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Research Article

RESEARCH ARTICLE

Vibrio elicits targeted transcriptional responses from copepod hosts

Amalia A. Almada and Ann M. Tarrant*

Department of Biology, Woods Hole Oceanographic Institution, 45 Water Street, Mailstop 33, Woods Hole, MA 02543, USA

*Corresponding author: Department of Biology, Woods Hole Oceanographic Institution, 45 Water Street, Mailstop 33, Woods Hole, MA 02543, USA. Tel: (+1)-508-289-3398; Fax: (+1)-508-457-2134; E-mail: atarrant@whoi.edu

One sentence summary: The copepod *Eurytemora affinis* exhibits consistent changes in gene expression following exposure to a zooplankton-associated *Vibrio* species (F10 9ZB36); this may reflect an ability of *Eurytemora* to recognize and regulate its associated vibrio community.

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ABSTRACT

Copepods are abundant crustaceans that harbor diverse bacterial communities, yet the nature of their interactions with microbiota are poorly understood. Here, we report that *Vibrio* elicits targeted transcriptional responses in the estuarine copepod *Eurytemora affinis*. We pre-treated *E. affinis* with an antibiotic cocktail and exposed them to either a zooplankton specialist (*Vibrio* sp. *F10 9ZB36*) or a free-living species (*Vibrio ordalii 12B09*) for 24 h. We then identified via RNA-Seq a total of 78 genes that were differentially expressed following *Vibrio* exposure, including homologs of C-type lectins, chitin-binding proteins and saposins. The response differed between the two *Vibrio* treatments, with the greatest changes elicited upon inoculation with *V.* sp. *F10*. We suggest that these differentially regulated genes play important roles in cuticle integrity, the innate immune response, and general stress response, and that their expression may enable *E. affinis* to recognize and regulate symbiotic vibrios. We further report that *V.* sp. *F10* culturability is specifically altered upon colonization of *E. affinis*. These findings suggest that rather than acting as passive environmental vectors, copepods discriminately interact with vibrios, which may ultimately impact the abundance and activity of copepod-associated bacteria.

Keywords: microbiome; innate immunity; crustacean; marine

INTRODUCTION

Animals have developed diverse mechanisms to initiate and regulate their interactions with microbiota in order to enrich for specific symbionts and prevent invasion by pathogens within microbially rich environments (Ezenwa *et al.* 2012; Buchon, Broderick and Lemaitre 2013). Those bacteria that successfully associate with hosts receive benefits including increased access to nutrients (Douglas 2009), protection against environmental stressors (Chowdhury *et al.* 1997), increased frequency of horizontal gene transfer (Meibom *et al.* 2005; Aminov 2011) and enhanced persistence in the environment (Huq *et al.* 1983). Bacterial communities associated with copepods exhibit increased growth rates and production relative to those bacteria free-living in the surrounding seawater (Griffith, Douglas and Wainright 1990; Carman 1994), in addition to access to unique environments provided by their migrating hosts (Grossart *et al.* 2010). Colonization of copepods by *Vibrio* bacteria is a relatively well-studied zooplankton–bacteria interaction due to the prevalence of pathogenic vibrios (e.g., *Vibrio cholerae*, *V. parahaemolyticus*) on these abundant chitinous organisms (e.g., Huq *et al.* 1983; Rawlings, Ruiz and Colwell 2007) and the dramatic impacts of these associations on the proliferation, virulence and physiology of vibrios (Kirn, Jude and Taylor 2005; Colwell 2009). However, whether copepods are in turn impacted by or further regulate colonizing vibrios is unknown. In light of copepods'

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abundance, prevalence across aquatic habitats, and enrichment with *Vibrio* associates, copepod physiology may be an important influence on *Vibrio* ecology that has not yet been fully explored.

Invertebrate host factors are increasingly recognized for their significant roles in symbiont acquisition and maintenance (Buchon, Broderick and Lemaitre 2013), often contributing to highly host-specific microbiomes (Franzenburg *et al*. 2013). As first lines of defense, the hard, chitinous exoskeleton and gut lining of arthopods such as copepods together form a physical and chemical barrier against pathogen attachment and invasion (Lemaitre and Hoffmann 2007; Vallet-Gely, Lemaitre and Boccard 2008). Chitinous surfaces are also known to induce genetic programs in *Vibrio* species, including the induction of natural competence (Meibom *et al*. 2005). Although invertebrates lack an adaptive immune system, focused studies have revealed a deeper level of complexity of the innate immune system than previously appreciated, including specific immune memory (Kurtz and Franz 2003; Little *et al*. 2003). Elements of the innate immune system, including C-type lectins and antimicrobial peptides (AMPs), enable invertebrate hosts to select for specific bacterial associates in addition to inhibiting growth of undesirable foreigners (Bulgheresi *et al*. 2006; Binggeli *et al*. 2014). For example, in the marine nematode *Laxus oneistus*, a mucus-secreted C-type lectin is produced to mediate symbiont association with the cuticle by directly binding to the symbiont's antigens and inducing symbiont aggregation (Bulgheresi *et al*. 2006). Once bacterial symbionts are acquired, innate immune elements such as AMPs can be crucial to further regulate interactions with microbiota, even by ensuring the proper localization of the symbionts within the host tissue (Login *et al*. 2011). Such finely tuned and localized innate immune responses to bacterial symbionts complement those highly conserved, systemic innate immune responses to invading microbes, including the prophenoloxidase (proPO) cascade and catalase activity. The proPO cascade is induced when host recognition proteins are activated by microbial compounds, including bacterial surface attachment proteins and cell wall components (Medzhitov 2007) that initiate the conversion of proPO into catalytically active phenoloxidase. Phenoloxidase in turn triggers the production of cytotoxic compounds and encapsulation of the microbial invaders (Cerenius, Lee and Söderhäll 2008 and references therein). In addition, catalases enzymatically decompose reactive oxygen species, specifically hydrogen peroxide, which is produced as part of the innate immune response (Ha *et al*. 2005; Wang *et al*. 2013).

In this study, we explored the responses of a copepod host to colonization by distinct *Vibrio* species. We chose as our model the copepod *Eurytemora affinis*, an invasive and abundant species that naturally associates with a diversity of pathogenic vibrios (Winkler, Dodson and Lee 2008; Zo *et al*. 2009) and has been consistently used in the few laboratory studies examining copepod-*Vibrio* interactions (Huq *et al*. 1983, 1984; Rawlings, Ruiz and Colwell 2007). However, to our knowledge, our study is the first to examine the potential of the copepod host to discriminately respond to *Vibrio* associations. The two *Vibrio* species tested in this study inhabit similar coastal environments to *E. affinis* (Huq *et al*. 1983; Preheim *et al*. 2011) and possess distinct physical characteristics and ecological specializations: *V.* sp. *F10* is classified as a zooplankton specialist that lacks the ability to degrade chitin (Preheim 2010; Preheim *et al*. 2011), while *V. ordalii* has been inferred to be 'almost exclusively free-living' because it is enriched in particle-free fractions of the water column and repeatedly absent from particles, zooplankton and larger invertebrates (Hunt *et al*. 2008; Preheim *et al*. 2011; Szabo *et al*. 2013). Here, we examined the global transcriptomic response elicited in *E. affinis* by these two ecologically distinct *Vibrio* species.

