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Ectosymbiotic Cleaners Induce Down-regulation of Crayfish Immune Response Genes

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ABSTRACT

Recent studies have revealed integral and complex relationships between hosts and their symbionts. Some of these findings demonstrate that symbionts can affect the gene expression of their hosts. We used a model cleaning symbiosis system of crayfish and their branchiobdellidan symbionts to examine whether symbionts could affect host gene expression through indirect means, by changing the interaction of the host crayfish with the environment. Previous research has shown this symbiosis to be a complex, context dependent relationship in which outcomes can shift between mutualism, when branchiobdellidans act as cleaners of their hosts, and parasitism, when damage to hosts' gills through branchiobdellidan feeding produces negative effects. These shifts are known to occur with changes in symbiont densities and environmental conditions. We manipulated densities of branchiobdellidans on host crayfish and examined the effect on expression of 3 candidate immune genes. We had 2 competing working hypotheses: 1) That branchiobdellidans would cause an increase expression in immune genes because of damage to the hosts' gills; and 2) That branchiobdellidans would decrease expression of immune genes by acting as cleaners and thus alleviating environmental stress. We found that the second hypothesis was strongly supported when branchiobdellidan activity decreased expression in 2 of the 3 candidate genes. One of these genes (astacidin) is related to generalized immune defense, while the other (prophenoloxidase) is a key part of the melanization cascade that provides defense in the case of physical damage and intrusion of microbes or foreign bodies. Given that there is no known mechanism through which branchiobdellidans can directly affect host physiology through chemical or genetic interactions, we concluded that this alteration in gene expression occurred through alleviation of environmental stress by the symbionts. Such indirect effects may be common in nature and continued study using easily manipulated systems like the crayfish-branchiobdellidan symbiosis may continue to produce insights regarding the importance of symbioses in ecological systems.

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INTRODUCTION

The more we know about symbiosis, the more we appreciate the ubiquity and importance of symbiotic interactions. While the study of symbiosis dates back at least to the ancient Egyptians who included intestinal symbionts in early medical texts circa 1550 BCE (Egerton 2014), modern science has revealed that the biology of symbionts and hosts are often far more intimately entwined than our early Egyptian pioneers would have ever imagined. We now know that symbionts can exert truly astonishing effects on their hosts. Some of these relationships are quite complicated and even bizarre, like fungi and parasitic worms that literally exert "mind control" over their insect hosts (Thomas et al. 2002; Mongkolsamrit et al. 2012). Even familiar and well-publicized symbioses like the relationships between plants and their pollinators, the influences of

mycorrhizal symbioses, and the interdependence between corals and their endosymbiotic dinoflagellates have proven to be far more complicated and varied than previously thought (Rodriguez-Lanetty et al. 2006; Karst et al. 2008; Carstensen et al. 2016). Recent work even suggests that our own evolutionary history, current health, and survival as a species are intimately related to our microbial symbionts (Backhed et al. 2005; Yatsunenko et al. 2012; Le Chatelier et al. 2013; Moeller et al. 2014).

One relatively recently discovered type of host-symbiont interaction is the influence of symbionts on host gene expression. These effects have been documented in a wide range of host-symbiont interactions, including bacteria and protozoans (Choi et al. 1997), plants and mycorrhizal fungi (Zouari et al. 2014), and bacteria that produce bioluminescence in several species of marine invertebrates (Almada and Tarrant 2016). We call these types of interactions "recently discovered" because, even though they have been suspected for some time, only recently has technology allowed a thorough investigation of gene expression in real time. To date, the majority of described cases in which symbionts affect the gene expression of hosts is through the symbiont directly interacting with host physiology, for example, the influence of *Wolbachia* on wasp hosts (Kremer et al. 2012), bacterial influence over plant flowering (Lutay et al. 2016), arbuscular mycorrhizal fungi affecting immune defense of grapes against nematodes (Li et al. 2006), or the protozoan *Trypanasoma* promoting susceptibility to transmission in mammals by altering expression in several host pathways (Garcia-Silva et al. 2014). These previous studies of symbiont effects on host gene expression mostly involve endosymbionts, usually bacteria or fungi, that affect host gene expression through direct interactions with host physiology. However, it is also probable that some types of symbionts can *indirectly* affect gene expression of their hosts by changing the interaction between the host and environment. One type of symbiotic interaction in which these types of indirect effects might be expected are cleaning symbioses in which ectosymbionts remove parasites or foreign material from hosts, providing a beneficial cleaning service (Limbaugh 1961; Grutter et al. 2002). In these types of interactions, cleaners rarely interact with hosts in such a way that host physiology is directly affected, but cleaners can change the way that hosts interact with their environments, and thus could be expected to alter expression of host genes that control a host's interaction with their environment.

