DIVERSE CONTRIBUTIONS TO EVOLUTIONARY KNOWLEDGE: INVESTIGATIONS OF SOCIAL IMMUNITY IN FAMILY LIFE AND COLLECTIVE KNOWLEDGE FOR TEACHING EVOLUTION

by

MICHELLE A. ZIADIE

(Under the Direction of Tessa Andrews and Allen Moore)

ABSTRACT

My dissertation is unique in that it is split into two fields: Genetics and Discipline-Based Education Research (DBER). My genetics work investigates the role of immune function in the evolution of parental care by looking at social immunity in beetles. Social immunity moderates the spread of pathogens in social groups and is especially likely in groups structured by genetic relatedness. The extent to which specific immune pathways are used is unknown. My work investigates the expression and social role of three functionally separate immune genes (pgrp-sc2, thaumatin, and defensin) during parental care in the beetle Nicrophorus vespilloides. These genes reside in different immune pathways, allowing me to test whether specific components of the immune system are targeted for social immunity. I develop this work by further investigating how changes in social environment, specifically family size, influence expression of social immunity in mothers and offspring. This project expands the concept of social immunity in the family beyond parental care by manipulating the extent of conflict between parents and offspring and among siblings and examining contributions to social immunity. In my DBER work I explore an essential type of instructor knowledge called pedagogical content knowledge (PCK) as a grounding framework to identify and analyze the kind of resources instructors need to help develop this critical knowledge base. An instructor's ability to facilitate student learning is influenced by their PCK, which is topic- specific knowledge for teaching and learning. My dissertation work demonstrates the utility of PCK as a framework for analyzing existing research by identifying gaps in the collective knowledge and proposing research priorities for maximum impact on evolution education practices. I collected and analyzed over 300 peer-reviewed publications. From my analyses I propose priorities for the research community and generate an open-access searchable database for instructors.

INDEX WORDS: Evolution, Education, Sociality, Parental care, Instructor knowledge, Gene expression, dual-thesis dissertation

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DEDICATION

For my students, past, present, and future.

I promise to continue to improve myself so that I can better support your learning and growth.

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CHAPTER 1

INTRODUCTION

My dissertation is unique in that it is split into two fields: Genetics and Discipline-Based Education Research (DBER). I arrived at UGA Genetics with the specific intention of being dually trained in classical science and science education research. I wanted to be trained in biology to satisfy my personal need to engage in the field and to acquire research expertise that I can share with my future students. It was equally important to me to be trained as a discipline-based education researcher so that I can contribute to knowledge that will influence the way students learn about evolution across the country and around the world.

My genetics work investigates the role of immune function in the evolution of parental care by looking at social immunity in beetles. Social immunity moderates the spread of pathogens in social groups and is especially likely in groups structured by genetic relatedness. The extent to which specific immune pathways are used is unknown. My work investigates the expression and social role of three functionally separate immune genes (pgrp-sc2, thaumatin, and defensin) during parental care in the beetle *Nicrophorus vespilloides*. These genes reside in different immune pathways, allowing me to test whether specific components of the immune system are targeted for social immunity. I develop this work by further investigating how changes in social environment, specifically family size, influence expression of social immunity in mothers and offspring. This project expands the concept of social immunity in the family

beyond parental care by manipulating the extent of conflict between parents and offspring and among siblings and examining contributions to social immunity. I make very specific predictions about how my manipulations would affect social immunity based on social theory and previous work in social immunity. My predictions are not met, revealing previously unknown constraints to social immunity in the context of family life.

In my DBER work I explore an essential type of instructor knowledge called pedagogical content knowledge (PCK) as a grounding framework to identify and analyze the kind of resources instructors need to help develop this critical knowledge base. An instructor's ability to facilitate student learning is influenced by their PCK, which is topic- specific knowledge for teaching and learning. My dissertation work aims to demonstrate the utility of PCK as a framework for analyzing existing research by identifying gaps in the collective knowledge and proposing research priorities for maximum impact on evolution education practices. Another aim of this research is to identify the collective knowledge that is available to undergraduate evolution instructors and to make that knowledge more accessible. I collected and analyzed over 300 peer-reviewed publications. From my analyses I propose priorities for the research community and generate an open-access searchable database for instructors.

The details of my dissertation work can be found in the following chapters and in the peer-review literature where those chapters are published. What is not immediately apparent is the intellectual and professional growth of the author.

In completing a dual thesis dissertation, I had to learn, first and foremost, time and project management. I had to balance the demands of each of my projects and thesis advisors in addition to completing my coursework, supervising undergraduates in the lab and teaching recitation sections. I struggled with this balance quite a bit at the beginning of my graduate

career. I often thought I would fail. My supervisors were firm, but supportive. They helped me to see that, given my unique situation, I had to learn time management faster than my peers and it has contributed greatly to my success. I also learned that completing my doctorate was going to be a collaborative effort between my advisors and myself, but that I was ultimately in control of my graduate career. This realization has empowered me throughout my degree and has motivated me to learn, achieve, and actively pursue opportunities for professional development.

I have learned the value of writing, as a form of science communication and as a tool for learning. Within weeks of arriving I began writing a National Science Foundation (NSF) Graduate Research Fellowship Proposal (GRFP) for my DBER work. It was to be first of many vigorous collaborative writing endeavors between my supervisors and myself. After two and a half months of writing, rewriting, rereading the literature, and arguing with my supervisor the proposal was submitted. Shortly thereafter I had to write another proposal, this time for my genetics research, as a part of my qualifying exam. Both proposals were successful– I was awarded the fellowship and I passed my qualifying exam. I also learned that the process of writing is just as valuable as the end product. Writing allowed me to explore and develop new ideas. While writing I could easily identify the limits of my knowledge. I was learning to be a better writer and a better scientific thinker at the same time. I therefore sought opportunities to develop as a writer. I joined a local science blog (Athens Science Observer), gaining experience writing science for the general public. I applied for funding from various sources, each time learning how to write for a slightly different, educated audience. I wrote and published manuscripts, learning how to communicate my research in two different fields. Writing has been critical to the development of my career and to my development as a scientist.

In my time as a graduate student I have been a teaching assistant (TA) for the introductory genetics course and evolution lab. As a TA I have had the opportunity to use what I have learned in my research to inform my teaching. My experience with DBER has taught me that effective instructors rely on specialized knowledge of teaching. Most of this knowledge is gained through experience, but some can be gleaned from the literature. As a TA I have been able to use what I have learned in the DBER literature to create a more dynamic learning experience for my students. Furthermore, my experience with DBER has made me more thoughtful and reflective of my own teaching and has enhanced my ability to construct valuable instructor knowledge. My genetics research has also informed my teaching. Many of the concepts and research techniques we discussed in class are ones I have had to think very carefully about and grapple with in my work. Because of this, I have a perspective that is somewhere between that of a novice and that of an expert. This allows me to relate to my students better and to bridge knowledge gaps that I otherwise might not recognize. I am grateful for my opportunities to teach, and they have reinforced my interest in undergraduate education. As I continue to develop as an educator, I look forward to applying the knowledge I have gained from my unique dual research experience.

While my experience with students in the classroom has been limited, my experience mentoring students has been bountiful. Over the course of five years I have mentored eight students in two different fields across three different projects. These students have come from diverse cultural, social, and educational backgrounds and some have been first in their families to attend university. I have guided these students as they learn to read scientific literature, design experiments, and present their work. I have supported them as they published papers, graduated, won scholarships, and applied to graduate school. Most importantly I have had the privilege of

watching students gain confidence in their ability to think and do and *be* science. It wasn't until I started graduate school that I really began to reflect on the challenges I faced as a woman of color pursuing a career in the sciences. My experience as a mentor has further allowed me to reflect on these challenges, recognize their absurdity, and push past them. I began to realize that I am not just a research mentor, but a role model and I take that title very seriously. Mentorship has been the best part of my graduate career and easily what I see as my most valuable contribution. Moving forward I hope to continue to build on my experience as a mentor, particularly (but not exclusively) for women and minorities.

Moving forward I would also like to expand on my research expertise, with the specific intention of ultimately being able to develop a research program that is amenable to undergraduate research and education. I came to graduate school with very little research experience. Throughout my time as a graduate student I have enjoyed building and completing a research project from research question to publication and I have learned the value of research in an educational setting. I would also like to expand on my teaching, specifically by gaining experience in curriculum development and implementation. Ultimately, I would like to combine what I have learned in my research and teaching to secure a faculty position at a small, primarily undergraduate institution.

CHAPTER 2

EVOLUTION OF PERSONAL AND SOCIAL IMMUNITY IN THE CONTEXT OF PARENTAL CARE[1]

[1] Ziadie, M. A., F. Ebot-Ojong, E. C. McKinney, A. J. Moore. (2019) The evolution of personal and social immunity in the context of parental care. American Naturalist, 193(2). Reprinted here with permission from the publisher

Abstract: Social immunity moderates the spread of pathogens in social groups and is especially likely in groups structured by genetic relatedness. The extent to which specific immune pathways are used is unknown. Here, we investigate the expression and social role of three functionally separate immune genes (*pgrp-sc2, thaumatin,* and *defensin*) during parental care in the beetle *Nicrophorus vespilloides.* These genes reside in different immune pathways, allowing us to test if specific components of the immune system are targeted for social immunity. To test for the evolution of specificity we manipulated the influence of social context and timing on gene expression and quantified the covariance of maternal immune gene expression and offspring fitness. Larvae reduced expression of all three genes in the presence of parents. Parental *pgrp-sc2* and *thaumatin* increased during direct parenting, while *defensin* was upregulated before larvae arrived. Parental expression of *pgrp-sc2* and *thaumatin* responded similarly to experimental manipulation of timing and presence of larvae, which differed from the response of *defensin.* We found a positive covariance between maternal expression and offspring fitness for *pgrp-sc2* and *thaumatin*, but not *defensin*. We suggest that social immunity can involve specific genes and pathways, which reflects evolution as an interacting phenotype during parenting.

Introduction

One of the major transitions in evolutionary history is the shift from solitary to social living (Szathmary and Maynard Smith 1995). Living in groups can afford benefits such as increased vigilance and enhanced foraging (Alexander 1974). However, sociality also engenders costs. One potential cost of group living is increased risk of pathogen infection (Meunier 2015; Cremer et al. 2018). Frequent and intimate interactions between individuals facilitates exposure and transmission, especially within animal families where high genetic relatedness makes group members more susceptible to the same pathogens (Meunier 2015; Cremer et al. 2018). Social immunity is one adaptive mechanism that helps to alleviate the risks of pathogen infection in group living individuals.

Unlike personal immunity, where increased immune function evolves to solely benefit the focal individual, social immunity describes an immune response mounted by an individual that evolves for and is maintained by the fitness benefit it provides to others (Cotter and Kilner 2010a). Social immunity is therefore predicted to evolve in animal families, particularly in the context of parental care where offspring fitness is often entirely dependent on parental contribution (Cotter and Kilner 2010a).While the costs of mounting an immune response can be high (Cotter et al. 2004; Cotter et al. 2010), failing to provide an immune defense for immunologically naïve offspring can result in reduced offspring fitness and survival which ultimately negatively impacts the parent's inclusive fitness (Cardoza et al. 2006; Rozen et al. 2008; Lam et al. 2009; Arida et al. 2012; Cotter et al. 2013). Provisioned immunity of any kind may also reflect parental investment; the two are not mutually exclusive (Cotter and Kilner 2010a). However. if social immunity is evolving we expect to see different patterns of selection,

including maternal selection, as the cross-generational effects of social immunity result in indirect genetic effects (Grindstaff et al. 2003).

Although social and personal immunity evolve through different selective pressures, it is often difficult to distinguish between the two, especially without manipulative studies (Cotter and Kilner 2010a; Meunier 2015; Cremer et al. 2018). A personal immune response increases the chance of survival for the individual mounting the response and thus the target of selection is the personal immunity. Other individuals may receive some immune benefit, perhaps as a by-product of being in close proximity to the focal individual; however, the immune response that is mounted is determined by the personal fitness of the individual mounting the response (Cotter and Kilner 2010a). In contrast, social immunity describes any collective or personal immune response that is directly selected to increase the fitness of other members of the group and may be in excess of what is required for personal immunity (Meunier 2015; Cremer et al. 2018). Social immunity evolves through kin or social selection (Cotter and Kilner 2010a). Thus, a minimal experimental demonstration of social immunity in the context of parental care requires evidence that offspring are relieved of mounting a full immune response, that parents upregulate their immune response in response to offspring beyond that required for personal benefit, and that the upregulation of parental immunity positively influences offspring fitness.

In this study we examine the social role of three immune genes in the burying beetle, *Nicrophorus vespilloides.* Burying beetles reproduce and care for young inside a vertebrate carcass that they manipulate to reduce decay; although it is not a sterile environment, the carcass is not rotting (Eggert and Müller 1997; Scott 1998, Duarte et al. 2017). Together or separately, parents process the carcass by stripping it of feathers or fur, coating it in anti-microbial peptides, forming it into a ball, and burying it to be used as a food resource for developing offspring

(Eggert and Müller 1997; Scott 1998). Larvae hatch in the soil and crawl to the carcass where parents regurgitate predigested carcass to their offspring. Social immunity is predicted to evolve in burying beetles and has been shown in studies that examine the social role of anti-microbial secretions in *N. vespilloides* (Cotter and Kilner 2010b; Vogel et al. 2011; Arce et al. 2012, 2013; McLean et al. 2014; Reavey et al. 2014a,b, 2015; Palmer et al. 2016; Duarte et al. 2018). However, the social implications of other parental immune responses have yet to be confirmed.

We ask if expression of different immune pathways during parental care all evolve to provide social immunity in *N. vespilloides*. Our approach builds from an earlier RNA-seq study that documented transcriptomic differences in *N. vespilloides* before, during, and after parental care (Parker et al. 2015). Those involved in immune function are among the genes most strongly differentially upregulated during parenting. Given the microbial and pathogen-rich environment which attract adult burying beetles for reproduction (Fialho et al. 2018), and upon which they live for several days during parenting, it is unsurprising that adults increase their immune response. However, we predict that the differential expression we see in immune genes during parenting is more extreme than needed for personal immunity and reflects social immunity. To test this, we examine the expression of *pgrp-sc2*, *thaumatin*, and *defensin* under natural and manipulative conditions.

The three immune genes we investigate here have different functional targets and are expressed in different immune pathways. The first is a peptidoglycan recognition protein (*pgrp-SC2*) which responds to gram-negative bacteria in the gut and provides protection against over-activation of the immune system in the Imd immune pathway (Broderick et al. 2009; Paredes et al. 2011, Guo et al. 2014). The second is an anti-fungal peptide (*thaumatin)* which is found in a limited number of species of insects and is involved in the Toll immune pathway localized in the

haemolymph and fat bodies (Broderick et al. 2009; Mylonakis et al. 2016). The third is an anti-microbial peptide (*defensin*) and is a common insect defense in the Toll immune pathway against gram-positive bacteria and is also localized in the hemolymph and fat bodies (Hoffmann and Hetru 1992; Broderick et al. 2009; Mylonakis et al. 2016).

We used an integrated series of experiments that together allow us to infer whether changes in gene expression are related to social immunity rather than personal immunity, testing for the three conditions we expect for social immunity during parenting. We first examined immune gene expression in offspring in the presence or absence of parental care. We then quantified changes in maternal gene expression associated with different stages in the transition to parenting. We next manipulated timing and presence of offspring to further partition social and environmental influences on maternal immune gene expression. Finally, we examined the covariance between maternal gene expression and components of offspring performance and fitness by measuring the maternal selection component (Kirkpatrick and Lande 1989). Our results support a social role for *pgrp-sc2* and *thaumatin*, but not for *defensin*. We argue that social immunity is more likely to evolve where interacting phenotypes are involved, such as during parent-offspring interactions, and thus its evolution is facilitated by the resulting social or kin selection.

Materials and Methods

Husbandry

Nicrophorus vespilloides used in these experiments were collected from the wild in Cornwall, UK and maintained as an outbred colony since 2011. Each summer new beetles are collected and interbred into the colony. Stock beetles are randomly mated with unrelated

individuals each generation. The colony is maintained with over 50 families. Each generation, an unrelated male and female are paired in a mating box (17.2 x 12.7 x 6.4 cm), allowed to mate and prepare a mouse carcass for offspring rearing, and adults are left with the offspring until parenting ceases and larvae disperse from the area where the carcass was consumed (fig. 1.1). At dispersal, we place larvae into individual plastic containers (9 cm diameter, 4 cm deep) filled halfway with moist soil. These individuals develop to pupae, then adults, in this container. After adult emergence, we feed beetles ad libitum organic ground beef once a week.

We used adult beetles 14-21 days post-eclosion for all experiments. In these experiments, we removed the male after the carcass was prepared and before the larvae arrived, so that all experiments involved uniparental female care. We focused on females because they remain with the brood for longer than males when both are present (Parker et al. 2015), and consistently rear broods as successfully as biparental females (Scott 1998; Parker et al. 2015). Females also invest more in antimicrobial defenses than do males (Cotter and Kilner 2010b).

Gene Expression under Manipulations of Social Context

We first sought to determine if there was any direct association between larval immune gene expression and parental immune gene expression. To do this, we collected larvae that were either parented (collected in the presence of parents) or not parented (isolated from parents by removing the parent), thus experimentally creating conditions where parental care and potential parental immunity was present or absent. In all experimental treatments, we paired males and females in a mating box filled halfway with moist soil and a fresh mouse carcass and then removed the males at 48 h. In half of the samples, we removed the female at 72 h, which reflects a time point after the carcass has been fully prepared for larval arrival but before the larvae hatch and arrive (fig. 1.1). In the other half of the samples females were left to parent newly arrived

larvae. We collected larval samples 24 hours after they arrived at the carcass. We pooled 4 larvae from each brood as a single sample; the sample size (N= 10 per treatment) reflects the number of families sampled. We placed all four of the larvae in a single tube and immediately froze the samples in liquid nitrogen to preserve tissues.

Next, we clarified the timing of the immune gene response over the reproductive bout to determine if the immune response was implicated in social immunity. Previous work by Parker et al. (2015) indicates that expression of immune genes *pgrp-sc2, thaumatin,* and *defensin* increases in adult beetles during parental care compared to virgins (supplemental figs. 9, 10 in Parker et al. 2015). This research therefore inspired the present study. Here, as in Parker et al. (2015), we collected samples of whole heads, which includes fat body as well as brain tissue (N = 10 per treatment). The expression of these genes is highly correlated in head and abdomen samples (*pgrp-sc2* $r = 0.57$, $N = 30$, $p = 0.001$; *thaumatin* $r = 0.53$, $N = 30$, $p = 0.0026$; *defensin* $r = 0.43$, $N = 30$, $p = 0.0177$).

We collected samples from females experiencing five different social conditions that represent key time points in the reproductive cycle: virgin, mated with no resources for reproduction, mated and preparing resources for reproduction, actively engaged in parental care, and post-care. These conditions reflect relevant behavioral and physiological changes in the reproductive cycle (fig. 1.1) and where gene expression changes that are associated with a transition to parenting have been examined in different contexts (e.g. Roy-Zokan et al. 2015; Cunningham et al. 2016; Mehlferber et al. 2017).