MATERIALS AND METHODS

Vibrio cultures

Vibrio growth was first measured over 24 h to confirm their ability to survive and grow under exposure conditions ideal for *E. affinis* (i.e., 15 PSU, 18°C) (Fig. S1, Supporting Information). In preparation for *E. affinis* exposure experiments, glycerol stocks of *Vibrio* cultures were streaked onto seawater complete (SWC) agar plates containing 15 PSU artificial seawater (ASW), peptone, yeast extract and glycerol before a 24-hour incubation at room temperature (RT). Several colonies were then transferred into 10 mL of SWC liquid media (15 PSU), shaken at 200 rpm and incubated for 19 h at 18°C (*V.* sp. *F10 9ZB36*) or 28°C (*V. ordalii 12B09*; 28°C was chosen for *V. ordalii* to ensure robust rapid growth; Fig. S1, Supporting Information). For the *E. affinis*—*Vibrio* exposure experiments, 1 mL of overnight *Vibrio* culture was transferred to 100 mL of SWC liquid media (15 PSU) and incubated for 19 h at 100 rpm. Cultures were then pelleted at 5500 × *g* for 5 min and rinsed twice with 0.22-μm sterile filtered artificial seawater (15 PSU, RT) before diluting to the desired cell density (2×10^7 CFU mL^{-1}). The final dilution factors for each strain were calculated from OD_{600} readings converted to colony forming units (CFU) concentrations using independently determined standard curves for each strain and test condition (data not shown).

To test whether *V.* sp. *F10* and *V. ordalii* secrete extracellular chitinases, overnight cultures were grown in SWC media, as described above, spread onto plates comprised of ∼2% (w/v) colloidal chitin in 1× marine agar (2216), and incubated at room temperature for 24–48 h. Colloidal chitin was prepared from crab shell chitin flakes (Sigma-Aldrich, St. Louis, MO, USA) (Murphy and Bleakeley 2012) and dyed with Remazol Brilliant Violet (Sigma-Aldrich) (Gomez Ramirez *et al*. 2004). When extracellular chitinases hydrolyze the chitin substrate and covalently linked dye, a clear halo is left surrounding the chitinase-producing culture. Those cultures that do not secrete chitinases under the conditions examined may grow on the plate but will not produce a clear halo.

Antibiotic treatment of the estuarine copepod *E. affinis*

Eurytemora affinis cultures that originated from the Baie de L'isle Verte in the St Lawrence estuary were generously provided by Carol Lee (University of Wisconsin). The copepod cultures were maintained at 12°C and 15 PSU on a 14 h light/10 h dark cycle with moderate air bubbling (1–2 bubbles per second). The cultures were fed with *Rhodomonas lens* three times a week at a concentration of 1×10^6 cells mL^{-1}.

Before *Vibrio* exposure, *E. affinis* were fed and treated for 24 h with an antibiotic mixture of ampicillin (0.3 mg mL^{-1}), streptomycin (0.1 mg mL^{-1}) and chloramphenicol (0.05 mg mL^{-1}) in moderately aerated, sterile seawater (15°C, 15 PSU). To initially validate the effectiveness of the antibiotic cocktail in reducing the natural flora of *E. affinis*, individual whole copepods, homogenized copepods, or 400 μL of seawater from flasks containing either antibiotic treated or untreated copepods were placed into 2 mL of marine broth. The absorbance of the marine broth from each of the treatments was measured after 48 h of incubation at 22°C. In ten independent experiments, the antibiotic treatment dramatically reduced the OD_{600} of all three sample types (Fig. S2A, Supporting Information). In all further *Vibrio* exposure

Table 1. Abundance of bacteria associated with the estuarine copepod *E. affinis* after 24 h of exposure. After 24 h of antibiotic pre-treatment, followed by inoculation with *Vibrio* culture or sterile seawater (control) for 24 h (18°C, 15 PSU), whole live copepods (5 per replicate) were rinsed with artificial seawater, homogenized, and stained with DAPI or plated on SWC agar (15 PSU) to obtain direct and plate (culturable) counts, respectively. Counts are listed as means ± standard error of two biological replicates. 'Density of total bacteria' attached to control copepods are not listed because the direct counts of these samples were consistently below detection (≤1 cell/field). [a]Indicates the culturable bacteria counts are approximate because they are below the statistical detection limit (<30 CFU/plate). [b]Indicates there is one biological replicate.

Vibrio strain	Inoculation density (CFU mL^{-1})	Density of culturable bacteria attached to copepods (CFU/copepod)	Density of total bacteria attached to copepods (direct cell counts/copepod)	Density of culturable bacteria on controls (CFU/copepod)	Sex of copepods in experiment
V. sp. F10 9ZB36	1.0×10^7	0 ± 0[a]	$2.3 \times 10^6 \pm 4.0 \times 10^5$	0.6 ± 0.6	Males and females (>400 μm)
V. sp. F10 9ZB36	2.0×10^7	18.5 ± 1.5[a]	$2.0 \times 10^6 \pm 5.0 \times 10^5$	2.5 ± 1	Mature, adult females
V. sp. F10 9ZB36	2.0×10^7	4.9 ± 1.1[a]	$7.4 \times 10^5 \pm 2.7 \times 10^5$	8.5 ± 1	Mature, adult females
V. sp. F10 9ZB36	2.0×10^7	15.6 ± 9.9[a]	$5.0 \times 10^6 \pm 1.1 \times 10^6$	0.75 ± 0.25	Mature, adult females
V. sp. F10 9ZB36	2.5×10^7	0 ± 0[a]	1.2×10^6[b]	0 ± 0	Males and females (>400 μm)
V. sp. F10 9ZB36	6.0×10^7	6.0 ± 2.0[a]	–	0.75 ± 0.75	Mature, adult females
V. ordalii 12B09	6.0×10^6	$2.1 \times 10^3 \pm 6.0 \times 10^2$	–	0.2 ± 0.2	Males and females (>400 μm)
V. ordalii 12B09	7.0×10^6	7.0×10^3[b]	–	2.8 ± 0.4	Males and females (>400 μm)
V. ordalii 12B09	2.0×10^7	$2.7 \times 10^4 \pm 9.0 \times 10^3$	$3.1 \times 10^4 \pm 4.5 \times 10^3$	2.5 ± 1	Mature, adult females
V. ordalii 12B09	2.0×10^7	$3.2 \times 10^4 \pm 1.4 \times 10^4$	$4.9 \times 10^4 \pm 3.5 \times 10^3$	8.5 ± 1	Mature, adult females
V. ordalii 12B09	2.0×10^7	9.0×10^4[a,b]	3.1×10^4[b]	0 ± 0	Mature, adult females
V. ordalii 12B09	2.0×10^7	$1.4 \times 10^4 \pm 6.0 \times 10^3$	$1.6 \times 10^5 \pm 1.5 \times 10^4$	0.75 ± 0.25	Mature, adult females
V. ordalii 12B09	3.0×10^7	$2.0 \times 10^4 \pm 1.0 \times 10^4$	$3.3 \times 10^4 \pm 8.5 \times 10^3$	1.25 ± 0.75	Males and females (>400 μm)
V. ordalii 12B09	6.0×10^7	$3.4 \times 10^4 \pm 2.0 \times 10^3$	$2.0 \times 10^5 \pm 2.0 \times 10^4$	0.2 ± 0.2	Males and females (>400 μm)
V. ordalii 12B09	6.0×10^7	$4.4 \times 10^4 \pm 1.0 \times 10^3$	–	0.75 ± 0.75	Mature, adult females
V. ordalii 12B09	6.0×10^7	$5.4 \times 10^4 \pm 2.5 \times 10^3$	–	0 ± 0	Mature, adult females
V. ordalii 12B09	7.0×10^7	$1.2 \times 10^5 \pm 6.1 \times 10^4$	$2.0 \times 10^4 \pm 2.5 \times 10^4$	2.8 ± 0.4	Males and females (>400 μm)