We investigated the potential for cleaners to indirectly induce changes in host gene expression using the cleaning symbiosis between freshwater crayfish (Arthropoda: Decapoda) and branchiobdellidans (Annelida: Oligochaeta). Branchiobdellidans are a group of ≈150 species of ectosymbiontic worms found on freshwater crustaceans in a Holarctic distribution (Gelder 1999). Until recently, the majority of branchiobdellidan species were considered to be commensals that had little effect on their hosts (Gelder 2010). However, a number of recent studies demonstrate that the relationship between crayfish and branchiobdellidans can be multifaceted and complex. The relationship can shift between a mutualism and parasitism, with worms sometimes promoting crayfish growth, but can also shift to parasitism under some conditions (Brown et al. 2002; Brown et al. 2012). This shift is thought to occur because branchiobdellidans clean crayfish body surfaces, particularly the gills, through their feeding behaviors, but can shift to direct consumption of host gill tissue when resources are limited (Brown et al. 2002; Brown et al. 2012). The relationship is context-dependent and can shift between mutualism, commensalism, and parasitism based on a variety of factors including symbiont density (Brown et al. 2012), host ontogeny (Thomas et al. 2016), and environmental conditions (Lee et al. 2009). Both host and symbiont can exert some influence over the relationship, with hosts using grooming to remove excess branchiobdellidans (Farrell et al. 2014; Skelton et al. 2014; Skelton et al. 2016), and the branchiobdellidans showing evidence of host choice (Brown and Creed 2004) and selective dispersal to optimize their own fitness (Skelton et al. 2015).

To examine the influence of branchiobdellidan symbionts on host immune function, we conducted an experiment in which we manipulated branchiobdellidan density on crayfish hosts, and measured the expression of 3 candidate immune genes in the host crayfish during a 6-day experiment. In this experiment, we created conditions in which there was relatively high potential for microbial gill fouling of crayfish, a phenomenon that can be harmful or even lethal to crustaceans (Bauer 1998), but in crayfish this fouling can be ameliorated to some degree by branchiobdellidans (Lee et al. 2009). We also used a fairly high density of branchiobdellidans as our experimental treatment so that significant gill damage to the host was also a possibility (Brown et al. 2012). We therefore had two competing working hypotheses regarding the effect of branchiobdellidans on host gene expression. 1) That we would measure decreased expression of immune-related genes in the branchiobdellidan treatments relative to the controls because the worms were alleviating gill fouling by cleaning. 2) That we would measure up-regulation of immune associated genes in response to tissue damage caused by branchiobdellidans at fairly high densities. Both of these hypotheses are compared to a null hypothesis of no effect of branchiobdellidans on host gene expression.

METHODS

Laboratory Experiment

We conducted a 6-day aquarium experiment in June of 2010 in which we manipulated branchiobdellidan density on 24 host crayfish. The presence or absence of branchiobdellidans was the only treatment in the experiment. We manipulated branchiobdellidan presence by field collecting host crayfish, then chemically killing their branchiobdellidan symbionts, and finally by adding branchiobdellidans back to the host at chosen treatment levels using methods established during prior experiments (Brown et al. 2002; Brown and Creed 2004; Brown et al. 2012). The host crayfish species was *Cambarus chasmodactylus* James and the branchiobdellidan symbiont was *Cambarincola ingens* Hoffman. We field captured *C. chasmodactylus* from the South Fork of the New River and selected 24 hosts that fell into a range of carapace lengths (CL) from 26.5 mm to 38.7 mm with a mean of 31.8mm. We chemically removed branchiobdellidans from host crayfish using a 10 minute immersion in a 10% magnesium chloride hexahydrate solution, a method demonstrated to kill the worms and their embryos contained in cocoons, but to have