We kept 'virgin' females in isolation until frozen at -80 C for expression analysis. Virgins serve as a negative control; this state represents a behaviorally, socially and physiologically neutral, baseline state. 'Mated' females provide a sample of socialized but not

reproductively active beetles– mated females do not begin to prepare for reproduction without a suitable resource (Trumbo 1997). For this social condition, we paired males and females in a mating box filled halfway with moist soil without a resource (mouse carcass). We allowed the pair to interact for 48 h, during which time mating occurs repeatedly (House et al. 2008), before freezing females for expression analysis. The 'resource preparation' individuals were allowed to prepare a carcass for 48 h but were otherwise treated the same. The presence of the mouse carcass stimulates ovarian development in females and indirect parental care in the form of carcass preparation and maintenance (Trumbo 1997). We collected and froze females at 48 h post-pairing for later analysis. Females in the 'parental care' sample were collected at a time point where a switch to direct care of larvae had occurred, which includes feeding and grooming along with carcass maintenance. For this social condition, we paired males and females in a mating box filled halfway with moist soil and a fresh mouse carcass. After 48 h we removed the male. We collected females for analysis 24 h after the larvae arrived (approx. 96 h post-pairing) and when they were observed directly interacting with the larvae. This social condition also served as a positive control as previous work by Parker et al. (2015) shows that there is an increase in immune gene expression at this point in the reproductive cycle. Females in the 'post-care' sample were collected and frozen approximately five days after the offspring arrived, when parenting is complete and the offspring and parent had fully dispersed from the carcass. For this social condition, we removed females from the mating box after they abandoned their carcass. We kept these females in individual containers for at least 24 h before collecting them for analysis, to allow them to return to a non-caring state. We immediately froze the sampled tissues (whole heads) in liquid nitrogen at the time of collection.

In the final experiment manipulating social context, we examined timing and expression. The resource in which *N. vespilloides* reproduce and provision to their offspring is microbe- and pathogen-rich (Fialho et al. 2018), and so it is possible that any increase in immune response observed during parental care is a personal immune response to the amount of time that parent(s) are exposed to microbes on the carcass rather than a response evolved to directly benefit their offspring. We therefore further manipulated timing and social environment to decouple these potential influences on maternal immune gene expression. This experiment had three treatments: females left 72 h on a carcass without larvae (72/-), 96 h on a carcass without larvae (96/-), and 96 h on a carcass with larvae (96/+). The latter time point is when larvae would normally be present (fig. 1.1). To achieve these treatments, we transferred females with their mouse carcasses into new boxes with fresh soil at 48 h, after the females had laid their eggs. We kept all original mating boxes and soil with eggs for observation. There is no apparent disruption of maternal behavior by moving females; all females continued to prepare their mouse carcass. Upon hatching, we transferred larvae from mothers in the 96/+ treatment to their respective carcasses for parental care. The first two treatments (72/-, 96/-) allow us to examine differences in immune gene expression due to timing, controlling for social environment. The final two conditions (96/-, 96/+) allow us to examine the differences in immune gene expression due to social environment, controlling for time exposed to carcass. We collected females at their respective time points (72 h post-pairing or 96 h post-pairing) and immediately froze the sampled tissues (whole heads) in liquid nitrogen.

Maternal Selection

While the first set of experiments suggests social roles of *pgrp-sc2, thaumatin,* and *defensin,* direct support requires evidence that offspring fitness covaries with the maternal trait.

We therefore conducted an experiment to determine the impact of maternal immune gene expression on offspring fitness, which allows calculation of the resulting maternal selection components.

For this experiment, we collected maternal gene expression and larval fitness and performance data from 111 families. We paired males and females in a mating box filled halfway with moist soil and a fresh mouse carcass. After 48 h on the carcass during the preparation phase, we removed the male. Females were then allowed to complete carcass preparation and lay eggs. Once larvae arrived, females cared for these offspring. We collected females for analysis between 48-72 h after the larvae arrived, when begging to be fed declines (Smiseth et al. 2003; fig. 1). We immediately froze the female sample tissues (whole heads) in liquid nitrogen at the time of collection.

We weighed each larva (N=1323) at dispersal, before putting them in individual containers filled halfway with moist soil. Dispersal mass was used as a measure of offspring performance (Lock et al. 2004), as larger individuals are more competitive for resources as adults. Larvae do not feed after dispersal and so adult size and mass is largely determined by dispersal mass. For fitness measures, we monitored larvae in these containers to determine offspring survival to pupae and to adult eclosion. Survival was determined by visual inspection: healthy pupae move and are pale yellow while larvae that have not survived to pupae are dark brown or black.

Quantification of Gene Expression

We immediately froze the sampled tissues in liquid nitrogen at the time of collection and stored each sample at -80 °C until RNA extraction. We extracted RNA from all samples using a Qiagen RNAeasy Lipid kit (Qiagen, Venlo, The Netherlands) per manufacturer's instructions.

Frozen samples were initially homogenized in 500 μL of Qiazol in a mortar chilled with liquid nitrogen. We quantified RNA in 1: 10 dilutions using a Qubit 2.0 florometer (Invitrogen Corporation, Carlsbad, CA, USA) and synthesized cDNA from 500 ng of RNA using Quanta Bioscience qScript reverse transcriptase master mix following manufacturer's instructions.

We quantified immune gene mRNA levels in three technical replicates for each biological sample by quantitative real-time PCR (qRT-PCR) using a Roche LightCycler 480 platform. We used a 10 μL reaction containing 2 μL of 1 :10 diluted cDNA, 5 μL of SYBR I Green Master Mix at a 60°C annealing temperature and 3 μL of a primer stock containing both sense and antisense primers at 2.67 μM. We designed qRT-PCR primers for each gene using PrimerQuest (Integrated DNA Technologies) with the following sequences: DefensinrtSense (TACGGTTCCGTCAACCATTC); DefensinrtAnti (CAATTGCAGACTCCGTCGAT); PGRP-SC2rtSense (CGAAGGTCAAGGTTGGGGTA); PGRP-SC2rtAnti (GTTCCGATGACACAGATGCC); ThaumatinrtSense (GAATCCGCCGCCTTCCAAT); ThaumatinrtAnti (ACTATTCTTGGGTGCGGCTCA). We used TATA-binding protein and alpha-tubulin as endogenous reference genes for all experiments, following MIQE guidelines; see Cunningham et al. (2014).

Statistical Analyses

Expression was measured quantitatively using qRT-PCR. For larval expression, changes across the transition from pre-parenting to parenting, and manipulation of larval and timing experiments we used the $\Delta\Delta C_T$ method to convert raw expression data to normalized expression values for analysis, with the two reference genes listed above (Livak and Schmittgen 2001). We graphed relative expression data to visually inspect the distribution. Based on this, we analyzed

log-transformed data, which were all normally distributed, and present these in the graphical displays.

We used a t-test to examine larval immune expression in the presence and absence of parental care for each of the three genes tested. For changes in immune expression over the development of parenting we tested the overall ANOVA model to examine how maternal gene expression changed before, during and after parenting. We then used Dunnett's test to test for pairwise comparisons, with samples compared to the two controls defined *a-priori*; the virgin social condition served as a negative control and the parental care social condition served as a positive control. Therefore, we report the two Dunnett's tests, one with pairwise comparisons of each social conditions to the negative control and one with pairwise comparisons of each social condition to the positive control. For the comparison of time versus social exposure, we report pairwise *a-priori* contrasts using Fisher's LSD. For this experiment, we focused on two statistical comparisons: 72/- compared to 96/- and 96/- compared to 96/+. We conducted all analyses using JMP Pro (v.13.0.0).

We calculated maternal selection components (Kirkpatrick and Lande 1989) by simple linear regression of maternal gene expression on offspring relative fitness or offspring performance (Lande and Arnold 1983) to quantify the covariance between gene expression in mothers and effects on their offspring. As our goal was to demonstrate the potential for mothers to influence their offspring, the experiment was designed to estimate only the maternal selection component and not the individual selection that might also act on offspring (Kirkpatrick and Lande 1989). We quantified expression as ΔC (i.e., relative to expression of reference gene) and then standardized to a mean of zero and a standard deviation of one, with higher expression positive and lower expression negative. We then fit a linear regression to estimate the

standardized maternal linear selection component (β_m) for each maternal immune gene (*pgrp-sc2, thaumatin, defensin)* and each measure of offspring performance (individual larval mass at dispersal) or component of offspring fitness (survival to pupae, survival to adult). We found no effect of female size or age, or carcass mass on maternal expression or offspring fitness.

Results

Gene Expression under Manipulations of Social Context

We collected immune gene expression data from larvae with and without parental care to determine if and how larvae were responding to changes in parental immune gene expression. We found a statistically significant effect of parental care on larval immune gene expression for each gene quantified. Further, the pattern of expression for *defensin, pgrp-sc2,* and *thaumatin* were nearly identical (fig. 1.2). For all three immune genes, larval expression was significantly higher when parental care was absent than when larvae were receiving parental care (*pgrp-sc2,* $F_{1,25} = 5.9853$, $p = 0.0218$; *thaumatin,* $F_{1,24} = 8.171$, $p = 0.0087$; *defensin,* $F_{1,25} = 5.3955$, $p = 0.0286$), suggesting that larvae increase their immune response in the absence of a parental immune response.

We collected expression data from females in different developmental and social conditions to determine at what point during the reproductive cycle immune gene expression increased (fig. 1.3). As expected, there was a statistically significant effect of social condition on maternal *pgrp-sc2* ($F_{4,45} = 22.7350$, $p < 0.0001$)*, thaumatin* ($F_{4,45} = 11.5664$, $p < 0.0001$), and *defensin* ($F_{4,45} = 18.7741$, $p < 0.0001$) expression. However, the specific pattern of expression was different for each gene quantified.

For *pgrp-sc2* we found that only females in the parental care social condition had significantly higher expression than females in the virgin negative control (fig.1. 3*A*; $p < 0.0001$). Females in the virgin, mated, preparing resource, and post-caring social conditions had significantly lower *pgrp-sc2* expression than females in the parental care positive control (all $p < 0.0001$). This suggests that parental *pgrp-sc2* expression is upregulated when larvae are present.

For *thaumatin* we also found that only females in the parental care social conditions had significantly higher expression than females in the virgin negative control (fig. 1.3*B*; $p < 0.0001$). Females in the virgin, mated, and post-caring social conditions had significantly lower *thaumatin* expression than females in the parental care positive control (all $p < 0.0001$). Females in the preparing resource social condition also had significantly lower *thaumatin* expression than females in the parental care positive control ($p = 0.0038$), though slightly higher than virgin, mated, and post-caring. These results suggest that parental *thaumatin* expression is also upregulated when larvae are present.

For *defensin* we found that females in the preparing resource and parental care social conditions had significantly higher expression than females in the virgin negative control (fig. 1.3*C*; $p < 0.0001$; $p = 0.0002$, respectively). Females in the virgin, mated, and post-caring social conditions had significantly lower *defensin* expression than females in the parental care positive control ($p = 0.0002$; $p = 0.0179$, $p = 0.0001$, respectively). Female *defensin* expression did not differ significantly between preparing resource and parental care social conditions. This suggests that parental *defensin* expression is upregulated before larvae arrive.

Timing and presence or absence of larvae affected the expression of all three genes (fig. 1.4): *pgrp-sc2* ($F_{2,27} = 25.3482$, $p < 0.0001$), *thaumatin* ($F_{2,27} = 23.0386$, $p < 0.0001$), and *defensin* ($F_{2,27} = 6.9226$, $p = 0.0037$). The pattern was very similar between *pgrp-sc2* and

thaumatin, and differed for *defensin*. Both *pgrp-sc2* (fig.1. 4*A*; $p < 0.001$) and *thaumatin* (fig. 1.4*B*; $p < 0.001$) expression increased from 72 to 96 h. The presence of larvae at 96 h resulted in slightly lower expression of *pgrp-sc2*, although not statistically significant ($p = 0.1473$) while lower *thaumatin* expression was statistically significant ($p = 0.0182$). For *defensin,* expression was not significantly affected by time from 72/- to 96/- ($p = 0.5755$). but was markedly and statistically significantly decreased in the presence of larvae (fig. 1.4*C*; $p = 0.0073$).

Maternal Selection

We calculated maternal *pgrp-sc2, thaumatin,* and *defensin* expression and relative offspring performance (mass at dispersal) and fitness (survival to pupae, survival to adult) to determine if offspring fitness was affected by maternal immune gene expression (fig. 1.5, fig. 1.6, fig. 1.7). We reared a total of 1323 larvae dispersed from 111 families. Mean mass at dispersal was 142.2 mg (SD = 29.1). 1087 offspring survived to the pupal stage. 989 offspring survived to adult eclosion. Expression of all three genes were correlated, although the correlation between *pgrp-sc2* and *thaumatin* ($r = 0.93$; $p < 0.001$) was considerably stronger than the correlation between *pgrp-sc2* and *defensin* ($r = 0.22$; $p = 0.022$), and *thaumatin* and *defensin* ($r = 0.23$; $p = 0.014$).

Overall, maternal selection arising from *pgrp-sc2* expression and *thaumatin* expression was similar, while there was little evidence of maternal selection arising from *defensin* expression. We found a statistically significant effect of maternal immune gene expression on larval mass at dispersal for all genes (fig. 1.5; all $p < 0.0001$), however for *pgrp-sc2* and *thaumatin* maternal selection was significantly positive (fig. 1.5*A*, 1.5*B*) while maternal selection on larval dispersal mass arising from *defensin* expression was negative (fig. 1.5*C*). We found significant positive maternal selection for *pgrp-sc2* expression affecting survival to pupation, but

no significant maternal selection for *thaumatin* and *defensin* expression for this component of fitness. Maternal selection associated with offspring survival to adult was significantly positive for both maternal *pgrp-sc2* and *thaumatin* expression, but again no evidence for maternal selection arising from *defensin* expression.

Discussion

In this study, we examined expression of three functionally-distinct immune genes (*pgrp-sc2*, *thaumatin*, and *defensin*) in parents and offspring of *N. vespilloides* to test if all immune function expressed during parental care reflected social immunity. Burying beetles reproduce on dead vertebrate carcasses, upon which they manipulate the pathogen load and care for their developing offspring (Jacobs et al. 2016; Vogel et al. 2017). Both the parents and the offspring live on the carcass while it is being consumed. Social immunity is therefore likely to be selected in burying beetles, as both the environmental conditions and parent-offspring relatedness exists. Here, we ask if parental immune responses from distinct immune pathways all are responding during parental care to provide social immunity. Using expression data to distinguish between social and personal immunity in the context of parental care requires evidence that offspring are relieved of mounting a full immune response, that parents upregulate their immune response in response to offspring beyond that required for personal benefit, and that the upregulation of parental immunity positively influences offspring fitness. The patterns we observed in our series of experiments suggest a social role for *pgrp-sc2* and *thaumatin*, but not for *defensin*.

All three genes we examined were down-regulated by offspring in the presence of a parent. All three were also up-regulated in the adult during parenting. However, only *pgrp-sc2* and *thaumatin* were most highly expressed during the period of time offspring were present, with

very similar patterns. In contrast, *defensin* was most highly expressed prior to the arrival of the offspring, and while still highly expressed at later time points we found a relative decrease during the time when larvae were present. Where *pgrp-sc2* and *thaumatin* expression in females were clearly influenced by the presence of offspring, *defensin* appeared to be more influenced by non-social environmental circumstances and timing. Patterns of maternal selection also supported a social role for *pgrp-sc2* and *thaumatin*. Offspring may decrease their expression of *defensin* in the presence of parents, but there was no observed fitness benefit. Our study therefore provides support for social immunity in two of the three genes examined, suggesting that different components of the immune system can be targeted to evolve social immune function separately.

The three genes we examined are expressed in different pathways and have different roles in insect immunity, which we suggest contributes to different forms of selection acting on them. PGRP-SC2 is a peptidoglycan-recognition protein involved in the Imd immune pathway that responds to gram-negative bacteria in the gut (Broderick et al. 2009). Its role is to prevent over-stimulation of the immune system in response to non-entomopathogentic bacteria (Paredes et al. 2011; Guo et al. 2014). In this way, *pgrp-sc2* expression maintains a healthy gut-microbiota and regulates the potentially lethal over-activation of the immune system. Thaumatin is an antifungal peptide that is produced by the Toll pathway in response to entomopathogentic fungi as well as sterile and septic injury (Broderick et al. 2009; Mylonakis et al. 2016). *Thaumatin* is not ubiquitous across insect orders or even found within all Coleoptera (Mylonakis et al. 2016). Interestingly, where it has been found, *thaumatin* is most common in Coleoptera, Blattodea, and Hemiptera; insect orders that also display social behavior and parental care. This suggests a more specialized function for *thaumatin*, restricted to more unique social circumstances. Defensin is an

antimicrobial peptide produced by the Toll pathway in response to gram-positive bacteria (Broderick et al. 2009; Mylonakis et al. 2016). Insect *defensins* are the most widespread group of inducible antibacterial peptides in insects and have been characterized in multiple insect orders (Hoffmann and Hetru 1992; Mylonakis et al. 2016).

Defensins appear to play a general and perhaps even generic role in insect immunity (Hoffmann and Hetru 1992; Mylonakis et al. 2016) and in *N. vespilloides* are used by parents on the carcass being processed prior to the arrival of offspring (Reavey et al. 2014b; Jacobs et al. 2016). The ubiquity of this gene amongst insects suggests strong selection for conserved functionality and may explain why *defensin* is implicated in personal and not social immunity. Both *pgrp-sc2* and *thaumatin*, on the other hand, have more specific roles in insect immunity (Broderick et al. 2009; Paredes et al. 2011; Guo et al. 2014; Mylonakis et al. 2016) and thus they may have fewer pleiotropic constraints. This suggests that social immunity where there is parental provisioning or environmental manipulation for the offspring may evolve to provide unique rather than common molecules to offspring.

We suggest that the upregulation of *pgrp-sc2* and *thaumatin* in the context of parent-offspring interactions, and the down-regulation in offspring, occurs so that parents can transfer this protection to their offspring directly. This interpretation follows Grindstaff et al. (2003), who suggest that maternally-provisioned immunity should evolve as an interacting phenotype; that is, provisioned immunity evolves both as a trait in the mother and a trait in the offspring through indirect genetic effects and social selection. The potential for direct transfer of immune molecules from parents to offspring exists in burying beetles because there is regurgitation of pre-digested food from the parent to begging offspring in this taxon. This direct interaction creates a social phenotype for offspring feeding, where offspring and parent phenotypes are

inextricably intertwined. Offspring feeding thus becomes an interacting phenotype (Moore et al. 1997), facilitating social selection (Wolf et al. 1999; McGlothlin et al. 2010).

The specific and statistically significant increase in *pgrp-sc2* and *thaumatin* expression in manipulative experiments occurred at 96 hours, a time point that corresponds to the expected presence of larvae. However, as these results show, larvae are not a necessary cue for parental increase in expression of these genes. Nevertheless, we suggest this reflects more than length of exposure to microbes. Using temporal cues may be general in *N. vespilloides* as social contexts occur in predictable sequences and times (fig. 1.1). This interpretation is supported by temporal kin recognition (Elwood 1994), which has been shown to occur in *N. vespilloides* in the context of parenting (Oldekop et al. 2007). Instead of relying on a cue derived from the offspring, such as odor or begging behavior, parents rely on consistent and accurate timing of offspring arrival. Both parents show time-dependent shifts from infanticide to parental care, which begins before and is independent of the arrival of offspring (Oldekop et al. 2007). The correlation of the shift to parental behavior and the expression pattern of *pgrp-sc2* and *thaumatin* suggest the increase in immune gene expression is correlated with the social context of parental care.

