Larvae and adults exhibit contrasting patterns of immune gene expression and infection resistance in wild flour beetle populations

Ann T. Tate*, Abby Perry, Derrick Jent

Department of Biological Sciences, Vanderbilt University, Nashville TN

Running title: Stage-structure in immune responses

*Correspondence: a.tate@vanderbilt.edu; 265 21st Ave S, Nashville, TN 37232 USA

Keywords: adaptive decoupling, life history evolution, ecological immunology, stage structure, Tribolium

Abstract

1. In nature, hosts face shifting patterns of parasite exposure and life history trade-offs as they develop from birth to old age. As a result, the net fitness benefit of immunological investment can change dramatically from one life stage to the next.
2. Previous work has revealed a puzzling diversity of relative immune investment patterns among juvenile and adult stages, and it is not clear whether lessons learned from one particular population or species can be generalized to wild populations, after accounting for local adaptation and other variance-generating processes.
3. In this study, we quantify larval and adult immune gene expression and resistance to bacterial infection in two flour beetle species (*Tribolium castaneum* and *T. confusum*) from two lab-adapted and five wild-derived populations.
4. Our results provide a clear signal of higher infection-induced immunological investment and resistance in adults relative to larvae, despite variation among species in immune gene regulation.
5. Better characterization of stage-specific investment in infection resistance in natural populations can inform our understanding of life history evolution and improve predictions of disease dynamics in the wild.

Introduction

The net contribution of different life history traits to host fitness changes across the ontogeny of the organism. While juveniles derive benefit from growing quickly to avoid predators and investing in survival to reach reproductive age, adults maximize fitness by finding mates and producing as many high-quality offspring as possible (Moran, 1994). Whether life stages share a niche or occupy different ones, juveniles and adults may face different hazards and trade-offs among life history traits (Gupta et al., 2021; Kirschman et al., 2018) that might favor trait divergence over evolutionary time. For example, juveniles may feed at different rates or from a different distribution of resources than adults, altering patterns of exposure to parasites, pathogens, and symbionts over host age (Clark et al., 2020; Combrink et al., 2020; Hammer & Moran, 2019). For organisms that undergo metamorphosis, such as holometabolous insects and amphibians, the dramatic reinvention of physiology can provide an opportunity to adaptively decouple juvenile and adult immune system regulation to better optimize fitness in stage-specific environments (Fellous & Lazzaro, 2011; Goedert & Calsbeek, 2019), leading to variation in immunity across ontogeny (Ashby & Bruns, 2018; Brown et al., 2019).

In honeybee males (*Apis mellifera*), for example, levels of the melanization enzyme phenoloxidase increase over development from larva to adult (Laughton et al., 2011), while in the tobacco hornworm (*Manduca sexta*), melanization activity peaks in the larval stage and declines thereafter (Trauer & Hilker, 2013) (see (Critchlow et al., 2019) for further examples). Most studies, however, employ insect populations reared (and evolving) in lab conditions. After accounting for the effects of local adaptation, can we still discern generalizable principles of stage-structured immunological investment?

To address this question, we used seven wild-derived populations of flour beetles (four *Tribolium castaneum,* three *T. confusum*). Previous research in *T. castaneum* has suggested that the regulation of immune gene expression differs among life stages in uninfected individuals (Critchlow et al., 2019), while, in larvae specifically, different populations of both species exhibit natural variation in immune gene expression and resistance to bacterial infection (Jent et al., 2019). To test whether immune gene expression and infection resistance in these populations differs among life stages, we injected larvae and adults from each population with the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) or sterile media. We quantified relative bacterial load and host expression of several immune genes previously shown to be among the most significantly differentially expressed during Bt infection (Tate & Graham, 2017), including the antimicrobial peptides *defensin-1 and attacin-1*, the recognition and peptidoglycan processing protein *pgrp-sc2,* and the melanization enzyme *ddc,* during acute infection. Our results highlight the importance of development for disease susceptibility and immune system evolution.