little influence on crayfish hosts (Brown et al. 2002). Each of the 24 hosts were assigned to one of 2 treatment levels, 0 or 10 branchiobdellidans. We then rinsed the crayfish in stream water and re-applied branchiobellidans (*C. ingens*) to the host using dissection probes (Brown et al. 2002; Brown et al. 2012). We chose 10 branchiobdellidans as our treatment because this density is high, but realistic for larger *C. chasmodactylus* (Brown and Creed 2004), and densities in this range have been demonstrated to have negative impacts on *C. chasmodactylus* (Brown et al. 2012). We watched each host with branchiobdellidans to ensure that the worms had attached and settled on to their hosts, then the crayfish were placed in aquaria. Prior to their placement in aquaria, half of each treatment group was randomly assigned to be collected at either 2-days or 6-days because sampling in this experiment was destructive, resulting in 6 replicate hosts for each treatment × time combination.

Once assigned to treatments and the branchibodellidan manipulations had been performed, we placed crayfish in assigned aquaria for the remainder of the experiment with each crayfish assigned to an individual aquarium. Aquaria (37 L) were filled with water from the South Fork of the New River in Boone, NC. We collected the water from just below the Boone waste water treatment effluent discharge pipe. Our reasoning was that bacterial loads in the effluent water might be higher than normal river water which would potentially lead to higher bacterial loads on the crayfish gills thus promoting a greater immune response. Aquaria were aerated and contained 3-hole bricks that crayfish could use as refugia. Aquaria were placed on wire racks in a room in which water temperature ranged from 16 – 17°C. Lights in the room were on a timer and on a 14:10 light:dark schedule which is the normal light cycle for summer in Boone, NC. Crayfish were provided with 2 shrimp pellets each day for nutrition.

Crayfish were sampled after 2 and 6 days. First, total remaining worm number was determined for crayfish stocked with worms. Crayfish were snap frozen whole in liquid nitrogen and stored at -80°C. At Clemson University, crayfish carapaces were removed, crayfish were allowed to thaw briefly, and hepatopancreas tissues were removed and stored in -80°C for further analysis. After harvesting the hepatopancreas, we also visually assessed any damage to the host gills. Branchiobdellidan damage to crayfish gills leaves characteristic melanization spots and trunctated ends of gill filaments (Figure 1; Quaglio et al. 2006; Brown et al. 2012; Rosewarne et al. 2012).

The day crayfish were placed in aquaria, we also field collected 3 *C. chasmodactylus* from the same sites as experimental crayfish and within the same size range. We immediately froze these 3 crayfish in liquid nitrogen to serve as a baseline for comparison with regards to gene expression of experimental crayfish. For simplicity, we refer to these crayfish in the Results and Discussion as field references. However, it should be recognized that these crayfish were not true controls since the experimental manipulation was presence or absence of branchiobdellidans, and each of the collected crayfish hosted *C. ingens*; it would be highly unusual to field-collect *C. chasmodacyltus* in this size range in the South Fork of the New River that do not host at least one *C. ingens* (Brown and Creed 2004).