The overall pattern of covariance of maternal immune gene expression and offspring performance or component of fitness further supports *pgrp-sc2* and *thaumatin* playing a social role. Moreover, it suggests a greater social impact of maternal *pgrp-sc2* expression as it is positively correlated with all three measures. We did not find any effect of maternal *thaumatin* expression on offspring survival to pupae but did find a positive effect on offspring mass at dispersal and survival to adult. In contrast, the pattern of expression of *defensin* is very different and suggests that any benefits derived by offspring are secondary to the parental expression. This lack of maternal selection implicates *defensin* in personal immune function. While social

immunity is clearly valuable in the context of parental care, our results show that personal immune function is not completely neglected during parenting.

Parental provision of immunity is also consistent with parental investment, but parental investment theory does not make predictions about specific types of immune genes that might evolve. We suggest that the evolution of social immunity in the context of parenting depends on the immune pathways involved and the opportunity for the specific immune gene evolution to be influenced by social interactions. We also suggest that while parenting may provide conditions likely for social immunity, these are most likely to arise when immune effects (fitness benefits) can be easily limited to offspring. This is consistent with the notion that maternal (kin) selection is generally weaker than individual (direct) selection. Our results are consistent with this; however, given the limited number of genes we could sample we encourage future work to investigate the social role of a greater variety of immune genes and different pathways.

A final caveat is that we are lacking functional analyses of these proteins. Like all gene expression studies using qRT-PCR, the data are correlational without manipulative studies showing gene function. Such studies are rare. Likewise, the maternal selection analyses show an association between maternal expression and offspring fitness but cannot show causality. There may be latent variables. Such manipulative genetic studies, and direct examination and manipulation of proteins, are needed to confirm our interpretations. Nevertheless, we suggest that the weight of evidence supports our predictions.

Figures and Legends

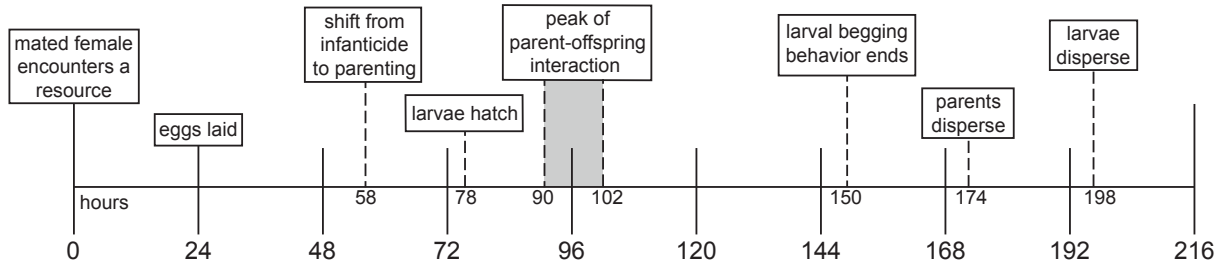

Figure 1.1. Timeline of reproductive cycle in Nicrophorus vespilloides. Timeline reflects laboratory observations and literature (Eggert et al. 1998; Lock et al. 2004; Smiseth et al. 2003, 2006).

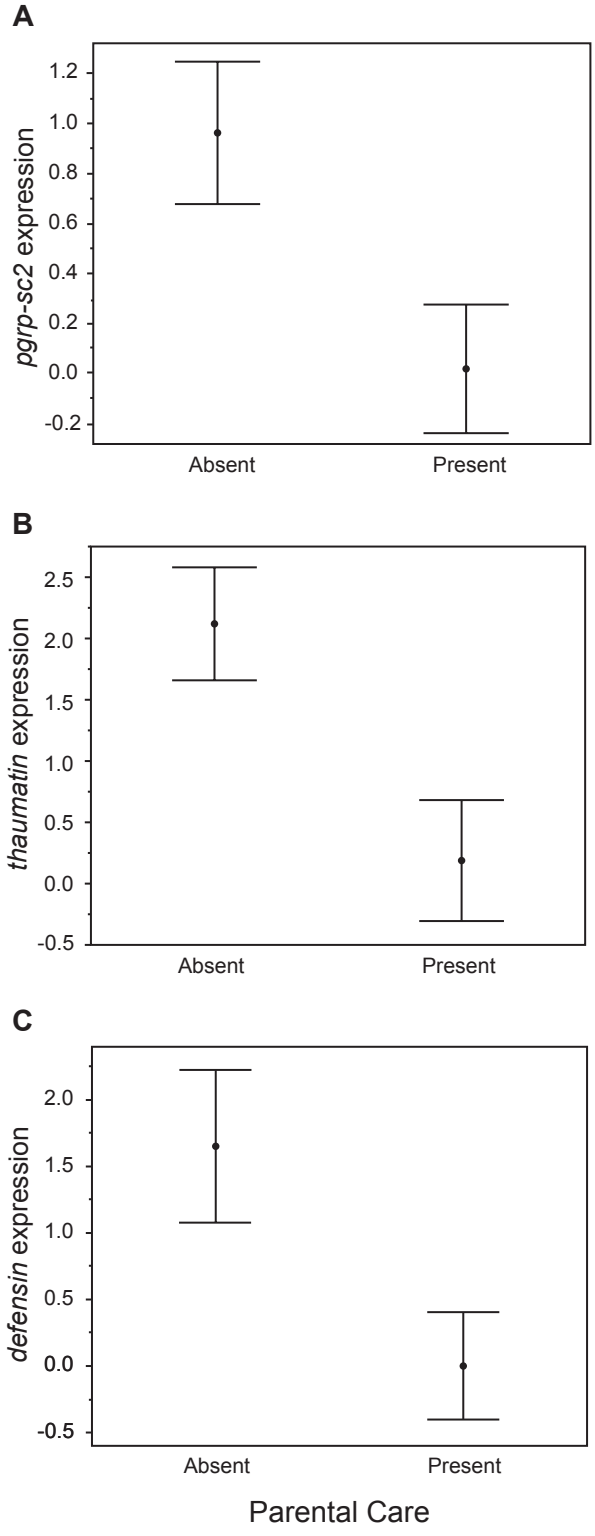

Figure 1.2. Larval immune gene expression in the presence and absence of parental care. Four larvae were pooled from each family for a single sample (N=10). A, Relative expression of pgrp-sc2, and immune gene responsible for suppressing immune response in the gut. B, Relative expression of thaumatin, an anti-fungal immune gene. C, Relative expression of defensin, an anti-bacterial immune gene. All comparisons are statistically significant.

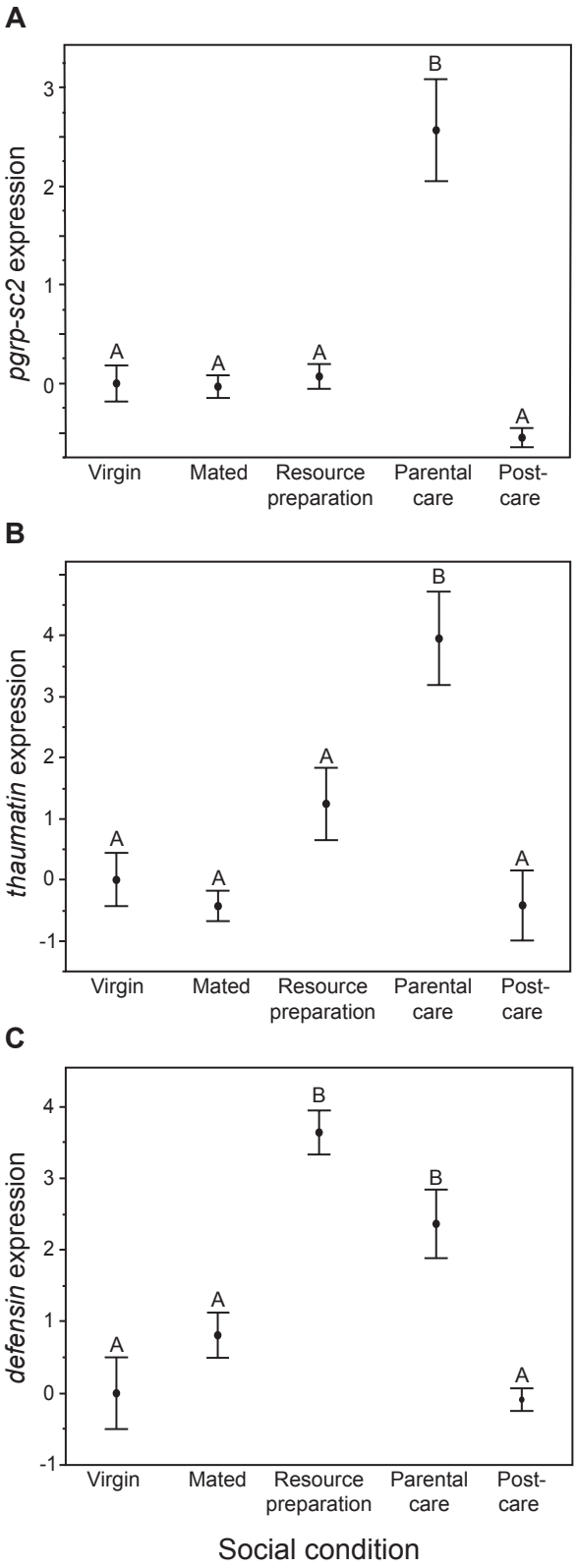

Figure 1.3. Maternal immune gene expression at five key social and physiological time points in the reproductive cycle. A single, whole head was used for each sample (N=10). Letters indicate statistically significant differences using Dunnets test. *A*, Relative expression of *pgrp-sc2*, and immune gene responsible for suppressing immune response in the gut. *B*, Relative expression of *thaumatin,* an anti-fungal immune gene. *C*, Relative expression of *defensin*, an anti-bacterial immune gene. All comparisons are statistically significant.

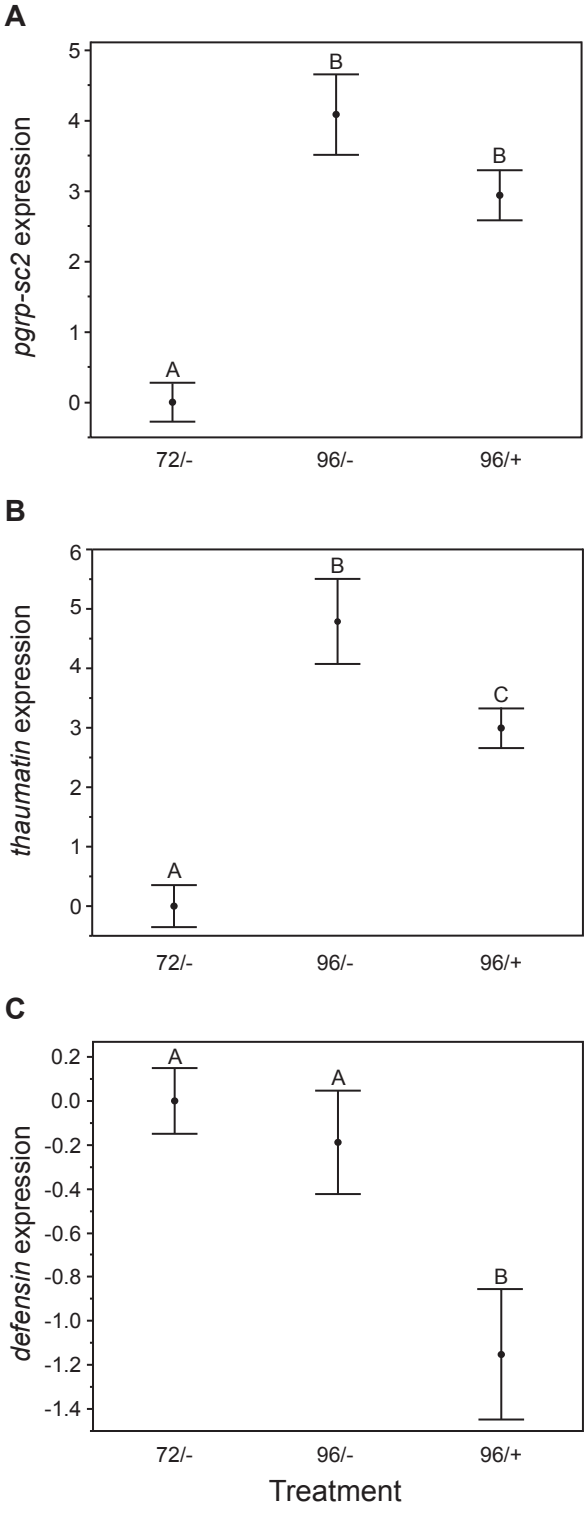

Figure 1.4. Maternal immune gene expression at 72 h on a carcass without larvae (72/-), 96 h on a carcass without larvae (96/-), and 96 h on a carcass with larvae (96/+). A single, whole head was used for each sample (N=10). Letters indicate statistically significant differences using Dunnets test. *A*, Relative expression of *pgrp-sc2*, and immune gene responsible for suppressing immune response in the gut. *B*, Relative expression of *thaumatin,* an anti-fungal immune gene. *C*, Relative expression of *defensin*, an anti-bacterial immune gene. All comparisons are statistically significant.

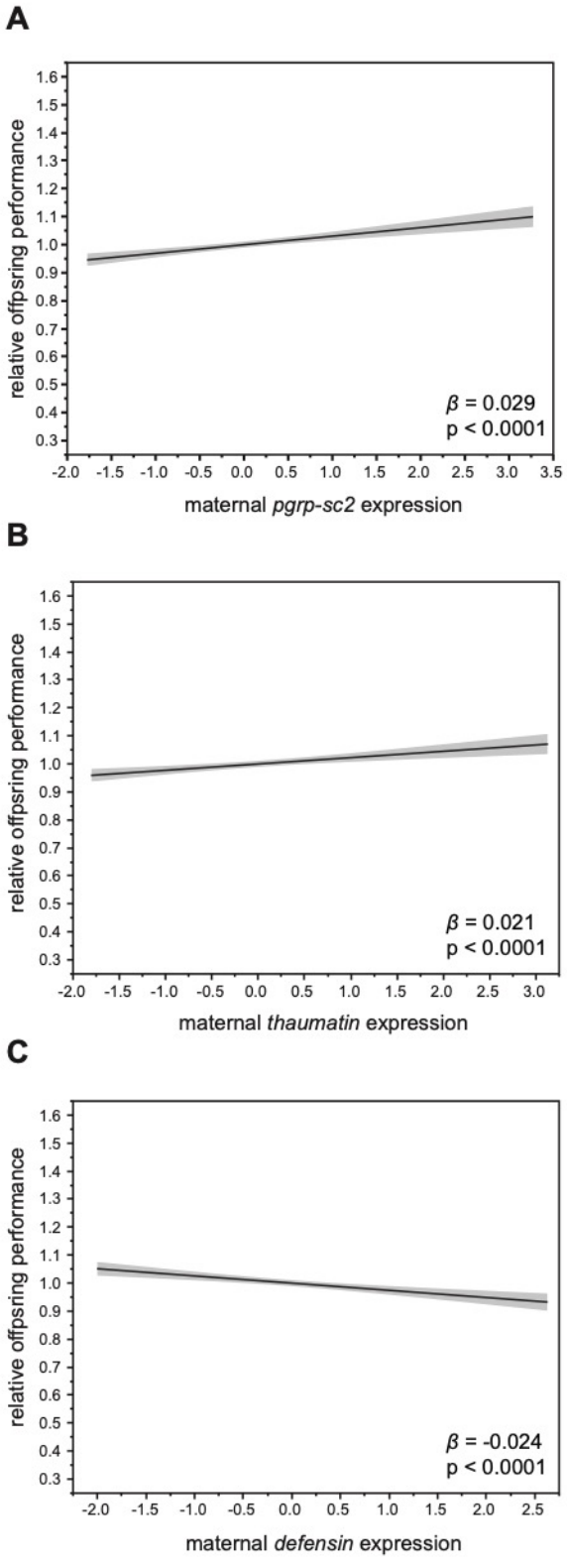

Figure 1.5. Maternal selection component (β_m), with 95% CI, of *pgrp-sc2* (fig. 5*A*), *thaumatin* (5*B*) and *defensin* (5*C*) on offspring performance (larval dispersal weight). Selection was calculated with relative performance measures and expression standardized to mean = 0 and SD = 1.

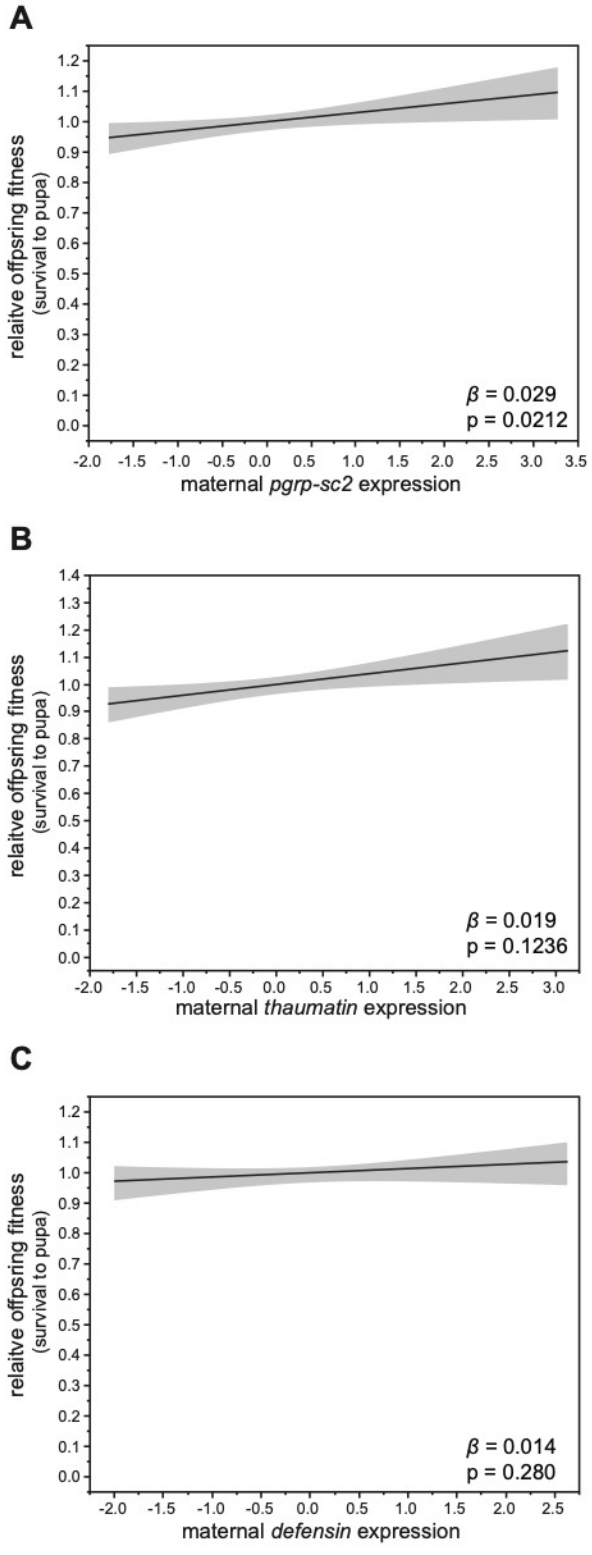

Figure 1.6. Maternal selection component (β_m), with 95% CI, of *pgrp-sc2* (fig. 6*A*), *thaumatin* (6*B*) and *defensin* (6*C*) on relative offspring fitness (survival to pupal stage). Selection was calculated with relative fitness and expression standardized to mean = 0 and SD = 1.