experiments, the effectiveness of the antibiotic treatment was monitored via plate and direct counts of copepods from the control treatments (antibiotic-treated and not inoculated with *Vibrio*), as described below, further demonstrating the success of the antibiotic treatment in reducing the native bacterial load (Table 1; Fig. S2B, Supporting Information; Fig. 2C).

Eurytemora affinis-Vibrio exposure experiments

After 24 h of antibiotic treatment, copepods were rinsed with sterile seawater onto an autoclaved 400-μm sieve and captured with a transfer pipette (USA Scientific, Ocala, FL, USA). In the RNA-Seq experiment, 20 mature, adult females were captured for each treatment replicate. 'Mature, adult females' were considered to include ovigerous females and non-ovigerous females with enlarged oviducts full of large oocytes, as previously defined (Boulange-Lecomte, Forget-Leray and Xuereb 2014). Follow-up quantitative PCR (qPCR) experiments were performed with pools (10–20 per replicate) of mature, adult females. For all exposures, copepods were placed into autoclaved 50 mL glass flasks containing *Vibrio* cultures diluted in sterile seawater (15 PSU, 15°C) and incubated at 18°C with moderate aeration for 24 h (14 h light/10 h dark cycle). After 24 h of *Vibrio* exposure, copepod samples used for the RNA-Seq and qPCR experiments were gently rinsed onto autoclaved 333-μm mesh, transferred using plastic pipettors into 1 mL of PureZOL (Bio-Rad, Hercules, CA, USA), and stored at −80°C until RNA extraction within ∼4 weeks.

Although the typical density of copepods' natural microbiota is $\sim 10^5$ cells copepod^{-1} within an ambient marine environment containing 1×10^5–10^6 total bacterial cells mL^{-1} (Möller, Riemann and Sondergaard 2007; Tang, Turk and Grossart 2010), we chose an inoculation density of 2×10^7 CFU mL^{-1} for the RNA-Seq and qPCR expression studies in order to increase the likelihood of eliciting a transcriptomic response in our test animals. Studies examining invertebrate host responses to bacteria frequently use a titer within or above this inoculation density and usually use more direct methods of infection (i.e., injection versus our approach of immersion, as in Vodovare *et al.* 2005; Watthanasurorot *et al.* 2011; Cha *et al.* 2015).

To quantify the abundance of bacteria associated with *E. affinis* after 24 h, live copepods from the three treatments (*V. sp. F10*, *V. ordalii*, control) were rinsed onto autoclaved 333-μm mesh sieves with sterile ASW (18°C, 15 PSU), and whole animals (5 per replicate) were homogenized in 200 μL of filter-sterilized ASW with sterile plastic pestles (Axygen Scientific, Corning, NY, USA). Homogenized copepods were then serially diluted and incubated for 20 h at RT on SWC or thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates before counting CFUs. Serial dilutions of the homogenized copepods were also preserved in formalin (1%) and stained with DAPI (10%) for direct counts on 0.22-μm black polycarbonate filters (EMD Millipore Isopore, Darmstadt, Germany) under blue light excitation. Samples from the control treatment were not serially diluted, in anticipation of low cell densities.

RNA extractions and library sequencing

Total RNA was extracted from *E. affinis* samples using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad). Samples were homogenized in 1 mL PureZOL (Bio-Rad) using a teflon homogenizer and processed according to the manufacturer's protocol, with final elution from columns in 40 μL of warmed elution buffer (Tris buffer), as described previously (Aruda *et al.* 2011). For qPCR, residual genomic DNA was removed with on-column DNase digestion. RNA yield and purity were quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and RNA quality was visualized on a denaturing agarose gel. Quality of RNA samples submitted for Illumina sequencing was further assessed using a Bioanalyzer. The

E. affinis samples, like many other arthropods, yielded one sharp peak on the Bioanalyzer due to a hidden break in their 28S rRNA that causes it to run at about the same size as the 18S rRNA.

Directional, polyA-enriched RNA libraries were built by the Hudson Alpha Genomic Services Laboratory with the NEBNext® Ultra Directionality Kit (New England BioLabs, Ipswich, MA, USA) from 1 μg of total RNA from each sample. The average fragment size of each library was ~300 bp. For transcriptome assembly, a library was constructed from a sample of pooled RNA made by combining ~200 ng from each sample (four biological replicates per treatment: control, *V.* sp. *F10*-exposed, and *V. ordalii*-exposed). The library constructed from this pooled sample was sequenced with 100 bp paired-end reads at a total sequencing depth of 111 million reads on a HiSeq 2000 (Illumina, San Diego, CA, USA). The libraries constructed from each of the twelve individual samples were multiplexed and sequenced across two lanes of the HiSeq 2000 with 50 bp paired-end reads at a total depth of 25 million reads per sample for differential expression analysis.

De novo transcriptome assembly and post-assembly analysis

Trimmomatic software (Bolger, Lohse and Usadel 2014) was used in paired-end mode to remove adaptor sequences, low quality sequences (phred score < 20 bp) and the first 12 bp of the 5′ end of the read, which often contains a biased nucleotide composition due to non-random hexamer priming (Hansen, Brenner and Dudoit 2010). Reads greater than 50 bp in length after quality trimming were retained for assembly, resulting in a total of 102 million reads for assembly. An *E. affinis* transcriptome was assembled *de novo* with the RNA-seq assembler Trinity (version r2013-08-14) using default parameters for paired-end, directional reads (Grabherr *et al.* 2011). The assembled transcriptome consisted of 138 581 contiguous consensus sequences (contigs) that were grouped into 82 891 Trinity components ('genes'). The size range of the transcripts was 201–23 627 bp with an N50 (weighted median) of 2087 bp. The *E. affinis* assembly is qualitatively similar to other recently reported copepod and amphipod transcriptomes (Table S1, Supporting Information). The assembled *E. affinis* transcriptome is accessible through the Transcriptome Shotgun Assembly database (TSA, Bioproject PRJNA242763).

