Title: Host blood meal identity modifies vector gene expression and competency

Running Head: Host blood meal alters tick vector competency

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Abstract

A vector's susceptibility and ability to transmit a pathogen — termed vector competency —determines disease outcomes, yet the ecological factors influencing tick vector competency remain largely unknown. *Ixodes pacificus*, the tick vector of *Borrelia burgdorferi* (*Bb*) in the western U.S., feeds on rodents, birds, and lizards. Unlike rodents and birds which are reservoirs for *Bb* and infect juvenile ticks, lizards are refractory to *Bb* and cannot infect feeding ticks. Additionally, the lizard bloodmeal contains borreliacidal properties, clearing previously infected feeding ticks of their *Bb* infection. Despite *I. pacificus* feeding on a range of hosts, it is undetermined how the host identity of the larval bloodmeal affects future nymphal vector competency. We experimentally evaluate the influence of larval host bloodmeal on *Bb* acquisition by nymphal *I. pacificus*. Larval *I. pacificus* were fed on either lizards or mice and after molting, nymphs were fed on *Bb*-infected mice. We found that lizard-fed larvae were significantly more likely to become infected with *Bb* during their next bloodmeal than mouse-fed larvae. We also conducted the first RNA-seq analysis on whole-bodied *I. pacificus* and found significant upregulation of tick antioxidants and antimicrobial peptides in the lizard-fed group. Our results indicate that the lizard bloodmeal significantly alters vector competency and gene regulation in ticks, highlighting the importance of host bloodmeal identity in vector-borne disease transmission and upends prior notions about the role of lizards in Lyme disease community ecology.

Introduction

Vector competency — the ability of a vector to successfully acquire and transmit a pathogen — and the factors that modulate it are increasingly the focus of efforts to control the emergence and spread of vector-borne zoonotic diseases (Beard, Cordon-Rosales, & Durvasula, 2002; Beerntsen, James, & Christensen, 2000; de la Fuente et al., 2017; Iturbe-Ormaetxe, Walker, & O' Neill, 2011; Pais, Lohs, Wu, Wang, & Aksoy, 2008). Manipulation of vector competency has been discussed as a disease prevention strategy in mosquitoes, teste flies, and triatome bugs (Beard et al., 2002; Iturbe-Ormaetxe et al., 2011; Kean et al., 2015; Pais et al., 2008; Weiss & Aksoy, 2011). In these vectors, rearing of naturally resistant populations, modifications of vector endosymbionts, and gene editing have been studied and implemented as applications of biological control to alter vector competency and reduce disease transmission (Beard et al., 2002; Iturbe-Ormaetxe et al., 2011; Kean et al., 2015; Pais et al., 2008). While strides have been made in understanding and manipulating vector competency in many systems, these studies highlight the complexity of vector-pathogen interactions and suggest that a more mechanistic understanding of disease transmission holds promise for disease control. For tick-borne pathogen systems in particular, the plasticity of vector competency and responsiveness to environmental or biological inputs remains poorly understood.

Tick-borne diseases constitute 40% of the emerging vector-borne diseases worldwide (Jones et al., 2008; Schwartz, Amy M., Hinkley, Alison F., Mead, Paul S., Hook, Sarah A., Kugeler, 2017; Swei, Couper, Coffey, Kapan, & Bennett, 2020) and are sensitive to changing abiotic and biotic interactions driven by land use change and increased globalization (de la Fuente et al., 2017; Keesing et al., 2010; Swei et al., 2020; van Baalen & Sabelis, 1995). In

93 the northern hemisphere, Lyme disease is the most common vector-borne disease, causing
94 an estimated 300,000 cases annually in the U.S. (Kilpatrick & Randolph, 2012; Mysterud,
95 Jore, Østerås, & Viljugrein, 2017; Swei et al., 2020). It is caused by the bacterial agent
96 *Borrelia burgdorferi* (*Bb*) and vectored by *Ixodes spp.* ticks, whose life history involves blood-
97 feeding on a wide range of hosts during each of their three life stages (larvae, nymph and
98 adult) (LoGiudice, Ostfeld, Schmidt, & Keesing, 2003). Tick blood-feeding induces a suite of
99 major physiological changes in the tick including antimicrobial activity (Smith, Navasa, Yang,
100 Marques, et al., 2016) and cuticular reconstruction (Gulia-Nuss et al., 2016; Perner et al.,
101 2016). In addition, the identity of the blood meal host has important consequences for
102 pathogen acquisition, tick survivorship, and microbiome composition (Landesman, Mulder,
103 Allan, Bashor, Keesing, Logiudice, et al., 2019; Muturi, Dunlap, Ramirez, Rooney, & Kim,
104 2018; Sonenshine, Hynes, Ceraul, Mitchell, & Benzine, 2005; Swei & Kwan, 2017).
105 Mounting evidence indicates that microbiome composition impacts vector competency
106 through induced immunological responses, morphological changes, or direct competition
107 between microbial components of the tick microbiome (Dong, Manfredini, & Dimopoulos,
108 2009; Narasimhan et al., 2014, 2017; Ross et al., 2018; Wang, Gilbreath III, Kukutla, Yan, &
109 Xu, 2011). However, the precise relationship between microbiome composition and tick
110 competency for *Bb* is not well understood (Eisen, 2020; Kwan, Griggs, Chicana, Miller, &
111 Swei, 2017). Tick *Bb* acquisition is a complex process that requires the pathogen to evade
112 numerous tick immune pathways and antimicrobial peptides (de la Fuente et al., 2017; Gulia-
113 Nuss et al., 2016; Hajdušek et al., 2013; Hayes et al., 2020; Liu et al., 2012; Shaw et al.,
114 2017; Smith, Navasa, Yang, Wilder, et al., 2016) followed by successful colonization of the
115 midgut (Pal et al., 2000; Zung et al., 1989). There is evidence that these interactions may be

