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Invasion history shapes host transcriptomic response to a body-snatching parasite

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Abstract

By shuffling biogeographical distributions, biological invasions can both disrupt long-standing associations between hosts and parasites and establish new ones. This creates natural experiments with which to study the ecology and evolution of host–parasite interactions. In estuaries of the Gulf of Mexico, the white-fingered mud crab (*Rhithropanopeus harrisii*) is infected by a native parasitic barnacle, *Loxothylacus panopaei* (Rhizocephala), which manipulates host physiology and behaviour. In the 1960s, *L. panopaei* was introduced to the Chesapeake Bay and has since expanded along the southeastern Atlantic coast, while host populations in the northeast have so far been spared. We use this system to test the host's transcriptomic response to parasitic infection and investigate how this response varies with the parasite's invasion history, comparing populations representing (i) long-term sympatry between host and parasite, (ii) new associations where the parasite has invaded during the last 60 years and (iii) naïve hosts without prior exposure. A comparison of parasitized and control crabs revealed a core response, with widespread downregulation of transcripts involved in immunity and moulting. The transcriptional response differed between hosts from the parasite's native range and where it is absent, consistent with previous observations of increased susceptibility in populations lacking exposure to the parasite. Crabs from the parasite's introduced range, where prevalence is highest, displayed the most dissimilar response, possibly reflecting immune priming. These results provide molecular evidence for parasitic manipulation of host phenotype and the role of gene regulation in mediating host–parasite interactions.

KEYWORDS
biological invasions, gene expression, host adaptation, immune priming, host-parasite interactions, parasitic manipulation, Rhizocephala

1 | INTRODUCTION

The geographical redistribution of biodiversity is one of the hallmarks of the current biological upheaval during the Anthropocene (Chen et al., 2011; Pecl et al., 2017; Pereira et al., 2010). Climate change, species introductions and changes in land use have all contributed to the ongoing shuffling of species and genes on the landscape (Chen et al., 2011; Guo et al., 2018; Vitousek et al., 1996). In the process, this reorganization has disrupted long-standing biotic interactions while simultaneously bringing new species into contact

(Gilman et al., 2010; Schweiger et al., 2008). Understanding how individual species and species assemblages will respond to these changes in biogeography is a central goal for contemporary studies of ecology, evolution and conservation (Bonebrake et al., 2018; Parmesan, 2006). By investigating the consequences of range shifts and invasions, scientists can use them as natural experiments for testing fundamental concepts of the ecology and evolution of species interactions, as well as for predicting potential responses under sustained environmental change (Moran & Alexander, 2014; Saul & Jeschke, 2015; Strauss et al., 2006).

One class of species interactions often altered by biogeographical shifts are those between hosts and their parasites (Brooks & Hoberg, 2007). In these intimate associations, the intense pressures exerted on either partner form an antagonism that can shape the evolutionary trajectory of both species. One particularly remarkable example is parasitic manipulation, where parasites exert control over their hosts by causing shifts in host physiology and behaviour that improve their own reproduction and/or transmission (Moore, 2002). For example, infections by the trematode *Euhaplorchis californiensis* cause its killifish intermediate hosts to exhibit erratic swimming behaviours, making them more prone to predation by the parasite's bird definitive hosts and thus promoting completion of the parasite's life cycle (Lafferty & Morris, 1996). These behavioural changes have been linked to the targeted modification of host neurochemistry, with an infection intensity-dependent alteration of serotonin activity that is distinct from a more generalized stress response (Shaw et al., 2009; Shaw & Øverli, 2012). While we often lack such detailed information on its underlying mechanisms, behavioural manipulation of hosts is not an isolated phenomenon, and may be relatively common, having been observed across many taxa of hosts and parasites (Andersen et al., 2009; Berdoy et al., 2000; Bethel & Holmes, 1977; Carney, 1969; Eberhard, 2000; Poulin & Latham, 2002; Thomas et al., 2002; reviewed in Lafferty & Shaw, 2013; Poulin, 2010; Thomas et al., 2005). Furthermore, examples of parasitic manipulation can extend beyond these conspicuous behavioural syndromes and include modification of a variety of host traits including immune function, reproduction, morphology, metabolism and development (Cézilly et al., 2013; Cornet et al., 2009; Thomas et al., 2010; Vance, 1996; Yanoviak et al., 2008). The taxonomic and functional diversity of parasitic manipulation of hosts highlights the broad range and success of host "body-snatching" as an evolutionary strategy. This phenomenon represents a particularly striking outcome of host–parasite coevolution—an extreme extended phenotype (Dawkins, 1982). However, determining whether these changes in host phenotype truly represent an adaptive strategy of the parasite, rather than a side effect of infection, is challenging and demands the integration of multiple lines of inquiry to link the causal molecular mechanisms to phenotypes and eventual ecological consequences (Hughes, 2013; Thomas et al., 2005).

Given the potential for dramatic fitness costs of infection, which can include castration or death, body-snatching parasites constitute an especially potent source of selection in the evolution of their hosts. The negative consequences of parasitic infection can shape host responses across a range of temporal scales. On deep evolutionary timescales, pressure from parasites has resulted in hosts evolving complex behavioural avoidance strategies and elaborate immune systems (Buck et al., 2018; Sheldon & Verhulst, 1996). On a more constrained microevolutionary timescale, adaptation to parasitism can result in host populations that have resistance to local parasite species/genotypes, while potentially exhibiting elevated susceptibility to unfamiliar parasites to which they lack historical exposure (Eizaguirre et al., 2012a; Roth et al., 2012). In the face of strong selection by parasites, resistance can evolve remarkably rapidly, even in just a handful of generations, provided sufficient standing genetic variation is available (Duffy & Sivars-Becker, 2007; Eizaguirre et al., 2012b; Wendling & Wegner, 2015). On shorter timescales, within the lifetime of an individual, host responses to infection are often contingent on *contemporary* exposure, with immune systems providing increased resistance on subsequent contact with a pathogen. Immunological memory and other forms of adaptive phenotypic plasticity, such as learned antiparasite behaviours, play a critical role in host fitness (Klemme & Karvonen, 2016; Schmid-Hempel, 2009). These plastic responses themselves are often influenced by other factors in the environment (Lazzaro & Little, 2009; Martin et al., 2021), adding layers of complexity that make their study in natural settings challenging.

The application of molecular methods has greatly facilitated the study of host responses to parasitism in nonmodel systems (Criscione et al., 2005). Transcriptome sequencing is of particular utility, as these responses, whether heritable or plastic, are often accompanied by changes in gene expression. These changes have been implicated in mediating a range of host–parasite interactions, including local adaptation between host and parasite (Barribeau et al., 2014; Feis et al., 2018; Lenz et al., 2013), rapid evolution of host resistance (Bonneaud et al., 2011; Grogan et al., 2018; Lohman et al., 2017) and parasitic manipulation of host phenotypes, including behaviour and immune function (Bankers et al., 2017; Feldmeyer et al., 2016; Geffre et al., 2017). In the context of the anthropogenic shuffling of biodiversity, transcriptomic approaches have the potential to provide unique insights into the molecular mechanisms mediating host–parasite interactions.