Trinity-supported protocols and scripts for downstream analyses were followed using default parameters (Haas *et al.* 2013) to align reads associated with each library to the assembled transcriptome and to estimate abundances of the assembled transcripts (RSEM). Abundance counts of genes were TMM- (trimmed mean of M-values) and FPKM- (fragments per kilobase per million reads mapped) normalized to account for differences in RNA production across samples (Robinson and Oshlack 2010) and gene length, respectively. The *E. affinis* genome was released in the midst of our analysis (Bioproject PRJNA203087), so a blastn search against the genome with a threshold e-value of 10^{-10} was performed to validate the origin of the transcripts as belonging to *E. affinis*. Principal component analysis (PCA) of the TMM- and FPKM-normalized abundance counts of all biological replicates across the three treatments, with *Vibrio* sequences removed, identified one outlier in the control treatment that was subsequently dropped from further analysis (Fig. S3, Supporting Information). Analysis of differentially expressed genes across the three treatments was performed with edgeR software (Robinson, McCarthy and Smyth 2010) with a minimum 2-fold difference in expression and a *P*-value cut-off for a false discovery rate (FDR) of 0.05. We chose a 2-fold threshold in light of previous findings that known modulators of host–microbiota interactions are often regulated within this range (Broderick, Buchon and Lemaitre 2014).

Representative sequences corresponding to the differentially expressed genes were provisionally annotated using blastx against the NCBI non-redundant (nr) database with a threshold e-value of 10^{-4}. The remainder of the transcriptome was annotated by blastx against the Swissprot database. Blast2GO (Conesa *et al.* 2005) was also used to gain further information about the gene ontology (GO) terms and conserved protein domains associated with the genes of interest.

Cloning and qPCR

To confirm the predicted sequences of the genes of interest and to generate standards for qPCR, 205–790 bp regions were cloned and sequenced as described previously (Aruda *et al.* 2011). All primer sequences are provided in Tables S7 and S8, Supporting Information. Material for cloning was obtained from mature, adult *E. affinis* females preserved in PureZOL at −80°C. Complementary cDNA (cDNA) was synthesized from 1 μg of total RNA per 20 μL reaction using the I-Script cDNA-synthesis kit (Bio-Rad) according to the manufacturer's instructions. PCR products were cloned into pGEM-T Easy (Promega, Madison, WI, USA) and sequenced. For qPCR experiments, cDNA was synthesized from 450 ng of total *E. affinis* RNA in a 20 μL reaction. The 20 μL cDNA synthesis reactions were each diluted with molecular biology grade water, such that each microliter of diluted cDNA corresponded to 10 ng of total RNA.

Gene expression was measured using SsoFast EvaGreen Supermix (Bio-Rad) on an iCycler iQ real-time PCR detection system (Bio-Rad). The 20 μL EvaGreen reaction mixture contained 10 μL master mix, 8 μL molecular biology grade water, 1 μL diluted cDNA and 1 μL of 10 μM primers. The PCR conditions were: 95°C for 2 min followed by 40 cycles of 95°C for 5 s and 62°C–64°C for 10 s. All samples and standards were run in duplicate wells on the same plate for each gene of interest. After amplification, PCR products from each reaction were subjected to melt-curve analysis to ensure that only a single product was amplified. Selected products were also visualized on agarose gels and consistently yielded single bands.

Gene expression was calculated relative to a standard curve of serially diluted plasmid standards encompassing the amplicon of interest followed by base-2 log-transformation. A normalization factor equal to the geometric mean of three normalizer genes (Vandesompele *et al.* 2002) was subtracted from the gene expression values. The normalizer genes were chosen from the Illumina data based on their moderate expression and low coefficient of variation between samples (i.e., thioredoxin domain-containing protein 5 (comp52262_c0), thyroid adenoma-associated protein homolog (comp59254_c0) and human leucine-rich repeat neuronal protein 2-like (comp53361_c0)). The normalizer genes exhibited stable expression throughout the study except for one *V.* sp. *F10*-exposed biological replicate that exhibited very low expression of all three normalizer genes and was subsequently removed from further analysis. Results from three independent *Vibrio* exposure experiments were combined to give a total of 8 biological replicates in the *V.* sp. *F10*-exposed treatments (after dropping one *V.* sp. *F10* replicate, as explained above), 9 replicates in the *V. ordalii*-exposed treatments, and 10 replicates in the control treatment. One-way ANOVAs were used to compare mean gene expression among treatments, except in the cases of C-type

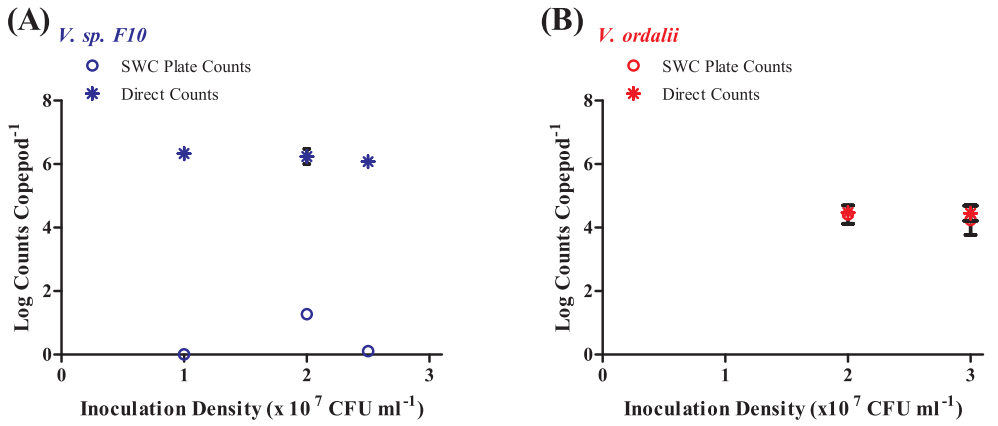


Figure 1. Association with the copepod *E. affinis* reduces *Vibrio* sp. *F10* culturability on seawater complete (SWC) agar across a range of inoculation densities. *V.* sp. *F10* (**A**) and *V. ordalii* (**B**) isolated from pools ($n = 5$) of homogenized adult, mature female *E. affinis* were either directly stained with DAPI (asterisks) or plated on SWC agar (circles) and incubated at room temperature for 20 h. Abundances are plotted as the base-10 log-transformed means of two biological replicates. Error bars indicate standard error and frequently fall within the area of the symbol. Note that the symbols associated with the direct and plate counts for *V. ordalii* lie on top of one another. The direct and plate counts of the copepods from the control treatments are not shown because they were consistently below detection (≤1 cell/field and <30 CFU/plate, respectively).