Methods

The collection of wild beetle populations and analysis of variation in immune gene expression among larvae has been described in detail in (Jent et al., 2019). The current study compares these larval data (available from (Tate et al., 2019)) to data from adults processed at the same time (available as Data S1 in Supplementary Material). Briefly, we created breeding groups from seven wild-derived stock colonies (*T. castaneum*: Snavely, Green River, Dorris, WF Ware; *T. confusum*: Snavely, Green River, Marshall) bred in the lab for 6 generations (except Snavely stocks, which were lab-reared for 4 years) under parasite-free conditions. Breeding groups laid eggs in whole wheat flour before passage to new flour every 48 hours. Adult cohorts were derived from the second egglay and larvae from the ninth. Egg

83 laying order within the first few weeks minimally affects Bt resistance traits in flour beetles
84 (Tate & Graham, 2015b).

85 Bacterial aliquots were derived from a mix of overnight and log phase cultures of
86 *Bacillus thuringiensis* Berliner (ATCC 55177) as previously described (Jent et al., 2019). We
87 injected both 4mm larvae and unmated adults (7 days post eclosion) with either sterile
88 Nutrient Broth or one of four two-fold-diluted Bt doses (1.25*10^7 CFU/mL to $1*10^8$
89 CFU/mL; estimated by plating on agar, N = 6-8 indivs/dose/stage/pop), using a micropin
90 dipped in solution. We sacrificed injected beetles at 8 hours post injection (when the immune
91 response is induced but the beetles have not yet started to die) by flash freezing. We extracted
92 RNA and quantified gene expression via RT-qPCR as previously described (Jent et al., 2019)
93 using degenerate primers with equal species efficiency (>95% efficiency) for Bt 16s rRNA,
94 the reference gene *rps18,* and four immune genes: *attacin-1*, *defensin-1*, *pgrp-sc2,* and *ddc*.
95 The ΔCt values (target gene Ct – reference gene Ct) for each individual were linearized (2^{-}
96 $^{\Delta Ct}$) prior to analysis and then log-transformed for normality, as in (Tate et al., 2017; Tate &
97 Graham, 2017).

98 To analyze the effect of life stage on resistance and gene expression at 8 hours post
99 infection, we employed linear mixed models of relative bacterial load or gene expression
100 using life stage as a fixed effect, population as a random effect, and initial dose or
101 log2(bacterial load) as additional fixed effects where appropriate (see Table 1 notes). Species
102 and species-by-bacterial load interactions were added as fixed effects if they significantly
103 improved model fit (log likelihood ratio test). Because p-values cannot be reliably calculated
104 for mixed models, we instead rely on confidence intervals (2.5%, 97.5%) to determine
105 significance, where a lack of overlap with 0 encourages the rejection of the null hypothesis.
106 All statistical analyses were conducted using the lmer function from the lme4 package in R
107 (v.4.0.3) and code is available as Data S2 in the online supplementary material.

Results

After injection with sterile media, life stage did not significantly predict expression of any gene (**Table 1**). The expression of *defensin-1* by different life stages across populations is depicted in **Fig. 1A**. While model selection did not generally favor the inclusion of species for most genes, the effect of species on *attacin-1* was significant, with *T. confusum* exhibiting higher expression than *T. castaneum*.

At 8 hours post injection with live bacteria, adults exhibited significantly higher expression of all immune genes relative to larvae (**Table 1**), which was relatively consistent across populations (**Fig. 1B**). The antimicrobial peptide *defensin-1* exhibited the biggest effect size between stages after accounting for variance among populations (**Fig. 1B**). As expected, the main effect of relative bacterial load on expression was significant for all genes. The species and the bacterial load-by-species interaction terms were significant only for *defensin-1*, where gene expression in *T. confusum* was lower for both genes (**Fig. S1**) and, unlike in *T. castaneum*, not strongly correlated to bacterial load.

Both initial dose and life stage significantly predicted relative bacterial load at 8 hours post infection (**Table 1**). The bacterial load increased with initial dose, and larval bacterial load was about 1.5 times greater than adult bacterial load (**Fig. 1C**).