Biological invasions offer such an opportunity to investigate novel responses to parasitism in natural populations, as they often result in the disruption of existing host–parasite relationships and/or the establishment of new ones (Dunn, 2009; Goedknegt et al., 2016; Prenter et al., 2004). The loss of parasites is frequently observed in species introductions and has been implicated as a factor promoting the establishment and spread of introduced species (the enemy/parasite release hypothesis; Keane & Crawley, 2002; Torchin et al., 2003). In their new range, non-natives can acquire unfamiliar parasites (Blakeslee et al., 2020; Criscione & Font, 2001; Gérard & Le Lannic, 2003; Torchin et al., 1996; Wells et al., 2015; reviewed in Kelly et al., 2009); similarly, introduced species may bring with them exotic parasites that can then infect naïve hosts in the new range (parasite spillover; Daszak et al., 2000; Elsner et al., 2011; Font & Tate, 1994; Miller et al., 2018; Prenter et al., 2004).

The establishment of new associations through invasion can result in the rapid evolution of both hosts and parasites (Feis et al., 2016; Wendling & Wegner, 2015). Previous studies have focused mostly on parasites infecting completely novel hosts, where each partner is encountering the other for the first time. However, when a host has a broader geographical range than its parasite, a situation can arise in which a parasite may be introduced into naïve populations of its host's range. By spilling over into a new population of a species to which it has already adapted, the parasite may be primed for success in the newly expanded range (Bushek & Allen, 1996; Tepolt, Darling, et al., 2020). Hosts within these populations, on the other hand, lack previous exposure to the parasite and are likely to be at an evolutionary disadvantage. This asymmetry in evolutionary history provides a natural experiment with which to examine host responses to novel parasites.

Loxothylacus panopaei (Rhizocephala) is a parasitic barnacle native to the Gulf of Mexico that infects mud crabs of the family Panopeidae (Boschma, 1955). It is a cryptic species complex, with lineages differing in patterns of host use (Kruse & Hare, 2007; Kruse et al., 2012). The ER clade (hereafter simply *L. panopaei*) specifically infects the host species *Eurypanopeus depressus* and *Rhithropanopeus harrisii*, both of which have broad geographical ranges in North America, extending from the Gulf of Mexico to New England (Williams, 1984). *L. panopaei* was introduced to the Chesapeake Bay in the mid-20th century, first observed in 1964 (Van Engel et al., 1966). Since its initial introduction, the parasite has continually spread south until meeting the putative northern edge of its native range at Cape Canaveral (Hines et al., 1997; Kruse et al., 2012). In contrast, *L. panopaei* has not yet expanded into host populations north of Chesapeake Bay, with the exception of one isolated introduction in Long Island Sound (Freeman et al., 2013). The host crabs thus inhabit three distinct zones in relation to their parasite: where the parasite is native, where the parasite is introduced and where the parasite is absent (Figure 1). Populations across these three regions differ in their history of exposure to the parasite, ranging from millennia to decades (<60 years) to none at all, providing an opportunity to investigate the host response to parasitism across temporal scales.

Infection by *L. panopaei* probably represents a strong selective force in the evolution of its crab hosts. Rhizocephalans are large, conspicuous parasites that exert dramatic fitness effects on their hosts (Høeg, 1995). After colonization by the infective larval stage, rhizocephalans grow an extensive internal "root" system throughout the host; at later stages of the infection, the parasite's reproductive organ (externa) emerges through the abdominal wall, mimicking the female crab's egg mass (Høeg, 1995). Rhizocephalan infections represent compelling cases of adaptive parasite manipulation, as they often result in the castration of both sexes, expression of female traits in males (feminization), disruption of the moult cycle and alteration of feeding and habitat choice (Belgrad & Griffen, 2015; Høeg, 1995; Mouritsen & Jensen, 2006; O'Shaughnessy et al., 2014; Takahashi et al., 1997; Toscano et al., 2014; Waser et al., 2016). In addition to the utility of *L. panopaei's* introduction history for studying how the host response to infection varies with its history of

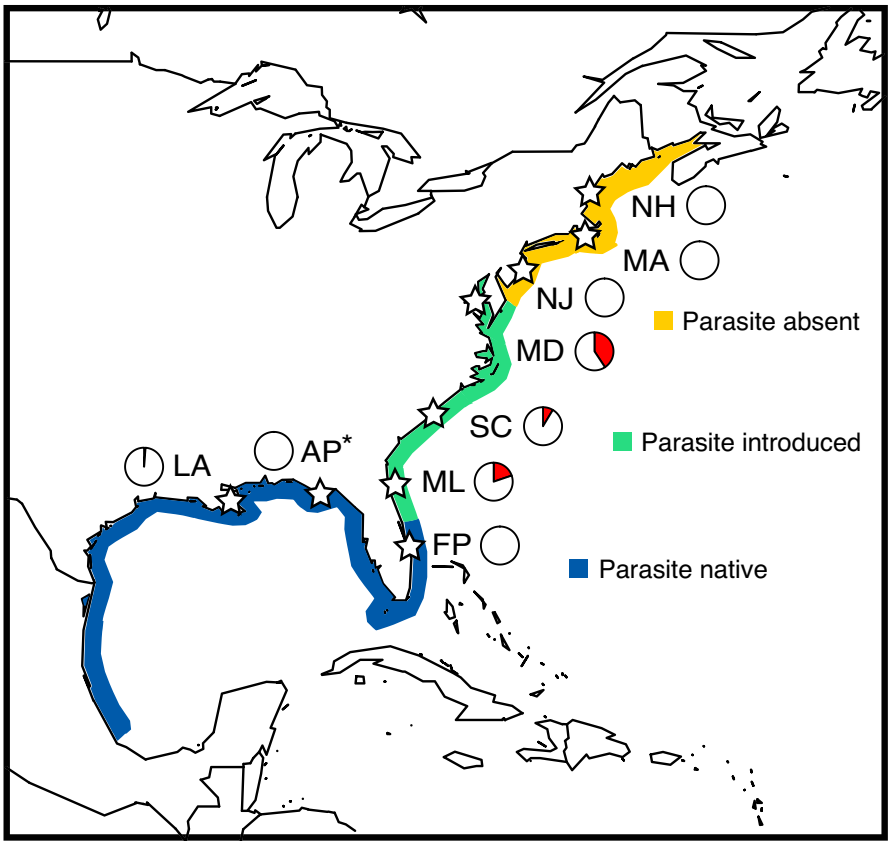

FIGURE 1 Map of *Loxothylacus panopaei* invasion history and survey data from Tepolt, Darling, et al. (2020). Pie charts represent parasite prevalence in *Rhithropanopeus harrisii* at each site in summer 2015. *At AP, no parasitized *R. harrisii* were found, but the presence of ER clade *L. panopaei* was confirmed by infections in co-occurring *Eurypanopeus depressus* hosts [Colour figure can be viewed at wileyonlinelibrary.com]

parasitism, this system is ideal for investigating the molecular basis of parasitic manipulation of host phenotype.

Here we test the transcriptomic response to *L. panopaei* infection of *R. harrisii* hosts from populations with different histories of exposure to the parasite. We previously conducted experimental infections in this system, and found differences in susceptibility among the geographical source populations (Tepolt, Darling, et al., 2020). Naïve crabs from sites with no history of parasitism were more susceptible to infection than those from sites where the parasite is native, providing evidence of the potential for host adaptation to parasitism in the native range (Tepolt, Darling, et al., 2020). Surprisingly, there was no difference in susceptibility between host crabs from regions where the parasite is introduced and where it is native. However, because we used adult crabs from natural populations, it is unclear whether this similarity in susceptibility reflects rapid adaptation of host resistance in the introduced range or a plastic response arising from previous exposure to the parasite. In this study, we take advantage of tissue samples collected during these susceptibility experiments to explore differences in the transcriptomic response to parasitism by these hosts across the species' range. Using mRNA sequencing of these samples, here we compare infected and uninfected hosts to investigate the molecular basis of potential parasitic manipulation. We further compare host populations from regions where the parasite is native, introduced and absent to investigate whether and how differences in gene expression may correspond with the observed differences in susceptibility. We hypothesize that rhizocephalan infection is accompanied by widespread, coordinated changes in host gene expression corresponding to putatively manipulated host phenotypes. Furthermore, we hypothesize that the transcriptomic responses exhibited within crab populations will reflect the observed differences in susceptibility and vary according to their history of exposure to the parasite. Such patterns could be reflective of host adaptation to parasitism, though we acknowledge that this possibility cannot be disentangled from plastic responses driven by other ecological factors. By examining a host's transcriptomic response to rhizocephalan infection, and how this may vary depending on a host population's history of exposure, this work contributes to a growing understanding of the molecular mechanisms of parasitic manipulation and host–parasite interactions more broadly.