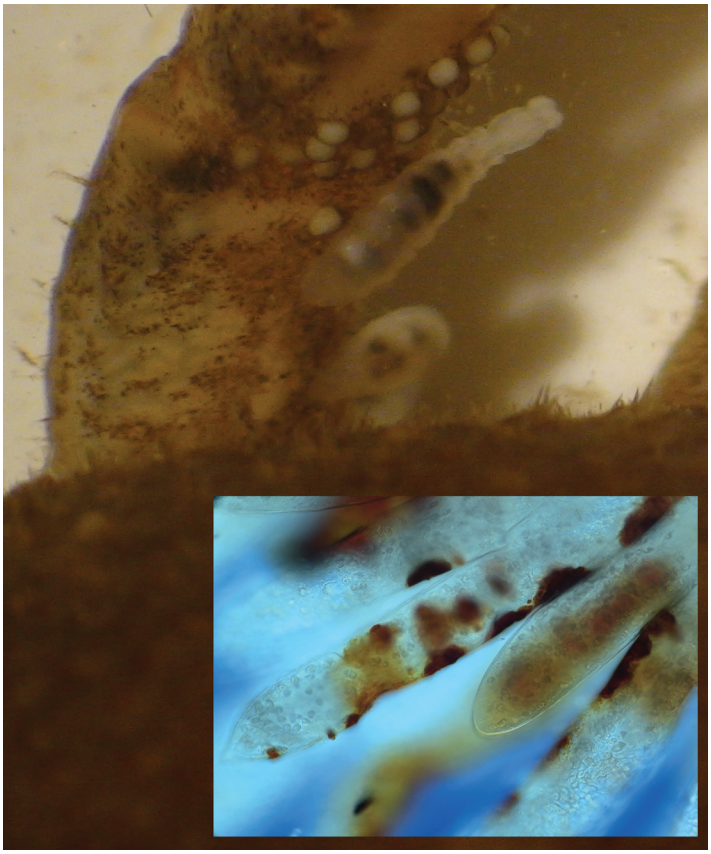

Figure 1. Branchiobdellidans (*Xironodrilus appalachius*) on the walking legs of a crayfish host (*Cambarus bartonii*). The off-white spheres are branchiobdellidan cocoons, each of which will produce a single juvenile worm. Inset: micrograph (40x) of melanization spots on crayfish gills indicating past damage. Damage of this sort is possible from branchiobdellidan activity.

Candidate Gene Isolation

Primers for amplification of the candidate immune and housekeeping genes were designed based on conserved sequences identified in red swamp crayfish (*Procambarus clarkii*), lobster (*Homarus* spp.), and other crustaceans. Briefly, initially identified sequences from phylogenetically-close relatives of *C. chasmodactylus* were used in BLASTN searches of the Genbank nr database. Multiple alignments of recovered sequences were performed to identify highly conserved sequences across phylogenies, and primers to those regions designed in Lasergene (DNAStar). Genes for which primers were designed include: *astacidin*, 5'–GTG TTG CCC TCG TTG CTC TTA TGG–3' and 5'–ACG TCG GCC TAC TTG CTC TTG AAC–3'; *crustin*, 5'–CCC GAG GGC CCA AAC AAG–3' and 5'–TGA ACA AGC GAG CCA ACA ACC TAT–3'; *prophenoloxidase*, 5'–CCG GGC GTG GTG CTT GAC A–3' and 5'–CAG GCC ACC CAC ACC CAC AGA A–3'; and *GAPDH*, 5'–ACA ATG AAA TGA AAC CAG AAA ACA–3' and 5'–TTG CCA AGG CGG ACA GT–3'.

From a single *Cambarus chasmodactylus* individual on ice, claw, gill, and hepatopancreas tissues were dissected and pooled, frozen with liquid nitrogen, and pulverized. Total RNA was extracted from the homogenate (Ambion RNAqueous kit), and oligo-dT primed for first strand cDNA synthesized (New England