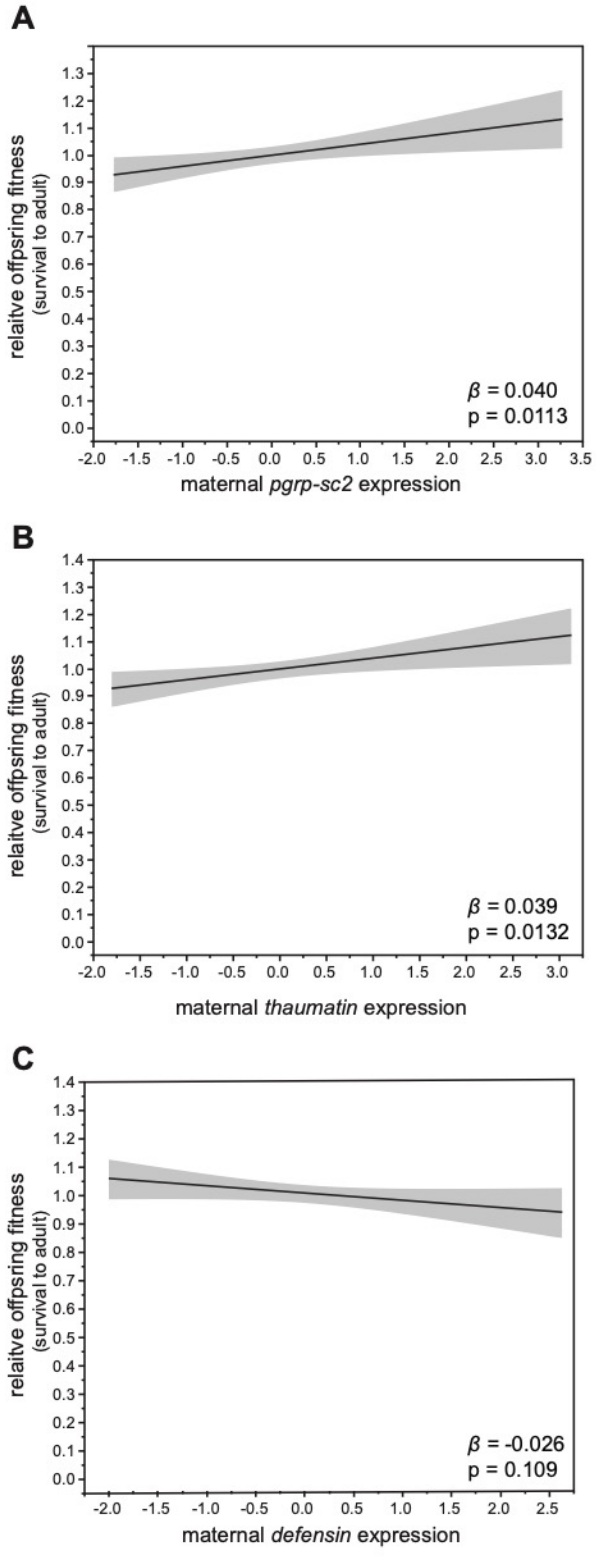

Figure 1.7. Maternal selection component (β_m), with 95% CI, of *pgrp-sc2* (fig. 7*A*), *thaumatin* (7*B*) and *defensin* (7*C*) on relative offspring fitness (survival to adult). Selection was calculated with relative fitness and expression standardized to mean = 0 and SD = 1.

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CHAPTER 3

BROOD SIZE MANIPULATIONS REVEAL CONSTRANTS TO SOCIAL IMMUNITY IN FAMILY LIFE[2]

[2] Ziadie, M. A., F. Ebot-Ojong, E. C. McKinney, A. J. Moore. Submitted to *Behavioral Ecology* 2/2020.

Abstract: Social immunity can be a part of group living and family life. As such, social immunity may be subject to changes in social factors that affect family dynamics and composition. One such social factor is family size. In this study we investigate the role family size plays in the expression of social immunity. We do this by measuring social immune gene expression in mothers and offspring in response to changes in family size in the burying beetle, *Nicrophorus vespilloides*. We hypothesized that genes influencing individual social immunity, collective social immunity, and personal immunity would differ in expression in response to changing family environments. We therefore measured the expression of three different immune genes reflecting these different social functions (*pgrp-sc2, thaumatin,* and *defensin*). We predicted that *pgrp-sc2*, a gene involved in an individual social immune response, would show increases in maternal expression with increasing family size, but larvae would not vary. We predicted that the gene involved in collective social immunity, *thaumatin,* would decrease in expression in both mothers and offspring as family size increased. We predicted no change in either mothers or offspring in the personal immune gene *defensin* in response to changing family size. Contrary to most of our predictions, the only response to changes in family size was an increase in *pgrp-sc2* expression by offspring. We now hypothesize that maternal gene expression is a threshold, and that further research on potential constraints to immune gene expression is needed to understand this lack of flexibility.

Introduction

The transition from solitary to social life affords group members benefits such as increased vigilance and enhanced foraging (Alexander 1974). However, group living also engenders costs, including the increased risk of disease (Meunier 2015; Cremer et al. 2018). Intimate interactions between group members facilitates pathogen exposure and transmission, especially within families where genetic relatedness increases the susceptibility to the same pathogens (Meunier 2015; Cremer et al. 2018). In response to this inherent risk of pathogen infection, social systems, including families, often evolve social immunity.

Social immunity describes any immune response that evolves for and is maintained by the fitness benefit it provides to others (Cotter and Kilner 2010a). Social immune responses are costly (Cotter et al. 2004, Cotter et al. 2010) and range from altruistic individual responses to mutualistic collective responses. Individual social immune responses are those mounted by an individual for the benefit of another group member. This occurs, for example, in the provisioning of antibodies to offspring in nursing mammals: mothers are mounting an immune response (generating costly antibodies) for the immune benefit of her offspring (Oddy 2001). Collective social immune responses are those mounted by the group as a whole for the benefit of the group as a whole. One example of a collective social immune response is behavioral fever in honeybees: multiple members of the hive will vibrate in unison to raise the temperature of the hive and eliminate a pathogenic threat for all (Starks et al. 2000).

Social immunity as a part of group living and family life is predicted to evolve in the context of parent-offspring interactions, where offspring health and survivorship is dependent on parental contribution (Cotter and Kilner 2010a). Non-social factors, such as sex, individual health, previous reproductive success, and trade-offs with personal immune function have been

shown to influence the extent to which parents will invest in social immunity in any given breeding bout (Cotter et al. 2010, Cotter and Kilner 2010b, Steiger et al 2011 Cotter et al. 2013). However, the extent to which social factors, such as family size, affect parental contributions to social immunity is not well understood. Yet family size is known to influence parent-offspring interactions (Smiseth and Moore 2002; Smiseth and Moore 2007; Smiseth et al. 2007; Gardner and Smiseth 2011 Schrader et al. 2015; Sieber et al. 2017; Andrews et al. 2017). Here we use experimental manipulation of family size to investigate how this social factor influences different forms of social immunity.

Given the known effects of family size on parent-offspring interactions, we can make specific hypotheses for how family size should influence different forms of social immunity. Family size matters when resources and costs are shared. Parental care is predicted to evolve to provide shared resources to offspring, despite an inequality of cost (Smiseth et al 2007). An example of this is the provisioning of parentally-derived immune molecules from parent to offspring as a form of individual social immunity. Parental provisioning of resources has been shown to increase with larger family (brood) size (Smiseth et al 2007). Therefore, we hypothesize that as family size increases, the provisioning of individual social immunity will also increase. In a collective social immune response, resources and costs are shared among all contributors. As family size increases, so does the number of contributors. Thus, in a collective immune response each individual should contribute proportionately and therefore lower their individual contribution to the collective response as family size increases. We test these hypotheses by manipulating brood size and quantifying immune gene expression in mothers and offspring of the burying beetle, *Nicrophorus vespilloides*.

Burying beetle families are typically composed of either a mated female or a mated pair and a single brood of larvae living in a prepared vertebrate carcass (Eggert and Müller 1997; Scott 1998; Parker et al. 2015). Before larval arrival, parents strip the carcass of fur or feathers, roll it into a ball, and bury it in the soil. During the processing of the carcass, parents repeatedly mate, and the female lays eggs in the surrounding soil. Larvae hatch in the soil approximately 50h after deposition (Smiseth et al. 2006), and crawl to the fully prepared carcass where they are regularly groomed and fed predigested carrion by regurgitation from their parents for 48-72h (Smiseth et al. 2003). Throughout the breeding bout, parents actively guard the carcass and coat it with antimicrobial secretions (Arce et al. 2012; Duarte et al. 2018). Larvae also contribute to the antimicrobial secretions that maintain the integrity of the carcass (Arce et al. 2013; Reavey et al. 2014).

Parental care in this species can be split into two categories: direct care and indirect care (Walling et al. 2008). Direct care includes all parental behaviors that involve direct parent-offspring interactions, including allogrooming and provisioning of food. The provisioning of food during direct care may include individual social immunity in the form of immune molecule transfer from parent to offspring during feeding (Grindstaff et al 2003; Ziadie et al 2019). Indirect care includes all parental behaviors that involve preparation and maintenance of the carcass for offspring consumption. The coating of the carcass with antimicrobial secretions during indirect care is a form of collective social immunity; both parents and offspring contribute to the secretions that reduce pathogen load and increase overall health of the family (Cotter et al. 2010; Cotter and Kilner 2010b; Arce et al. 2013; Cotter et al. 2013; Reavey et al. 2014; Palmer et al. 2016; Duarte et al. 2016)

The effects of family composition on larval and parental behaviors in *N. vespilloides* is well documented (Smiseth and Moore 2002; Smiseth and Moore 2007; Gardner and Smiseth 2011 Schrader et al. 2015; Sieber et al. 2017; Andrews et al. 2017). However, our understanding of how changes in family composition affect social immunity in *N. vespilloides* is limited (but see Duarte et al. 2016). We measure three different immune genes shown previously to have varying levels of social immune function (Ziadie et al., 2019) and make predictions about how gene expression will change in response to changing brood size based on whether the genes are involved in individual or collective social immunity (table 2.1).

The first gene we measured is a peptidoglycan recognition protein, *pgrp-sc2*, which responds to gram-negative bacteria in the gut (Broderick et al. 2009; Paredes et al. 2011; Guo et al. 2014). Maternal *pgrp-sc2* expression is an example of an individual social immune response– mounted by the parent for the benefit of the offspring. This peptidoglycan recognition protein is thought to help maintain healthy gut microbiota (Paredes et al. 2011; Guo et al. 2014) and is provisioned from parent to offspring during feeding (direct care; Ziadie et al. 2019). Because *pgrp-sc2* is involved in individual social immunity, we predicted maternal *pgrp-sc2* expression would increase as brood size increased and there were more larvae to provision to. We therefore predict that larval *pgrp-sc2* will remain constant across brood size treatments. We also measure expression of *thaumatin*, an antifungal peptide present in the secretions parents spread over the carcass during indirect care (Broderick et al. 2009; Mylonakis et al. 2016; Jacobs 2016). *Thaumatin* is also expressed by larvae (Ziadie et al. 2019). *Thaumatin* is involved in a collective social immune response, in which all family members can contribute to through secretions on the carcass. We predicted that both larval and maternal *thaumatin* expression would decrease as brood size increased and there were more individuals to contribute to the collective response.

Finally, we measured expression of *defensin*, a very common insect anti-microbial peptide found in the hemolymph that responds to gram-positive bacteria (Hoffmann and Hetru 1992; Broderick et al. 2009; Mylonakis et al. 2016). Expression of *defensin* appears to function for personal, not social immunity (Ziadie et al. 2019), and therefore we predicted that its expression in mothers and larvae would be unaffected by brood size.

Materials and Methods

Husbandry

Nicrophorus vespilloides used in these experiments were collected from the wild in Cornwall, UK and Edinburgh, UK. Beetles are maintained as an outbred colony at the University of Georgia, Athens, GA, USA as described in Ziadie et al, 2019.

All experiments involved uniparental female care. We focus on females for these experiments to control the quality and quantity of parental care. Parental care is most often uniparental female, and even in biparental broods females remain with the brood for longer than males (Parker et al. 2015). Single females rear broods as successfully as biparental females (Scott 1998; Parker et al. 2015). We used adult female beetles 18 days post-eclosion for all experiments. We paired females with sexually mature, unrelated males and removed the male after the carcass was prepared and before the larvae arrived.

Brood size manipulations

In this study, we sought to determine if there was any direct association between family size and immune gene expression. To do this we collected gene expression data from females caring for either 5, 10, or 20 larvae, which are family sizes that fall within a normal range (Smiseth and Moore 2002). We collected expression data from a total of 59 families, (N=21, 18,

and 20 for families of 5, 10, and 20 larvae, respectively). In all experimental treatments, we paired non-sibling males and females in a mating box (17.2 cm x 12.7 cm x 6.4 cm) filled halfway with moist soil and a fresh mouse carcass. The size of the carcass was tightly controlled (mean weight = 19.23g, SD = 0.14) to ensure gene expression was not influenced by amount or quality of resource. Female size was also tightly controlled (mean weight = 0.209g, SD = 0.036; mean pronotum length = 5.11mm, SD = 0.31). Maternal expression of *thaumatin* was significantly correlated with female pronotum length ($p = 0.0366$). We corrected for this correlation in our statistical analysis, however including pronotum length in the analysis does not change the statistical significance of the result.

We checked the box daily for the presence of eggs. If eggs were found, the mated pair and carcass were moved to a new box with fresh soil. We then carefully collected the eggs from the original box and placed them in a clean petri dish with a moist paper towel. We examined each female's new box for eggs at regular intervals. If any eggs were present, we again moved the pair to a new box with fresh soil and added the additional eggs to the original set in the petri dish. We did this as many days as was necessary to collect all eggs and kept track of which eggs belonged to each female.

When eggs hatched, we moved the female and carcass to a new box with fresh soil and checked the old box for eggs one last time. Males were also removed at this time. Larvae that hatched in the same 12-hour period were mixed and assigned randomly to females in groups of 5, 10, or 20 (N=20 per treatment). We used mixed broods to disrupt any parent-offspring coadaptation which could mask the effects of treatment (cite?). Larvae were placed directly on the prepared carcass. Females only received larvae if their own eggs had hatched in the same 12-hour interval. All females included in this study accepted their foster broods.

After assigning brood size treatments, we check families at regular intervals for the presence of parental care. Parental care was determined by direct parental care (grooming or feeding). We collected females within 12 hours of observed parental care and immediately froze sample tissues (whole heads) in liquid nitrogen to preserve tissues. Samples were stored at -80ºC until ready for gene expression analysis.

We also collected gene expression data the larvae in the manipulated brood size treatments. Larvae were collected at the same time as their foster mothers. There was natural variation in larval survival, however we ensured all broods analyzed were successful by only including broods were more than half of the larvae survived. The average survivorship for broods of 5, 10, and 20 were 4.67, 9.34, and 17.95 larvae, respectively. Each larva was placed in an individual tube and immediately frozen in liquid nitrogen to preserve tissues. Samples were stored at -80ºC. We analyzed expression data from 3 individual larvae per family to generate a family average.

Quantification of Gene Expression

We extracted RNA and quantified gene expression exactly as we did previously in Ziadie et al. 2019. Briefly: we stored each sample at -80 °C until RNA extraction. We extracted RNA from all samples using a Qiagen RNAeasy Lipid kit (Qiagen, Venlo, The Netherlands) per manufacturer's instructions. Frozen samples were initially homogenized in 500 microl of Qiazol. We quantified RNA in 1: 10 dilutions using a Qubit 2.0 florometer (Invitrogen Corporation, Carlsbad, CA, USA) and synthesized cDNA from 500 ng of RNA using Quanta Bioscience qScript reverse transcriptase master mix per manufacturer's instructions.

We quantified immune gene expression in three technical replicates for each biological sample by quantitative real-time PCR (qRT-PCR) using a Roche LightCycler 480 platform. We

used a 10 μL reaction containing 2 μL of 1 :10 diluted cDNA, 5 μL of SYBR I Green Master Mix at a 60°C annealing temperature and 3 μL of a primer stock containing both sense and antisense primers at 2.67 μM. We used the same qRT-PCR primers described in Ziadie et al. 2019. We used TATA-binding protein as and endogenous reference gene for both experiments.

Statistical Analyses

We measured gene expression quantitatively using qRT-PCR and we used the $\Delta\Delta C_T$ method to convert raw expression data to normalized expression values for analysis (Livak and Schmittgen 2001). We tested the overall ANOVA model to examine how maternal and larval immune gene expression changed as a function of brood size. In instances where the ANOVA reported a significant effect of treatment, we used Tukey-Kramer HSD test to determine which pairs were significantly different. We conducted all analyses using JMP Pro (v.14.1.0).

Results

Effects of family composition on pgrp-sc2 *expression*

Maternal expression of *pgrp-sc2* was not statistically significantly affected by brood size treatment ($F_{2,56} = 0.083$, $p = 0.921$ Fig 2.1a). There was a statistically significant effect of brood size on larval *pgrp-sc2* expression ($F_{2,56} = 3.404$, $p = 0.040$ Fig 2.1b). Larval *pgrp-sc2* expression increased as brood size increased from 5 to 20 (Tukey-Kramer HSD, $p = 0.033$), however changes in brood size from 5 to 10, and from 10 to 20 were not significant (Tukey-Kramer HSD, $p = 0.262$, $p = 0.634$, respectively).

Effects of family composition on thaumatin *expression*

We found no significant effect of brood size treatment on maternal *thaumatin* expression ($F_{2,55} = 0.125$, $p = 0.883$, Fig 2.2a). Larval *thaumatin* expression was not significantly affected

by brood size treatment ($F_{2,56}$ = 0.433, p = 0.651, Fig 2.2b).

Effects of family composition on defensin *expression*

Maternal expression of *defensin* was not significantly affected by brood size treatment ($F_{2, 56}$ = 2.440, p = 0.096, Fig 2.3a). There was no significant effect of brood size on larval *defensin* expression, either ($F_{2, 56}$ = 0.182, p = 0.834, Fig 2.3b).

Discussion

In this study we sought to determine if social immune function in a family setting could be affected by family composition. More specifically, we wanted to know how changes in brood size would affect gene expression involving different forms of social immunity in the burying beetle, *Nicrophorus vespilloides*. We manipulated brood size and measured the expression of three immune genes with varying levels of social immune function in mothers and offspring. We predicted a positive relationship between brood size and maternal gene expression where there is a contribution to individual social immunity, an inverse relationship between brood size and both maternal and larval gene expression and contributions to shared social immunity, and no relationship between brood size and contributions to personal immunity. We found that these simple predictions were not supported.

Prgp-sc2

Pgrp-sc2 is a peptidoglycan recognition protein that responds to bacteria in the gut (Broderick et al. 2009; Paredes et al. 2011; Guo et al. 2014). We previously found that maternal expression of *pgrp-sc2* is an individual social immune response, likely provisioned with regurgitated food (Ziadie et al. 2019). Individual social immune responses are mounted by an individual for the benefit of others. We therefore predicted that maternal *pgrp-sc2* expression

would increase with increasing brood size, indicating an adaptive maternal effect. As brood size increases, so does offspring demand and we predicted that mothers would increase supply to match offspring need. This would be consistent with patterns of maternally provisioned social immunity in mammals, for example, when mothers increase the production of milk (and immunoproteins in milk) with increased offspring (Saint et al. 1986). Immune expression is costly and providing immune support for developing offspring (enough to match offspring demand) would allow offspring the selective advantage of investing instead in development (Reavey et al. 2014). In contrast, as larvae cannot contribute to social immunity with this genes, we further predicted that larval *pgrp-sc2* expression would remain constant across treatments. This combination of results would indicate that mothers are responding to offspring demand and compensating for changes in brood size.