Discussion

Theory predicts that evolutionarily optimal levels of juvenile investment in immunity depend on the precise magnitude of life history parameters like life span and juvenile mortality rate (Ashby & Bruns, 2018). At the same time, changes in parasite exposure across stages likely contribute to ontogenetic variation in immunological investment (Tate & Graham, 2015a). Since natural populations may experience very different selective pressures from parasites and other ecological factors, it is difficult to predict *a priori* whether

132 decoupling of immunological traits will show consistent patterns in the wild. *Tribolium*
133 larvae and adults both dwell within the same food medium but occupy different
134 microenvironments (adults on top of the food column, larvae within it), feed at different rates,
135 and prioritize development or reproduction, respectively. These differences likely contribute
136 to different rates of parasite exposure and selection for immune investment in each life stage.
137 Our results provide a clear signal of life stage-specific variation in resistance-associated
138 traits: adult flour beetles invest more in the inducible expression of all assayed immune genes
139 and reap a modest but detectable benefit with regard to resisting bacterial proliferation.

140 Is the observed stage-specific variation actually adaptive, or could it simply reflect a
141 developmental constraint on the deployment of the larval immune response? The dominance
142 of the adult response to infection does not extend to the wounding response, as differences in
143 gene expression among life stages appear to be population-specific and exhibit no consistent
144 trend (e.g. Fig. 1A). Thus, there does not appear to be any fundamental developmental
145 constraint manifest in the baseline inducible response. In the absence of studies that
146 manipulate the expression of key genes independently in each life stage, however, it is
147 difficult to disentangle the relative roles of adaptation and constraint in ontogenetic
148 decoupling of immunity.

149 In summary, our study provides evidence of stage-structured variation in immune
150 responses across natural populations. Our two species diverged 60 million years ago
151 (Angelini & Jockusch, 2008) and occupied different continents, but re-converged at the dawn
152 of domestication to specialize on agricultural products. Whether ontogenetic decoupling of
153 immunity is ancestral to these beetles, or whether it arose more recently as ecological
154 conditions converged, remains an interesting question for future study in this and other host
155 taxa. These observations also raise the question of whether these differences evolved
156 adaptively against stage-specific parasite pressures, or whether trade-offs or developmental

constraints dominate the optimization of immune system investment. Future work investigating the mechanistic basis of immune system regulation across host development could help to disentangle the ecological and evolutionary contributors to ontogenetic decoupling of immune responses.

Data Accessibility Statement

The RT-qPCR data for larvae are deposited in Data Dryad (Tate et al. 2019: https://doi.org/10.5061/dryad.dbrv15dx3) and the data for the adults are available in the supplementary material (Data_S1.txt). R code used to analyze the data is available in the Supplementary material (Data_S2.R).

Acknowledgements

We thank Nora Schulz, Stephanie Birnbaum, and two anonymous reviewers for helpful comments on previous versions of the manuscript. This work was supported in part by NSF DEB grant no. 1753982 to A.T.T.

Author Contributions

A.T.T. conceived and designed the study and experiments. A.P. and D.J. conducted the experiments. A.T.T. and A.P. analyzed the data. A.T.T. wrote the manuscript.

Conflict of Interest Statement

The authors declare no conflicts of interest associated with the work conducted in this manuscript.

Supporting Materials

Figure S1: The impact of life stage and species on the inducible expression of the antimicrobial peptide *defensin-1* in infected flour beetles.

Data S1: RT-qPCR data for adult gene expression

Data S2: R code used to analyze data and reproduce figures

References

Angelini, D.R. & Jockusch, E.L. (2008) Relationships among pest flour beetles of the genus Tribolium (Tenebrionidae) inferred from multiple molecular markers. *Molecular phylogenetics and evolution*, **46**, 127-141.

Ashby, B. & Bruns, E. (2018) The evolution of juvenile susceptibility to infectious disease. *Proceedings of the Royal Society B: Biological Sciences*, **285**.

Brown, L.D., Shapiro, L.L.M., Thompson, G.A., Estévez-Lao, T.Y., & Hillyer, J.F. (2019) Transstadial immune activation in a mosquito: Adults that emerge from infected larvae have stronger antibacterial activity in their hemocoel yet increased susceptibility to malaria infection. *Ecology and Evolution*, **9**, 6082-6095.

Clark, J., McNally, L., & Little, T.J. (2020) Pathogen Dynamics across the Diversity of Aging. *The American Naturalist*, **197**, 203-215.

Combrink, L., Glidden, C.K., Beechler, B.R., Charleston, B., Koehler, A.V., Sisson, D., Gasser, R.B., Jabbar, A., & Jolles, A.E. (2020) Age of first infection across a range of parasite taxa in a wild mammalian population. *Biology Letters*, **16**, 20190811.