2 | MATERIALS AND METHODS

2.1 | Collections and experimental infections

All collections and experiments were carried out during the work described in Tepolt, Darling, et al. (2020). Briefly, uninfected *Rhithropanopeus harrisii* adults were collected from nine estuaries along the Gulf and Atlantic coasts, three from each of three regions differing in their degree of historical exposure to *L. panopaei*: the parasite's native range (Terrebonne Bay, LA [LA]; Apalachicola, FL [AP]; St. Lucie River, FL [FP]), the parasite's introduced range (Pellicer Creek, FL [ML]; Ashley River, SC [SC]; Chesapeake Bay, MD [MD]) and outside of the parasite's current range (Mullica River, NJ [NJ]; Moonakis River, MA [MA]; Squamscott River, NH [NH])] (Figure 1). Crabs were kept individually in 50 ml of 15 PSU artificial seawater at 20°C and a 12-h:12-h light–dark cycle. Control crabs were never exposed to the parasite in the laboratory, while "treatment" crabs were exposed to infective parasite larvae within 24 h of moulting, when they are most susceptible to infection (Alvarez et al., 1995). Naturally infected crabs from MD with mature parasite externae were used as the source of parasite larvae for all experimental exposures. Crabs were maintained in the laboratory through an additional moulting cycle, after which their infection status was determined by visual inspection for parasite externae. Tissue samples from 82 crabs, 51 control and 31 parasitized (Tables 1 and S1), and two "reference" parasite externae from LA and MD were collected and stored in RNAlater at −80°C.

2.2 | RNA extraction, library preparation and sequencing

Sequence data from these specimens were generated previously, as described in Tepolt, Blakeslee, et al. (2020). As the largest and most accessible neural organ, the thoracic ganglion was chosen for sequencing due to its probable functional importance to the parasite's impact on host behaviour. RNA extractions were performed using Trizol with 1-bromo-3-chloropropane, and cDNA libraries were generated using Illumina TruSeq Stranded mRNA Library Prep Kits. Libraries were sequenced across seven lanes of an Illumina HiSeq 2000 in 50-bp single-end reads at the University of Utah High Throughput Genomics Core Facility. Further methodological details, including the results of a population genomic investigation of the host crab, can be found in Tepolt, Blakeslee, et al. (2020).

2.3 | Sequence processing, de novo transcriptome assembly and functional annotation

With the exception of sequence read trimming and assembly of the parasite transcriptome, code for all steps of the bioinformatic pipeline, downstream analyses and plotting is given in a publicly accessible GitHub repository (http://www.github.com/tepoltlab/RhithroLoxo_DE/). Please see the included README.md file for a more detailed description of the steps briefly outlined here. The pipeline was constructed and executed using the workflow engine SNAKEMAKE (Köster & Rahmann, 2012), and all computation was performed on the Poseidon high-performance computing cluster at Woods Hole Oceanographic Institution.

QC and trimming of raw reads were performed with TRIM GALORE! Version 0.4.0 (Krueger, 2015), a Perl wrapper for CUTADAPT (Martin, 2011) and FASTQC (Andrews, 2010). Read ends were trimmed of Illumina adapters and bases with Phred quality scores of less than 20; reads shorter than 20 bp after trimming were discarded. One

TABLE 1 Summary of *Rhithropanopeus harrisii* individuals included in infection experiment

		Control			Parasitized		
Range	Site	Female	Male	Total	Female	Male	Total
Native	LA	3	3	6	0	2	2
	AP[a]	2	4	6	3	1	4
	FP	3	3	6	3	3	6
Introduced	ML	3	3	6	1	1	2
	SC	3	3	6	1	2	3
	MD	1	4	5	1	1	2
Absent	NJ	3	3	6	5	1	6
	MA[b]	0	4	4	0	0	0
	NH	3	3	6	3	3	6
Total		21	30	51	17	14	31

[a]One AP control female removed as expression outlier.

[b]Four MA controls were removed, including three as expression outliers and one due to sequencing failure.

sample, MA_C_3, displayed low quality scores and was removed from subsequent analyses. To identify potential contamination from reads originating from the parasite, sequences were aligned to a *Loxothylacus panopaei* transcriptome that had been previously assembled from sequencing of two parasite externae, one each from the parasite's native (LA) and invasive (MD) ranges. The parasite transcriptome was assembled as per Tepolt, Blakeslee, et al. (2020) and reads from host libraries were screened against it using MAGIC-BLAST (Boratyn et al., 2019); reads aligning across their entire length with ≥98% identity were removed using BBTOOLS (https://sourceforge.net/projects/bbmap).

De novo assembly of the *R. harrisii* transcriptome was performed using TRINITY version 2.8.5 (Grabherr et al., 2011; Haas et al., 2013). To minimize inclusion of any residual parasite-derived reads in the assembly, only cleaned reads from the uninfected individuals were used. A range of parameter values was used for assembly, and the optimal transcriptome was selected based on the N50 and ExN50 quality metrics generated by utility scripts within TRINITY. Only the longest isoform per "gene" was retained and contigs less than 200 bp were discarded. Contigs were then queried against the NCBI nt database with BLASTN using BLAST+ version 2.7.1 (Camacho et al., 2009) to screen for potential contaminant taxa including fungi, bacteria, platyhelminthes and nematoda. Contigs with alignments to contaminant taxa references sequences with e-values of 1^{-10} or lower were removed.

Functional annotation was performed using the software ENTAP version 0.9.0-beta (Hart et al., 2020), which uses DIAMOND version 0.9.9 (Buchfink et al., 2015) to perform similarity searches against user-specified reference sequence databases and retrieve gene family assignments and associated gene ontology (GO) information using the EGGNOG version 4.5 database (Huerta-Cepas et al., 2016). Six-frame translated contigs were queried against the following protein databases: UniProt's Swiss-Prot, TrEMBL and UniRef90, and NBCI's nr and RefSeq (NCBI Resource Coordinators, 2017; UniProt Constortium, 2018). In addition to those identified as contamination in the nucleotide BLAST search, transcripts matching contaminant reference sequences in protein space were removed from the finalized transcriptome prior to analyses.

2.4 | Transcript quantification, differential expression analysis and co-expression network analysis

Transcript quantification was performed using the pseudo-aligner SALMON version 0.14.2 (Patro et al., 2017) from within TRINITY. Differential expression analysis was performed in R version 3.6.1 (R Development Core Team, 2017) using the package DESEQ2 version 1.26.0 (Love et al., 2014). Prior to the analysis, initial screening and filtering of latent contaminant transcripts from *L. panopaei* and other parasites was carried out and four expression outliers (MA_C_1, MA_C_2, MA_C_4 and AP_C_1) were identified by a principal components analysis and removed, as such samples can impede estimation of dispersion and model fitting. These four outlier samples probably harboured intense field-derived fungal infections, as transcripts driving this outlier pattern were homologous to sequences from fungal genera (*Microsporidium*, *Hepatospora*, *Enterospora*, etc.). We also performed expression-based filtering, removing transcripts with normalized counts of less than 10 in over 90% of samples. After these steps, we conducted three separate sets of analyses to test the host's general response to infection, and how this response differed depending on host sex and history of parasitism (native, introduced and absent).