116 influenced by biotic interactions such as host bloodmeal identity or microbiome interactions
117 (Landesman, Mulder, Allan, Bashor, Keesing, Logiudice, et al., 2019; Narasimhan et al., 2017;
118 Swei & Kwan, 2017). Manipulation of the microbiome in laboratory-reared *Ixodes scapularis*
119 found that lower microbiome diversity reduced *Bb* colonization through induced changes in
120 tick midgut morphology (Narasimhan et al., 2014). In *Ixodes pacificus*, greater microbiome
121 diversity was associated with *Bb* colonization in one study but not another (Kwan et al., 2017;
122 Swei & Kwan, 2017). The life history of *Ixodes pacificus*, the Lyme disease vector in the
123 western U.S., provides a unique opportunity for natural microbiome manipulation. Juvenile *I.*
124 *pacificus* feed predominantly on the western fence lizard, *Sceloporus occidentalis*, a *Bb*-
125 refractory host, but will also parasitize reservoir competent hosts, typically rodents such as
126 *Peromyscus* spp. mice, western gray squirrels (*Sciurus griseus*), and dusky-footed woodrats
127 (*Neotoma fuscipes*) (Eisen, Dolan, Piesman, & Lane, 2003; Robert S Lane, Mun, Eisen, &
128 Eisen, 2005; Robert S Lane, Mun, & Stubbs, 2009). In addition to varying greatly in reservoir
129 competency, blood meals from these host species can lead to stark differences in tick
130 microbiome composition (Swei & Kwan, 2017). Lizard-feeding results in a significant reduction
131 in *I. pacificus* microbiome diversity relative to rodent-feeding (Swei & Kwan, 2017).
132 Given recent findings that lizard-feeding significantly reduces microbiome diversity
133 (Swei & Kwan, 2017) and experimental evidence of tick microbiome diversity affecting *Bb*
134 colonization success (Narasimhan et al., 2014), we sought to determine the direct effect of
135 blood meal identity on *I. pacificus* vector competency in a tick pathogen acquisition
136 experiment. We fed larval ticks on either lizards or mice then subsequently fed those ticks on
137 *Bb*-infected mice and found that the ticks with previous lizard bloodmeals were significantly
138 more susceptible to *Bb* infection. We then investigated mechanisms by which host blood meal

may alter vector competency by conducting the first RNA-seq analysis on whole-bodied *I. pacificus* nymphs and comparing gene expression profiles for *I. pacificus* ticks following mouse or lizard larval blood meals. We find significant differences in tick vector competency based on larval host blood meal identity and detect multiple immune and metabolic factors that may alter *I. pacificus* vector competency for the Lyme disease pathogen.

Materials and methods:

Ixodes pacificus collection

Fed *I. pacificus* larvae were collected from either western fence lizards (*Sceloporous occidentalis*) or deer mice (*Peromyscus maniculatus*). As lizards have naturally high larval burdens of *I. pacificus* (mean=25; Swei, Briggs, Lane, & Ostfeld, 2012), we collected ticks from *S. occidentalis* by capturing and holding lizards in drop off cages suspended over water for 3-4 days in a temporary field lab to collect replete ticks. We then transferred all collected, replete larvae to the lab facilities at San Francisco State University. Natural burdens of *I. pacificus* larvae on *Peromyscus spp*. are very low (Swei et al., 2012). Because of low natural tick burdens, we experimentally attached up to 200 larval *I. pacificus* (BEI Resources, Manassas, VA) to *Peromyscus maniculatus* (Peromyscus Genetic Stock Center, Columbia, SC) in the lab. We collected and stored all replete ticks from lizard and mouse drop-off procedures under standard rearing conditions of 23°C and 90% relative humidity until they molted eight weeks later.

Experiments involving animals

All experiments involving *Peromyscus maniculatus* and C3H/HeJ mice at San Francisco State University were pre-approved by Institutional Animal Care and Use Committee (IACUC) under the protocol number AU19-01 and researchers were properly trained by the university veterinarian.

Host inoculation and tick Bb acquisition experiments

Borrelia burgdorferi cultures were grown until they reached a concentration of 10^6 spirochetes/mL (Polovinchik, 2002). Mice were inoculated intradermally with 100 µL of *Bb* culture (10,000 total spirochetes). Eight weeks post inoculation, successful *Bb* acquisition in the mice was determined via nested PCR of ear tissue targeting the 5S-23S rRNA spacer region (Robert S Lane, Steinlein, & Mun, 2004).

Five C3H/HeJ mice were used to feed nymphs (Jackson Laboratory, Bar Harbor, Maine). Three of the C3H/HeJ mice were inoculated with *Bb* leaving the remaining two mice as uninfected controls. Nymphs that fed as larvae on either lizards or mice were then placed on either *Bb*-infected or uninfected C3H/HeJ mice for nymphal feeding. Host-to-tick acquisition experiments were conducted in three separate trials. The first trial was conducted at Indiana University, where lizard-fed and mouse-fed nymphs were fed on C3H/HeJ mice infected with *Bb* at a concentration of 10^5 spirochetes/mL (1,000 total spirochetes). The second and third trials were conducted in the animal facilities at San Francisco State University where lizard-fed and mouse-fed ticks were subsequentially fed on C3H/HeJ mice infected with 10^7 and 10^6 spirochetes/mL (100,000 and 10,000 spirochetes total), respectively.

Nucleic acid isolation and pathogen testing

After completing their bloodmeal, nymphs were placed under standard rearing conditions for 24 hours, flash frozen, and stored at -80°C until nucleic acid extraction. Prior to extraction, ticks were thoroughly surface sterilized with successive 1 mL washes of 3% hydrogen peroxide, 70% ethanol, and de-ionized H_2O to remove surface contaminants. The tick was then lysed and homogenized using the Qiagen TissueLyser II (QIAGEN, Valencia, CA, USA). Tick samples were extracted simultaneously for total DNA and RNA using the Qiagen AllPrep DNA/RNA Micro Kit (QIAGEN, Valencia, CA, USA). DNA and RNA concentrations were measured using a Quibit Fluorometer (ThermoFisher Scientific, Walthman, CA, USA) in preparation for pathogen testing and library preparation. RNA content and quality were evaluated using a Bioanalyzer (Agilent, Santa Clara, CA, USA). DNA from engorged *Bb*-fed nymphs was tested for infection in triplicate by qPCR (Barbour et al., 2009).

Statistical analyses

To determine if larval bloodmeal host was a predictor of nymphal pathogen acquisition, we used a generalized mixed-effect model (GLMM) with a binomial error distribution. We used larval host bloodmeal (lizard or mouse feeding) as a fixed effect and trial as a random effect to account for experimental variation between trials. Analyses were performed using the glmm package (v.1.4.2) in R (Knudson, Benson, Geyer, & Jones, 2021).

Tick transcriptome analysis

Ticks from the third pathogen transmission experiment were used for transcriptome analysis. Unfed nymphs and engorged nymphs were divided into six experimental groups (Figure 1). Our six experimental groups were composed of ticks that either fed on a lizard or a

mouse as a larva. Then, molted nymphs from either larval bloodmeals either remained unfed, fed on an uninfected mouse, or fed on a *Bb*-inoculated mouse (Figure 1). Each experimental group will hereinafter be described with the following abbreviations: unfed nymphs are referred to as "UF" and fed nymphs are referred to by whether they were fed on a *Bb*-positive "+Bb," or *Bb*-negative "-Bb" C3H/HeJ mouse. Additionally, the larval bloodmeal (lizard or mouse) in each experimental group is indicated in the subscript following the abbreviation.