Critchlow, J.T., Norris, A., & Tate, A.T. (2019) The legacy of larval infection on immunological dynamics over metamorphosis. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **374**, 20190066.

Fellous, S. & Lazzaro, B.P. (2011) Potential for evolutionary coupling and decoupling of larval and adult immune gene expression. *Molecular Ecology*, **20**, 1558-1567.

Goedert, D. & Calsbeek, R. (2019) Experimental Evidence That Metamorphosis Alleviates Genomic Conflict. *The American Naturalist*, **194**, 356-366.

Gupta, V., Frank, A., Matolka, N., & Lazzaro, B. (2021) Reliance on polyfunctional tissue leads to a reproduction-immunity tradeoff due to inherent constraint. *bioRxiv*, 2021.01.28.428655.

Hammer, T.J. & Moran, N.A. (2019) Links between metamorphosis and symbiosis in holometabolous insects. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **374**, 20190068.

Jent, D., Perry, A., Critchlow, J., & Tate, A.T. (2019) Natural variation in the contribution of microbial density to inducible immune dynamics. *Molecular Ecology*, **28**, 5360-5372.

Kirschman, L.J., Crespi, E.J., & Warne, R.W. (2018) Critical disease windows shaped by stress exposure alter allocation trade-offs between development and immunity. *Journal of Animal Ecology*, **87**, 235-246.

Laughton, A.M., Boots, M., & Siva-Jothy, M.T. (2011) The ontogeny of immunity in the honey bee, Apis mellifera L. following an immune challenge. *Journal of Insect Physiology*, **57**, 1023-1032.

Moran, N.A. (1994) Adaptation and constraint in the complex life cycles of animals. *Annual Review of Ecology and Systematics*, **25**, 573-600.

Tate, A., Jent, D., Perry, A., & Critchlow, J. (2019) Data From: Natural variation in the contribution of microbial density to inducible immune dynamics. In Data Dryad. https://doi.org/10.5061/dryad.dbrv15dx3.

Tate, A.T., Andolfatto, P., Demuth, J.P., & Graham, A.L. (2017) The within-host dynamics of infection in trans-generationally primed flour beetles. *Molecular Ecology*, **26**, 3794–3807.

Tate, A.T. & Graham, A.L. (2015a) Dynamic Patterns of Parasitism and Immunity across Host Development Influence Optimal Strategies of Resource Allocation. *The American Naturalist*, **186**, 495-512.

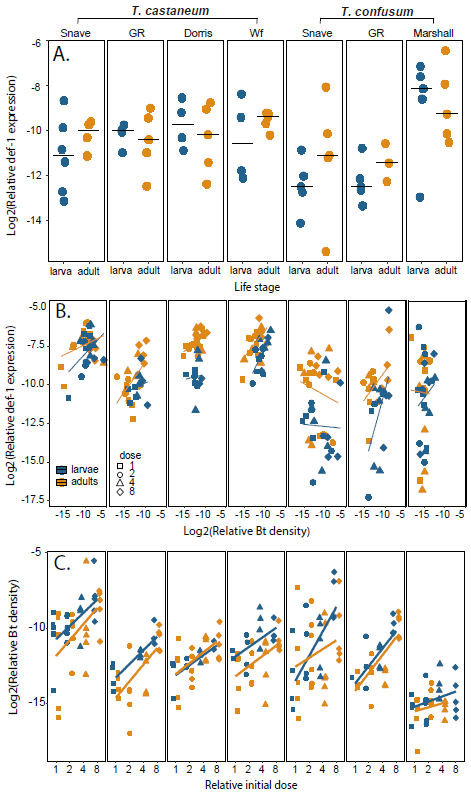
Tate, A.T. & Graham, A.L. (2015b) Trans-generational priming of resistance in wild flour beetles reflects the primed phenotypes of laboratory populations and is inhibited by co-infection with a common parasite. *Functional Ecology*, **29**, 1059-1069.

Tate, A.T. & Graham, A.L. (2017) Dissecting the contributions of time and microbe density to variation in immune gene expression. *Proceedings of the Royal Society B: Biological Sciences*, **284**.