Our results do not support these predictions but indicate that maternal *pgrp-sc2* expression behaves as a threshold trait, and that larvae are carrying the burden of compensating for increases in brood size through an increase in personal immunity protection. That is, maternal *pgrp-sc2* appears to be a limited resource, and an increase in demand from larvae does not increase maternal supply. Instead, larvae increase their personal *pgrp-sc2* expression to compensate for the increased demand on maternal resources. This pattern of expression could reflect a safeguard against the cost of mounting a social immune response for parents, as previous work has shown that mounting a social immune response decreases lifetime reproductive success in *N. vespilloides* (Cotter et al. 2010). This pattern is also consistent with work that examines the effect of brood size on begging and parental behavior in *N. vespilloides*. While increasing brood size increases larval begging, larger broods do not elicit more direct parental care (Smiseth and Moore 2002, Andrews et al. 2017). We believe this suggests

potential constraints to individual social immune responses. This pattern of expression does not represent an example of sibling competition, however, because an increase in offspring's personal immunity does not come at the expense of their siblings' personal fitness (Mock and Parker 1998).

Thaumatin

Thaumatin is an antifungal peptide present in the anal secretions of burying beetles and expressed in both larvae and adults to reduce fungal growth on a carcass (Broderick et al. 2009; Mylonakis et al. 2016; Jacobs 2016, Ziadie et al. 2019). Previous work suggests that expression of *thaumatin* may function as a collective social immune response (Ziadie et al). In a collective immune response all group members contribute to group immunity and all members reap immune benefit (Cotter and Kilner 2010a, Cremer et al 2018). Previous work examining other collective social immune responses in *N. vespilloides* found that mothers and offspring both decrease their individual contribution to brood-level lysozyme activity as brood size (and therefore number of contributors) increases (Duarte et al. 2016). We therefore predicted that *thaumatin* expression would decrease in mothers and larvae as brood size increases, reflecting cooperation for collective social immunity.

Counter to our predictions there was no statistically significant effect on maternal or larval *thaumatin* expression. We suggest that maternal *thaumatin* expression is more likely tied to the size of the resource provided rather than the number of offspring. That changes in brood size have no effect on larval *thaumatin* expression is more difficult to interpret. Our previous work shows that larvae upregulate their *thaumatin* expression when parents are absent, confirming that larval *thaumatin* expression can respond to changes in social condition (Ziadie et

al. 2019). It may be that larvae have no way of assessing the contributions of others to brood-level *thaumatin*. Thus, like their mothers, larvae have a threshold-level of *thaumatin* expression.

Defensin

Defensin is a common anti-microbial peptide found in the Toll immune system of most insects (Hoffmann and Hetru 1992; Broderick et al. 2009; Mylonakis et al. 2016). In *N. vespilloides*, maternal *defensin* expression increases while females are manipulating the carcass, independent of parent-offspring interactions, with no signature of social immune function (Ziadie et al., 2019). We therefore predicted that *defensin* expression in mothers and offspring would be unaffected by changes in brood size.

As predicted, we found no evidence of an effect of brood size on *defensin* expression in mothers or offspring, as predicted. This is consistent with our original assertion that *defensin* does not serve a social immune function (Ziadie et al. 2019).

Conclusions

Our study is the first to examine the effects of family composition on social immune gene expression. Our findings suggest that while social immunity is an adaptive trait, there is limited flexibility and individual contributions to social immunity may have constraints. This is supported by our results for *pgrp-sc2.* Though an increase in maternal *pgrp-sc2* expression positively covaries with offspring fitness (Ziadie et al. 2019), mothers do not respond to an increase in brood size with increased expression. This likely reflects a cost associated with mounting a social immune response and may be one mechanism employed to defend against that cost.

Our previous work found that larvae upregulate expression for all three immune genes in the absence of parental care (Ziadie et al. 2019), indicating that flexible expression in response to

different social environments is possible. It is therefore surprising that brood size manipulations did not have a greater impact on larval immune response across immune genes. This suggests that the presence or absence of parental immunity may be more important than the presence or absence of sibling immunity. This is evidenced by our results for *thaumatin.* While larvae respond to the absence of parental care with increased *thaumatin* expression, an increase in the number of siblings does not influence larval *thaumatin* expression.

Overall, our work suggests limits to the flexibility of genes influencing social immunity, with threshold expression of immune genes more common than refined regulation in response to social cues. We therefore suggest that future work in this area consider limitations and constraints to social immunity, and perhaps immune function in general. In addition to understanding how social immunity evolves as an adaptive trait, we also need a better understanding of what constraints there are on social immunity and what factors contribute to those constraints.

Figures, Tables, and Legends

Table 2.1. Predictions for changes gene expression in mothers and offspring based on type of social immune function.

Gene	Level of social immunity	Predicted expression in mothers	Predicted expression in offspring
Pgrp-sc2	Individual social immunity	Increase with increasing brood size	Remain constant across brood size treatments
Thaumatin	Collective social immunity	Decrease with increasing brood size	Decrease with increasing brood size
Defensin	Personal immunity	Remain constant across brood size treatments	Remain constant across brood size treatments

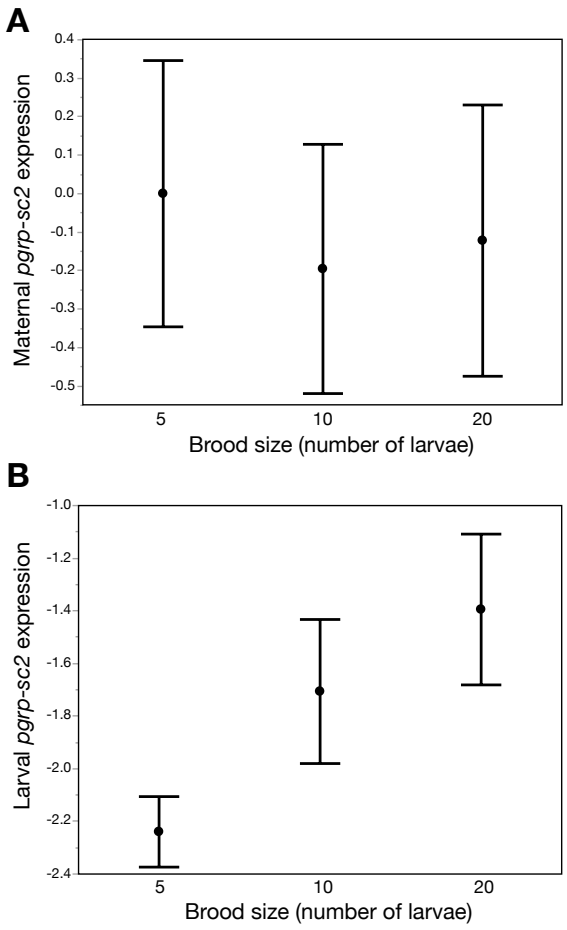

Figure 2.1: *pgrp-sc2* expression in response to changes in brood size. *A,* $-\Delta\Delta C_T$ values of expression for mothers caring for broods of 5, 10, and 20 larvae (N= 21, 18, and 20, respectively). *B,* family average $-\Delta\Delta C_T$ values of expression for larvae. Expression values from three larvae per family were used to get a family average. Error bars represent ±1 SE.

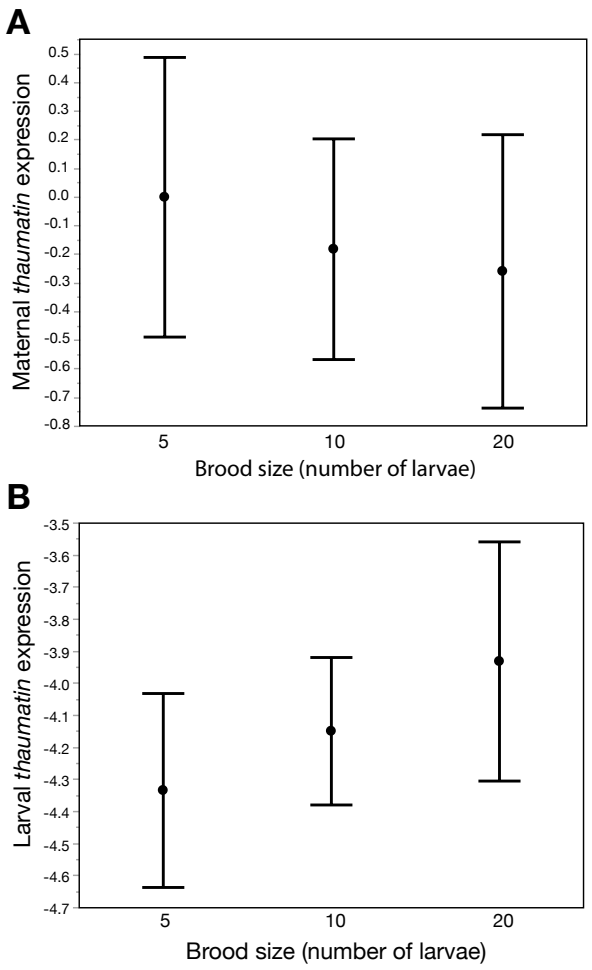

Figure 2.2: *thaumatin* expression in response to changes in brood size. *A*, $-\Delta\Delta C_T$ values of expression for mothers caring for broods of 5, 10, and 20 larvae (N= 21, 18, and 20, respectively). *B,* family average $-\Delta\Delta C_T$ values of expression for larvae. Expression values from three larvae per family were used to get a family average. Error bars represent ±1 SE.

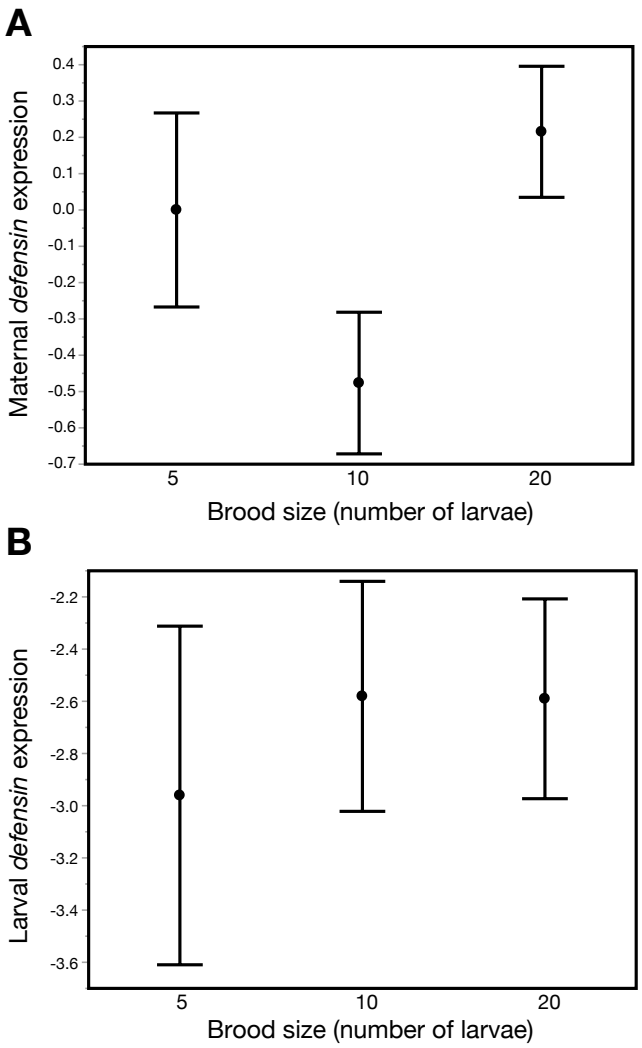

Figure 2.3: *defensin* expression in response to changes in brood size. *A,* $-\Delta\Delta C_T$ values of expression for mothers caring for broods of 5, 10, and 20 larvae (N= 21, 18, and 20, respectively). *B,* family average $-\Delta\Delta C_T$ values of expression for larvae. Expression values from three larvae per family were used to get a family average. Error bars represent ±1 SE.

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CHAPTER 4

MOVING EVOLUTION EDUCATION FORWARD: A SYSTEMATIC ANALYSIS OF LITERATURE TO IDENTIFY GAPS IN COLLECTIVE KNOWLEDGE FOR TEACHING[3]

[3] Ziadie, M. A., Andrews, T. C. (2018) Moving evolution education forward: a systematic analysis of literature to identify gaps in collective knowledge for teaching. CBE-Life Sciences Education, 17(1). Reprinted here with permission from the publisher.

Abstract: Evolution is a unifying theory in biology, and it is challenging for undergraduates to learn. An instructor's ability to help students learn is influenced by pedagogical content knowledge (PCK), which is topic-specific knowledge of teaching and learning. Instructors need PCK for every topic they teach, which is a tremendous body of knowledge to develop alone. However, investigations of undergraduate thinking and learning have produced collective PCK that is available in peer-reviewed literature. Currently, it is unclear if the collective PCK available adequately addresses the topics in evolution that college instructors teach. We systematically examined existing literature to determine what collective PCK for teaching evolution is available and what is missing. We conducted an exhaustive literature search and analyzed 316 relevant papers to determine: the evolutionary topics addressed; whether the focus was student thinking, assessment, or instructional strategies; and the type of work (e.g., empirical, literature review). We compared the collective PCK available in the literature to the topics taught in a sample of 32 undergraduate evolution courses around the country. Based on our findings, we propose priorities for the evolution education research community, and propose that PCK is a useful lens for guiding future research on teaching and learning biology.

Introduction

Evolution is a unifying and explanatory theory for all of biology and is therefore a core concept in undergraduate biology education (AAAS, 2011). However, it is also challenging to learn (e.g., Nehm & Reilly, 2007; Andrews, Leonard, Colgrove & Kalinowski, 2011; Price & Perez, 2016). Students often possess intuitive conceptions about the world that do not align with a scientifically-accurate understanding of evolution (Nehm, Rector, & Ha, 2010; Smith, 2010; Coley & Tanner, 2012). Additionally, evolution requires knowledge of abstract concepts that are hard for people of all ages, such as randomness in mutation and genetic drift (e.g., Lecoutre, Rovira, Lecoutre, & Poitevineau, 2006; Garvin-Doxas & Klymkowsky, 2008). Student difficulties translate into challenges faced by instructors. Undergraduates retain inaccurate ideas about evolutionary concepts even after carefully-planned lessons (e.g., Phillips, Novick, Catley, & Funk, 2012; Price et al., 2014). One factor that influences the effectiveness of instruction is teacher knowledge (e.g., Hill, Rowan, & Ball, 2005; Sadler, Sonnert, Coyle, Cook-Smith, & Miller, 2013). Helping students learn evolution likely requires more than just content knowledge.

One type of teacher knowledge associated with student learning is pedagogical content knowledge (e.g., Hill et al. 2005; Sadler et al., 2013). Pedagogical content knowledge (PCK) is knowledge of teaching and learning used in the everyday work of instructors (Shulman, 1987). PCK is topic-specific, meaning that an instructor needs distinct PCK for each topic they teach (e.g., natural selection, speciation; Gess-Newsome, 2015). This knowledge goes beyond disciplinary knowledge scientists use in their day-to-day research (Ball, Thames, & Phelps, 2008). For example, an evolutionary biologist thinks carefully about whether changes in populations result from adaptive or non-adaptive processes. In addition to knowing this, an undergraduate evolution instructor benefits from knowing that students commonly think all

evolutionary change results from natural selection, being able to identify when students express this idea, and having strategies to help students develop more accurate conceptions. The education research community lacks consensus on what components make up PCK, but widely-cited work includes these four components (Figure 3.1): knowledge of student thinking, knowledge of assessment, knowledge of instructional strategies, and knowledge of curriculum (e.g., Shulman, 1987; Magnusson, Krajcik, & Borko, 1999; Park & Oliver, 2008).

Knowledge of student thinking includes awareness of difficulties students are likely to have in thinking about a specific topic, how they will express their thinking, and how their ideas are likely to change as a result of instruction (Schneider, 2015). This component of PCK encompasses what makes topics easy or difficult. For example, undergraduates learning about natural selection often think that new traits arise in a population because individuals need them to survive and that new traits are always beneficial (Gregory, 2009). Anticipating this misconception allows an instructor to plan questions to reveal this thinking and instruction that helps students construct scientifically-accurate ideas about natural selection.

Knowledge of assessment includes knowledge of the dimensions of a topic that are important to assess, methods to assess student knowledge of a topic, and how to interpret results of assessment (Park & Oliver, 2008). Continuing with the example of natural selection, there are multiple research-based approaches to assessing undergraduates' thinking about key concepts in natural selection. Forced-response instruments include the Conceptual Inventory of Natural Selection (Anderson, Fisher, & Norman, 2002) and the Conceptual Assessment of Natural Selection (Kalinowski, Leonard, & Taper, 2016). Alternatively, instructors can ask constructed-response items from an assessment collection called ACORNS (Assessing COntextual Reasoning about Natural Selection) and use an online portal to automatically analyze students'

written responses (Moharreri, Ha, & Nehm, 2014). These instruments are carefully designed to reveal both misconceptions and scientifically-accurate ideas. Instructors can use them to gauge students' prior knowledge, measure what they know following instruction, and assess learning gains resulting from instruction.

Knowledge of instructional strategies includes knowledge of topic-specific approaches that help students reevaluate problematic ideas and construct scientifically-accurate ideas, including examples, models, illustrations, analogies, problems, demonstrations, and simulations. For example, a series of simulations in which undergraduates design experiments and collect data about snail shell thickness and predation corrected misconceptions about natural selection among beginning and advanced undergraduates (Abraham et al., 2009). An inquiry-based curriculum based on the digital evolution platform Avida-ED (http://avida-ed.msu.edu) can increase the level of complexity of undergraduates' explanations about the relationship between mutation and selection (Bray-Speth, Long, Pennock, & Ebert-May, 2009). An instructor with awareness of these instructional strategies can employ them to facilitate development of their students' ideas about natural selection.

Knowledge of curriculum includes knowledge of goals and standards for students learning a topic and knowledge of specific curriculum for teaching a topic at a particular level (Magnusson et al., 1999). This component must be tailored to be relevant to undergraduate education because state and national standards do not exist for undergraduate education and college instructors rarely adopt a full curriculum developed by someone else. Nonetheless, instructors benefit from knowledge of learning goals for particular topics. Therefore, knowledge of curriculum at the undergraduate level is best thought of as knowledge of learning goals. One example of work that generates knowledge of learning goals is the BioCore Guide (Brownell,

Freeman, Wenderoth, & Crowe, 2014). The BioCore Guide is a framework of specific concepts a graduating general biology major should know. Building on Vision & Change (AAAS, 2011), researchers gathered input from more than 240 biologists to arrive at general principles and specific statements about what students should learn about evolution and four other core concepts in biology. These provide guidance to instructors as they develop objectives for what they aim to help their students achieve. Articulating learning objectives is the first step for instructional design (Wiggins & McTighe, 1998).