Groups one and two, which represent our unfed nymphs, "UF_{lizard}" and "UF_{mouse}" (Figure 1), were set aside to examine the effect of the lizard or mouse larval bloodmeal on *I. pacificus* gene expression. The remaining four groups were engorged nymphal ticks. Uninfected control groups three and four, "-Bb_{lizard}" and "-Bb_{mouse}" were nymphs that fed on uninfected C3H/HeJ mice during their nymphal bloodmeal (Figure 1). Groups five and six, "+Bb_{lizard}" and "+Bb_{mouse}" were nymphs that fed on *Bb* infected C3H/HeJ (Figure 1).

We prepared three replicates from each of the six experimental tick feeding conditions (Figure 1) for a total of 18 libraries. We pooled three individual ticks for each experimental replicate. RNA-seq libraries were prepared from total RNA extracted from ticks followed by rRNA depletion using Depletion of Abundant Sequences by Hybridization (DASH) (Gu et al., 2016). The NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, E7760S) was used to make RNA-seq libraries following the standard manual protocol. After constructing RNA-seq libraries from total RNA, reads containing rRNA sequences from *Ixodes spp.*, *Bb*, and mouse were depleted using DASH, which targets Cas9 to abundant sequences in RNA-seq libraries. We utilized previously designed guide RNAs against mice and *Ixodes spp.* rRNAs (Dynerman et al., 2020), and we designed additional

complementary guides to improve rRNA depletion of tick and *Bb* sequences using RNA-seq libraries made from total RNA from an *I. scapularis* nymph and *Bb* B-31 culture (Table S1).

rRNA depletion with DASH

Guide RNAs were designed using DASHit (http://dashit.czbiohub.org/), and prepared from DNA oligos as in Gu et al. (2016) following the protocol for *In Vitro* Transcription for dgRNA Version 2 (dx.doi.org/10.17504/protocols.io.3bpgimn). The complete protocol for rRNA depletion with DASH can be found in the supplementary methods. Following DASH, RNA-seq libraries were sequenced on an Illumina NextSeq with paired-end 75 base pair reads. Fastq files and raw read counts have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE173109.

RNA-seq sequence processing and analysis

RNA-seq reads were trimmed of adapters and bases with quality score lower than 20 using Cutadapt (Martin, 2011) via Trim Galore! v0.6.5 and then mapped to the *Ixodes scapularis* ISE6 genome (assembly GCF_002892825.2_ISE6_asm2.2_deduplicated) accessed from NCBI RefSeq, using STAR (v2.7.3a) [ref. 40]. Reads mapping to predicted genes (gtf-version 2.2, genome build: ISE6_asm2.2_deduplicated, NCBI genome build accession: GCF_002892825.2, annotation source: NCBI Ixodes scapularis Annotation Release 100) were tabulated using Subread FeatureCounts (v2.0.0) [ref. 41], counting primary hits only. DESeq2 v1.26.0 (Love, Huber, & Anders, 2014), was used to determine differential expression between groups. Volcano plots from the 'Enhanced Volcano" package in R, were used to visualize significant differential gene expression between experimental

groups (Blighe, Rana, & Lewis, 2019). The visualization tools heat map and principle coordinate analysis plots in Deseq2 were utilized to visualize similarities across our gene expression profiles (Love et al., 2014). All code in our Deseq2 analysis is available at: https://github.com/choulabucsf/Ipac_DE_Ring_et_al_2021

Results

Host-to-tick Bb acquisition experiment

The effect of larval host bloodmeal on *I. pacificus* nymphal vector competency was examined in a host-to-tick pathogen acquisition experiment where replete larval *I. pacificus* were obtained from mice or lizards and then subsequently fed on *Bb*-infected C3H/HeJ mice (Figure 1). A total of 36 lizard-fed ($+Bb_{lizard}$) and 46 mouse-fed ($+Bb_{mouse}$) *I. pacificus* nymphs were used across three experimental trials (Figure S1).

I. pacificus nymphs that fed on *Bb*-inoculated C3H/HeJ mice were significantly more likely to become infected if they previously fed on lizards as larvae than if they fed on mice (χ^2 (1, N = 82) = 7.8266, p =.0051). During their nymphal bloodmeal, 64% of lizard-fed ticks (N= 23/36) became infected with *Bb* compared to 30% of the mouse-fed ticks (N=14/46; Figure 2**)**. Even after accounting for trial as a random effect, our GLMM analyses found that the lizard larval bloodmeal is a significant, positive predictor of *Bb* acquisition in *I. pacificus* (Table 1). These results support our hypothesis that the host bloodmeal source may shape intrinsic vector competency of ticks across at least one life stage transition.

Experimental transcriptomic differences

To investigate potential explanations for the differences in vector competency between lizard-fed and mouse-fed ticks, we conducted an RNA-seq analysis to compare gene expression between ticks with different blood histories and pathogen exposure. A total of 18 RNA-seq libraries were prepared from the six experimental groups, each represented by three replicates and resulting in over 370 million total reads (Figure 1). With no available annotated *Ixodes pacificus* genome, we aligned our reads to the ISE6 *Ixodes scapularis* genome (Miller et al., 2018). Mapping rates among replicates averaged at 45% with 15 million reads per library. Sequencing statistics for each replicate are presented in Table S2**.**

To visualize overall differences in gene expression profiles across the experimental groups, we created a heatmap and a PCA plot of our 18 replicates. The heatmap, generated from sample-to-sample distances, is based on read counts for all genes and showed that tick engorgement status (unfed vs. engorged) induced significant changes in *I. pacificus* gene expression (Figure 3a). Additionally, the PCA plot indicated significant distinction of overall gene expression between unfed ticks of either bloodmeal type, UF_{lizard} and UF_{mouse} (Figure 3b). The engorged experimental groups (groups three to six) had similar gene expression profiles and did not distinctly cluster together by experimental condition (Figure 3b).

Differential gene expression

To investigate the mechanism through which host blood alters tick vector competency, we took a global transcriptomic approach to identify key genes or pathways modulated by mouse or lizard hosts. Differential gene expression analyses focused on several pairwise comparisons to examine transcriptomic differences between 1) the lizard versus mouse

296 bloodmeal in the unfed group 2) unfed versus fed ticks, and 3) bloodmeal identity distinctions
297 between *Bb* exposed groups.

298 The comparison between our unfed nymphs (UF_{lizard} vs. UF_{mouse}), demonstrated that
299 the lizard bloodmeal induced distinct transcriptomic changes in *I. pacificus* with 468
300 significantly differentially expressed genes (DEGs) (Table S3). While many of the DEGs
301 remain undescribed, some of the highest upregulated genes induced by the lizard bloodmeal
302 in the unfed group included antioxidants and antimicrobial peptides (Figure 4a). The
303 antioxidant glutathione peroxidase was the most significant DEG and was upregulated 48.5-
304 fold after the lizard bloodmeal compared to mouse bloodmeal. Other tick antioxidants that
305 were upregulated after the lizard bloodmeal include peroxidase (upregulated 21-fold) and
306 glutathione-S-transferase (upregulated four-fold; Figure 4a). We also found several DEGs that
307 are related to the regulation of antimicrobial peptides but have never been described in *I.*
308 *pacificus* ticks, such as acanthoscurrin-1, acanthoscurrin-2-like, micropulsin and micropulsin
309 isoform, which were upregulated by 27.9, 104, 4, and 22.6-fold, respectively (Figure 4a; Table
310 S3).