Trauer, U. & Hilker, M. (2013) Parental Legacy in Insects: Variation of Transgenerational Immune Priming during Offspring Development. *PloS One*, **8**, e63392.

Figures

Figure 1. The impact of life stage on bacterial load and inducible expression of the antimicrobial peptide *defensin-1* in seven flour beetle populations. Larvae (blue) and adults (orange) were injected with sterile saline or one of four two-fold increasing doses (1,2,4,8) of Bt, and sacrificed at 8 hours post infection. Host *defensin-1* expression was estimated via RT-qPCR relative to host reference gene expression after a sterile saline jab (**A**) or Bt infection (**B**; plotted against bacterial load), as was bacterial load from Bt-infected individuals (**C**; plotted against initial dose). Shapes reflect initial dose, and lines reflect linear fits using the "lm" method of the geom_smooth function in ggplot2.



247 **Tables**

Table 1. The effect of life stage on immune gene expression and bacterial load at 8 hours post injection with sterile saline or Bt

	Estimate	Std. error	t value	2.5CI	97.5CI
Relative bacterial load (Bt)					
Intercept	-14.73	0.66	-22.18	-16.06	-13.40
Initial dose	0.86	0.09	9.33	**0.68**	**1.04**
Stage	-0.62	0.20	-3.05	**-1.02**	**-0.22**
Attacin-1 (sterile)					
Intercept	-13.33	0.48	-27.98	-14.37	-12.28
Stage	-0.07	0.38	-0.20	-0.82	0.68
Species	1.57	0.67	2.35	**0.03**	**3.07**
Defensin-1 (sterile)					
Intercept	-10.75	0.38	-28.01	-11.58	-9.91
Stage	0.61	0.35	1.75	-0.08	1.32
Pgrp-sc2 (sterile)					
Intercept	-11.62	0.65	-17.76	-13.09	-10.15
Stage	0.37	0.23	1.63	-0.08	0.82
Ddc (sterile)					
Intercept	-11.44	0.20	-57.38	-11.84	-11.01
Stage	0.56	0.27	2.06	-0.02	1.11
Attacin-1 (infected)					
Intercept	-5.83	0.82	-7.08	-7.48	-4.20
Stage	0.82	0.17	4.93	**0.49**	**1.14**
Bt load	0.40	0.06	6.52	**0.28**	**0.52**
Species	-2.04	1.24	-1.65	-4.48	0.46
Bt x Species	-0.12	0.09	-1.38	-0.29	0.05
Defensin-1 (infected)					
Intercept	-5.40	1.03	-5.24	-7.48	-3.36
Stage	1.29	0.24	5.45	**0.82**	**1.76**
Bt load	0.31	0.09	3.63	**0.14**	**0.48**
Species	-5.79	1.53	-3.79	**-8.85**	**-2.62**
Bt x Species	-0.28	0.12	-2.34	**-0.52**	**-0.04**
Pgrp-sc2 (infected)					
Intercept	-9.00	1.02	-8.80	-11.12	-6.84
Stage	0.37	0.15	2.55	**0.08**	**0.66**
Bt load	0.11	0.06	2.09	**0.01**	**0.22**
Species	0.18	1.55	0.12	-3.07	3.44
Bt x Species	0.05	0.08	0.62	-0.10	0.20
Ddc (infected)					
Intercept	-6.96	0.63	-11.03	-8.21	-5.68
Stage	0.54	0.14	3.76	**0.26**	**0.83**
Bt load	0.27	0.05	5.08	**0.16**	**0.37**
Species	-0.51	0.94	-0.55	-2.36	1.36
Bt x Species	0.06	0.07	0.86	-0.08	0.21

Notes: Linear mixed model for relative bacterial load is log2(bacterial load) ~ dose + stage + (1|pop). Adding species as a factor did not improve model fit (as determined by log likelihood ratio test) and was excluded from the model. Linear mixed models for immune gene expression (using maximum likelihood method) are of the general structure: gene expression ~ log2(Bt load) + stage + (1|pop). For infected analyses, all genes either had no difference among models with and without species, or were improved with the inclusion of species and a Bt load x species interaction term, so species and Bt x species was retained for all. Bold confidence intervals indicate lack of overlap null expectation. For all analyses larvae are the baseline stage and *T. castaneum* is the baseline species.

248