Instructors need PCK for every topic in evolution they teach, which is a tremendous body of knowledge to develop alone. However, empirical investigations of undergraduates' thinking and learning have produced knowledge that is available in peer-reviewed literature. PCK generated by researchers and practitioners and made accessible for study and use by instructors is referred to as "collective" PCK[4]. Currently, it is unclear if the collective PCK available adequately addresses the topics undergraduates need to learn. Some propose that education research has focused on natural selection at the expense of other important evolutionary concepts (Padian, 2010; Novick, Schreiber, & Catley, 2014). The BioCore guide identifies mutation, gene flow, genetic drift, speciation, common ancestry, phylogenetics, evolutionary trade-offs, and sources of phenotypic variation as additional evolutionary topics important for undergraduates to master (Brownell et al., 2014).

The goals of this study were to determine what collective PCK for undergraduate evolution instruction is available and what is missing, to produce a searchable database of

[4] A recently-proposed "consensus" model of PCK refers to this as topic-specific professional knowledge (Gess-Newsome, 2015), but this name has not yet been widely adopted. We, like other researchers, find "collective PCK" to be a more intuitive name (Smith, Esch, Hayes, and Plumley, 2016).

available collective knowledge, and to demonstrate that PCK is a useful framework to guide future research on teaching and learning in undergraduate biology education. Specifically, we addressed two research questions:

1. What collective PCK for undergraduate evolution education is available in peer-reviewed literature?
2. How do the topics covered by undergraduate evolution instructors compare to the topics for which collective PCK is currently available?

Methods

Identifying peer-reviewed literature

We aimed to identify all peer-reviewed literature potentially relevant to undergraduate evolution education. We started by searching the Education Resources Information Center (ERIC) database because this database focuses on education research and information. We conducted a single Boolean search with 26 term representing topics in evolution (e.g., natural selection, speciation), 19 terms about teaching and learning (e.g., student thinking, instruction), and 14 terms referring to study population (e.g., undergraduate, post-secondary) (Table S3.1). We used the operators "OR" and "AND" so that each search result contained at least one term from each category.

The ERIC database indexes education journals, and we anticipated that relevant literature had also been published in other journals. Therefore, we also searched within specific journals. We reviewed every published volume of *Evolution: Education and Outreach* because we expected many articles in this journal to be relevant. We also searched *Science*, *Evolution*, *Genetics*, *PLOS* and *BioScience*. We used more general terms for these journals, including

"evolution" and "education." Some of these journals organize papers related to education into searchable collections. In those cases, we reviewed every paper within the collection. We conducted all searches between July and October 2016, and added a few papers published later in 2016.

Screening peer-reviewed literature for inclusion

We reviewed every article produced by these searches by reading titles, then abstracts, and full papers as necessary. If there was any question about the relevance of a publication, we included it for further analysis. We excluded papers that clearly did not address the teaching or learning of *biological* evolution, such as those that discussed the "evolution" of a history curriculum and papers about legal proceedings, court litigation, and evolution education policy. We also excluded papers that primarily focused on students' beliefs, acceptance, and attitudes regarding evolution. We recognize these papers can be highly valuable to instructors and that affective factors may impact learning, but our aims focused exclusively on cognitive components of evolution education. We also excluded papers whose primary objective was to examine a pedagogical approach (e.g., case study teaching, argumentation), rather than to learn about teaching and learning evolution. For example, one study investigated the impact of a fully-flipped versus partially-flipped classroom on student performance, withdrawal rates, and attitudes toward active learning. This study took place in a course about evolution, but teaching and learning evolution were not the focus of the research questions (Adams, Garcia, & Traustadóttir, 2016). We excluded papers that exclusively presented content knowledge about a topic in evolution. These papers clearly aimed to contribute to instructor knowledge of a particular topic, but did not provide insights into PCK. Ultimately, we also excluded papers published prior to 1990 and those that described computer resources that were no longer

discoverable using an internet search because we determined the utility of these papers to instructors to be minimal.

Originally, we intended to include papers about evolution education at the high school level. We expected these papers to be useful in at least two ways. First, papers presenting instructional strategies for high school students could provide ideas for instructors, especially instructors of introductory and smaller college classes. Second, we anticipated that papers investigating student thinking could be useful because advanced high school students and those early in their college career may not differ substantially. We collected and analyzed these papers in the same way as we analyzed papers that focused on undergraduates. Ultimately, we determined that papers about high school did not fill gaps in collective PCK specific to undergraduate education. Therefore, we did not include these in our final analysis and results. Some of these papers may provide new ideas for college instructors, so we have included them in our searchable database (see *Creating searchable database of collective knowledge).*

We next evaluated the efficacy of our literature search. We searched reference sections of papers in our collection for relevant work we had not yet identified. Our first round of searching references involved selecting 17 papers we expected to be most likely to cite papers we had not yet identified. We included literature reviews and other papers that extensively reviewed prior work (n = 9). We also included papers on evolution topics underrepresented in our sample (n = 8), such as evolutionary developmental biology and biodiversity. We examined all peer-reviewed literature cited by these papers, and determined which were relevant to our study. If a paper was cited by more than one of these 17 papers, we only counted it once. We found 138 citations relevant to our study. We had already identified 83.3% (n = 115) of these papers. This provided a

conservative estimate of the proportion of all relevant literature that we had successfully identified. We added 23 new papers to our collection using this approach.

We repeated this process with a randomly-selected sample of 50 papers from our collection, to calculate a more general estimate of our search efficacy. We reviewed the citations as described above, and identified 154 papers that were relevant to our study. We again determined which papers were already part of our collection. We had already identified 96.7% (n = 149) of these papers. We added five new papers to our collection, and concluded that our literature search had revealed the vast majority of relevant publications. The final collection included 316 papers relevant to undergraduate evolution education. These publications span 41 peer-reviewed journals, including 29 education journals and 13 discipline-specific journals. Most of these publications focus on U.S. student populations, however we also found work that studies students in South America, Europe, Australia, and Asia.

Systematic analysis of the literature

We analyzed each of these 316 papers to characterize the collective PCK available by PCK component, type of work, and evolutionary topic.

Identifying PCK component

The authors independently read abstracts and full papers as necessary to determine what component(s) of PCK each paper addressed, and then discussed any disagreements until we reached consensus. Most papers addressed a single PCK component (student thinking, assessment, instructional strategies, or goals), but some addressed two. Most commonly, papers that addressed two components focused on student thinking and either assessment or instructional strategies. As part of this analysis, we also analyzed the type of work for each

paper. We categorized each paper as descriptive, empirical, literature review, or author's perspective (Table 3.1).

Identifying evolution topic

We determined the evolution topic(s) addressed in each paper. We began this process by creating a list of evolutionary topics, drawing on evolution textbooks and our own disciplinary expertise. The authors and undergraduate research assistants independently read each abstract, and papers as necessary, to determine the topic(s) addressed. We gathered as a research team to discuss disagreements until we reached consensus. We iteratively refined this list of topics throughout the analysis process. As the list of evolution topics changed, we re-analyzed papers we had previously considered. After every abstract had been categorized for topic and the list of evolution topics was no longer changing, we examined all papers within a single topic. We examined related topics at the same time to clarify the boundaries between topics. This approach helped us to refine the descriptions of each topic.

Some topics in our final list are organized into overarching categories. This structure was necessary to make comparisons between peer-reviewed literature and topics covered in evolution courses. We grouped large-scale patterns in evolution, major transitions in the history of life, and deep time into the overarching category of macroevolution. We also organized tree-thinking and systematics together under phylogenetics. Lastly, we organized population genetic modeling, allelic interactions, Hardy-Weinberg Equilibrium, and genetic drift within the overarching category of population genetics.

Creating searchable database of collective knowledge

We organized the data produced by these analyses into an excel file to create a searchable database. The database includes the 316 relevant papers, and is organized by PCK component,

type of work, evolution topic, journal, and publication year. The database also includes a worksheet with 93 papers about high school evolution education organized in the same way (Supplemental Materials).

Identifying and comparing topics taught in undergraduate evolution courses

We aimed to compare the collective PCK accessible in the peer-reviewed literature to what is relevant to undergraduate evolution instructors. We used course syllabi from evolution courses as a proxy for what evolution instructors see as important topics in undergraduate evolution education. We limited our search to courses that taught evolution broadly, excluding courses with more specific foci (e.g., Macroevolution, Evolution of flowering plants, Molecular evolution) and more broad foci (e.g., Introductory biology, Cell biology, Zoology). We focused on upper-division evolution courses because we expected them to cover a greater diversity of topics in evolution that lower-division courses. We collected syllabi from around the country, focusing on large, public universities. We searched university websites for publicly available syllabi but found few. Therefore, we used course schedules and class bulletins to identify the course number for upper-division evolution courses and instructors who had recently taught the course. We emailed instructors directly to ask if they would be willing to share their most recent syllabus. We collected syllabi from 32 upper-level evolution courses, spanning 25 states and 27 institutions. We analyzed each syllabus to determine the topics taught in each course. In the analysis presented here, we focused on topics taught in at least 40% of the courses we surveyed. We compared these topics to what we found in our collection of peer-reviewed papers. We made comparisons between overarching topic categories for macroevolution, phylogenetics, and population genetics because syllabi often did not describe these topics at a finer grain size.

Results

Analysis of peer-reviewed literature by PCK component and type of work

Out of 316 papers about undergraduate evolution education, 75% presented instructional strategies (n=239), 21% addressed student thinking (n=64), and 8% dealt with assessment (n=24). Six papers (2%) concentrated on goals for undergraduate evolution instruction. Fifteen papers addressed more than one component of PCK.

Student Thinking. These papers investigated or summarized student thinking about specific topics in evolution. Most of these studies were empirical (Table 3.1). For example, one paper examined the effects of college students' prior knowledge on their ability to reason from information depicted in cladograms, and found that students demonstrated more sophisticated reasoning when the taxa were unfamiliar and they had to rely solely on the diagrammatic information presented rather than prior knowledge (Novick & Catley, 2014). Other papers reviewed empirical work, providing a distilled resource for instructors. For example, one paper describes the process of natural selection, discusses possible causes of misconceptions, and reviews the most common misconceptions undergraduates possess about natural selection and adaptive evolution (Gregory, 2009). Lastly, some papers presented author's professional perspectives about the origin and causes of difficulties students have in learning topics in evolution.

Assessment. Papers about assessment described the development and validation of instruments to measure student understanding about a topic in evolution, further evaluated previously-published instruments, and addressed theoretical questions about assessment. Most assessment papers were empirical (Table 3.1). For example, one paper described the EvoDevo CI, which measures student thinking about six core concepts in evolutionary developmental

biology (Perez et al., 2013). Other researchers conducted a distracter analysis of the Conceptual Inventory of Natural Selection using Item Response Theory and suggested test items that require revision (Battisti, Hanegan, Sudweeks, & Cates, 2010).

Instructional Strategies. These papers addressed strategies for teaching topics in evolution. Some papers described specific strategies, ranging in grain size from a single activity or class period to a full course. For example, one paper describes and evaluates a laboratory exercise in which students develop and test simple hypotheses about sperm competition in humans (Cotner & Gallup, 2011). Another paper describes and evaluates an entire evolutionary biology course that uses emerging infectious diseases as a case study to appeal to students who aspire to become health professionals at a historically black college (Pai, 2009). Papers discussing instructional strategies described strategies, with or without collecting empirical data to assess efficacy (Table 3.1). Some presented author perspectives on broader issues. For example, one essay argued that origin of life and prebiotic evolution should be part of the undergraduate biology curriculum (Lazcano & Peretó, 2010).

Goals. We identified six papers that dealt with goals for undergraduate evolution education. In addition to the BioCore Guide, which outlines learning goals for multiple topics in evolution, we found five papers that outlined goals for specific topics. For example, one paper outlined four central points that students need to understand about coevolution (Thompson, 2010), and another identified core concepts for teaching developmental aspects of evolution (Hiatt et al., 2013). Two papers about goals were empirical (Hiatt et al., 2013, Brownell et al., 2014) and four presented author's perspectives (Brewer, 1996; Baum & Offner, 2008; Thompson, 2010; Gregory, Ellis, & Orenstein, 2011).

Analysis of peer-reviewed literature by evolutionary topic

We identified 22 distinct evolutionary topics and most papers (78%) addressed one or more of these (Table 3.2). The other 22% of papers addressed evolution broadly without specifying topic more narrowly. Natural selection, phylogenetics and evolution broadly accounted for 69% of published papers. Eight topics were addressed by five or fewer papers (Table3. 3). One hundred and seven papers addressed more than one topic. The majority of this overlap occurred within overarching categories (macroevolution, phylogenetics, and population genetics).

We also examined topic representation by PCK component to more richly characterize gaps in available collective PCK (Table 3.3). The representation of evolutionary topics across papers addressing instructional strategies was reflective of our complete collection of papers, but this was not true for papers focusing on student thinking or assessment. Student thinking papers largely focused on tree-thinking, natural selection, and evolution broadly. Ten topics in evolution were not addressed by a single student thinking paper (Table 3.3). Assessment papers focused primarily on natural selection and tree-thinking, and 14 topics were not addressed by a single assessment paper. Papers about goals for undergraduate evolution education addressed coevolution (n=1), evolutionary developmental biology (n=1), phylogenetics (n=2), and evolution broadly (n=2).

Comparison between topics covered by evolution instructors and available collective PCK

We identified seventeen topics that were covered in at least 40% of the 32 upper-division evolution courses we sampled from around the country. These topics were not equally represented in the peer-reviewed literature on undergraduate evolution education (Figure 3.3). Nearly all courses covered natural selection, macroevolution, speciation, phylogenetics, and

population genetics, but the number of papers addressing these topics varied considerably (Figure 3.3). Over 140 papers addressed natural selection or phylogenetics, but only 50 papers addressed macroevolution, speciation, or population genetics (Figure 3.3). Eighty-one papers addressed the remaining 12 topics, and two topics covered in almost 60% of courses were not addressed by any peer-reviewed paper (Figure 3.3).

Discussion

One aim of this paper was to demonstrate the utility of pedagogical content knowledge as a framework for analyzing existing research and providing a roadmap for future work. We have addressed this aim by identifying gaps in the collective PCK currently available for undergraduate evolution education, and now propose priorities for the research community. We first outline priorities by evolution topic. Next, we propose that research on student thinking is fundamental and therefore should be prioritized. Lastly, we consider the value of different types of work, including literature reviews, empirical investigations, and descriptions of instructional strategies, and propose priorities for types of work going forward.

Evolution topics that are top priority for future research

As anticipated, natural selection has received much more attention than other evolution topics. This is despite the breadth of topics taught in undergraduate evolution courses (Figure 3.3) and evidence that learning natural selection does not prepare students to do well on other topics in evolution (e.g., Padian, 2010; Novick, Schreiber, & Catley, 2014; Price & Perez, 2016). Though natural selection is a central mechanism in evolution, it is insufficient to focus primarily on adaptive evolution in an undergraduate evolution course. Focusing primarily on natural selection runs the risk of exacerbating an existing problem: undergraduates often mistakenly

conflate evolution and natural selection (e.g., Jakobi, 2010; Begrow & Nehm, 2012). Furthermore, undergraduates' understanding of natural selection may improve as a result of effective instruction for other evolutionary topics (Price & Perez, 2016).

We propose that four evolutionary topics warrant immediate attention from the research community: macroevolution, speciation, quantitative genetics, and population genetics. We generated this list by considering: topics that were taught in at least 60% of the classes we surveyed, topics for which collective PCK is largely unavailable (Figure 3.3), and topics that have been identified as core ideas in biology by the community (e.g., Padian, 2010; AAAS, 2011; Brownell et al., 2014; Cary & Branchaw, 2017). It is important to note that there are many finer-grain concepts encompassed by each of these topics. Our analysis did not reveal many papers about learning goals, but a critical first step for the research and education community will be identifying a list of learning goals undergraduates should achieve for these topics. Once the community has generated a list of key concepts, researchers can investigate student thinking about each concept within a topic, develop and refine assessments, and begin testing instructional approaches. Another potentially promising approach to discovering PCK for these critical topics is interviewing experienced and effective undergraduate evolution instructors to discover what they know about teaching and learning these topics. Instructors generate their own PCK through careful reflection on their own instruction (Gess-Newsome 2015) and are likely to be a rich source of knowledge that can be useful to other instructors.

There are other topics that are commonly taught in college evolution courses and for which limited collective PCK is available. The evolution of behavior, molecular evolution, sexual selection, and coevolution were each taught in over 60% of the classes we surveyed, but have been the focus of little or no research on student thinking and assessment. These are not

explicitly included in lists of core ideas for biology (e.g., Brownell et al., 2014; Cary & Branchaw, 2017), however, they are clearly important for upper-division evolution courses, which are required for many undergraduate life sciences majors. Therefore, we encourage the evolution education research community to consider these four topics in need of attention as well.

Research on student thinking is foundational to both teaching and education research

We propose that research on student thinking take priority over other PCK components because it is foundational to both teaching and education research. First, the development of research-based assessments and instructional strategies depends on knowing the difficulties students are likely to have in learning a topic and how their thinking can change throughout instruction. Thus, researchers need empirical data on student thinking to develop assessment and instructional strategies. Research on student thinking and research on ways to reveal their thinking (i.e., assessment) can often be conducted in tandem, meaning that progress in generating collective PCK about student thinking can occur simultaneously with progress in generating collective PCK about assessment. Second, even if extensive collective PCK for assessment and instructional strategies were available, college biology instructors would still create formative and summative assessments and lessons of their own design. Therefore, college instructors also stand to benefit disproportionately from research on student thinking compared to research on assessment and instructional strategies. Additionally, knowledge of student thinking is crucial for effective teaching, and this is especially true for student-centered instruction. For example, college math instructors learning to use an inquiry-based curriculum struggled because they could not anticipate the difficulties students were likely to have and were unable to make sense of student reasoning during class discussions (Wagner, Speer, & Rossa, 2007; Speer & Wagner,

2009; Johnson & Larsen, 2012). These instructors were constrained by their lack knowledge of student thinking.

Literature reviews are scarce, but most likely to be high-impact

We propose that the research community also continue to produce literature reviews about student thinking and assessment for topics in evolution. We suspect that literature reviews are most useful to instructors because they distill collective PCK from numerous empirical investigations, considerably minimizing the number of papers an instructor needs to read. For example, one literature review summarizes common misconceptions about phylogenetics, the relationships among these misconceptions, and their cognitive origins (Meisel, 2010). An instructor can access knowledge generated by over 20 empirical investigations of student thinking about phylogenetics by reading this single paper. Currently, there are only a few topics with a sufficient body of research on student thinking to warrant a literature review, and *Evolution: Education & Outreach* has led the way by publishing literature reviews written with instructors in mind (e.g., Gregory, 2008; Gregory, 2009; Meisel, 2010). We found no reviews for assessment, but college instructors would likely benefit from a paper that reviewed all published, research-based assessments for topics in evolution. Instructors need to know the key concepts and misconceptions addressed in each assessment, the target population, and considerations related to validity and reliability in order to select assessment appropriate for their students and instructional goals. We encourage the community to continue to write reviews with college instructors in mind as more empirical work accumulates regarding student thinking and approaches to revealing student thinking.