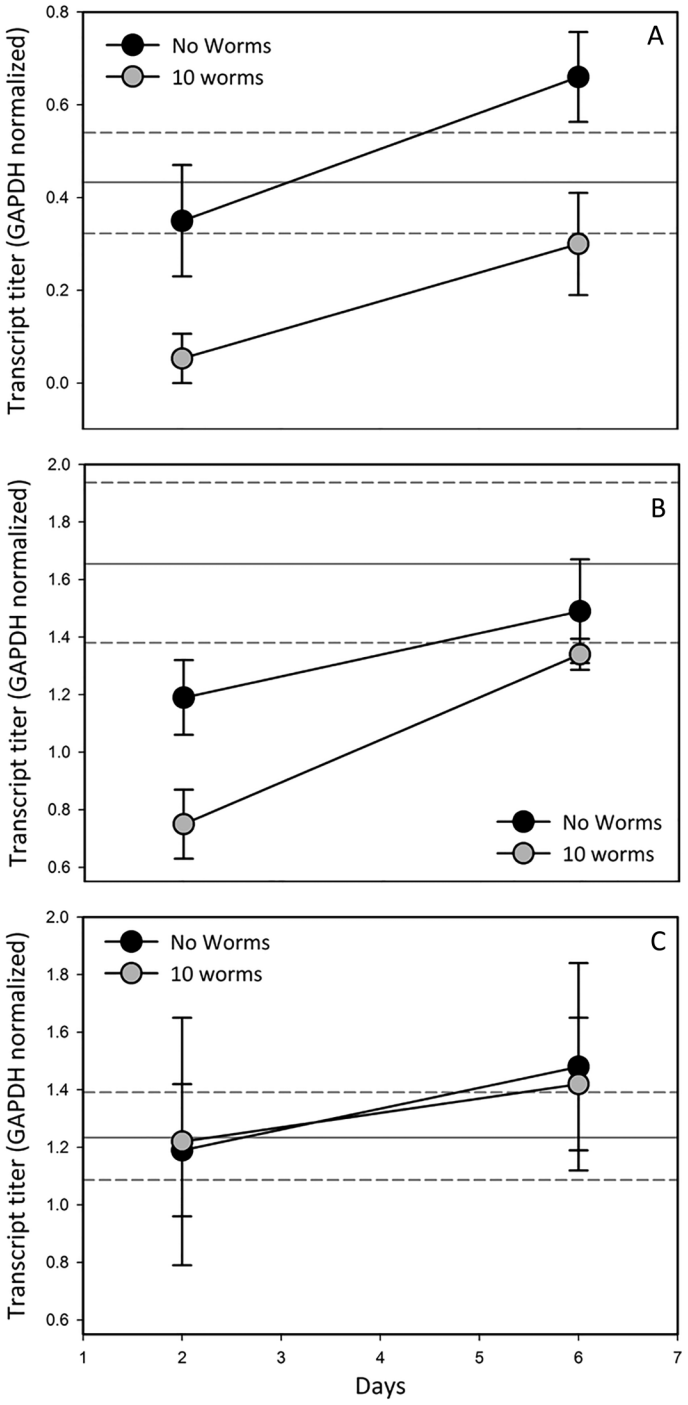

Figure 2. Effect of branchiobdellidans on expression of candidate genes during the 6-day experiment. Points show mean normalized number of transcripts and error bars represent +1SE. Grey lines indicate mean (solid line) and standard error for expression in field references. A) Expression for *PPO*. B) *Astacidin*. C) *Crustin-2*.

Biolabs Protoscript First Strand Synthesis kit). Four microliters of cDNA was used in a 50 µL volume PCR with GoTaq Green Master Mix (Promega) with 200 nM of forward and reverse primers. Cycling conditions were as follows: 3 min at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C; 4°C. Bands of the expected size were excised from a low melt agarose gel. DNA was column purified from gel bands (Qiagen Gel Extraction kit), ligated to pGEMT-Easy (Promega), and clones sequenced (Clemson University Genomics Institute). Sequences were analyzed and consensus sequences developed in Lasergene. BLASTN searches were performed to identify homologues, and multiple alignments and pairwise comparisons made in Lasergene.

Semi-Quantitative PCR

Total RNA was isolated as above from individual hepatopancreas dissected from crayfish. DNase I (Promega)-treated RNA was used to synthesize oligo-dT primed cDNA as above, and diluted to 50 µL with nuclease free water. Polymerase chain reactions were performed in 10 µL volumes, with GoTaq Green Master Mix and 333 nM forward and reverse primers and 1 µL template. Templates consisted of experimental cDNAs, pGEMT-Easy clones (positive control), DNase I-treated RNA (to verify absence of gDNA), and nuclease free water (negative control). PCR conditions consisted of an initial 3 min denaturation at 94°C followed by 35 cycles of 94°C, 60°C, 72°C (30 sec each). Products were separated by electrophoresis through 2% agarose gel, stained with ethidium bromide, and visualized by UV-light. Images were captured by digital camera and band intensity analyzed with NIH ImageJ. Pixel intensity was determined for the band in each lane and the region above and below the band. Intensity values were imported to Microsoft Excel, and background intensity (average of regions above and below bands) subtracted from band intensity. Intensity values for each experimental gene within a given cDNA pool were normalized to the housekeeping gene GAPDH amplimer (normalized value = experimental gene intensity value / GAPDH intensity value).

Data Analysis

We used 2-way Analysis of Variance (ANOVA) to examine expression of individual genes with treatment and sample date as factors. Although this experiment was designed to produce data in a time series, a repeated measures analysis or within-subjects design was not appropriate since there was destructive sampling on each date, and therefore no assumed autocorrelation between dates. Residuals of responses were normally distributed within tolerance, and given our balanced design and no loss of experimental units, the assumption of inequality of variance was not problematic (Box 1954).

