	30	11.14	**0.0023**
	Female hemolymph PO activity	30	6.21	**0.0185**
	Brood size	30	4.74	**0.0375**
	Female oral PO activity*Treatment	30	0.28	0.5997
	Brood size*Treatment	30	1.51	0.2291
	Female anal PO activity*Treatment	30	6.04	**0.0199**
	Female hemolymph PO activity*Treatment	30	0.00	0.9719
Larval hemolymph LLA				
	Treatment	30	0.31	0.5825
	Female oral LLA	30	0.51	0.4788
	Female anal LLA	30	1.32	0.2599
	Female hemolymph LLA	30	15.69	**0.0004**
	Brood size	30	8.27	**0.0073**

Table 3 continued.

	Female oral LLA*Treatment	30	0.07	0.7886
	Brood size*Treatment	30	3.99	0.0548
	Female anal LLA*Treatment	30	0.19	0.6654
	Female hemolymph LLA*Treatment	30	15.19	**0.0005**
Larval hemolypmh PO activity				
	Treatment	30	0.84	0.3664
	Female oral PO activity	30	0.03	0.8732
	Female anal PO activity	30	0.08	0.7854
	Female hemolymph PO activity	30	0.34	0.5651
	Brood size	30	2.88	0.0999
	Female oral PO activity *Treatment	30	0.31	0.5806
	Brood size*Treatment	30	1.46	0.2361
	Female anal PO activity *Treatment	30	0.24	0.6283
	Female hemolymph PO activity *Treatment	30	1.46	0.2364

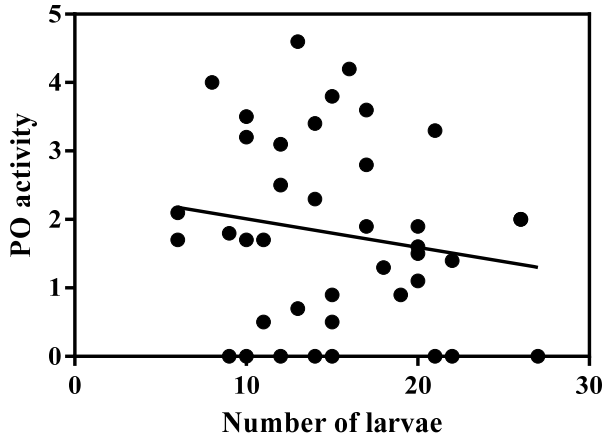

Figure 6. Effect of brood size on PO activity of larval oral secretions. Results of statistical analysis are reported in table 3.

CHAPTER 4: DISCUSSION

The source of LLA in larval oral secretions of the burying beetle *N. orbicollis* could be (a) from their parents as a result of receiving regurgitates or feeding on carrion treated by the parents, or (b) as a result of their own production. Results from Experiment 1 demonstrate the later. In the absence of their parents, larvae in Experiment 1 maintained higher levels of LLA in their oral secretions when exposed to a bacterial signal, while control larvae did not (table 1; fig 2a). In the context of larvae feeding on the carcass, this represents an adaptive social immune response which benefits themselves, their siblings and parents, similar to that of parental secretions. Previous work has demonstrated the presence of LLA in larval exudates but did not clearly demonstrated the source (Arce *et al.* 2013; Reavey *et al.* 2014)

I demonstrate the social immune response observed in Experiment 1, with higher levels of LLA in the high bacterial load treatment, comes as a trade-off with personal immunity (table 1). Greater decline in LLA levels of the larval oral secretions were seen in larvae that were immune compromised (fig 2a). This suggests that when the threat of personal immune challenge through wounding and internal bacterial signal is present, the adaptive production of a social immune response is no longer maintained. This result is consistent with observations in adult *N. vespilloides*, which have decreased LLA of their anal exudates in response to a personal immune challenge (Cotter *et al.* 2013).

PO levels in larval oral secretions decreased less in the immune-compromised larvae than those who were not, independent of bacterial load (table 1; fig 2b). One possible explanation is immune-compromised larvae switch their investment pattern in social immunity after immune compromise with more investment to PO than LLA. This switch in investment is not supported with the lack of response in larval oral PO activity to bacterial load in Experiment 1 (table 1) or fresh versus aged carcass treatments in Experiment 2 (table 3). However, larval oral PO activity did respond by increased investment with decreasing brood size in Experiment 2 (table 3). The larval response to number of participants supports the ability to adapt their investment in PO activity in social immunity. Alternatively, the increase of PO activity in the oral secretions in response to immune challenge (table 1) could be from perceived threat of infection that is directed towards the gut and exuded in secretions. Elevation of midgut PO activity in response to immune challenge has been shown in the damselfly, *Mnais costalis* (Siva-Jothy *et al.* 2001) and African armyworms, *Spodoptera exempta* larvae (Wilson *et al.* 2001).