311 To analyze the DEGs between engorged and un-engorged ticks, we combined the two
312 unfed groups (UF_{lizard} & UF_{mouse}) as our reference 'unfed' group and compared this group to
313 the 'engorged' nymphs (i.e. $-Bb_{lizard}$, $-Bb_{mouse}$, $+Bb_{lizard}$, & $+Bb_{mouse}$). Engorgement induced
314 significant gene expression differences, producing 6730 significant DEGs with most of the
315 difference in expression being upregulated genes in the engorged groups (Figure 4b). Of the
316 top 100 most significant DEGs, 25% were related to cuticle formation (Table S4). Other
317 notable genes that were differentially expressed include the antioxidant and detoxifying
318 genes, glutathione peroxidase and sulfotransferase, which were both upregulated 4096-fold in

the engorged group (Table S4). Over a hundred DEGs between unfed and engorged ticks remain uncharacterized.

Despite significant differences in pathogen acquisition success between the $+Bb_{lizard}$ and $+Bb_{mouse}$ in pathogen transmission experiments, only 25 genes were differentially expressed between the two groups (Figure 4c). The two most significantly DEGs included exonuclease V-like (upregulated 32-fold) and 4-coumarate–CoA ligase (downregulated 8-fold) in $+Bb_{lizard}$ (Figure 4c). No genes that are known to be related to immune function were detected as differentially expressed in the $+Bb_{lizard}$ vs. $+Bb_{mouse}$ comparison, with 7 of the 25 differentially regulated genes classified as uncharacterized (Table S5).

Discussion

Vector competency is considered an intrinsic property of a vector that determines its ability to acquire, maintain, and transmit pathogens (Beerntsen et al., 2000), but the extent to which it is modulated by biotic or abiotic factors is poorly understood, especially in tick-borne pathogen systems. Here, we conducted a tick *Bb* acquisition experiment and transcriptome analysis on *I. pacificus* to determine if and by what potential mechanisms host bloodmeal history affects *I. pacificus* vector competency for the Lyme disease pathogen, *Bb.* Through *Bb* feeding experiments, we found that larval bloodmeal history significantly affects *I. pacificus* pathogen acquisition, a key component of vector competency. When ticks that fed on either lizards or mice as larvae fed on *Bb*-infected mice as nymphs, the previously lizard-fed ticks were twice as likely to acquire the pathogen. Further, significant transcriptomic signatures were detected between ticks with different bloodmeal histories. Gene expression analysis identified an upregulation of tick antioxidants and antimicrobial peptides in *I. pacificus* that fed

342 on lizards, which may play a role in altering tick vector competency for *Bb*. Our results initiate
343 a potential mechanistic understanding of how host blood meal affects *I. pacificus* gene
344 expression and the ecological factors that control *I. pacificus* susceptibility to *Bb.*
345 Recent studies suggest that tick microbiome composition can impact vector
346 competency (Narasimhan et al., 2017) and that host bloodmeal source can shape microbiome
347 community structure (Landesman, Mulder, Allan, Bashor, Keesing, Logiudice, et al., 2019;
348 Swei & Kwan, 2017). These two recent findings motivated this study to test whether the lizard
349 bloodmeal host that has been previously shown to reduce tick microbiome diversity (Swei &
350 Kwan, 2017) can have subsequent effects on vector competency. Ticks with prior lizard or
351 mouse bloodmeal histories displayed significant differences in pathogen acquisition when fed
352 on mice infected with *Bb.* Across three separate experimental trials, we found that a prior
353 lizard bloodmeal significantly increased the acquisition of *Bb* in nymphal *I. pacificus*. These
354 results were surprising, especially given that infected *I. pacificus* that feed on *S. occidentalis*
355 are cleared of their infection (Kuo, Lane, & Giclas, 2000; R S Lane & Quistad, 1998). The *Bb*-
356 refractory nature of *S. occidentalis* has long been held as evidence of the lizard's importance
357 in maintaining lower prevalence of Lyme disease in the western U.S. and it likely contributes
358 to lower disease risk relative to the northeastern U.S. However, whether this *Bb*-refractory
359 property could be sustained transstadially in *I.* pacificus was unknown. Our results indicate
360 that lizard-feeding does not preclude *Bb* infection in future life stages of *I. pacificus*, but rather
361 enhances pathogen acquisition success relative to ticks with a prior mouse bloodmeal (Table
362 1). These results indicate that the acute and long-term consequences of a lizard bloodmeal on
363 pathogen transmission are divergent.

364 The role of the microbiome in tick vector competency is unresolved (Kwan et al., 2017;
365 Landesman, Mulder, Allan, Bashor, Keesing, LoGiudice, et al., 2019; Narasimhan et al.,
366 2014). In a prior study, lower microbiome diversity in *I. scapularis* was associated with lower
367 *Bb* colonization success due to decreased expression of genes involved in gut epithelium
368 renewal, which enhances *Bb* colonization (Narasimhan et al., 2014). Therefore, we predicted
369 that lizard-fed ticks, previously shown to have significantly lower microbiome species diversity
370 than mouse-fed ticks (Swei & Kwan, 2017), would similarly have lower *Bb* infection
371 prevalence. However, our *Bb* acquisition experiment found that *I. pacificus* microbiome
372 diversity, resulting from lizard-feeding (Swei & Kwan, 2017), and pathogen transmission
373 success are negatively correlated. This finding may be due to species-specific differences
374 between *I. scapularis* and *I. pacificus* or be driven using different experimental procedures
375 used to manipulate the vector microbiome. Additionally, lizard feeding may affect tick vector
376 competency through altering specific microbes rather than altering overall microbial diversity.
377 Ultimately, the role of microbiome diversity and composition on pathogen acquisition success
378 in *Ixodes* spp. remains uncertain and future studies are needed to disentangle the
379 complicated interactions of these microbes.
380 Our RNA-seq analysis of *I. pacificus* with different bloodmeal histories revealed
381 potential mechanisms that could be driving the differences observed in tick pathogen
382 acquisition. Larval bloodmeal identity and engorgement have large impacts on *I. pacificus*
383 gene expression (Figure 3). Unfed nymphs clustered significantly by larval bloodmeal type
384 (lizard vs. mouse; Figure 3b), indicating that larval bloodmeal source induced distinct
385 transcriptomic alterations in *I. pacificus*. We analyzed gene expression profiles in unfed
386 nymphs right after they molted from larvae to nymph. Our analysis suggests that the effect of