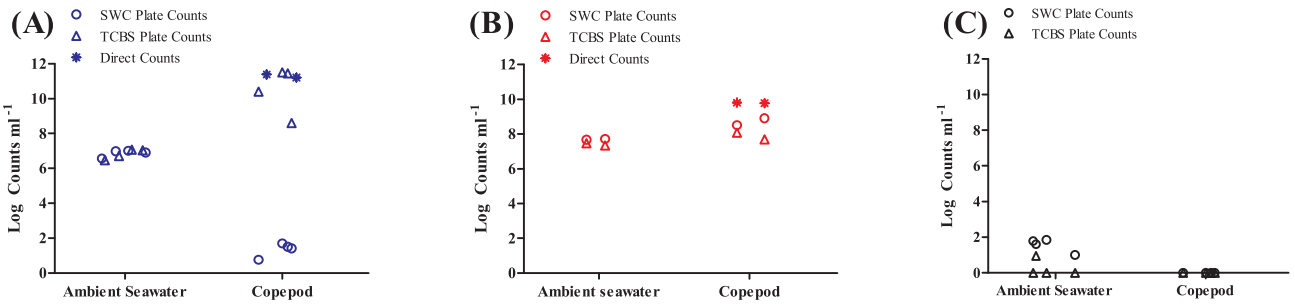


Figure 2. Reduction of *Vibrio* sp. *F10* culturability upon colonization of the *E. affinis* surface is media-specific. Equivalent bacterial concentration of antibiotic pre-treated copepods 24 h after inoculation with a bacterial density of 2×10^7 CFU mL^{-1} of *V.* sp. *F10* (**A**), *V. ordalii* (**B**) or no bacterial inoculation control (**C**). Bacteria isolated from pools ($n = 5$) of homogenized adult, mature female *E. affinis* or from the ambient seawater were either directly stained with DAPI (asterisks), plated on SWC agar (circles) or plated on TCBS agar (triangles) and incubated at room temperature for 20 h. Equivalent concentration of copepod-associated bacteria was calculated by dividing counts per copepod by the approximate volume of *E. affinis* (~2.5×10^{-5} mL) to qualitatively compare ambient seawater and copepod-associated bacterial concentrations (Tang *et al.* 2010). SWC plate counts of *V.* sp. *F10* isolated from *E. affinis* were consistently below the statistical detection limit (<30 CFU/plate) and therefore were not normalized to *E. affinis* body volume. The direct and plate counts of the copepods from the control treatments were consistently below detection (≤1 cell/field and <30 CFU/plate, respectively). All counts were base-10 log-transformed and replicates were jittered along the *x*-axis to improve readability. In the control panel (**C**), all negative log-transformed values and zero counts (undefined log value) were replaced with a zero for ease of presentation.

lectin-like (comp47544, comp46353, comp49674) and Saposin-like (comp58868) genes, for which Welch ANOVAs were used due to unequal variances between treatments. Unplanned post hoc comparisons (Tukey's test) in genes with significant ANOVA results ($P < 0.05$) compared all possible pairs of treatment means.

RESULTS

Characterization of *Vibrio* cultures' chitinolytic ability and association with *E. affinis*

Metabolic characterization of *V.* sp. *F10 9ZB36* and *V. ordalii 12B09* using colloidal chitin plates suggested that *V.* sp. *F10* does not secrete exogeneous chitinase under the conditions examined (Fig. S4, Supporting Information), in accordance with previous findings that *V.* sp. *F10* does not metabolize chitin (Preheim 2010). Conversely, *V. ordalii* does appear to secrete chitinase (Fig. S4, Supporting Information), although the molecular basis for this physiological difference between the two *Vibrio* species is not clear. We also observed that unlike the copepod-associated *V. ordalii* colonies, the copepod-associated *V.* sp. *F10* colonies were yellow on TCBS media, suggesting sucrose metabolism.

Exposure to *V.* sp. *F10* did not cause *E. affinis* mortality at any of the inoculation densities tested in this study (1–7 × 10^7 CFU mL^{-1}) (Table S2, Supporting Information). In some initial experiments, exposure to *V. ordalii* caused low levels of *E. affinis* mortality (5%–10%) at inoculation densities of 7 × 10^6–7 × 10^7 CFU mL^{-1}. However, no mortalities were observed during any of the transcriptome and qPCR expression studies (Table S2, Supporting Information; densities of 2 × 10^7 CFU mL^{-1}). We quantified the abundance of bacteria associated with live *E. affinis* in comparison with that of ambient seawater through direct counts (DAPI staining) and plate counts (*Vibrio*-selective TCBS and SWC agar) of whole, homogenized copepods. The direct and plate counts of the copepods from the control treatments (antibiotic-treated, uninoculated) were consistently below statistical limits of detection (≤1 cell/field and <30 CFU/plate, respectively) (Table 1; Fig. 2C; Fig. S2, Supporting Information). The direct counts and plate counts on *Vibrio*-selective TCBS media were highly consistent for both *V. ordalii* and *V.* sp. *F10* treatments (Fig. 2).

The direct and plate counts on SWC of *V. ordalii*-exposed copepods were also highly consistent with one another (Figs 1 and 2; Table 1); conversely, there was great discrepancy