I found no difference in PO activity of the hemolymph between immune-compromised and control larvae (table 1). However, a melanized plug did form at the site of wounding (Riley, personal observation) during the two hour period between wounding and when the sample was taken. In wound healing, PO is known to have a rapid response which may explain why its levels were no longer elevated at the time of sampling (Cerenius *et al.* 2008). There was marginally less decline in PO activity in larval hemolymph in response to bacterial load (table 1). This could be a response to perceived

increased chance for infection through the intestinal tract which has the most vulnerable physical barrier through which pathogens enter (Siva-Jothy *et al.* 2005).

My results demonstrate that both the female and her offspring cooperate in maintaining the carcass. However, their strategies appear to differ. For females, LLA increased with decreasing brood size but was not affected by treatments (table 2), suggesting a flexible strategy based on number of participants. Data suggests larvae take a different approach. Experiment 1 demonstrates that larvae are capable of reducing the level of LLA in their oral secretions in response to a decreased bacterial signal (table 1). Larval LLA did not differ between treatments (aged vs fresh) or across brood sizes in Experiment 2 (table 3) suggesting a fixed maximum level of effort. However, larval oral PO activity increased with decreasing brood size but was not affected by carcass treatment in Experiment 2 (table 2). This suggests that although larvae have a fixed investment in LLA towards social immunity they do have an adaptive response with PO activity based on participation of others.

In the context of parent-offspring conflict, parents adjust their level of oral LLA investment in response to offspring effort and larvae adjust their level of oral PO activity based on the contribution of siblings. Parent and offspring joint social immunity in burying beetles represents a potentially valuable alternative model system to study parent-offspring conflict. There are costs in mounting a social immune response that may make it beneficial for parents to contribute as little as possible (Cotter *et al.* 2010). However, benefits provided through preparation of a carcass increases fitness through offspring survival (Arce *et al.* 2012; Rozen *et al.* 2008). The ability of larvae to also

contribute through a social immune response affects the strategies for both parents and offspring as observed in my experiments, though further experimentation is needed to determine the consequences of their investment to social immunity.

Experiment 2 results (tables 2 and 3) with parent and offspring investment in social immunity adapting to number of participants could impact optimal brood size. Experimentally increasing brood size of female burying beetles results in decreased fecundity and lifespan (Creighton *et al.* 2009). When more larvae are present than is optimal for a given carcass size burying beetle parents will adjust brood size through filial cannibalism (Bartlett 1987; Creighton 2005). However, when broods are too small individual larval mass is greater but carcass utilization decreases (Müller 1987; Wussler & Müller 1994). My results offer another explanation for smaller broods being suboptimal with parents having to increase effort directed towards social immunity of LLA, and larvae increasing contribution to PO activity in social immunity.

I found no response to carcass treatments in female (table 2) or larval (table 3) LLA or PO activity of secretions. This may be a result of sampling methodology. Samples were collected when the larvae were four days old. This means both the female and larvae had time to respond to any difference between treatments in bacterial load. For females, LLA in anal secretions peaks during carcass preparation prior to arrival of larvae (Cotter *et al.* 2010; Steiger *et al.* 2011). I found that female oral secretions had greater LLA than anal secretions on day four. This may represent transition from treatment of carcass surface to treatment of the carcass interior, provided more through oral secretions (Eggert & Müller 1997; Hoback *et al.* 2005). One day old larvae in *N. vespilloides* have

higher LLA in their exudates with LLA decreasing over time (Arce *et al.* 2013; Reavey *et al.* 2014). This change may reflect decreasing bacterial load over this time period. Further studies examining the change in both parent and larval secretions throughout the breeding cycle may establish the potential effect of carcass quality on secretion levels.

Costs to personal immunity of parental females measured in their hemolymph showed marginally significant change between treatments in PO activity with higher values when on aged carcasses (fig 4). This corresponds with females' overall increase in PO activity of hemolymph, oral and anal secretions on the aged compared to fresh carcass treatments (table 2). This increase could be a response to increased bacterial load or opportunistic nematode infection. Honey bee larvae, *Apis mellifera*, have increased PO activity in their hemolymph in response to *Paenibacillus larvae* infection (Chan *et al.* 2009) and various insect species show increased PO response with nematode infection (Castillo *et al.* 2011).