Papers about instructional strategies should identify and empirically test what components are critical to student learning

Papers about instructional strategies may provide ideas and inspiration to instructors but most do not contribute to an overall body knowledge about how to facilitate learning about topics in evolution. We identify two areas for improvement for future papers presenting instructional strategies. First, existing papers often do not carefully consider the components of a strategy that are essential for student learning. We propose that a fidelity of implementation framework is a fruitful approach for considering critical components of an instructional strategy (e.g., Stains & Vickrey, 2017). Critical components of an instructional strategy include (a) the procedures of how a strategy is intended to be implemented, (b) what knowledge instructors must possess to effectively implement the strategy, (c) how the instructor should behave and interact with students while implementing the strategy, and (d) how students should interact with the instructor, peers, and learning materials during implementation (Stains & Vickrey, 2017). Identifying what components make a strategy effective is important for the developer and other instructors who may use the strategy, regardless of whether the strategy has been empirically tested. Considering critical components encourages the developer to be more reflective about the instructional strategy and how it influences student outcomes, which is a valuable exercise for developing personal teaching expertise (e.g., McAlpine, Weston, Beauchamp, Wiseman & Beauchamp, 1999). Even more importantly, other instructors are more likely to be successful in implementing a published instructional strategy if the developer has clearly articulated their thinking about what is essential for student learning.

Second, instructional strategies that have been empirically demonstrated to be effective at facilitating student learning in one instructional context generally require further study to make them generalizable to other instructional contexts. Ultimately the critical components of an instructional strategy must be determined empirically. As an example, Kalinowski, Leonard,

Andrews and Litt (2013) demonstrated that six classroom exercises for teaching natural selection can be highly effective at facilitating student learning in one instructional context. They addressed the four critical components in their paper and proposed hypotheses about what made these classroom exercises effective. These are important first steps, but additional empirical work is necessary to test their hypotheses. Without this follow-up, we fail to move the field toward generalizable principles for instructional strategies for teaching natural selection to undergraduates. Wide adoption of the fidelity of implementation framework by the education research community will allow us to begin making comparisons across instructional strategies. Only then will we have generated collective PCK for instructional strategies that is generalizable beyond single classrooms.

Limitations and Conclusions

We encourage readers to consider three limitations of this work. First, the reality of any analysis of prior literature is that is immediately out of date because new work is always being published. We hope this paper lays the groundwork for continuing to monitor the progress of evolution education research. Second, there are likely some articles we did not identify. This is most problematic if the missing papers contribute to filling the gaps we identified. We conducted a reference check with this in mind, but we recognize there are limitations to that approach for testing our efficacy as well. Third, most of the work we were able to find focuses on undergraduates in the United States and therefore may not be generalizable to other student populations.

Our work is the first to demonstrate the utility of PCK as a lens for analyzing existing work relevant to undergraduate biology education. We found that collective PCK available in peer-reviewed literature does not adequately address the topics in evolution that college

instructors teach. Many topics for which little or no collective PCK is available are taught in the majority of upper-division evolution courses. Given the importance of teacher knowledge to effective instruction, and the centrality of student thinking to evidence-based instructional practices, identifying and filling the gaps in our collective knowledge is critical to maximizing the utility of education research to college instruction.

Figures, Tables, and Legends

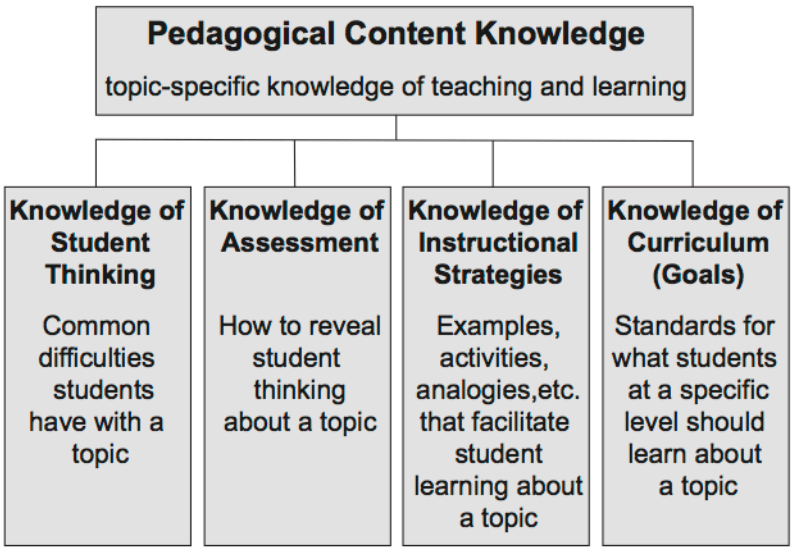

Figure 3.1. Components of pedagogical content knowledge (PCK).

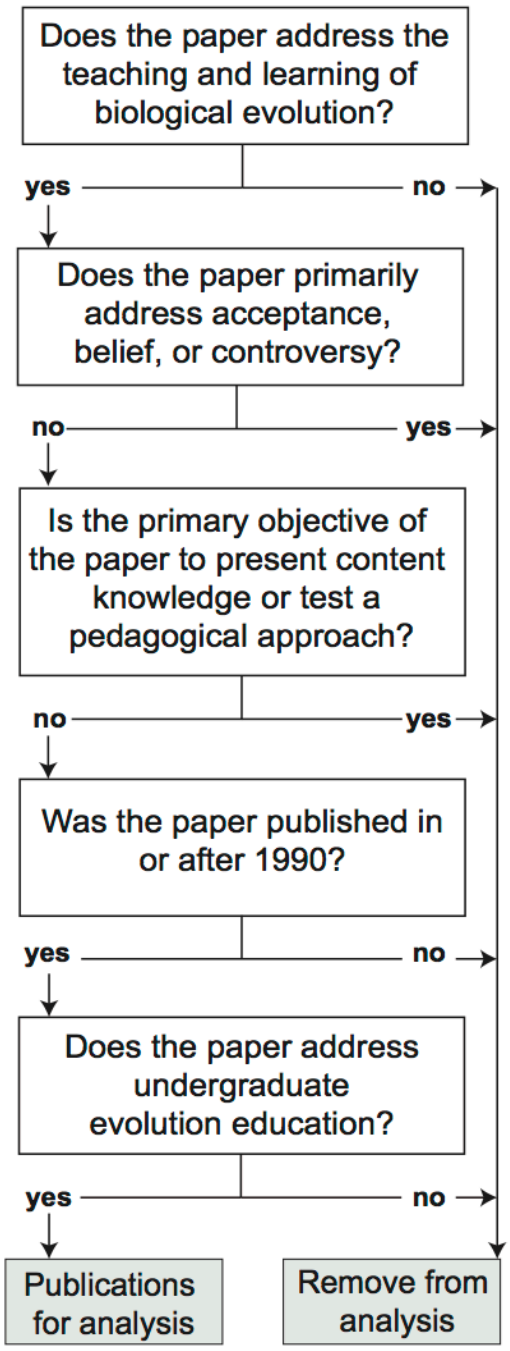

Figure 3.2. Decision tree for screening papers for analysis.

Table 3.1. Descriptions of types of papers, and representation by PCK component

Type of paper	Definition	% of all papers (n=317)	% of student thinking (n=65)	% of assessment (n=24)	% of instructional strategies (n=239)
Descriptive	Describes an activity, lesson, unit, or course. May provide resources and student self-report data.	51	0	0	67
Empirical	Presents data collected and systematically analyzed to answer a research question.	37	83	92	23
Author's perspective	Presents an argument, drawing on existing literature and professional experience.	10	8	8	9
Literature review	Extensively reviews existing empirical literature.	2	9	0	1

Table 3.2 Descriptions of evolution topics, some of which are grouped (indicated by indent).

Topic	**Description of how topic was operationalized in papers**
Natural selection	Natural selection, heritable variation, differential fitness/reproductive success,
Macroevolution	
Macro patterns	Phyletic gradualism and punctuated equilibrium, biogeography
Major transitions	Origin of life, origin of the cells, evolution of multicellularity, extinction
Deep time	Timeframe of the history of earth, including geological and paleontological evidence
Speciation	Species concepts, fossil evidence of speciation
Phylogenetics	
Tree-thinking	Interpreting evolutionary trees, relatedness, common ancestry
Systematics	Building trees with morphological and molecular data; homology and homoplasy
Population genetics	
Pop gen modeling	Mathematical models of population genetics, effective population size
Allelic	Dominance in allelic pairs, heterozygosity, heterozygote advantage
Genetic drift	Random sampling of alleles that results in changes in allele frequencies
Hardy-Weinberg	Calculating, interpreting, and reasoning about Hardy-Weinberg Equilibrium
Origin of variation	Mutation, horizontal gene transfer, meiosis, randomness of mutation
Evolution of behavior	Animal behavior, human behavior, sociality, cooperation, morality
Human evolution	Human & primate evolution, human social behavior, human disease evolution, race
Molecular evolution	Rate of mutation, chromatin evolution, protein evolution, molecular clock
Sexual selection	Mate choice, male-male competition, sexual behavior, sexual and natural selection
Coevolution	Predator-prey and plant-herbivore interactions, coevolutionary arms race, Red Queen
Quantitative genetics	Variation in quantitative traits
Evolutionary	Application of evolution to the study of human health
Biodiversity	Intra-species diversity, biogeography
EvoDevo	Evolutionary developmental biology, heterochrony, heterotopy, organogenesis
Human impact	Human impacts on contemporary evolution
Evolution broadly	Papers in this category did not focus on any particular topic in evolution.

Table 3.3. Number of papers (n = 315) by PCK component and evolution topic. Shading indicates number of papers.

Topic	Student thinking (n=64)[A]	Assessment (n=24)[A]	Instructional strategy (n=238)[A]
Natural selection	16	10	69
Macroevolution			
Macro patterns	1	0	3
Major	1	0	5
Deep time	2	2	3
Speciation	0	2	8
Phylogenetics			
Tree-thinking	18	8	12
Systematics	4	1	27
Population			
Pop gen	0	0	6
Allelic	0	1	2
Genetic drift	3	1	8
Hardy-	0	0	5
Origin of variation	2	0	10
Evolution of	1	0	11
Human evolution	2	0	13
Molecular	0	0	12
Sexual selection	0	0	5
Coevolution	0	0	5
Quantitative	0	0	1
Evolutionary	0	0	5
Biodiversity	1	0	5
EvoDevo	1	1	7
Human impact	0	0	2
Evolution broadly	20	1	48

Key

0-4	5-9	10-14	15-19	20+

[A]107 papers were coded for multiple topics: 81 were coded for two topics, 21 were coded for 3 topics, 1 was coded for 4 topics, 2 were coded for 5 topics, and 2 were coded for 6 topics

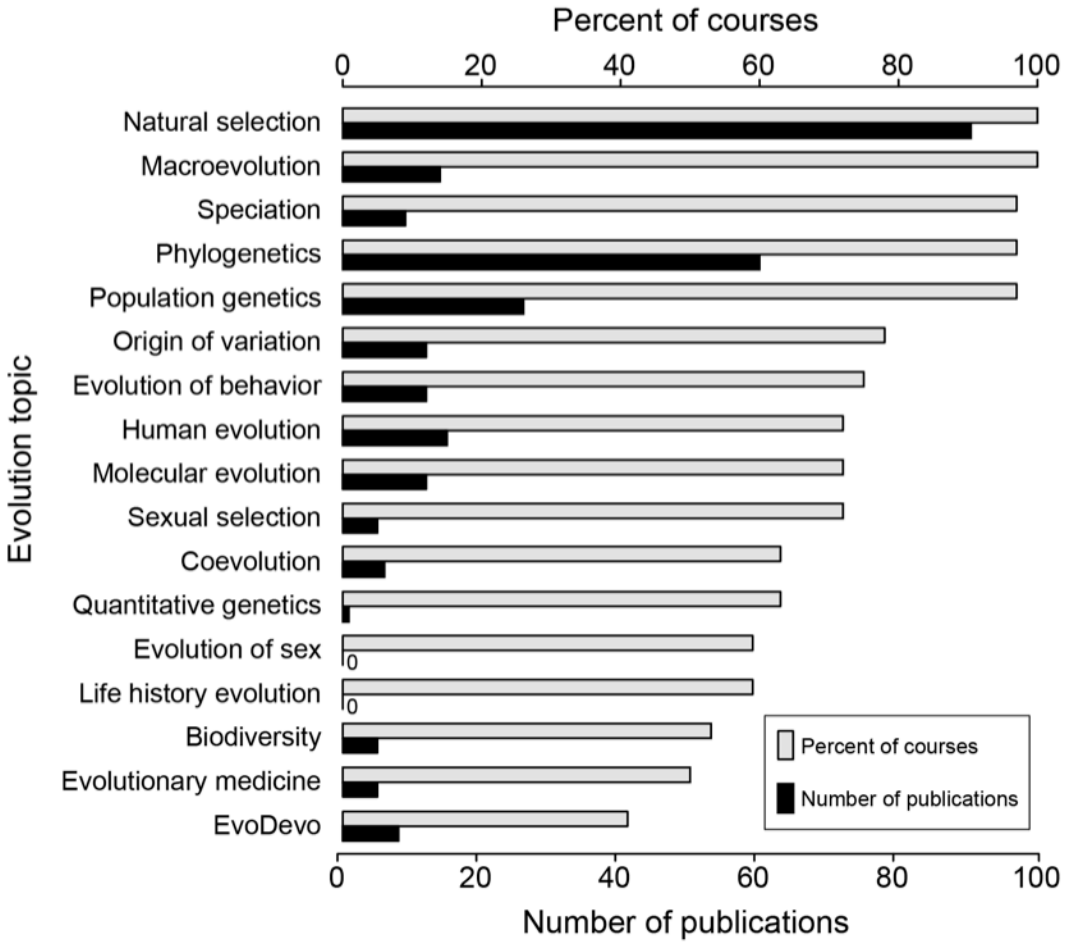

Figure 3.3. Topics taught in evolution courses compared to topic representation in the peer-review literature. Topics were included if they were listed in more than 40% of course syllabi (n=32).

Supplementary Materials

Table S3.1. Exact terms used in Boolean search in ERIC database. Shortened words (e.g. Lab) and abbreviations (e.g. PCK) allow for a more inclusive search.

Evolution topic	Teaching and learning	Study population
Natural selection	Student learning	K-12
Genetic drift	Student thinking	High school
Gene flow	Misconception	Primary
Evolutionary tree	Naïve conception	Secondary
Phylogenetic tree	Case study	Post-secondary
Phylogenetics	Active learning	College
Macroevolution	Instruction	Undergraduate
Evo-Devo	Prior knowledge	University
Speciation	Teaching strategy	Higher education
Sexual selection	Pedagogical content knowledge	Graduate
Human evolution	PCK	Instructor
Molecular evolution	Pedagogy	Teacher
Kin selection	Subject matter knowledge	Faculty
Plasticity	SMK	Professor
Gene by environment	Threshold concept	
Hardy Weinberg	Lesson	
Population genetics	Activity	
Dominance	Exercise	
Allele	Lab	
Lamarck		
Tree thinking		
Adaptation		
Genome		
Variation		
Heritability		
Artificial selection		

Table S3.2. Number of papers (n =92) by PCK component and evolution topic.

Topic	Student thinking (n=17)	Assessment (n=3)	Instructional strategy (n=76)
Natural selection	7	2	35
Macroevolution			
Macro patterns	0	0	4
Major transitions	0	0	0
Deep time	2	2	3
Speciation	0	0	1
Phylogenetics			
Tree-thinking	1	0	2
Systematics	4	0	4
Population genetics			
Pop gen modeling	0	0	0
Allelic interactions	0	0	0
Genetic drift	0	0	2
Hardy-Weinberg	0	0	3
Origin of variation	0	0	1
Evolution of behavior	0	0	0
Human evolution	1	0	8
Molecular evolution	0	0	1
Sexual selection	0	0	1
Coevolution	0	0	2
Quantitative genetics	0	0	0
Evolutionary medicine	0	0	1
Biodiversity	0	0	1
EvoDevo	0	0	0
Human impact	0	0	0
Evolution broadly	9	1	30

Supplemental text regarding collective knowledge for teaching high school

Out of 92 papers about high school evolution education, 86% presented instructional strategies (n=76), 18% addressed student thinking (n=17), and 3% dealt with assessment (n=3). One paper (1%) concentrated on goals for high school evolution instruction. Five papers addressed more than one component of PCK. We identified 16 distinct evolutionary topics and most papers (59%) addressed one or more of these (Table S3.2). The other 41% of papers addressed evolution broadly without specifying topic more narrowly. Natural selection, and evolution broadly accounted for 87% of published papers. Twelve topics were addressed by five or fewer papers. Twenty seven papers addressed more than one topic. The majority of this overlap occurred with natural selection and another topic (e.g. natural selection and human evolution).

Directions for using the Collective PCK for Undergraduate and High School Evolution Education Database

We have made the database generated by this research available as a resource for education researchers and instructors. This database is stored in two Excel worksheets, one for work that includes undergraduates and one for work on high school students. You can open it using a free program like Google Sheets if you do not have access to Excel.

Both worksheets are formatted the same way. Each line of the worksheet corresponds to one paper and lists the full APA citation, journal title, publication year, PCK component(s), type of work, and evolution topic(s). Some evolution topics are organized into overarching categories, as shown in Table 2. Overarching categories are indicated using all caps for column titles.

You can use the “Sort” function in Excel to find papers of interest to you. For example, imagine you want to learn about student thinking related to tree-thinking. Select the full data set by selecting the cell in the uppermost left. Select the dropdown list “Data,” then select “Sort.” This opens a box. Make sure “My list has headers” is selected. Sort first by the Column titled “Tree-thinking” and then the column titled “Student thinking.” The rows will rearrange and the those at the top will be papers describing undergraduate’s thinking about tree-thinking. You can use the journal, publication year, and type to give you more information about the paper to determine if it meets your needs.

This searchable file is freely available as a supplemental material with Ziadie and Andrews (2018) at https://www.lifescied. org/doi/10.1187/cbe.17-08-0190.

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CHAPTER 5

DON'T REINVENT THE WHEEL: CAPITALIZING ON WHAT OTHERS ALREADY KNOW ABOUT TEACHING TOPICS IN EVOLUTION[5]

[5] Ziadie, M. A., & Andrews, T. C. (2019). Don't Reinvent the Wheel: Capitalizing on What Others Already Know about Teaching Topics in Evolution. The American Biology Teacher, 81(2), 133-136. Reprinted here with permission from the publisher.

Abstract: What knowledge do you need to be an effective instructor? One key type of knowledge is pedagogical content knowledge (PCK), which includes awareness of how students are likely to think about a topic and where they will struggle as they learn that topic. We propose PCK as a valuable framework for reflecting on your own knowledge for teaching topics in evolution. We have created a searchable file that uses PCK as a framework to organize over 400 peer-reviewed papers from 40+ journals to give you better access to relevant resources for teaching evolution to undergraduates and advanced high school students. None of us have time to read 400 papers to inform our teaching, so we provide tips to maximize your use of this collective knowledge in the time you have available. We have written these to be useful to instructors across career stages.

Take a moment to reflect on the knowledge that you use when you teach evolutionary topics. Most obviously, you use knowledge of the discipline of evolutionary biology. You also use pedagogical content knowledge (PCK). PCK combines content knowledge of a specific topic with knowledge about how students will interact with that topic as they learn (Magnussen et al., 1999; Park & Oliver, 2008; Gess-Newsome, 2015). Most often we build PCK through teaching experience, but could we also benefit from the published work of veteran evolution instructors and education researchers? We think so. Our aim in this article is to guide you to recognize the PCK that you may already have and to encourage you to capitalize on collective knowledge to continue to build PCK for teaching topics in evolution.