the larval bloodmeal on *I. pacificus* gene expression is carried through the transstadial molt and is present prior to the initiation of the nymphal bloodmeal. Among the unfed ticks, bloodmeal history drove divergence of 468 significantly expressed genes between un-engorged lizard and mouse fed ticks (Table S3). The most significant DEG between unfed ticks with different bloodmeal histories was glutathione peroxidase being upregulated in the lizard-fed group (Figure 5a). Glutathione peroxidase is an important anti-oxidative enzyme, that works by reducing H_2O_2 and detoxifying OH radicals and prevents oxidative stress and cell damage in the tick (Galay et al., 2017; Lubos, Loscalzo, & Handy, 2011). Two other known anti-oxidative enzymes, peroxidase and glutathione S-transferase were significantly upregulated after the lizard bloodmeal (Figure 5b&c). A nutritional dependence on blood has required ticks to evolve and produce anti-oxidants to digest an inherently toxic meal containing high levels of iron and pro-oxidant levels (Galay et al., 2017). Notably, glutathione peroxidase is homologous to SALP25d, a tick antioxidant produced in the salivary glands that has been shown to promote the transmission of *Bb* from tick to host and protects *Bb* from harmful hydroxyl radicals *in vitro* (Narasimhan et al., 2007). The exact mechanism by which the lizard blood initiates the production of antioxidants in feeding ticks is unknown, but unlike mammals, reptiles have nucleated red blood cells (Claver & Quaglia, 2009). Enucleation — the evolutionary loss of a nucleus in red blood cells — is unique to mammals, and is thought to evolved to elevate hemoglobin levels to improve oxygen transport (Ahmed, Ghatge, & Safo, 2020; Ji, Murata-Hori, & Lodish, 2011). We speculate that the upregulation of antioxidants in the lizard-fed ticks may be a result of a response to the lower oxygen levels and potentially higher reactive oxygen species in the nucleated lizard bloodmeal compared to the enucleated mammalian bloodmeal (Salin et al., 2015). The upregulation of glutathione peroxidase and

other antioxidants in lizard-fed ticks has the potential to directly benefit *Bb* colonization from host to tick during the nymphal bloodmeal by increasing antioxidant concentration and protecting *Bb* from the harmful oxidative components of blood.

There was also a strong signal of microbial defense signals in unfed tick comparison. The antimicrobial peptides (AMPs) acanthoscurrin-1, acanthoscurrin-2**,** micropulsin, and a micropulsin isoform were all significantly upregulated in the unfed nymphs with prior lizard bloodmeals relative to prior mouse bloodmeals (UF_{lizard}; Figure 5d-g). Acanthoscurrin is a glycine-rich cationic AMP, known to be expressed in the hemocytes of tarantula spiders, *Acanthoscurria gomesiana*, and has activity against the yeast, *Candida albicans,* and gram-negative bacteria (Lorenzini, da Silva Junior, Fogaça, Bulet, & Daffre, 2003). Micropulsin is a cysteine-rich AMP with histidine-rich regions, found in the hemolymph of the cattle tick, *Rhipicephalus microplus,* with high activity against gram-positive bacteria and fungus (Silva et al., 2009). Neither of these AMPs have been detected in *Ixodes pacificus* prior to this study, but these results indicate that they may play an important role in pathogen acquisition and warrant further study. While lizard blood feeding contributes to the expression of AMPs, it is unclear what upstream components initiate their production. The antimicrobial activity may be an outcome of an initiated humoral immune response or derived from host immune effector molecules, as demonstrated when *Ixodes scapularis* feeds on a *Bb*-infected mouse (Smith et al., 2016). To understand how the expression of these AMPs occur, future gene expression studies should examine the tick immune response to the lizard larval bloodmeal at multiple time points to track the immune response at different stages of feeding. Interestingly, the upregulation of AMPs with broad activity against microbes coincides with a previously described study showing that a lizard bloodmeal significantly reduces *I. pacificus* microbiome

diversity after feeding (Swei & Kwan, 2017). Our results indicate that the lizard bloodmeal is associated with the production of AMPs that may reduce microbe-microbe competition for *Bb* colonization in future bloodmeals.

To further characterize the physiological changes that occur during *I. pacificus* host feeding, we analyzed unfed versus fed ticks, 24 hours after ticks completed their nymphal bloodmeal. Our study confirmed that engorgement induces a large number of transcriptional changes to the physical structure of the tick (Perner et al., 2016; Figure 3b). The greatest number of DEGs was between fed and unfed ticks (Figure 4). Genes related to cuticle formation, antioxidant production, and detoxification were all significantly upregulated in fed ticks (Table S4) and are consistent with structural reformation that occurs during the engorgement process when ticks must rapidly synthesize a new cuticle over the course of taking a large bloodmeal (Gulia-Nuss et al., 2016). Glutathione peroxidase and sulfotransferase were highly upregulated during engorgement and are critical for detoxifying the massive host bloodmeal and protect ticks from harmful oxidative stress inherent in blood feeding (Gulia-Nuss et al., 2016; Perner et al., 2016). These results, while unsurprising, indicate that transcriptomic changes during *I. pacificus* engorgement are like the physiological alterations found in *Ixodes scapularis* and *Ixodes ricinus* (Gulia-Nuss et al., 2016; Perner et al., 2016).

Gene expression of *I. pacificus* is heavily shaped by engorgement status and bloodmeal history in unfed ticks but among the engorged nymphal ticks (groups 5&6; Figure 1), there was not a strong signal of bloodmeal history or infection status (Figure 3b). Despite the significant differences in pathogen acquisition between host bloodmeal experimental groups (Figure 2), only 25 genes with no known pathogen or immune function were

differentially expressed between these groups. Comparing these results to our gene expression analysis from unfed nymphs, the strongest divergence in gene expression is present in the unfed ticks. This suggests that the physiological changes induced by the larval bloodmeal has lasting effects into the nymphal stage.

We document a strong correlation between host bloodmeal and vector competency, but there were limitations to our study. Naturally low burdens prevented us from using field-collected ticks for the mouse bloodmeal (Swei et al., 2012) and use of field-collected questing larvae is problematic because it is difficult to verify whether a tick had a previous, incomplete bloodmeal (Eisen, 2020). Our transcriptomic results indicate that bloodmeal history was only significantly different in the unfed nymphal group while fed nymphs were less apparent, which strongly suggests that larval host blood meal identity played a larger role in gene expression than tick source (Figure 3).