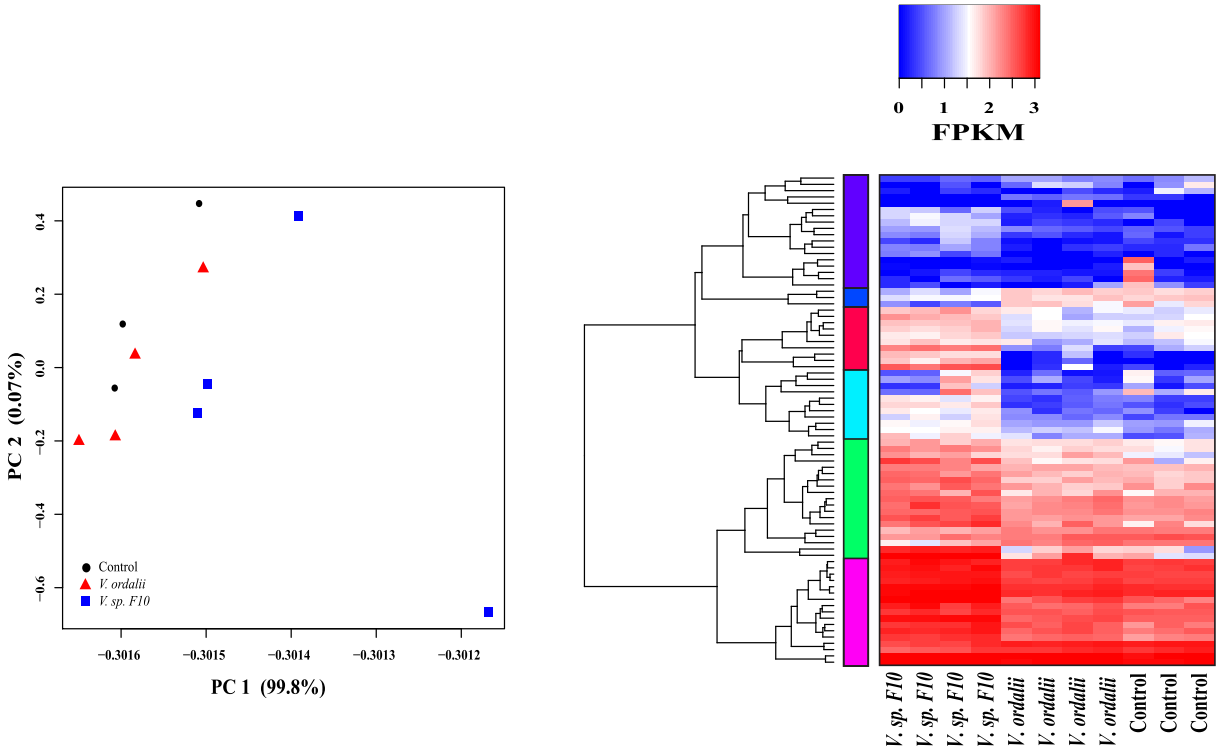


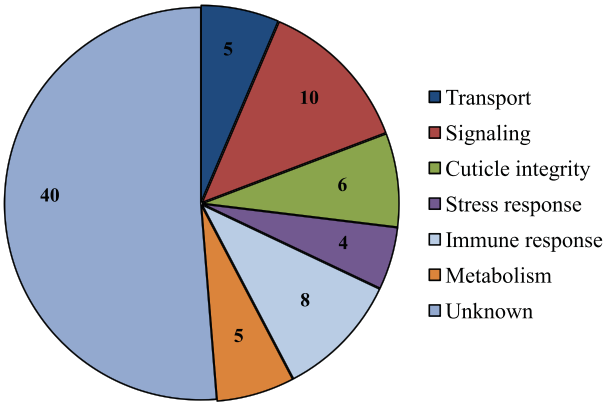
Figure 3. *Vibrio* species elicit distinct transcriptional profiles in *E. affinis*. (Left) PCA demonstrates strong distinction between the *V.* sp. *F10*-exposed and control treatments, with little distinction between the *V. ordalii*-exposed and control treatments. (Right) A heat map representing the base-2 log-transformed FPKM expression values of the 78 differentially expressed genes (fold change > 2, FDR > 0.05) across the three *Vibrio* exposure treatments demonstrates a similar trend. Colors to the left of the heat map represent clades of transcripts with similar expression patterns across biological replicates. Horizontal groupings indicate hierarchical clustering of biological replicates by transcript expression patterns.

(10^6-fold difference) between the direct and SWC plate counts for *V.* sp. *F10*-exposed copepods across all inoculation titers tested (Figs 1 and 2; Table 1). The culturability of copepod-associated *V.* sp. *F10* on SWC agar was consistently below detectable levels (<30 CFU/plate), while direct counts remained high. We observed that the *V.* sp. *F10* free-living in the ambient seawater of the incubation flasks did not have reduced culturability on SWC agar, suggesting that the change in the *V.* sp. *F10* culturability is specific to association with copepods (Fig. 2A). The culturability of *V.* sp. *F10* appears to rapidly decrease upon association with copepods, as there was a 300-fold decrease in culturability on SWC agar between 6 and 24 h of *E. affinis* inoculation (Fig. S5, Supporting Information). Interestingly, copepod-associated *V.* sp. *F10* demonstrated highly consistent direct and plate counts when the samples were cultured on TCBS agar (Fig. 2A), suggesting that the reduced culturability of copepod-associated *V.* sp. *F10* is media-specific.

RNA-Seq differential expression analysis

We used RNA-Seq to identify changes in gene expression in *E. affinis* following exposure to either *V.* sp. *F10* or *V. ordalii*. Overall, relative to the control treatments, the global gene expression pattern of the *V.* sp. *F10*-exposed treatment was the most distinct (Fig. 3). The global expression pattern of the *V. ordalii*-exposed treatment was similar to that of the control treatment (Fig. 3). A total of 78 genes were differentially expressed with a fold change >2 and an FDR > 0.05 in pair-wise comparisons of the three treatments (Table S3–S5, Supporting Information). The differentially expressed genes were annotated through blastx-based searches of the NCBI nr database, and putative functions were inferred based on associated GO terms. Among the differentially expressed genes, 38 could be annotated and were associated with diverse predicted functions, including cell signaling, immune function, maintenance of cuticle integrity, cellular transport, metabolism and stress responses (Fig. 4). Many of these functions, notably maintenance of cuticle integrity, immune response, and stress response, are specifically associated with invertebrate host responses to microbes.

The majority of the 78 differentially expressed transcripts originated from the *V.* sp. *F10*-exposed treatment (61 genes; 47 up-regulated, 14 down-regulated). The genes up-regulated by *V.* sp. *F10* exposure are primarily involved in stress response, cuticle integrity (chitin metabolism, chitin binding) and the innate immune response (C-type lectins, saposin-like) (Fig. 4; Table S3, Supporting Information). *V.* sp. *F10* exposure also induced mild up-regulation of several cell transport and cell signaling genes, as well as mild down-regulation of several cell signaling, metabolism, stress response and immune elements. Exposure to *V. ordalii* induced few transcriptional changes in *E. affinis*, with strong down-regulation (6–8-fold change compared to control) of two transcripts of unknown function which were also down-regulated by *V.* sp. *F10* exposure, and mild down-regulation of a knottin-like inhibitory protein unique to the *V. ordalii* exposure treatment (Table S4, Supporting Information). A total of 53 genes were differentially expressed between the *V.* sp. *F10*- and *V. ordalii*-exposed treatments, 16 of which were unique to this comparison (Table S5, Supporting Information) and were primarily up-regulated in the *V.* sp. *F10*-exposed treatment. The majority of these genes were of unknown function, with a few involved in cell signaling and maintenance of cuticle integrity (Table S5, Supporting Information). Interestingly, two genes were similarly



Function	Transcript ID	log_2FC (*V. sp. F10*)	log_2FC (*V. ordalii*)
RESPONSE TO STRESS			
Injury response	comp32809_c1	1.59	-
Detoxification	comp55690_c0	-1.50	-
Detoxification	comp46208_c1	-1.04	-
Inhibitory protein	comp44575_c0	-	-2.81
CUTICLE INTEGRITY			
Chitin metabolism	comp55805_c0	1.28	-
Chitin-binding	comp35157_c0	1.88	-
Chitin-binding	**comp43891_c0**	2.10	-
Chitin-binding	**comp47090_c0**	2.03	-
IMMUNE SYSTEM PROCESSES			
C-type lectin-like	comp43463_c0	-1.16	-
C-type lectin-like	comp50187_c1	-1.80	-
Saposin-like	**comp58868_c1**	4.52	-
C-type lectin-like	comp46353_c0	8.21	-
C-type lectin-like	**comp46353_c1**	7.64	-
C-type lectin-like	**comp49674_c0**	6.93	-
C-type lectin-like	**comp47544_c0**	4.61	-
C-type lectin-like	comp40027_c0	5.99	-
UNKNOWN			
Unknown	comp51822_c0	-5.53	-5.90
Unknown	comp40339_c0	-8.35	-7.66