RESULTS

There were significant differences in expression of two of the three candidate immune genes — *astacidin* and *PPO* — in response to the branchiobdellidan treatment. In agreement with our cleaning hypothesis, both genes had lower expression in the presence of branchiobdellidan symbionts when compared to the 0-worm controls. On both sample day-2 and day-6, *PPO* responded to the branchiobdellidan treatment with 3 to 4 times as many transcripts in the 0-worm control as compared to the 10-worm treatment (Figure 2A, Table 1); there was also a significant direct effect of time ($P = 0.0099$) with expression increasing from day 2 to day 6, but there was not a treatment × sample interaction (Figure 2A, Table 1). In comparison to the Field reference crayfish, expression of *PPO* in the 10-worm treatment was always lower,

Table 1. Results of 2-way Analysis of Variance models testing for the effects of symbiont presence on gene expression in genes associated with *astacidin* and prophenoloxidase.

	Model Effect	df	Sums of Squares	F-ratio	*P* (>F)
Astacidin	Branchiobdellidans	1	0.52	5.025	0.036
	Time	1	1.19	11.44	0.0030
	Branch x Time	1	0.12	1.45	0.30
	Residuals	20	2.07		
Prophenoloxidase	Branchiobdellidans	1	0.65	11.72	0.0027
	Time	1	0.45	8.12	0.0099
	Branch x Time	1	0.0057	0.10	0.75
	Residuals	20	1.11		
Crustin-2	Branchiobdellidans	1	0.36	0.57	0.46
	Time	1	0.001	0.001	0.97
	Branch x Time	1	0.013	0.02	0.89
	Residuals	20	12.50		

while the no-worm treatment fell within the Field reference range on day 2, but was higher than the Field reference on day 6 (Figure 2A).

Expression of *astacidin* followed a similar pattern with significant effects of both branchiobdellidans ($P = 0.036$) and time ($P = 0.0030$) but not the treatment × time interaction (Figure 2B, Table 1). Like *PPO*, *astacidin* was significantly lower in the 10-worm treatment than in the 0-worm control on both dates and expression increased from day 2 to day 6. One difference between *astacidin* and *PPO* was how expression compared to expression in the field reference crayfish. Like *PPO* expression, *astacidin* expression in the 10-worm treatment was always lower than in the field reference. However, unlike with *PPO*, expression in the no-worm treatment on day 2 was also lower than the field reference, while day 6 was well within the range of the field reference. Crustin-2 was the only gene whose expression changed with neither treatment nor time and all observations were either within, or very close to the range of the field reference values (Figure 2C).

DISCUSSION

Branchiobdellidan symbionts significantly affected the expression of two immune function genes (*astacidin* and *PPO*). Within the context of our experiment, three results are particularly pertinent to the ultimate interpretation of these outcomes. The first result is the treatment effect that resulted in lower gene expression in the 10-worm treatment, the second is the significant increase in expression through time measured in *astacidin* and *PPO*, and the third is the relative level of expression with respect to the field references. However, to clearly interpret these effects first requires placing the results in a physiological context. What do changes in these genes actually signify for a host crayfish immune system?

Both *astacidin* and *PPO* are known to be key elements of the invertebrate immune system. *PPO* is a well-described contributor to the melanization cascade that is a fundamental immune defense mechanism in invertebrates (Aspán et al. 1995; Cerenius and Soderhall 2004). Melanization is a key defense mechanism in organisms with chitinous exoskeletons in which damaged tissues or foreign bodies are isolated through the melanization of tissue which physically shields intrusion and actively prohibits biotic invasion through the secondary production of toxic quinones (Cerenius and Soderhall 2004). During the melanization cascade, prophenoloxidase is activated to phenoloxidase through cleavage by serine proteinases, which then catalyzes the production of quinones with melanin as a downstream product. (Cerenius and Soderhall 2004). *Astacidin* is a relatively recently discovered antimicrobial peptide isolated from the haemolymph of crayfish that inhibits growth of both Gram-positive and Gram-negative bacteria (Lee et al. 2003). Unlike *PPO*, the relationship between immune function and production of this peptide appears to be direct and not part of a complex cascade, so that there is a direct correlation between increased transcripts and increased presence of *astacidin* in crayfish haemolymph (Lee et al. 2003). Similar to *astacidin*, *crustin* is an antimicrobial peptide, expressed by members across Crustaceans (Smith et al. 2008). In red swamp crayfish, *Procambarus clarkii*, *crustin* transcript and peptide titers are upregulated upon intrahemocoelic pathogen challenge (Liu et al. 2016). To the best of our knowledge, increased expression of these genes results in a general increase in immune function. *Astacidin* and *crustin* are straightforward protein products of expression and so there is a more direct, monotonic relationship between expression and the abundance of antimicrobial peptides (Lee et al. 2003). In the case of *PPO*, up-regulation is a precursor to increasing activity of the phenoloxidase enzyme that catalyzes the ultimate steps of the melanization cascade (Boucias and Pendland 1998).