Despite these intriguing results, our study highlights the importance of a more complete annotation of the reference transcriptome for *Ixodes spp.* ticks. A large proportion of DEGs remain uncharacterized indicating additional investigation into tick molecular function and transcriptomics is needed. The lack of differentially expressed genes in our comparison *of Bb*-exposed nymphs with different bloodmeal histories could be attributed to the timing of RNA sampling (24 hours after completed bloodmeal). Examination of gene expression before, during, and immediately after feeding would improve insight into the mechanism of pathogen colonization into *I. pacificus.* Additionally*,* future experiments should focus on understanding the role of antioxidants and the AMPs identified in this study in modifying tick vector competency. We found a strong association between lizard bloodmeal history and antioxidant activity as well as AMP production. These responses coupled with naturally high natural tick

burdens and preferential feeding on lizards may suggest an evolutionary benefit to feeding on the western fence lizard for *I. pacificus* and perhaps *Bb.* Additional research should investigate whether changes in vector competency are propagated through additional life stages (i.e., nymphal to adult and adult to eggs), and its effect on the epigenetic memory of ticks over time (De et al., 2020).

The public health burden of Lyme disease is increasing, and diagnosis and treatment are expensive and imperfect. The complexity of tick-host-pathogen interactions involve many competing interactions, making intervention or prevention of disease transmission very difficult. A better understanding of the molecules, microbes, and antigens involved in vector competency presents a different approach to prevention (Bhowmick & Han, 2020; Galay et al., 2017; Iturbe-Ormaetxe et al., 2011; Pais et al., 2008). Identifying molecular and microbial drivers of tick survival and vector competency are attractive targets for novel control methods (de la Fuente et al., 2017). Using the perturbation of natural host bloodmeal, our study identified multiple molecular components that may be important in the successful acquisition of *Bb* in *I. pacificus* and identifies potential new targets for manipulating and preventing the transmission of tick-borne diseases.

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Data accessibility

Fastq files and raw read counts have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE173109.

Author contributions

K.R., L.I.C., S.C, and A.S. conceived the study. K.R. and L.I.C. conducted field work. K.R., L.I.C., K.C, and F.Y. performed experimental tick feedings. A.L.S and K.R. executed library preparation, created the bioinformatic pipeline, implemented DASH, and analyzed the RNA-seq data. C.M., A.L.S., and K.R. wrote and edited the bioinformatic code. K.R. wrote the manuscript and A.S., S.C., L.I.C., A.L.S, and F.Y. edited and approved the final manuscript.

Figures

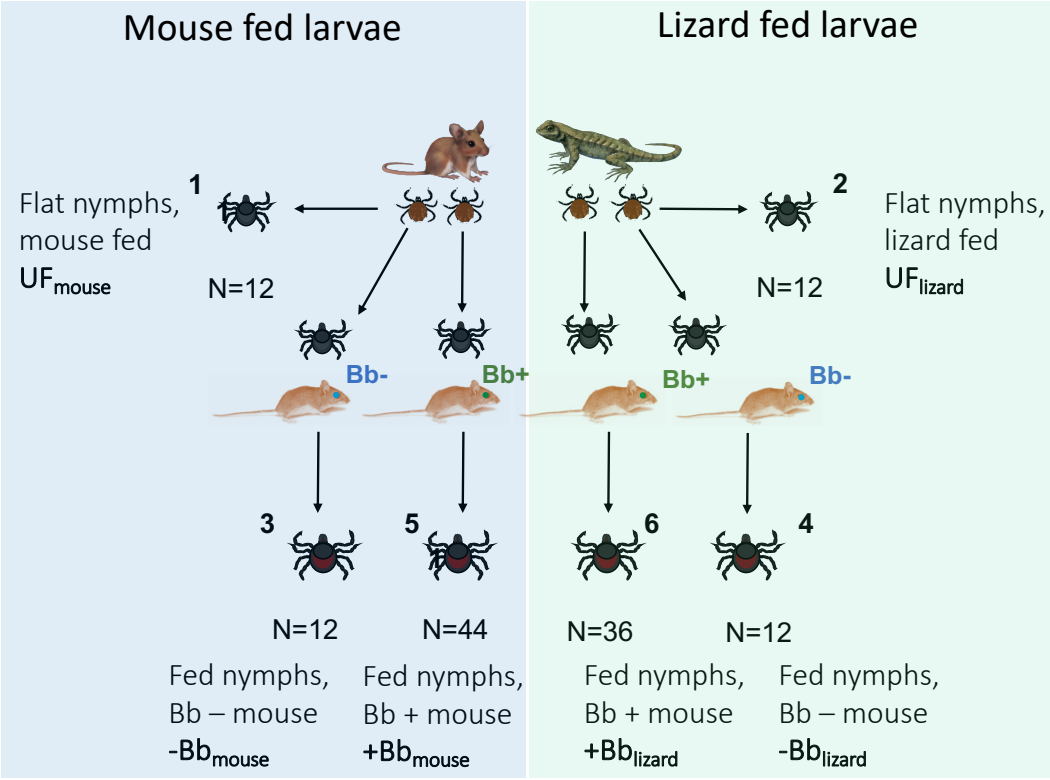
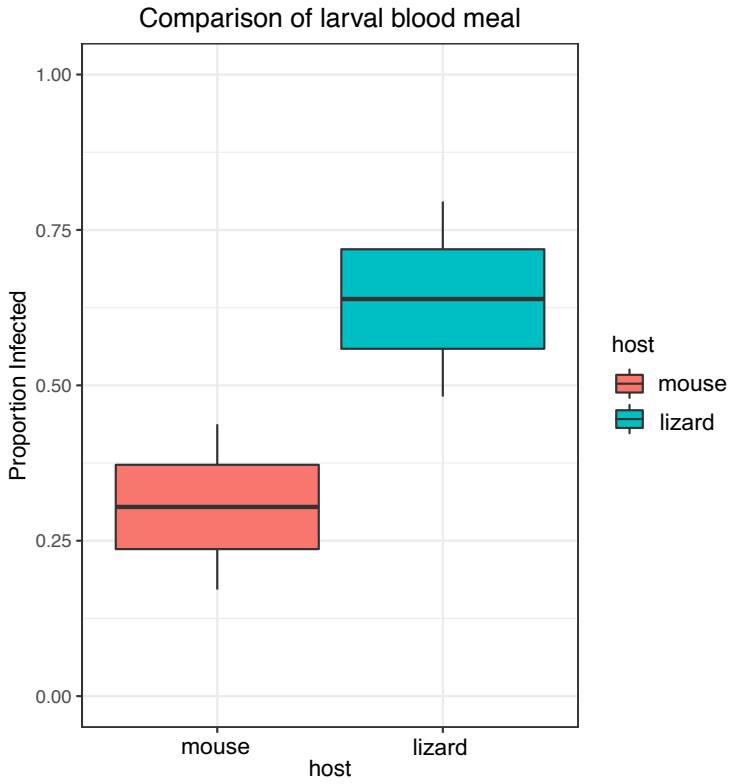


Figure 1 Transmission experiment design. Replete larval *I. pacificus* were obtained from *P. maniculatus* mice or *S. occidentalis* lizards. Successfully molted ticks from both groups were then either immediately sacrificed as unfed nymphs (groups 1&2) or fed on uninfected (groups 3 & 4) and *Bb*-infected (groups 5 & 6) C3H/HeJ mice. The *Bb*-fed ticks were analyzed via qPCR for *Bb* infection status. RNA from all groups was used to make RNA-seq libraries for transcriptomic analysis.