2.4.1 | General response to infection

First, using the trimmed data set of 77 individuals (original 82 less one failed library and four outliers), we tested for differentially expressed transcripts between parasitized and control crabs,

irrespective of their site of origin. We used a likelihood ratio test to evaluate a generalized linear model of site and infection status as coefficients against a null model with site alone to account for site-specific differences.

2.4.2 | Sex-specific response to infection

Second, using this same data set, we tested for differences in the magnitude of response to infection between males and females as a means of investigating signatures of host feminization. We used Wald tests to identify where expressed transcripts differed significantly in each of the two sexes. Because the sexes differed in their number of control and parasitized crabs, and thus power to resolve regulatory differences, we randomly down-sampled each sex to 21 control and 14 parasitized crabs, their respective minima in either sex. This randomization procedure was repeated across 1000 iterations, and we used a Welch's *t* test to examine the difference in the mean number of differentially expressed genes (upregulated, downregulated and overall) between males and females in their response to infection. We estimated effect sizes using Cohen's *d*.

2.4.3 | Range-specific response to infection

Third, we tested for differences in the response to infection according to range. This analysis used a reduced data set of 58 individuals across six populations, including two populations from each of the three ranges. We excluded FP because although it has historically been considered as part of *L. panopaei's* native range, this was based on a single historical record that (i) pre-dates recognition of the cryptic species complex and (ii) may have occurred in a different panopeid species misidentified as *Eurypanopeus depressus* or *R. harrisii*. Because MD was used as the source of *L. panopaei* larvae in the experimental infections, we excluded this site to avoid potentially confounding signatures of strong, estuary-level adaptation of the parasite to local hosts. In addition, after removal of the aforementioned sample outliers, no crabs remained from MA. We first investigated differences in the magnitude of transcriptional response to infection, using Wald tests to identify transcripts that were differentially expressed *within* each range separately (native, introduced or absent) while controlling for site-specific effects. As in the case of sex-specific differences, each range differs in their number of control and infected crabs. We therefore repeated the same randomization procedure, down-sampling to five control crabs and two infected crabs, their respective minima across the sites, but repeating this across 5000 iterations. Significance testing of differences in the magnitude of response among ranges was performed using an analysis of variance (ANOVA), followed by Tukey's honestly significant difference post-hoc tests for pairwise comparisons. We again used Cohen's *d* to estimate effect size. We then tested for differences *among* ranges in their response to infection by using a likelihood ratio test to evaluate a full model including range, infection status and their interaction as coefficients against a null model of range and infection status alone. Further subcomparisons were made using Wald tests to reveal transcripts displaying a significant range × infection status interaction *between pairs* of ranges. Significance testing for all differential expression analyses was performed with adjusted *p*-values using the Benjamini–Hochberg method (Benjamini & Hochberg, 1995).

2.4.4 | Co-expression analysis

We also used weighted gene co-expression network analysis (WGCNA) to characterize transcriptome-wide expression patterns and relate them to infection status and host sex (Langfelder & Horvath, 2008). Rather than test each transcript individually, this approach clusters transcripts exhibiting similar expression patterns across all samples, agnostic to experimental conditions. The overall expression signatures (eigengene) of the resulting co-expression modules are then tested for significant correlations with sample characteristics, here infection status and sex.

2.5 | Functional enrichment analysis

To investigate enrichment of functions across the various differential expression and co-expression analyses described above, we used the R package GO_MWU (Wright et al., 2015). This approach uses Mann–Whitney *U* tests to determine which GO terms are significantly overrepresented among the up- or downregulated transcripts, or within WGCNA co-expression modules. We included only GO terms for transcripts mapping to arthropod clusters of orthologous genes (COGs) with e-values of 1^{-50} or less, except when analysing WGCNA modules, for which we used GO terms for transcripts mapping to COGs with e-values of 1^{-10} regardless of taxonomy. Mann–Whitney *U* tests were performed on $-\log_{10}p$-values from the differential expression analyses and on the transcript module membership scores (kMEs) of select WGCNA modules. Significance testing was performed using Benjamini–Hochberg adjusted *p*-values for differential expression results and a permutation-based procedure for inclusion of GO terms in WGCNA modules.

3 | RESULTS

3.1 | Transcriptome assembly and annotation

Sequencing yielded 1.19 Gb of cleaned sequence data from 50 control and 31 parasitized crabs, of which 0.82 Gb from control crabs were used for de novo transcriptome assembly. After the removal of 3179 putative contaminant contigs, the final transcriptome assembly consisted of a total of ~87 Mbp, containing 146,332 contigs with an average length of 577 bp, a total N50 of 774 bp and an ExN50 of 1119 bp for those transcripts which constituted 90% of

the total expression. Of these, 19,217 contigs (13.1%) had an alignment to reference sequences and 24,483 (16.7%) were assigned to gene families, with 21,550 (14.7%) having at least one GO term. After further contaminant screening and expression-based filtering, 60,488 transcripts were retained in the first differential expression data set (general response and sex-specific response) and 59,579 were retained in the second data set, which encompassed six sites (range-specific response).

3.2 | Transcriptional response to infection

The overall response to infection, regardless of range, was investigated by comparing the expression of 46 control and 31 parasitized individuals. A likelihood ratio test identified 852 upregulated and 743 downregulated transcripts ($p_{adj} < .05$) in the parasitized group, with 71 and 65 significant transcripts having absolute $\log_2$-fold changes (LFCs) > 2, respectively (Figure 2). Of these 1595 transcripts, 269 (16.9%) mapped to decapod references (Figure S1). A Mann–Whitney *U* test for overrepresentation of GO terms from high-confidence, arthropod-specific gene family assignments among differentially expressed transcripts indicated enrichment of molecular functions including chitin binding ($p_{adj} = 1.22E^{-5}$), RNA binding ($p_{adj} = 1.13E^{-6}$) and molecular transducer activity ($p_{adj} = 4.90E^{-5}$); biological processes such as cell surface receptor signalling pathway ($p_{adj} = 5.70E^{-5}$), carbohydrate metabolic process ($p_{adj} = 1.28E^{-6}$) and RNA processing ($p_{adj} = 2.88E^{-5}$); and cellular localizations including extracellular region ($p_{adj} = 1.66E^{-11}$), ribonucleoprotein complex ($p_{adj} = 5.39E^{-9}$) and actin cytoskeleton ($p_{adj} = 2.17E^{-5}$; Figure 2a; Table S2).

WGCNA identified 16 co-expression modules, two of which were negatively associated with infection (Figure S2). One infection-associated module ($r = -.41$, $p = 2E^{-4}$, 180 transcripts) was enriched for GO terms associated with nervous system processes, such as regulation of glutamatergic synaptic transmission ($p_{adj} = .033$) and positive regulation of neuron projection development ($p_{adj} = .065$, Table S3). The second ($r = -.34$, $p = .002$, 175 transcripts) was enriched for GO terms associated with cytoskeletal functions, such as cytoskeleton organization ($p_{adj} = 0$), actin filament-based process ($p_{adj} = 0$), and microfilament motor activity ($p_{adj} = 0$) among others.