Given this physiological background, we can begin to interpret the results of this experiment with regard to the influence

of branchiobdellidans on crayfish immune function. First, in our experiment there were clearly effects of the branchiobdellidan *C. ingens* on gene expression of two immune-related genes, *astacidin* and *PPO*, on the host crayfish *C. chasmodatcylus*. In both cases, branchiobdellidan presence led to decreased expression which was counter to the gill damage hypothesis. The logic behind the damage hypothesis was based on the knowledge that branchiobdellidans can damage their hosts' gill tissues (Quaglio et al. 2006; Rosewarne et al. 2012), including the species under study (Brown et al. 2012), and that this damage can result in negative effects on growth of host crayfish, including the host species in this study (Brown et al. 2012). This damage occurs through opportunistic feeding of branchiobdellidans which will scrape hosts' gills with a pair of sclerotized toothlike structures (Brinkhurst and Gelder 2001). In many cases, this rasping may only remove biofilm substrates from gills, but in other cases, entire pieces of gill filaments are consumed (Rosewarne et al. 2012), creating significant damage to crayfish gills that can result in significant loss of haemolymph (Quaglio et al. 2006) and potential entry of bacterial pathogens (Cerenius and Soderhall 2004). We expected this physical damage, the associated loss of haemolymph, and the possibility of invasion by pathogens could precipitate up-regulation in the presence of branchiobdellidans. Instead, expression of *astacidin* and *PPO* was lower with the worms present.

What does decreased expression in the presence of branchiobdellidans indicate? There are two basic possibilities. The first is that branchiobdellidans are actively affecting the physiology of their hosts and inducing decreased expression of immune-related genes through direct chemical or genetic interaction. Such interactions have been described in a variety of host-symbiont pairs. In some cases, selective induction of expression clearly benefits the host and often results in host selection for very specific symbiont species or strains, for example for strains of *Vibrio* in marine copepods (Almada and Tarrant 2016) and bacteria in *Hydra* (Franzenburg et al. 2013). In other cases, selective induction of expression benefits the symbiont and is cited as a general strategy of bacteria to circumvent insect defenses (Vallet-Gely et al. 2008). If branchiobdellidans were having these effects, the purpose would likely be to decrease the efficacy of the melanization cascade that may inhibit branchiobdellidan feeding on host gills. However, this mechanism is not likely due to the lack of an obvious means through which branchiobdellidans can directly affect specific host gene expression through chemical or genetic interaction. Unlike the previously cited examples, branchiobdellidans are strictly ectosymbionts whose only contact with the interior of their hosts would be very limited exposure to haemolymph on damaged gill tissue. It is possible that branchiobdellidans may have some oral secretion that interacts with host haemolymph, but to the best of our knowledge, no such secretions have been described.

A much more likely possibility is that, in the case of this experiment, branchiobdellidans were actively alleviating stress on the host by removing microbial and particulate material from the crayfish exoskeleton, resulting in decreased expression of immune associated genes. There is also a well-supported mechanism for these effects. In multiple experiments, branchiobdellidans have increased growth of their hosts relative to branchiobdellidan-free controls (Brown et al. 2002; Lee et al. 2009; Brown et al. 2012; Ames et al. 2015; Thomas et al. 2016); the mechanism in these cases appears to be cleaning of the host exoskeleton and, particularly, the gill filaments, through the opportunistic feeding of the worms (Brown et al. 2002). While branchiobdellidans are known to damage the gills of their hosts (Quaglio et al. 2006; Brown et al. 2012; Rosewarne et al. 2012) it is likely that they only do significant damage in high densities with the exception of some species that are known gill parasites (Skelton et al. 2013). However, *C. ingens* is not known to be exclusively parasitic, and our own observations of its gut contents suggest a primarily predatory ecology. An additional argument for this mechanism is that, as described in the Methods, the aquarium environment in the experiment was intentionally created to be a high-fouling environment, and positive effects of branchiobdellidan cleaning on crayfish are more likely to occur in higher fouling environments (Lee et al. 2009).