761

762 **Figure 2** Comparison of infection status of *Bb*-fed *I. pacificus* nymphs with prior larval
763 bloodmeals on either mice (mouse-fed; +Bb_{mouse}) or lizards (lizard-fed; +Bb_{lizard}). Lizard-fed
764 larvae were significantly more likely to become infected when subsequently feeding on a *B.*
765 *burgdorferi* infected mouse as nymphs than nymphs that previously fed on mice as larvae,
766 with 64% of lizard-fed ticks infected compared to 30% of the mouse-fed ticks

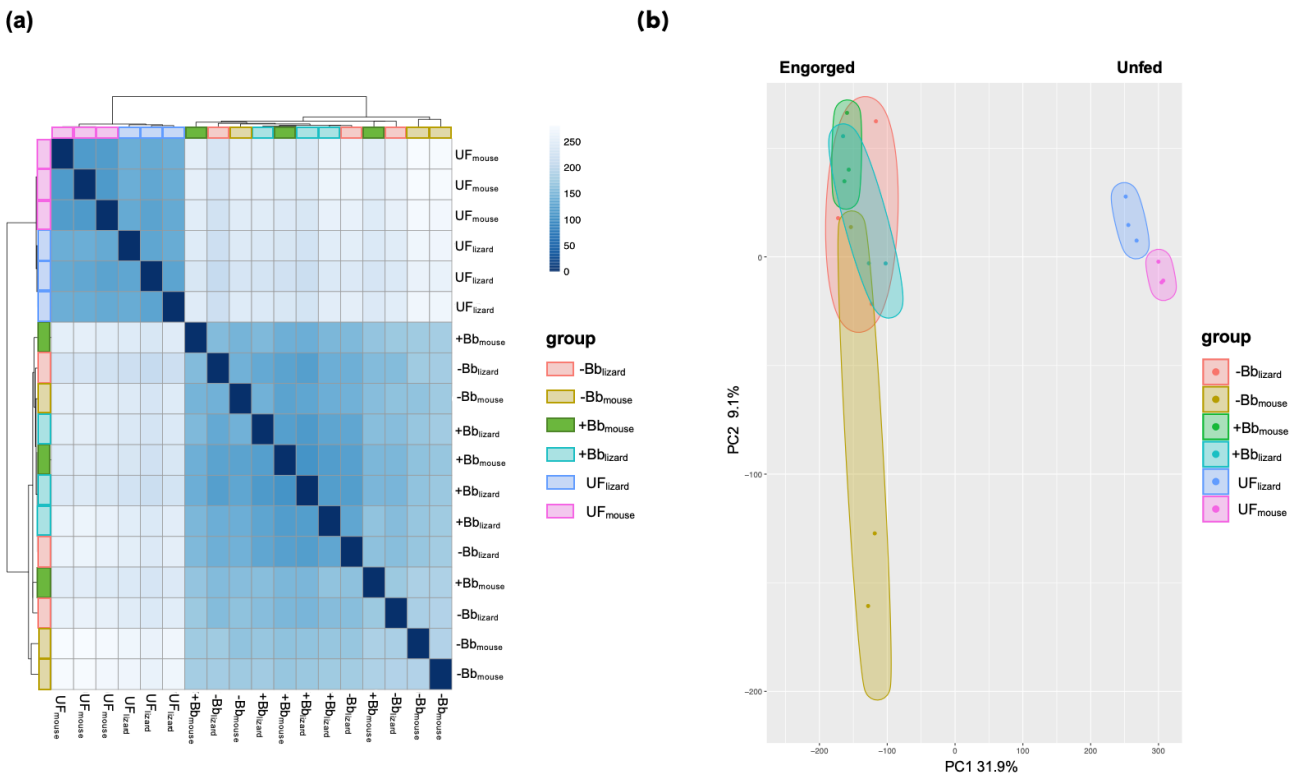


Figure 3 The similarity of transcriptomic profiles based on sample-to-sample distance shown by **(a)** a heat map plot of all samples. Visualization of the overall effect of experimental conditions shown by clustering in a **(b)** principal coordinate analysis on all transcriptomic profiles with plotted 95% confidence ellipses around experimental replicates.