3.3 | Sex-specific response to infection

Male and female crabs differed in the magnitude of their transcriptional response to *Loxothylacus panopaei* infection. Across 1000 random iterations to account for differences in sample size, females averaged 120 upregulated and 206 downregulated transcripts in response to infection, whereas males exhibited a significantly higher number of differentially expressed transcripts, with an average of 663 upregulated and 537 downregulated (Figure 3). In addition to those modules discussed above, WGCNA recovered

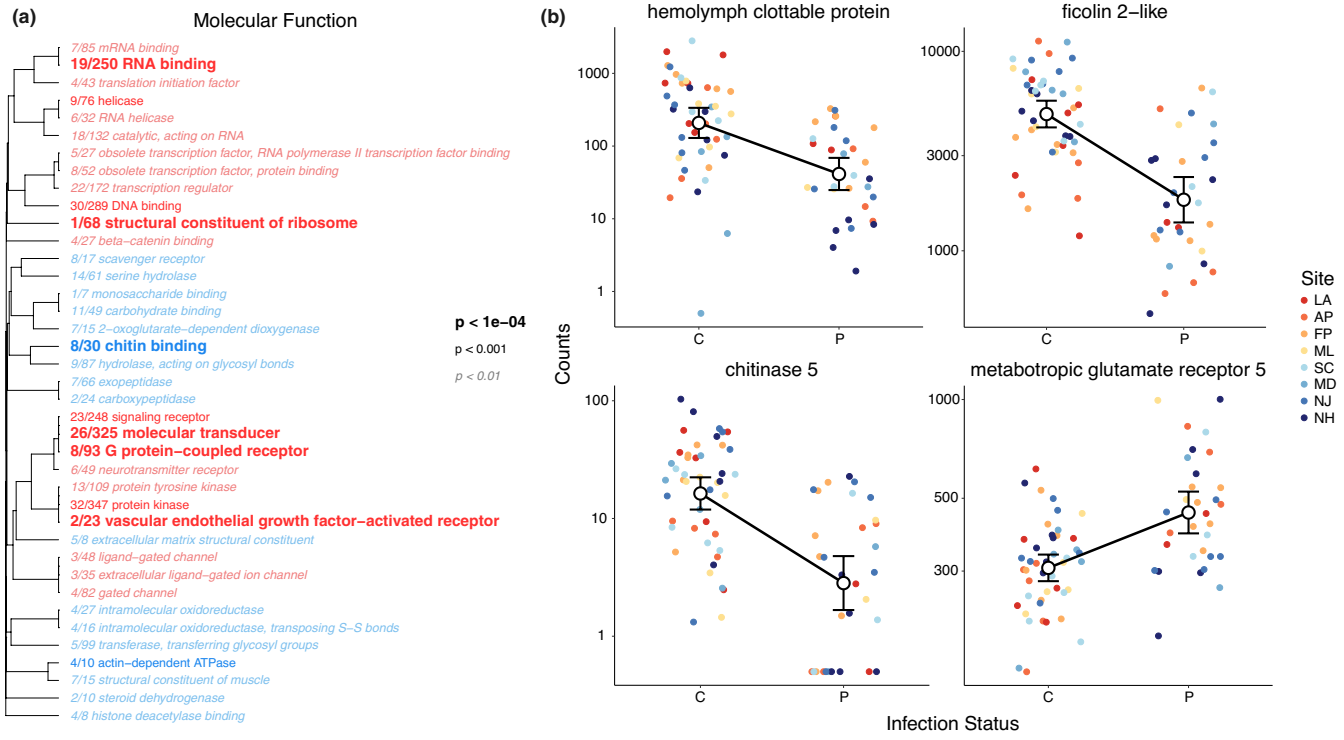

FIGURE 2 (a) Hierarchical clustering of Molecular Function GO terms significantly enriched among upregulated (red) and downregulated (blue) transcripts between infected and control crabs, as determined by a Mann–Whitney *U* test. (b) Curated subset of highly significant transcripts involved in innate immunity, ecdysis and neurotransmission, corresponding to GO term categories shown in (a). Error bars represent 95% confidence intervals. Note $\log_{10}$ *y*-axis scale [Colour figure can be viewed at wileyonlinelibrary.com]

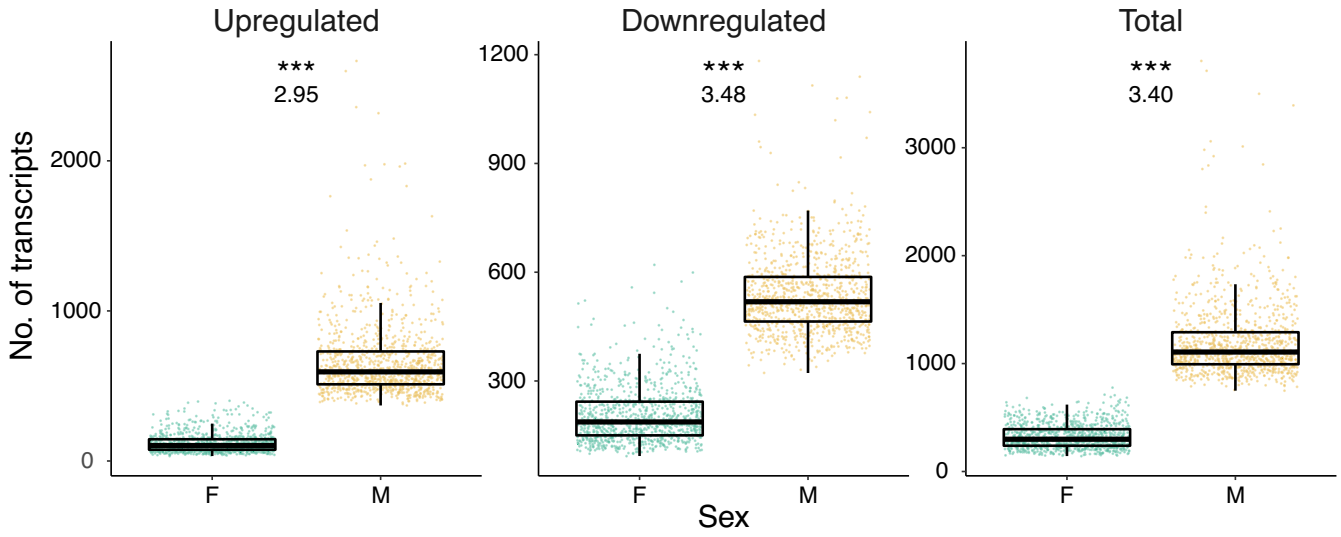

FIGURE 3 Number of differentially expressed transcripts in response to *Loxothylacus panopaei* infection by host sex, across 1000 iterations of random downsampling. ***p << .001, numbers indicate Cohen's d effect size [Colour figure can be viewed at wileyonlinelibrary.com]

an additional module containing 467 transcripts that exhibited associations consistent with feminization of male hosts (Figure S3). This sex-associated module exhibited lower expression in uninfected males relative to uninfected females (r = −.34, p = 2E^{-3}), but was more highly expressed in infected males relative to uninfected males (r = .47, p = 1E^{-5}). GO term enrichment of this module revealed an overrepresentation of a diverse set of functions, but none with obvious links to host feminization. These included GO terms such as response to interleukin-1 (p_{adj} = 0), cell surface receptor signalling pathway (p_{adj} = .0125) and antioxidant activity (p_{adj} = 0; Table S3).