A second piece of evidence that can help explain the results of this experiment is that for *astacidin* and *PPO*, there was a significant increase in gene expression from day-2 to day-6. This increase occurred in both the 0-worm control and in the 10-worm treatment, indicating that this effect was independent of branchiobdellidan activity. Again, the most likely explanation for this result is that the aquaria represented a high-fouling environment and pressure from physical fouling and potential microbial invasion increased through time with increased exposure. Interestingly, while branchiobdellidans alleviated these effects to some extent, they were not able to completely compensate and consequently expression of *astacidin* and *PPO* increased in both the control and treatment groups from day-2 to day-6. Effects of the $MgCl_2$ used to remove branchiobdellidans at the beginning of the experiment might have decreased microbial load on the hosts and contributed to this pattern (Hotchkiss 1923). However, this potential effect of $MgCl_2$ doesn't actually change the interpretation of the increase in expression, except that hosts might have been moving from a low to normal microbial load rather than a normal to high microbial load in the 6-day experimental period.

A comparison between the experimental results and field references also supports the argument that crayfish were in a stressful environment and that branchiobdellidans were able to alleviate that stress to some extent. An enlightening result is that for *astacidin* and *PPO*, expression in the control group largely overlapped with the field references and at least one of the two control samples fell within the range of the field reference, with the other sample lying close to the range of the field reference. However, for *astacidin* and *PPO*, the 10-worm treatment never fell within the range of the field reference, indicating that branchiobdellidans were likely causing a *decrease* in expression as opposed to the 0-worm control showing *increased* expression. Of course, this interpretation begs the question: If all of the field reference crayfish hosted branchiobdellidans, why did the field references overlap with the 0-worm controls? Again, the likely answer is that the aquaria represented a high-fouling environment and that stream dwelling crayfish rarely encounter such conditions, at least when water is abundant and flowing as was the case when these crayfish were collected.

Taken together, the evidence from the experiment strongly suggests that a) the high fouling aquarium environment was stressful to crayfish, and b) that branchiobdellidans were able to alleviate some, but not all, of this environmental stress. These results also agree with a variety of previous work suggesting that the crayfish-branchiobdellidan symbiosis can be a cleaning mutualism under some environmental conditions, but that the relationship is context dependent and may shift to a commensalism or parasitism in other conditions (Lee et al. 2009; Brown et al. 2012; Thomas et al. 2013). One unexpected result is that there was a strong response of *astacidin* while there was no significant response of *crustin*. We anticipated that these two genes would have very similar responses to treatments since they are both antimicrobial peptides that are part of the generalized immune defense. It is possible that *astacidin* may be more sensitive to environmental stimuli or have greater responsiveness to particular microbial strains that were found in the experimental environment, but the exact reason for this lack of response in *crustin* cannot be ascertained from this experiment.

This study adds to a growing appreciation for the importance of symbiosis, and for the range of effects that symbionts can have on their hosts. It also does so with an interesting twist: it demonstrates that ectosymbionts can indirectly induce changes in gene expression of hosts by changing the host-environment interaction. While previous studies have demonstrated clear evidence of altered gene expression in animal symbioses, the majority of these results have come from endosybmionts, primarily bacteria, directly interacting with host physiology (Vallet-Gely et al. 2008; Franzenburg et al. 2013; Almada and Tarrant 2016). Continuing investigation of these sorts of gene × environment interactions mediated by symbionts should strongly contribute to our growing knowledge regarding both symbiont effects on hosts, and how symbionts can modify host interactions with environment. These studies can also be greatly enhanced through the use of systems like the crayfish-branchiobdellidan symbiosis. The system has a number of advantages including ubiquity of organisms, ease of manipulation of both symbionts and environmental conditions, ease of husbandry, and a rapidly growing background on the ecological relationships between hosts and symbionts, and we suspect that continued investigations of the interaction between gene expression, environmental conditions and symbiosis in this system would prove very fruitful.

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