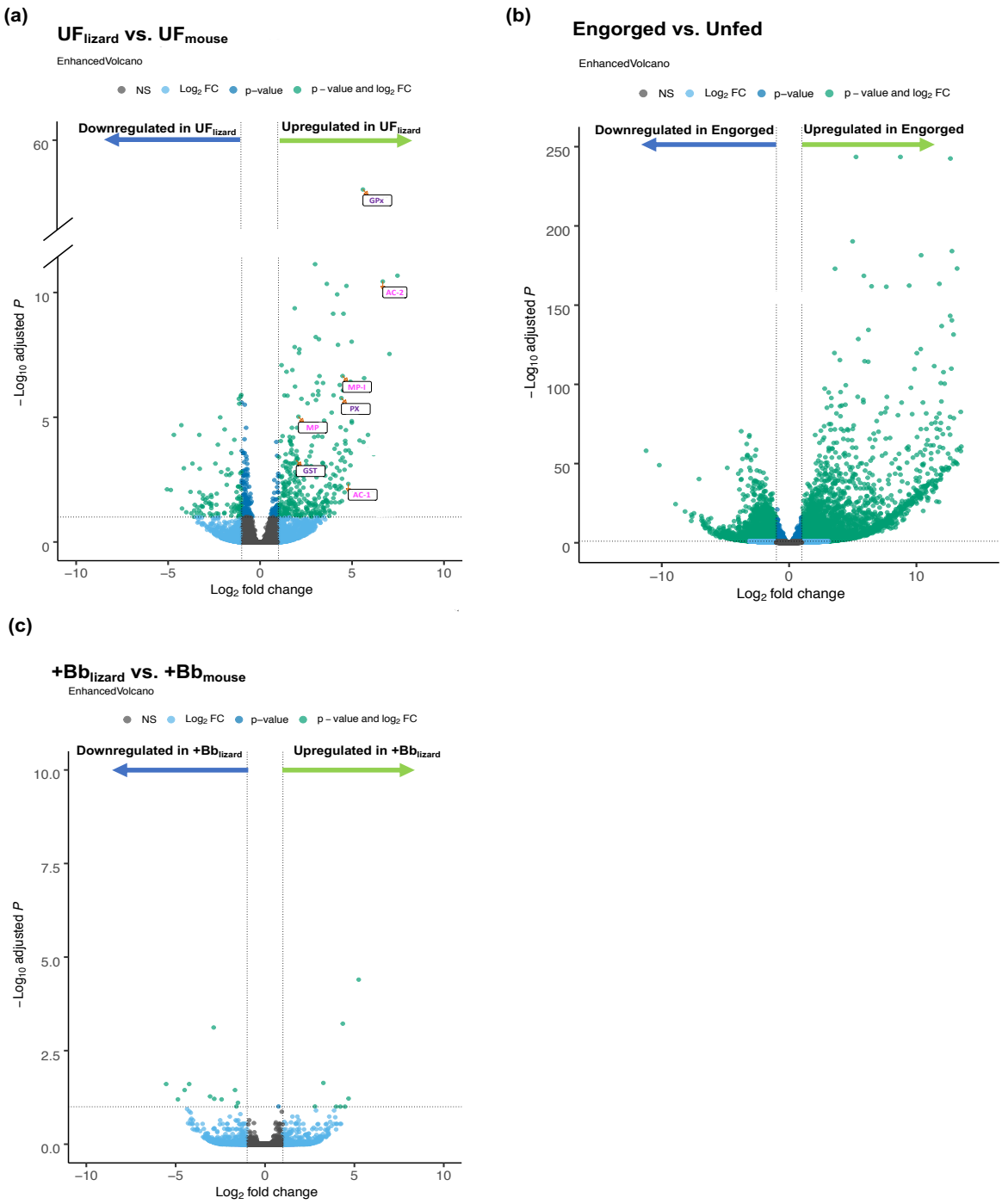


Figure 4 Volcano plots showing significant differential gene expression between the following: **(a)** UF_{mouse} (reference) vs. UF_{lizard} (comparison). Key annotated genes include tick antioxidants (purple print) glutathione peroxidase (GPx), glutathione S transferase (GST), peroxidase (PX) and antimicrobial peptides (pink print) acanthoscurrin-1 (AC-1), acanthoscurrin-2-like (AC-2), micropulsin (MP) and micropulsin isoform (MP-1). **(b)** Engorged (reference) vs. Unfed (comparison) and **(c)** $+Bb_{mouse}$ (reference) vs. $+Bb_{lizard}$ (comparison). Green points above the dotted x-axis represent genes significantly up or downregulated (padj value < 0.05 and log2FC > |1|).

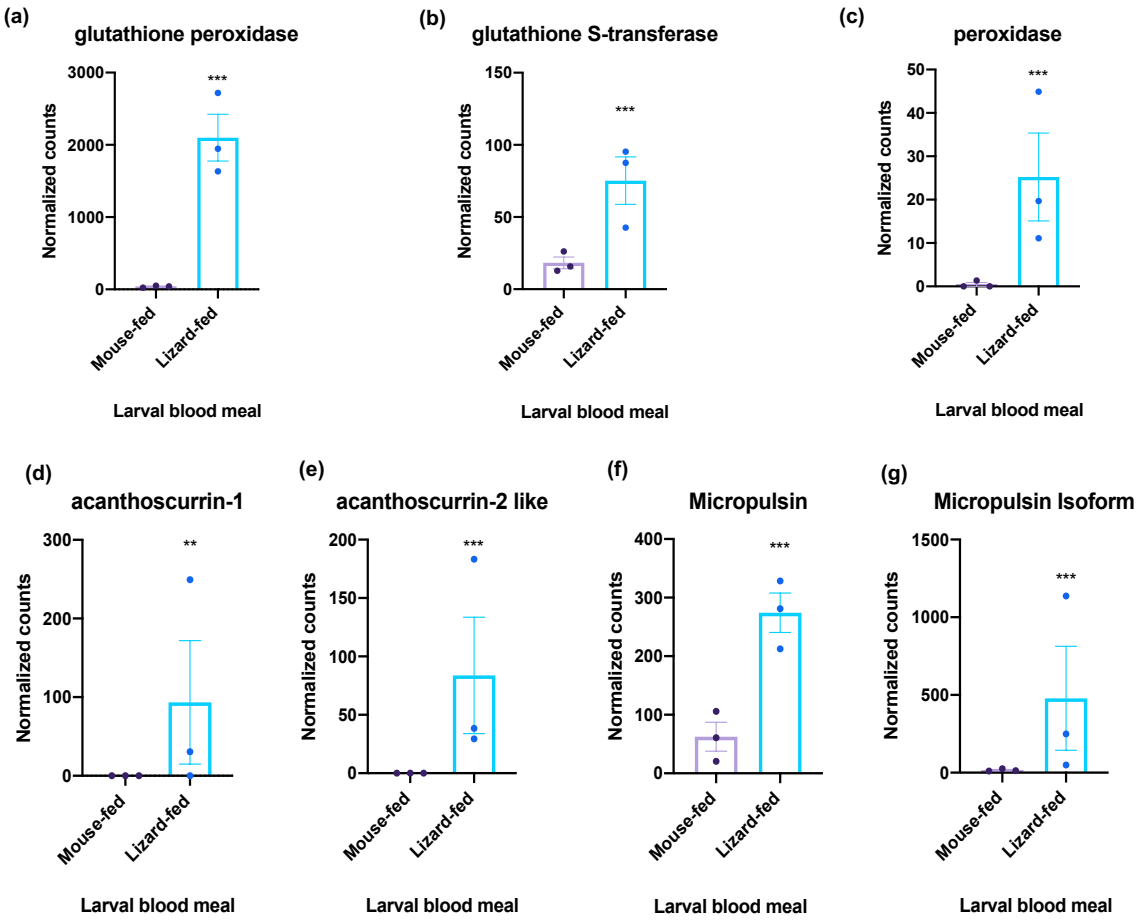


Figure 5 Comparison of key significant DEGs in unfed comparison UF_{lizard} vs. UF_{mouse}. Graphs show the comparison of significantly DEGs using Wald's test on normalized transcript for tick antioxidants **(a)** glutathione peroxidase (padj value= 2.79e-59) **(b)** glutathione-S-transferase (padj value= .0005) **(c)** peroxidase (padj value = 1.34e-6) and antimicrobial peptides **(d)** acanthoscurrin-1 (padj value=.003) **(e)** acanthoscurrin-2 like **(f)** micropulsin (padj value= 1.04e-5) **(g)** micropulsin isoform (padj value = 1.22e-7).

802 **Table**

Response	Fixed effect	Estimate	Standard Error	z value	p value
Infection status	(Intercept)	-1.02	.51	-2.023	<.05*
Infection status	Lizard larval bloodmeal	1.51	.51	2.95	<.01**

803 **Table 1**. Summary of results from a generalized mixed-effect model (GLMM) with binomial
804 distribution examining the correlation between lizard larval blood host and the probability of
805 infection in the nymphal stage. Inoculum load of host was included as a random effect to
806 account for differences between experimental trials.