You have been using and building PCK since you started learning to teach. For example, imagine you pose this question to your students and they write down their thoughts: "A species of fish lacks fins. How would biologists explain how a species of fish without fins evolved from an ancestral fish species with fins?" (Nehm et al., 2012). Now reflect: What kinds of answers do you expect from your students? Could you predict a difficulty your students would have with this question? Maybe you predicted that undergraduates would have a much harder time answering this question accurately than one about how traits become common through natural selection (Nehm & Ha, 2011). Or maybe you thought about how students would be likely to explain that fins evolved away because the fish didn't "need" them anymore (Bishop & Anderson, 1990). If so, you were relying on PCK for teaching natural selection.

PCK is central to many parts of teaching. We use PCK when we decide what learning objectives for a topic are important and reason- able for students to achieve and what objectives are less crucial and can be cut if we run out of time. We employ PCK when predicting what makes a topic particularly hard to learn and where students might get stuck. During instruction

we use PCK when drawing on specific analogies, visual representations, or activities that we know are useful in helping students construct accurate understandings. Additionally, we rely on PCK when writing in-class questions and exam questions that reveal what students actually know about a topic. Importantly, what is challenging about learning (and therefore teaching) one topic is often entirely different than what is challenging about learning the next topic, so we depend on distinct PCK for each topic we teach.

As a result, the body of PCK we need as evolution instructors is staggering! What if we could supplement our personal PCK by drawing on the collective knowledge others have already built through experience and research? This knowledge can be referred to as "collective PCK." Collective PCK is generated by researchers and instructors and made publicly available for others. We have taken steps to make collective PCK in peer-reviewed literature more readily available. We hope this makes it more useful to college and AP Biology instructors at all career stages.

We created a searchable file that organizes over 400 peer- reviewed papers about undergraduate and high school evolution instruction from over 40 different journals (see https://www.life scied.org/doi/suppl/10.1187/cbe.17-08-0190). You can read more about how we identified, screened, and analyzed these papers in Ziadie & Andrews (2018). None of us have time to read 400 papers to inform our teaching, so here are some tips to maximize your use of this collective knowledge in the time you have available.

Tip 1: Use the Searchable File to Strategically Identify Peer-Reviewed Papers That Meet Your Specific Needs

The searchable file organizes each paper by several characteristics so that you can find just what you are looking for. Papers are organized by the area of instruction (student thinking,

instructional strategy, assessment, learning goals), the type of work (empirical, descriptive, author's perspective, literature review), evolution topic(s) (e.g., genetic drift, speciation, population genetics, human evolution), publication year, and journal. For example, if you are preparing to teach a lesson about phylogenetics and you want an evidence- based activity to challenge your students, you can sort the file by "phylogenetics," "type," and "instructional strategies." You would find eight papers that describe empirical investigations (i.e., type = empirical) of an instructional strategy for teaching phylogenetics to undergraduates and another 24 papers that describe instructional strategies but do not investigate their effectiveness (i.e., type = descriptive). This searchable file is freely available as a supplemental material with Ziadie and Andrews (2018) at https://www.lifescied. org/doi/10.1187/cbe.17-08-0190.

Tip 2: Prioritize Papers about Student Thinking

An awareness of how students are likely to think about a topic is central to all facets of teaching. Knowing what prior ideas students will have and what difficulties they may experience as they learn a topic will help you design student-centered learning objectives, assessments, and instruction. There are different types of work that present collective PCK about student thinking. We recommend starting with literature reviews, which condense what researchers have discovered and thus provide high return on invested time. For many evolutionary topics, there have been too few empirical investigations of undergraduate thinking to warrant a literature review (Ziadie & Andrews, 2018). In those cases, there is significant value in reading a single study that describes in detail the ideas students commonly have about a topic.

Tip 3: Not Sure Where to Start? Here Are Five Papers That We Highly Recommend

Gregory (2009). Though natural selection seems logical – even intuitive – to a biologist, it is consistently challenging for under- graduates to learn. Many students retain major misconceptions about natural selection, even after carefully planned instruction (e.g., Nehm & Reilly, 2007; Andrews et al., 2011). This literature review summarizes the specific difficulties students encounter in learning natural selection. This is particularly useful because the misconceptions that students invoke as they think about other topics, such as genetic drift and evolutionary development, are often rooted in misunderstandings of natural selection (Andrews et al., 2012; Hiatt et al., 2013; Price & Perez, 2016).

Gregory (2008) and Meisel (2010). Being able to read phylogenetic trees is a key step in developing understanding of evolutionary relationships. It is also very hard. Without targeted instruction many students leave college courses unable to interpret even simple trees (e.g., Novick & Catley, 2007). For example, students often think that the order of terminal nodes in a tree indicates relatedness and so assume that two nodes that are physically closer to each other are more closely related (Baum et al., 2005; Meir et al., 2007). Gregory (2008) reviews accurate and inaccurate ways to read phylogenetic trees and describes common misconceptions. Meisel (2010) focuses on the two most common misconceptions and suggests approaches to help- ing students overcome these challenges.

Mead & Scott (2010a) and Mead & Scott (2010b). Terms used in evolutionary biology often have different meanings in everyday life. For example, scientists use the term random to refer to unpredictability of a given event but students often interpret random to mean purposeless or meaningless. In fact, it is com- mon for students to think that random processes are not important in biological systems (Garvin-Doxas & Klymkowsky, 2008). This two-part essay series highlights problematic terms in teaching evolution and suggests research-based solutions.

Keeping in mind how the terminology we use might be heard by students prevents inadvertently promoting inaccurate ideas.

Tip 4: Create Opportunities to Learn from Your Students

What topics are particularly difficult for your students? Do you know why they struggle? Pick a topic that you expect to be challenging and that you would like to rethink in your teaching, and use your students as confidential informants to learn how they think about this topic. You can learn about student thinking in class by asking all students to write a response to an open-ended question on notecards (Angelo & Cross, 1993). A quick read through these cards will reveal a wide variety of thinking and some patterns that you might not anticipate. You can learn even more in conversations with students. Invite students with a range of performance to office hours and ask them probing questions with the goal of uncovering their thinking. Some prompts that we find useful are "What do you mean when you say...?" and "Tell me more about that." It is also informative to ask students to discuss how one concept relates to another. Try to get a complete picture of what a student is thinking before giving any feedback. You may be surprised by how much you learn!

Conclusion

Our work focused on cognitive components of evolution education rather than work related to students' beliefs, acceptance, and attitudes
regarding evolution. We recognize that such work can be highly valuable to instructors, but it was outside the scope of the research that produced the searchable file. We recommend a recent essay that presents a framework, reviews relevant research, and recommends teaching practices to reduce perceived conflict between evolution and religion and increase acceptance of evolution among students (Barnes & Brownell, 2017).

Figures and Legends

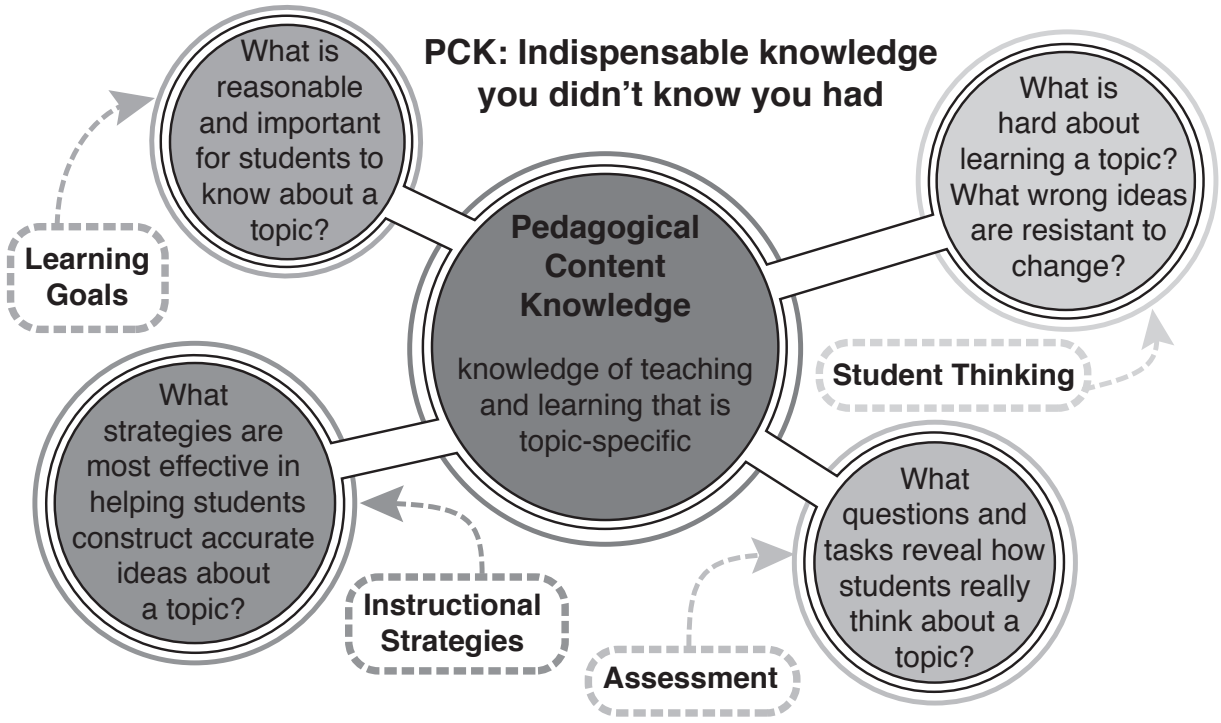

Figure 4.1: PCK: the indispensable knowledge you didn't know you had.

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CHAPTER 6

CONCLUSIONS

Genetics: Social immunity in the evolution of parental care and family dynamics

The first chapter of my dissertation examines the role of social immunity in the context of parental care. Social immunity describes any collective or personal immune response that is directly selected to increase the fitness of other members of the group and may be in excess of what is required for personal immunity (Cotter and Kilner 2010). Social immunity moderates the spread of pathogens in social groups and is especially likely in groups structured by genetic relatedness (Meunier 2015; Cremer et al. 2018). I investigated the expression and social role of three immune genes (*pgrp-sc2, thaumatin,* and *defensin*) during parental care in the beetle *Nicrophorus vespilloides*. The three immune genes I investigate have different functional targets and are expressed in different immune pathways. To determine the social role of immune gene expression, I first had to delineate three conditions for social immunity in the context of parental care (Ziadie et al. 2019): (1) evidence that offspring are relieved of mounting a full immune response, (2) evidence that parents upregulate their immune response in response to offspring beyond that required for personal benefit, and (3) evidence that the upregulation of parental immunity positively influences offspring fitness.

I used an integrated series of experiments that together allow me to infer whether changes in gene expression are related to social immunity rather than personal immunity, testing for the three conditions I expect for social immunity during parenting. I first examined immune gene expression in offspring in the presence or absence of parental care. I then quantified changes in maternal gene expression associated with different stages in the transition to parenting. Finally, I examined the covariance between maternal gene expression and components of offspring performance and fitness by measuring the maternal selection component. My results support a social role for *pgrp-sc2* and *thaumatin*, but not for *defensin*.

I expand on this work in my second chapter by further investigating the role of social immunity in the family. This project develops social immunity beyond parental care by manipulating the extent of conflict between parents and offspring and among siblings and examining individual contributions to social immunity. I asked how changes in social environment, specifically family size, influence expression of social immune genes in mothers and offspring. I measured the same three immune genes as in the previous study and made predictions about how my manipulations would affect expression of these genes based on the social role of the gene. *Pgrp-sc2* and *thaumatin* were previously shown to be involved in social immunity, however they appear to be involved in different kinds of social immune responses. *Pgrp-sc2* expression is involved in individual social immunity while *thaumatin* expression is involved in collective social immunity. I therefore predicted that as family size increased, expression of *pgrp-sc2* in mothers would also increase, reflecting an increase in the provisioning of individual social immunity. I also predicted that as family size increased, *thaumatin* expression in mothers and offspring would decrease, reflecting a decrease in individual contribution to a collective resource as the number of contributors increases. *Defensin* is

involved in personal immunity. I predicted that expression of *defensin* would not respond to changes in social environment.

With the exception of *defensin,* my predictions were not met, revealing previously unknown constraints to social immunity in the context of family life. Maternal *pgrp-sc2* expression remained constant across family size treatments while offspring expression increased with increasing family size. This result suggests that maternal contribution to social immunity functions as a threshold trait and the burden of compensating for sibling competition lies on the offspring. *Thaumatin* expression was unaffected by changes in family size in both parents and offspring. This suggests that, in this case, the level of collective immune response is dependent more on the size of the resource and less on the number of contributors. Overall, this work suggests limits to the flexibility of genes influencing social immunity, with threshold expression of immune genes more common than refined regulation in response to social cues. By exposing incongruities between fact and theory, this work also provides valuable scaffolding for future work in social immunity to build upon.

Significance and Broader Impacts.

The shift from solitary life to group living is considered to be one of the major evolutionary transitions (Szathmary and Maynard Smith 1995). Group living can be found in almost all animal taxa and ranges in complexity from simple mutual attraction between individuals, to temporary family associations and parental behavior, to permanent societies of related and unrelated individuals, to eusocial groups with reproductive division labor (Meunier 2015) Understanding the causes and consequences of this transition is critical to understanding variation of social systems in the natural world as well as our own evolutionary history as a highly social species.

The evolution of social immunity is an essential part of the transition from solitary to social life. The success of social groups is typically attributed to the fitness benefits group living provides such as increased vigilance and enhanced foraging (Alexander 1974). However, group living also engenders costs, including the increased risk of disease (Meunier 2015; Cremer et al. 2018). Intimate interactions between group members facilitates pathogen exposure and transmission, especially among related individuals where genetic similarity increases the susceptibility to the same pathogens (Meunier 2015; Cremer et al. 2017). Social groups evolve various forms of social immunity to alleviate this increased risk of pathogen infection (Cotter and Kilner 2010; Meunier 2015; Cremer et al. 2018). Thus, a complete understanding of the evolution of group living would not be possible without also understanding the evolution of social immune function.

To date, eusocial insects, primarily bees, wasps, ants, and termites, have served as the main biological models to study social immunity (Meunier 2015). These studies have provided clear evidence for the importance of social immunity in complex social groups. However, because eusocial societies are the most derived of social systems, these studies are of limited relevance to our understanding of the role of social immunity in the evolution of social living. It is therefore critical that studies of social immunity be conducted in non-eusocial systems, such as *Nicrophorus vespilloides.* Non-eusocial systems encompass the vast majority of social systems on the planet, including those that represent ancestral social states (Wilson 1971; Costa 2006). Furthermore, non-eusocial systems are, by definition, not associated with reproductive division of labor. Because of this, direct fitness benefits can easily be assigned to all individuals (because all individuals can reproduce) and any trade-offs between direct and indirect fitness can be quantified.

My work provides insight into how social immunity functions in a sub-social species. I was able to shed light on how different parts of a personal immune system may have evolved social roles in a fairly ancestral social state. I also revealed previously unknown restraints on social immunity in the context of parental care and family life. This work helps to fill the gap in knowledge about how social immunity works in non-eusocial species. In doing so, it also contributes to our understanding of the evolution of group living and sociality.

DBER: Pedagogical Content Knowledge (PCK) as a lens to critically analyze and catalog collective knowledge for teaching evolution.

The third chapter of my dissertation uses pedagogical content knowledge (PCK) as a framework to critically analyze collective knowledge for teaching and learning evolutionary biology found in the peer-reviewed literature. PCK is instructional knowledge that is topic-specific, meaning evolution instructors build distinct PCK for each topic in evolution they teach (Magnussen et al., 1999; Park and Oliver, 2008; Gess-Newsome, 2015). PCK is also comprised of four components: knowledge of student thinking, knowledge of assessment, knowledge of instructional strategies, and knowledge of curriculum (Shulman, 1987; Magnusson et al. 1999; Park and Oliver, 2008). This is a tremendous body of knowledge that an instructor typically gains through years of experience and careful reflection. However, research and scholarship generated by the DBER and practitioner communities has generated a collective knowledge base in the peer-reviewed literature (Smith et al 2016, Ziadie and Andrews 2018). The goals of this study were to examine the literature and determine what collective PCK for undergraduate evolution instruction is available and what is missing, to produce a searchable database of

available collective knowledge, and to demonstrate that PCK is a useful framework to guide future research on teaching and learning in undergraduate biology education.

After examining thousands of publications, my final collection included 316 papers relevant to undergraduate evolution education. These publications span 41 peer-reviewed journals, including 29 education journals and 13 discipline-specific journals. Out of 316 papers about undergraduate evolution education, 75% presented instructional strategies, 21% addressed student thinking, and 8% dealt with assessment. Six papers (2%) concentrated on goals (knowledge curriculum) for undergraduate evolution instruction. I identified 22 distinct evolutionary topics and most papers (78%) addressed one or more of these. The other 22% of papers addressed evolution broadly without specifying topic. Natural selection, phylogenetics and evolution broadly accounted for 69% of published papers. Eight topics (36%) were addressed by five or fewer papers. One hundred and seven papers addressed more than one topic. The majority of this overlap occurred within overarching categories (macroevolution, phylogenetics, and population genetics). I also examined topic representation by PCK component to more richly characterize gaps in available collective PCK and compared topic representation in the literature to topic representation in the classroom, by collecting data on topics taught in undergraduate evolution classrooms from around the country.

Significance and Broader Impacts.

These data allowed me to determine what PCK is available and what is missing for teaching evolution. More importantly, using PCK as a framework in combination with these data allowed me to make very specific, empirically supported research priorities for the DBER and SOTL (scholarship of teaching and learning) communities. By considering topics that were taught in at least 60% of the classes surveyed, topics for which collective PCK is largely

unavailable, and topics that have been identified as core ideas in biology by the community, I was able to propose four evolutionary topics that warrant immediate attention from the research community: macroevolution, speciation, quantitative genetics, and population genetics. I further proposed that research on student thinking take priority over other PCK components because it is foundational to both teaching and education research. College instructors also stand to benefit disproportionately from research on student thinking compared to research on assessment and instructional strategies because knowledge of student thinking is crucial for effective teaching, especially in student-centered instruction.

I have presented this work at education and evolution specific conferences, both domestically and abroad. The results and resources generated by this work are currently being used by the international biology education research community to provide support for future investigations of student learning in priority research areas. Furthermore, the dissemination of this work provides an invaluable resource to college and advanced high school evolution instructors who are looking to increase their effectiveness and adopt evidence-based practices in their teaching. As a supplement of this work, I have generated an open-access, searchable file that organizes over 400 resources about undergraduate and high school evolution instruction for the practitioner community. As of this writing, this resource has been downloaded 885 times.

In an effort to reach as much of the practitioner community as possible I have also written an article that translates my research and findings for the broader science-educator community (Chapter 4). This chapter was an exercise in educational outreach that allowed me to distil the most critical concepts of PCK and collective knowledge, how they relate to evolution education, and more importantly how to utilize these resources into less than 1300 words for a non-research audience. By publishing this work, I make the findings of my previous work, including the

collective PCK database, more accessible to a broader audience of evolution instructors. This article also helps to highlight the principles of PCK to a practitioner community that uses and generates personal PCK in their daily work as instructors, though they may not be aware of it. Hopefully by making instructors more aware of their own PCK, we can encourage them to be more reflective their teaching and to utilize collective knowledge in the literature to supplement their experiential knowledge.

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