Figure 4. *Vibrio* exposure alters expression of genes putatively involved in invertebrate host response to microbiota. (Left) Functional GO terms associated with the 78 differentially expressed genes identified by Illumina sequencing. The total gene number in each category is indicated on the pie chart. (Right) Highlight of the *E. affinis* genes that were most altered by exposure to *Vibrio* identified by Illumina sequencing. Base-2 log-transformed fold changes (log_2FC) in gene expression for each *Vibrio* exposure condition are relative to the control treatment. Positive and negative log_2FC values reflect genes up-regulated and down-regulated, respectively, compared to the control treatment. Genes highlighted with bolder colors are more intensely altered by *Vibrio* exposure, with red hues indicating up-regulation and blue hues indicating down-regulation. Those genes further profiled by qPCR are in bold.

regulated in direction and magnitude in the *V.* sp. *F10*- and *V. ordalii*-exposed treatments (Fig. 4; Table S5, Supporting Information). These two genes had no significant match to the nr or InterProScan databases, although a BLAT (BLAST-like Alignment Tool) search against the *E. affinis* genome confirmed their origin as *Eurytemora* (99%–100% nucleotide match to *E. affinis* genome; data not shown).

Eurytemora affinis gene expression profiling via qPCR

Eight genes with predicted innate immune function were selected for further qPCR profiling. Six of these genes were differentially expressed within the RNA-Seq study (three C-type lectin-like transcripts, a saposin-like transcript, and two chitin-binding transcripts). The three C-type lectin-like genes selected for further study are predicted to have mannose-binding domains (Hunter *et al.* 2012), and two of them (comp49674, comp46353) are also predicted to have signal peptides, suggesting they may be secreted (Petersen *et al.* 2011). The saposin-like gene is also predicted to have a signal peptide and to be secreted. Finally, the two chitin-binding genes selected are both predicted to have chitin-binding domains (InterPro), which are often found in genes involved in maintaining the integrity of the arthropod cuticle and gut lining to prevent against invasion of microbes and their toxins (Buchon *et al.* 2009; Kuraishi *et al.* 2011).

The RNA-Seq results were strongly supported by the qPCR studies, with consistency in the magnitude and direction of induction of the target genes across all *E. affinis* treatments (Table 1). The three C-type lectin-like and the saposin-like genes were similarly and highly up-regulated across independent *V.* sp. *F10*-exposed samples (Fig. 5), suggesting tight regulation of these innate immune genes in response to *V.* sp. *F10* exposure. The chitin-binding genes were more subtly and variably up-regulated in the *V.* sp. *F10*-exposed treatment (Fig. 5), implying that they may be less tightly regulated than the C-type lectin genes under *V.* sp. *F10* exposure. Two genes that were not differentially expressed in the transcriptome analysis, proPO and catalase, were selected for qPCR profiling in light of their highly conserved roles in the innate immune response. In accordance with the RNA-Seq results, proPO and catalase were not differentially expressed upon *Vibrio* exposure via qPCR (Fig. S6, Supporting Information).

DISCUSSION

In this study, we investigated the potential of an ecologically significant invertebrate host, the estuarine copepod *E. affinis*, to transcriptionally respond to *Vibrio* exposure. We found that distinct *Vibrio* species elicited discriminate and targeted transcriptional responses in the copepod host and that association with *E. affinis* triggered a change in the culturability of *V.* sp. *F10*.

Vibrio elicits distinct transcriptional responses from *E. affinis*

The immune response genes up-regulated by *V.* sp. *F10* association, specifically saposin-like genes and C-type lectins, belong to families that are characteristically involved in symbiont acquisition and maintenance (Bulgheresi *et al.* 2006; Fraune *et al.* 2010; Heath-Heckman *et al.* 2014). Saposins can act as pore-forming AMPs in response to microbial infection in a diversity of invertebrates (Banyai and Patthy 1998; Aguilar *et al.* 2005; Roeder *et al.* 2010), while also functioning as selective host regulators of highly stable and specific microbiome communities of organisms such as the freshwater cnidarian *Hydra* (Franzenburg *et al.* 2013). In turn, mannose-binding C-type lectins can function as pattern recognition proteins to initiate acquisition of bacterial symbionts from the environment (Bulgheresi *et al.* 2006; Kvennefors *et al.* 2008; Bright and Bulgheresi 2010). Additionally, C-type lectins internally inhibit the proliferation of endogenous