3.4 | Range-specific responses to infection

Crabs from locations where the parasite is native, introduced or absent differed in the number of transcripts differentially expressed according to infection status. Across 5000 iterations, there were an average of 128 upregulated and 173 downregulated transcripts in the native range, 805 and 407 in the introduced range and 1074 and 410 where the parasite is absent (Figure 4a). A likelihood ratio test revealed 4449 transcripts that displayed a significant interaction between range and infection status, of which 547 (12.3%) aligned to decapod reference sequences (Figure S4). Interestingly, most significant interactions involved the introduced range, where transcripts tended to exhibit the opposite response relative to the native range and/or where the parasite is absent (Figure 4b,c). This set of transcripts was enriched for molecular functions including RNA binding (p_{adj} = 3.85E^{-3}), oxidoreductase activity (p_{adj} = .0194) and structural constituent of ribosome (p_{adj} = 1.89E^{-13}); biological processes such as mitotic cell cycle process (p_{adj} = 8.08E^{-5}), ATP synthesis-coupled electron transport (p_{adj} = 3.52E^{-4}) and cellular response to stress (p_{adj} = .03); and cellular localizations including cytosolic ribosome (p_{adj} = 1.27E^{-10}), transcription repressor complex (p_{adj} = .0331) and mitochondrial protein complex (p_{adj} = 2.32E^{-7}) (Figure S5, Table S4).

In the analysis of transcripts displaying a significant range × infection status interaction *between pairs* of ranges, there was considerably less overlap between pairs including the introduced range than between the absent and native range (Figure S6). Functional enrichment of transcripts exhibiting an interaction when comparing the native and introduced ranges revealed an overrepresentation of the GO terms immune response (p_{adj} = .0187), neurotransmitter receptor activity (p_{adj} = 1.63E^{-4}) and chitinase activity (p_{adj} = .0267; Table S5). Despite the greater overlap in the response of transcripts between the native range and absent range, functional enrichment analysis recovered hundreds more divergent GO terms in this comparison than for the introduced-native range pair (899 vs. 106 <0.1 false discovery rate [FDR], Table S6). Similar results were found for the absent–introduced range pair (598 GO terms <0.1 FDR, Table S7), perhaps resulting from the greater and more balanced sample size in the absent range.

4 | DISCUSSION

The redistribution of biodiversity through species invasions has disrupted existing host–parasite relationships and established new ones, creating opportunities to study novel host–parasite interactions in the wild (Goedknegt et al., 2016). Building upon our previous work demonstrating differences in *Rhithropanopeus harrisii's* susceptibility to *Loxothylacus panopaei* across a mosaic of historical parasite exposure (Tepolt, Darling, et al., 2020), here we use transcriptomic analyses to investigate the molecular mechanisms underlying the host response. We demonstrate that rhizocephalan infection has a widespread effect on patterns of host gene regulation, with changes in expression including patterns that may reflect

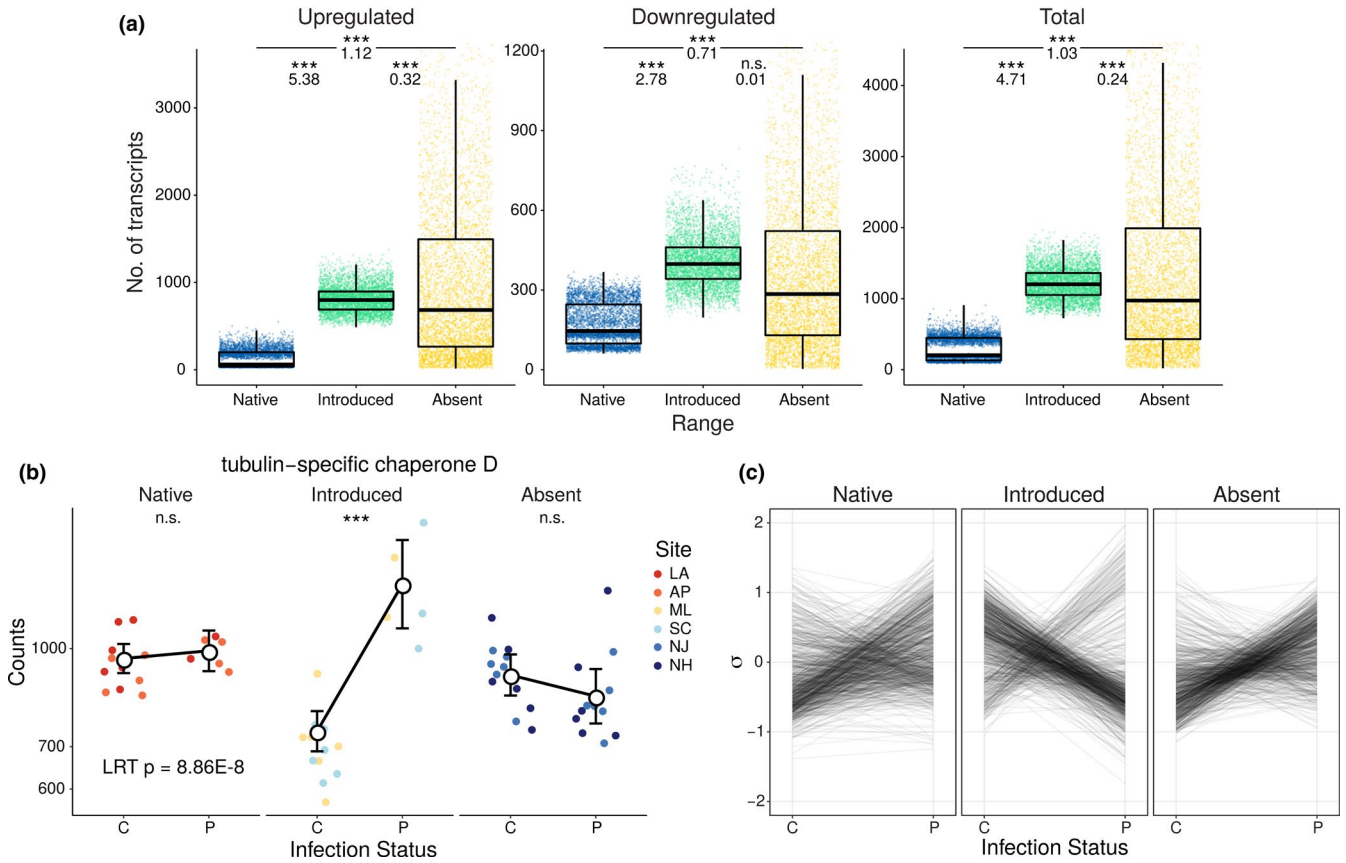

FIGURE 4 (a) Number of differentially expressed transcripts in response to L. panopaei infection according to parasite status in the host range, across 5000 iterations of random downsampling. *** = p << 0.001, numbers indicate Cohen's d effect size. Some datapoints for the absent range not shown due to truncation of y-axis limit for display purposes. (b) Most significant transcript in likelihood ratio test of range:infection status interaction. Error bars represent 95% confidence intervals. Significant within-range t-test result indicated by *** (p < 0.001). (c) Composite response of the 848 transcripts exhibiting a significant range:infection status interaction with p < 0.01. Each line represents a single transcript, with y-axis indicating the mean expression level within each group relative to the overall mean, in units of standard deviation. [Colour figure can be viewed at wileyonlinelibrary.com]

the modification of key biological processes implicated in parasitic manipulation, including host immunity and moulting. Male crabs exhibited a stronger response than females, which may be indicative of sex-specific changes associated with host feminization. On a regional level, hosts differed in their response to infection depending on their degree of historical exposure to the parasite, a pattern consistent with previously observed differences in susceptibility in the laboratory and prevalence in the field. By exploring *R. harrisii's* gene regulatory response to *L. panopaei* infection, we contribute to a growing understanding of the molecular basis of parasitic manipulation and host–parasite interactions more broadly.