In conclusion, my results show that *N. orbicollis* larvae have antimicrobial oral secretions that are an adaptive social immune response to microbial challenge. I also determined a direct trade-off with social and personal immunity in the larvae. Female parents and larvae were determined to adjust their social immune investment based on brood size. Investigation of the conflict between parental and larval social immune investment should be a focus of future work.

REFERENCES

REFERENCES

Adamo, S. A. (2004). Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis. Journal of Insect Physiology,* 50, 209-216.

Arce, A. N., Johnston, P. R. Smiseth, P. T., & Rozen, D. E. (2012). Mechanisms and fitness effects of antibacterial defences in a carrion beetle. *Journal of Evolutionary Biology,* 25, 930-937.

Arce, A. N., Johnston, P. R. Smiseth, P. T., & Rozen, D. E. (2013). Antimicrobial secretions and social immunity in larval burying beetles, *Nicrophorus vespilloides*. *Animal Behaviour,* 86(4), 741-745.

Bartlett, J. (1987). Filial cannibalism in burying beetles. *Behavioral Ecology and Sociobiology,* 21, 179-183.

Bartlett, J., & Ashworth, C. M. (1988). Brood size and fitness in *Nicrophorus vespilloides* (Coleoptera: Silphidae). *Behavioral Ecology and Sociobiology,* 22, 429-434.

Castillo, J. C., Reynolds, S. E., & Eleftherianos, I. (2011). Insect immune responses to nematode parasites. *Trends in Parasitology*, 27(12), 537-547.

Cerenius, L., Lee, B. L., & Söderhäll, K. (2008). The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology,* 29(6), 263-271.

Chan, Q. W., Melathopoulos, A. P., Pernal, S. F., & Foster, L. J. (2009) The innate immune and systemic response in honey bees to a bacterial pathogen, *Paenibacillus larvae. BioMed Central Genomics*, 10(1), 387.

Cotter, S. C. & Kilner, R. M. (2010a). Personal immunity versus social immunity. *Behavioral Ecology,* 21(4), 663-668.

Cotter, S. C. & Kilner, R. M. (2010b). Sexual division of antibacterial resource defence in breeding burying beetles, *Nicrophorus vespilloides. Journal of Animal Ecology,* 79, 35-43.

Cotter, S. C., Littlefair, J. E., Grantham, P. J, & Kilner, R. M. (2013). A direct physiological trade-off between personal and social immunity. *Journal of Animal Ecology,* 82(4), 846-853.

Cotter, S. C., Topham, E., Price, A. J. P., & Kilner, R. M. (2010). Fitness costs associated with mounting a social immune response. *Ecology Letters,* 13, 1114-1123.

Creighton, J. C. (2005). Population density, body size, and phenotypic plasticity of brood size in a burying beetle. *Behavioral Ecology,* 16, 1031-1036.

Creighton, J. C., Heflin, N. D., & Belk, M. C. (2009). Cost of reproduction, resource quality, and terminal investment in a burying beetle. *The American Naturalist,* 174(5), 673-684.

Eggert, A.-K., & Müller, J. K. (1997). Biparental care and social evolution in burying beetles: lessons from the larder. In: The evolution of social behavior in insects and arachnids (Choe, J. C., Crespi, B. J., eds). Cambridge: *Cambridge University Press*, 216-236.

Flatt, T., Heyland, A., Rus, F., Porpiglia, E., Sherlock, C., Yamamoto, R., Garbuzov, A., Palli, S.R., Tatar, M., & Silverman, N. (2008). Hormonal regulation of the humoral innate immune response in *Drosophila melanogaster. The Journal of Experimental Biology,* 211, 2712-2724.

Hamilton, C., Lejeune, B. T., & Rosengaus, R. B. (2011). Trophallaxis and prophylaxis: Social immunity in the carpenter ant *Camponotus pennsylvanicus. Biology Letters,* 7, 89-92.

Hoback, W. W., Bishop, A. A., Kroemer, J., Scalzitti, J., & Shaffer, J. J. (2004). Differences among antimicrobial properties of carrion beetle secretions reflect phylogeny and ecology. *Journal of Chemical Ecology,* 30(4), 719-729.

Kalinová, B., Podskalská, J., Růžička, J, & Hoskovec, M. (2009). Irresistible bouquet of death how are burying beetles (Coleoptera: Silphidae:Nicrophorus) attracted by carcasses. *Naturwissenschaften,* 96(8), 889-899.