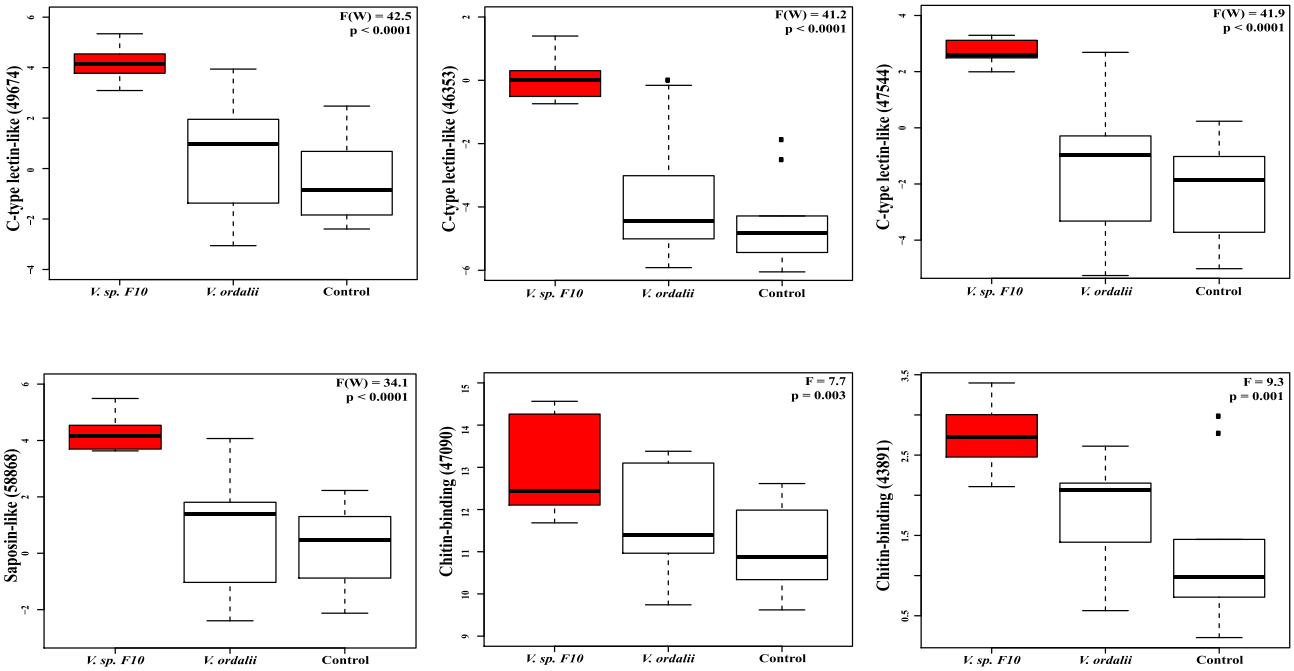


Figure 5. qPCR validation of RNA-Seq gene targets up-regulated upon *V.* sp. *F10* exposure. Gene expression was measured in pooled samples of adult female *E. affinis* (10–20 individuals per sample), and results from three independent *Vibrio* exposure experiments were combined for a total of $n = 10, 9, 8$ biological replicates in the control, *V. ordalii* and *V.* sp. *F10* treatments, respectively. Expression values were normalized to housekeeping genes and base-2 log-transformed. The F-statistics ('F(W)') and *P*-values from Welch ANOVAs are listed for each profiled gene with unequal variances between treatments. Tukey's post hoc comparisons demonstrated that the *V.* sp. *F10* treatment, labeled and indicated in red, was significantly different from the *V. ordalii* and control treatments in each of the genes profiled here.

bacteria by modulating the expression of AMPs (Wang *et al.* 2014) or directly binding to bacteria and acting as antimicrobial agents (Cash *et al.* 2006). Components of highly conserved and systemic innate immune pathways such as the Toll and IMD signaling pathways and the proPO cascade (Franzenburg *et al.* 2013; Binggeli *et al.* 2014; Valenzuela-Munoz and Gallardo-Escarate 2014) were not up-regulated by *V.* sp. *F10* exposure, highlighting the targeted nature of the immune response elicited by *V.* sp. *F10*.

The mild up-regulation of genes with chitin-binding properties upon *V.* sp. *F10* exposure may reflect the renewal of the peritrophic membrane to restrict the bacteria from invading the host through the gut (Buchon, Broderick and Lemaitre 2013). A potentially vulnerable point of entry into the host, the gut is lined with the chitinous peritrophic matrix, which acts like a sieve that surrounds and prevents bacteria, bacterial toxins and hard food fragments from contacting the intestinal epithelium (Lehane 1997). When the thickness and permeability of the peritrophic matrix is compromised in *Drosophila*, there is higher susceptibility to infection by pathogenic bacteria or mortality from bacterial toxins (Kuraishi *et al.* 2011). Furthermore, ingestion of bacteria elicits a stronger immune response in *Drosophila* with a compromised peritrophic matrix, demonstrating the important role that this barrier defense contributes to host immunity (Kuraishi *et al.* 2011). The renewal of the host's chitinous surfaces under an immune response may in turn have significant effects on the physiology of the colonizing vibrios, in light of the dramatic impacts of chitin association on *Vibrio* genetic programs (Kirn, Jude and Taylor 2005; Meibom *et al.* 2005). Further transcriptomic studies could explore whether other naturally associating, chitinolytic vibrios (e.g., *V. cholerae*) trigger stronger up-regulation of chitin-renewal genes in *E. affinis* than do non-chitinolytic zooplankton specialists like *V.* sp. *F10*.

Exposure to *V. ordalii* induced a limited transcriptomic response in *E. affinis*, despite our observations that *V. ordalii 12B09* can digest chitin and abundantly colonize *E. affinis*. One mildly down-regulated transcript was identified as a knottin-like inhibitory protein, which is commonly involved in the stress and antimicrobial responses of invertebrates (Zhang *et al.* 2014). Two of the genes that were strongly down-regulated by *V. ordalii* exposure were similarly down-regulated in the *V.* sp. *F10* treatment, suggesting that these unknown transcripts may be candidate markers of *Vibrio* exposure in *E. affinis* (Table S4, Supporting Information). Characterization of the function of these two genes and examination of their expression patterns upon copepod exposure to other *Vibrio* species warrant further study. Further examination of the localization of *V.* sp. *F10* and *V. ordalii* on *E. affinis* via FISH or gfp-labeling could provide important context for the observed differences in the *E. affinis* transcriptomic response to these species, particularly if they are differentially distributed on the internal versus external 'hot spots' of the copepod (i.e., chitin-lining of the gut and anus versus mouthparts and carapace) (Sochard *et al.* 1979; Huq *et al.* 1983).

Association with copepods alters culturability of a natural zooplankton specialist

A zooplankton specialist that does not degrade chitin, *V.* sp. *F10* heavily colonizes *E. affinis*. Attachment to *E. affinis* alters the metabolism of *V.* sp. *F10* by quickly and dramatically reducing its culturability on SWC agar to below detection. This phenomenon is not observed in the free-living *V.* sp. *F10* collected from the ambient seawater, suggesting that this process is specific to close association with copepods and is not likely caused by a broadly secreted factor. The association of bacteria that are non-culturable on standard media but are detectable by immunological or PCR-based methods (i.e., viable but non-culturable, VBNC) with copepods and other zooplankton has been frequently observed in environmental samples (Huq *et al.*

1983; Signoretto *et al*. 2005; Thomas *et al*. 2006). The VBNC phenomenon is thought to enhance bacterial survival during unfavorable environmental conditions, including dramatic shifts in salinity and temperature (Colwell 2009).

Many previous studies describe VBNC vibrios as non-culturable on TCBS agar (Chowdhury *et al*. 1997; Signoretto *et al*. 2005; Halpern *et al*. 2007), a highly selective medium often used for isolation and enumeration of vibrios. In contrast, we found that the *V.* sp. *F10* associated with copepods are culturable on TCBS agar but non-culturable on SWC agar. Further study is needed to identify which components unique to TCBS media, potentially sucrose and bile salts, lead to the observed differences in the culturability of copepod-associated *V.* sp. *F10* on SWC and TCBS agar plates. Even upon entering the VBNC state, *Vibrio* species can be highly sensitive to bile salts (Su, Jane and Wong 2013), which are known to affect the physiology of many bacteria (Begley, Gahan and Hill 2005) and can serve as stimuli for biofilm formation, increased motility and activation of virulence genes in *Vibrio* (Hung *et al*. 2006; Gotoh *et al*. 2010; Hay and Zhu 2015). In light of *V.* sp. *F10*'s strong association with living zooplankton in the natural environment (Preheim *et al*. 2011), future work should also investigate whether physiological changes associated with altered culturability of copepod-associated *V.* sp. *F10* confer a fitness advantage to *V.* sp. *F10*.

To conclude, our study demonstrates that the estuarine copepod *E. affinis* dynamically and discriminately interacts with *Vibrio* species. Specifically, we have shown that *E. affinis* can distinctly respond to *Vibrio* through targeted up-regulation of immune elements that may be involved in the recognition and maintenance of symbiotic *Vibrio* associates. The effect of *E. affinis* association on *V.* sp. *F10* culturability highlights our limited understanding of the impacts of copepod association on vibrios. We propose that continued study of the dynamics of copepod-*Vibrio* interactions may reveal that copepod physiology is a significant influence on *Vibrio* activity and abundance in the natural environment.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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