4.1 | General response to infection: signatures of parasitic manipulation?

Individuals from all parts of *R. harrisii's* range, regardless of historical exposure to the parasite, displayed a core response to *L. panopaei* infection. Perhaps the most striking component of this pattern is the widespread downregulation of transcripts involved in immunity. Crustaceans, like all invertebrates, canonically lack an acquired (adaptive) immune system, instead relying on nonspecific innate immunity (Cerenius et al., 2010; Iwanaga & Bok, 2005; but see Melillo et al., 2018). Central processes in crustacean innate immunity include pattern recognition, prophenoloxidase (proPO) activation, encapsulation and clotting, among others (Vazquez et al., 2009). Pattern recognition is dominated by proteins that bind carbohydrates and glycoproteins on the cell surface of pathogens, with lectins playing a central role (Drickamer & Taylor, 1993; Marques & Barracco, 2000). In this experiment, infected crabs exhibited a reduction in GO terms associated with carbohydrate binding, with several transcripts encoding for lectins orthologous to haemocytin, ficolin 2 and C-type lectins significantly downregulated during infection (Figure 2). We also found evidence for disruption of the clotting response, with a significant reduction in transcripts for haemolymph clottable protein and haem binding protein 1 (Theopold et al., 2004). Additionally, potential disruption of the proPO activation system, the invertebrate analogue to the complement system (Vazquez et al., 2009), may be reflected in the reduction of the peptidase and oxidoreductase GO categories in parasitized individuals.

The pronounced reduction in immune-associated functions in parasitized individuals is consistent with active immunomodulation

by *L. panopaei*. Evasion of the host's immune response is a common feature of infection across parasitic taxa, either through passive detection avoidance, for example, by infecting minimally surveilled tissues, or actively by directly interfering in immune pathways (Maizels & Yazdanbakhsh, 2003; Sacks & Sher, 2002). While passive immune evasion may occur simultaneously, rhizocephalans, with their conspicuous externae and internally ramifying "root" systems, probably have little recourse for escaping detection and thus would be expected to rely principally upon active mechanisms (Viney & Cable, 2011). To date, there have been no comprehensive studies of a host's immune response to rhizocephalan infection. However, suppression of host immunity has been observed in other parasitic arthropods, including parasitoid wasps and ticks (Schmidt et al., 2001; Wikel, 1999). Additionally, other parasites of crustaceans have been shown to interfere with host immune systems (Cornet et al., 2009; Helluy & Thomas, 2010).

In addition to host immunity, we found several differentially expressed transcripts coding for proteins involved in ecdysis (Figure 2). Transcripts for cryptocyanin, haemocyanin and a chitinase, all of which have been implicated in the crustacean moult cycle (Rocha et al., 2012; Terwilliger, 2012), were significantly downregulated in *L. panopaei*-infected crabs. By contrast, other studies have found elevated levels of haemocyanin in the haemolymph of rhizocephalan-infected crabs (Manwell & Baker, 1963; Shirley et al., 1986). This could be due to differences in expression among tissues, as the source of mRNA for the present study was the thoracic ganglion. Furthermore, haemocyanin and cryptocyanin are part of the larger haemocyanin gene family, of which there are many constituent genes, paralogues and isoforms with diverse functions (Burmester, 2002). In fact, prophenoloxidase, of the aforementioned proPO system, is itself a member of the haemocyanin family. This, along with evidence for the potential involvement of haemocyanin and cryptocyanin in immunity (Lei et al., 2008; Liu et al., 2006), makes it difficult to disentangle the effects of homologous transcripts potentially involved in both immunity and moulting. Nonetheless, the observed response may be reflective of inhibition of moulting by the parasite, in what has been termed parasitic anecdysis (O'Brien & Van Wyk, 1984).

Rhizocephalan infection is intimately associated with the moult cycle (Høeg, 1995). In general, hosts that have recently moulted are susceptible to penetration by cyprid larvae, with emergence of the parasite externa typically occurring after the following moult, beyond which ecdysis is often inhibited (Alvarez et al., 1995; O'Brien & Skinner, 1990). This can be considered a putatively adaptive manipulation, as host ecdysis would divert resources from parasite growth and reproduction, result in greater exposure to predation, and may cause severe physical damage to parasite tissues. While the underlying mechanisms are unclear, parasitic anecdysis probably relies on modulation of the host neuroendocrine system (Høeg, 1995). Moulting inhibition in rhizocephalan-infected crabs has been associated with the regression of the host Y-organ (Chassard-Bouchaud & Hubert, 1976), a neuroendocrine gland which mediates the ecdysis cycle (Chang & Mykles, 2011). Correspondingly, we observed the upregulation of many transcripts involved in neuronal and signalling processes, such as neuroparsins and cell-surface receptors, and the enrichment of molecular function GO categories including neurotransmitter receptor activity and G-protein-coupled receptor activity. Our WGCNA also recovered a differentially expressed co-expression module that was enriched for terms associated with neuronal processes, further highlighting the potential for host neuromodulation by the parasite (Figure S2, Table S3). However, rhizocephalan infection results in the modification of numerous aspects of behaviour beyond moulting (Mouritsen & Jensen, 2006; Toscano et al., 2014), and it is unclear how the changes in expression observed here may influence individual behavioural processes.

Another hallmark of host manipulation by rhizocephalans is the feminization of male hosts, which includes changes in behaviour (Brockerhoff et al., 2010; Høeg, 1995; Kristensen et al., 2012). We found that males exhibited a greater transcriptional response to infection compared to females, which may reflect differences in how *L. panopaei* manipulates its host depending on its sex. Furthermore, we found one WGCNA module that exhibited patterns of expression consistent with male feminization, albeit rather slight (Figure 3). The annotations of its constituent transcripts and the enrichment of GO terms did not appear to be dominated by any one biological function, obscuring its specific role in host feminization. Nonetheless, the coordinated change in expression of this set of genes points towards its involvement in this process, and this analysis reveals key candidates for further investigation.

4.2 | Variation in susceptibility, immune priming and host adaptation

Crabs from populations differing in their historical exposure to *L. panopaei* were expected to differ in their transcriptional response to infection. Specifically, we hypothesized that crabs from the parasite's introduced range would mount a response most similar to that of crabs from the native range, consistent with previous results demonstrating similar susceptibility (Tepolt, Darling, et al., 2020) and possibly reflecting recent parallel adaptation to parasite pressure. In contrast, we expected that crabs from locations without the parasite, which were more prone to infection, would exhibit a contrasting reaction. Contrary to expectations, the vast majority of transcripts that displayed a significant interaction involved range pairs that included the introduced range. In other words, it was crabs from the introduced range of the parasite that displayed the most dissimilar transcriptional response. While we cannot conclusively ascribe this seemingly paradoxical result to a specific mechanism, we provide two probable explanations.