Milne, L. J. & Milne, M. (1976). The social behavior of burying beetles. *Scientific American,* 235, 84-89.

Moret, Y. & Schmid-Hempel, P. (2000). Survival for immunity: The price of immune system activation for bumblebee workers. *Science,* 290, 1166-1168.

Müller, J. K. (1987). Replacement of a lost clutch: A strategy for optimal resource utilization in *Necrophorus vespilloides* (Coleoptera: Silphidae). *Ethology,* 76, 74-80.

Müller, J. K., Eggert, A.-K., & Dressel, J. (1990). Intraspecific brood parasitism in the burying beetle *Necrophorus vespilloides* (Coleoptera: Silphidae). *Animal Behavior,* 40, 491-499.

Pukowski, E. (1933). Ökologische untersuchungen an *Necrophorus*. *Zeitschrift für Morphologie und Ökologie der Tiere*, 27, 518-86.

Reavey, C. E., Beare, L., & Cotter, S. C. (2014). Parental care influences social immunity in burying beetle larvae. *Ecological Entomology,* DOI: 10.1111/een.12099.

Rozen, D. E., Engelmoer, D. J. P., & Smiseth, P. T. (2008). Antimicrobial strategies in burying beetles breeding on carrion. *Proceedings of the National Academy of Sciences,* 105(46), 17890-17895.

Scharf, I., Modlmeier, A. P., Beros, S., & Foitzik, S. (2012). Ant societies buffer individual-level effects of parasite infections. *The American Naturalist*, 180(5), 671-683.

Simmons, L. W. (2011). Resource allocation trade-off between sperm quality and immunity in the field cricket, *Teleogryllus oceanicus*. *Behavioral Ecology,* 23, 168-173.

Siva-Jothy, M. T., Moret, T., & Rolff, J. (2005). Insect immunity: An evolutionary ecology perspective. *Advances in Insect Physiology,* 32, 1-47.

Siva-Jothy, M. T., Tsubaki, Y., Hooper, R. E., & Plaistow, S. J. (2001). Investment in immune function under chronic and acute immune challenge in an insect. *Physiological Entomology,* 26, 1-5.

Steiger, S., Gershman, S. N., Pettinger, A. M., Eggert, A.-K., & Sakaluk, S. K. (2011). Sex differences in immunity and rapid upregulation of immune defence during parental care in the burying beetle, *Nicrophorus orbicollis*. *Functional Ecology,* 25, 1368-1378.

Turillazzi, S., Perito, B., Pazzali, L., Pantera, B., Gurfer, S., & Tancredi, M. (2004). Antibacterial activity of larval saliva of the European paper wasp *Polistes dominulus* (Hymenoptera, Vespidae). *Insectes Sociaux,* 51, 339-341.

Traniello, J. F. A., Rosengaus, R. B., & Savoie, K. (2002). The development of immunity in a social insect: Evidence for the group facilitation of disease resistance. *Proceedings of the National Academy of Sciences,* 99(10), 6838-6842.

Trumbo, S. T. (2009). Age-related reproductive performance in the parental burying beetle, *Nicrophorus orbicollis*. *Behavioral Ecology*, 20, 951-956.

Vogelweith, F., Thiery, D., Moret, Y., & Moreau, J. (2013). Immunocompetence increases with larval body size in a phytophagous moth. *Physiological Entomology,* 38, 219-225.

Wang, Z., Zhang, S., Tong, Z., Li, L., & Wang, G. (2009). Maternal transfer and protective role of the alternative complement components in zebrafish *Danio rerio*. *PLoS ONE,* 4(2), e4498. doi:10.1317/journal.pone.0004498.

Wilson, K., Cotter, S. C., Reeson, A. F., & Pell, J. K. (2001). Melanism and disease resistance in insects. *Ecology Letters*, 4, 637-649.

Wussler, G. L. & Müller, J. K. (1994). Optimal family planning through infanticide? *Verhandlungen der Deutschen Zoologischen Gesellschaft,* 87, 66.

Zanchi, C., Troussard, J.-P., Moreau, J., & Moret, Y. (2012). Relationship between maternal transfer of immunity and mother fecundity in an insect. *Proceedings of the Royal Society Biological Sciences,* 279, 3223-3230.

Zuk, M. & Stoehr, A. M. (2002). Immune defense and host life history. *The American Naturalist,* 160, S9-S22.