First, this may result from differences in contemporary parasite pressure among the populations. We previously found that naïve *R. harrisii* populations that lacked historical exposure to *L. panopaei* were more prone to infection than those from the parasite's native range, possibly due to evolution of host resistance over millennia of sympatry (Tepolt, Darling, et al., 2020). Interestingly, there was no difference in host susceptibility between the introduced and native

ranges, which at first glance might suggest rapid adaptation to parasitism in just dozens of generations. However, *L. panopaei* prevalence is substantially higher in its introduced range than in its native range (Tepolt, Darling, et al., 2020). As the crabs used in experimental infections were collected as adults from the wild, hosts from different populations also differ in their degree of *contemporary* exposure to the parasite. Crabs from the introduced range, where average prevalence was 25.9%, were much more likely to have already avoided or terminated infection than those from the native range, where prevalence averaged just 1.2% (Tepolt, Darling, et al., 2020). The dramatically higher prevalence in the introduced range probably resulted in the collection of crabs that were biased towards those with increased resistance, as variability in parasite susceptibility is commonly observed within populations, potentially mediated by innate genetic and/or plastic mechanisms (Ebert et al., 1998; Henter & Via, 1995). The idiosyncratic transcriptional response of crabs from the introduced range may thus reflect constitutive differences in gene expression among ranges and/or an elevated immune response potentiated by previous exposure.

Innate immunity, in contrast to the adaptive immune system exclusive to vertebrates, is classically perceived as lacking memory; that is, exposure to a pathogen should not elicit an improved response upon subsequent contact (Zinkernagel et al., 1996). Instead, pattern recognition in innate immunity is more general, relying on germline-encoded receptors that bind broadly to evolutionarily conserved pathogen antigens (Janeway & Medzhitov, 2002). However, a number of studies from the field of ecological immunity have established a potentiating effect of previous exposure within innate immunity, which has been termed trained innate immunity or immune priming (Kurtz, 2005; Moret & Schmid-Hempel, 2001; Moret & Siva-Jothy, 2003; Netea et al., 2016; Rodrigues et al., 2010). Host immune priming in *L. panopaei's* introduced range may help explain the divergent expression profile observed. In the parasite's introduced range, where prevalence is high, the response of many host transcripts to infection was opposite of that observed in both the native range and where the parasite is absent. Many of the most highly significant transcripts displaying a range × infection status interaction are involved in immunity, and mostly increased in abundance in the introduced range while decreasing in the others. Functional enrichment analysis in a comparison of range × infection status interaction transcripts between the native and introduced range revealed overrepresentation of the GO term "immune response," further supporting the potential for immune priming. While immune priming in rhizocephalan host–parasite systems has not been established, it has been observed in crustacean diseases such as vibriosis and white spot syndrome (Chang et al., 2018).

Alternatively, the differences in the expression patterns seen in the introduced range may indeed reflect rapid adaptation to parasitism. Our expectation was rooted in the assumption that putative evolution of host resistance in the parasite's introduced range may render the host's transcriptional response more similar to that seen in the native range. However, a growing body of evidence has indicated that parallel phenotypic evolution may be mediated by *nonparallel* molecular evolutionary trajectories (Bolnick et al., 2018; Elmer et al., 2014; Therkildsen et al., 2019; Wang et al., 2020). Thus, the unexpected host response in the parasite's introduced range may reflect the evolution of a novel reaction to rhizocephalan parasitism that is distinct from that in the native range. Independent evolutionary trajectories are particularly likely in *R. harrisii*; population structure in this species is very high, with each estuary in this study having a distinct population genomic signature (Tepolt, Blakeslee, et al., 2020). This strong structure, as well as the species' larval behaviour (Forward, 2009), suggests very limited larval dispersal among estuaries. Given this, rapid evolution would very likely act on the standing genetic variation present in each individual estuary rather than drawing from the variation present in a larger metapopulation.

Conversely, the heightened reaction in the introduced range may instead reflect a *lack* of evolved host resistance—a transcriptomic uproar indicative of an uncoordinated and overzealous immune response that may be maladaptive and/or be in response to secondary damage by the parasite. Studies of emerging wildlife diseases, such as chytrid fungus in amphibians and white-nose syndrome in bats, have shown that susceptible host lineages often launch more robust transcriptomic responses than their tolerant counterparts, with specific dysregulation of transcripts with immune functions (Davy et al., 2017; Eskew et al., 2018; Grogan et al., 2018; Savage et al., 2020). However, if this were the case in the present study, we would expect to observe a similar response by the crabs from where the parasite is absent. This observation, coupled with the results of the analysis including all crabs, irrespective of origin, demonstrating the overall downregulation of transcripts involved in immunity, renders the possibility of immune priming and/or nonparallel adaptation more likely.

An additional source of complexity is the potential for this pattern to be driven by evolution of the parasite. All parasites were sourced from naturally infected crabs in Maryland, and it is possible that the idiosyncratic response in the introduced range reflects strong adaptation of the parasite to local hosts. We excluded MD crabs from the analysis to avoid potentially confounding signals from tight, estuary-level adaptation of parasite to host, but cannot rule out local adaptation of the parasite across the introduced range as a potential driver of this pattern. Further study is required to determine how these processes, immune priming or rapid evolution of the host and/or parasite, directly influence susceptibility in the introduced range.

5 | CONCLUSIONS

Range shifts caused by rapidly changing environmental conditions and continued anthropogenic transport of exotic species are redistributing global biodiversity, blurring biogeographical lines, and bringing novel combinations of species and genes into contact. Understanding how species will respond to these novel encounters, spanning recent and distant timescales, is of critical importance to predicting and managing impacts to native ecosystems. Our study

investigated the gene regulatory response of *Rhithropanopeus harrisii* to infection by *Loxothylacus panopaei*, and how it differed among populations with different histories of parasitism. Our transcriptomic results demonstrated signatures consistent with parasitic manipulation and previous observations of differences in susceptibility and parasite prevalence among regions. (Tepolt, Darling, et al., 2020). Despite the constraints of limited annotation of the *R. harrisii* de novo transcriptome, we were able to observe meaningful changes in the regulation of key transcripts involved in innate immunity and the moulting cycle, two host processes with direct impacts on parasite viability. While follow-up experiments should be conducted to demonstrate causality, the data presented here provide insights into the molecular basis of an extreme extended phenotype—parasitic manipulation.

The differences in the host's transcriptional response to infection between the native range and where the parasite is absent may reflect long-standing host adaptation to parasitism in the native range. The unexpected pattern in the introduced range, however, could suggest alternative processes (e.g., immune priming, nonparallel evolution, local adaptation of parasite to hosts) occurring in place of rapid parallel adaptation of *R. harrisii* to a novel parasite. Continued empirical investigations, ideally with crabs reared for multiple generations in the laboratory and parasites from different source populations, will help to disentangle the potential contributions of heritable and plastic mechanisms. By examining *R. harrisii 's* transcriptomic response to *L. panopaei* infection across a mosaic of historical exposure, this study adds to a growing understanding of the foundational role of gene regulation in both host–parasite interactions and novel biotic encounters facilitated by human introductions.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest regarding this publication.

AUTHOR CONTRIBUTIONS

C.K.T., G.M.R., A.E.F., A.M.H.B., J.A.D., M.E.T. and A.W.M. conceived and designed this study. C.K.T. conducted the experimental work. Z.J.C.T. analysed the data. Z.J.C.T. drafted the manuscript with assistance from C.K.T., and all authors contributed to editing it.

DATA AVAILABILITY STATEMENT

Rhithropanopeus harrisii sequence reads are available in NCBI's Sequence Read Archive (SRA) under BioProject ID PRJNA633282. Individual BioSample IDs and SRA accession numbers are provided in Table S1. *Loxothylacus panopaei* sequence reads are included within BioProject ID PRJNA739649 (SRR 14872363-4). The host transcriptome, parasite transcriptome, gene expression matrix and sample metadata files can be found at https://doi.org/10.5061/dryad.rxwdbrv8m.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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