PARTNER CONTROL AND ENVIRONMENTAL FOULING IN THE CRAYFISH-BRANCHIOBDELLID SYMBIOSIS

A Thesis
by
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FOREWARD

The research detailed in each chapter of this thesis will be submitted for publication in a peer-reviewed scientific journal. Chapter 2, “Symbiont Distribution During Host Reproduction: Shifts in the Location and Abundance of Ectosymbiotic Worms on Reproductive Female Crayfish,” and Chapter 4, “Influence of Environment on Epibiotic Fouling: Direct Measures of Microbial Fouling of Crayfish Gills” will be submitted to *Invertebrate Biology,* an international peer-reviewed journal owned by Wiley-Blackwell and published by John Wiley & Sons Inc. Chapter 3, “Preventing Overexploitation in a Mutualism: Partner Control in the Crayfish-Branchiobdellid Symbiosis” will be submitted to *Oecologia*, an international peer-reviewed journal owned by Springer and published by Springer Science + Business Media. Each of these chapters will be submitted for publication with Dr. Robert Creed (ASU) and Dr. Bryan Brown (Virginia Tech) as co-authors, as they have both aided with experimental design, analysis, and editorial guidance in the preparation of the manuscripts, and provided research funding. Each chapter of the thesis has been prepared according to the guidelines of the journal to which it will be submitted.

ABSTRACT

PARTNER CONTROL AND ENVIRONMENTAL FOULING IN THE CRAYFISH-BRANCHIOBDELLID SYMBIOSIS. (May 2012)

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Previous research found that crayfish (*Cambarus chasmodactylus*) may be engaged in a cleaning mutualism with ectosymbiotic worms (Annelida: Branchiobdellidae), yet mechanisms for symbiosis establishment and maintenance remain unknown. While intermediate densities of worms improve crayfish survival and growth rates, relationships between worm colonization and crayfish reproduction have not been examined. In addition, it is unclear why a co-occurring crayfish species (*Orconectes cristavarius*) hosts almost no worms. The research in this thesis builds on over a decade of research into crayfish/ branchiobdellid symbioses by incorporating field surveys with laboratory and field experiments to assess additional aspects of the interaction.

I used field surveys to document the number of worms and worm eggs present on crayfish in local waterways. These surveys indicated that female *C. chasmodactylus* carrying eggs or recently hatched young had fewer worms than those not actively reproducing. In addition, the distribution of worms on the reproductive females differed from that of other crayfish. This observation provides indirect evidence that worms do not directly improve

reproductive fitness in the host crayfish, though they likely indirectly improve reproductive output by increasing crayfish growth and survival.

To address differences in worm loads between crayfish species, I conducted a lab experiment to document crayfish grooming behaviors directed at the branchiobdellid worms. By comparing the grooming behaviors of *C. chasmodactylus* and *O. cristavarius*, I found that the two crayfish species varied in the level of partner control behaviors directed at the symbiont worms. *O. cristavarius* removed worms more often than *C. chasmodactylus*, which agrees with field observations that *O. cristavarius* rarely harbors ectosymbiont worms. To assess a possible mechanism that could explain such differences, I conducted a hemolymph antimicrobial assay to determine whether hemolymph from *O. cristavarius* more effectively inhibited bacterial growth on the gills. The hemolymph from *O. cristavarius* did inhibit some bacteria more effectively, which suggests that this crayfish may be able to maintain clean gills on its own. This in turn would make cleaning by the worms less beneficial, and potentially more harmful if limited fouling material causes the worms to shift from feeding on fouling material to feeding directly on host gill tissues.

Finally, I experimentally quantified the impact of environmental factors on microbial fouling of crayfish gills. Crayfish exposed to only stream water in a laboratory experiment experienced much less fouling than crayfish in a field enclosure experiment. This provides the first direct evidence that gill fouling rate is influenced by contact with the substrate. By combining field observations with experiments to assess animal behavior, gill fouling, and innate antimicrobial ability, my research provides additional insight into, and potential mechanisms to explain, the symbiosis between crayfish and branchiobdellid worms.

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I would like to thank Dr. Robert Creed for his assistance in all aspects of this research, and for his continuing support of my research efforts. I would also like to thank my committee members, Dr. Jennifer Geib and Dr. Suzanna Bräuer, for their input and assistance in developing this thesis. I am grateful to Dr. Bryan Brown of Virginia Tech for his help with experimental design and for editorial suggestions on early drafts of this thesis, and to Dr. Guichuan Hou and Jim Sobeiraj for their invaluable assistance in technical aspects of scanning electron microscopy and microbial assays, respectively. Michael Thomas, April Meeks, Mark Rollins, David Young, and Joshua Lomonaco provided essential hands-on field and laboratory assistance in one or more of these projects. Finally, I would like to thank my parents, John and Maggie Farrell, for their unwavering encouragement and support. Funding for this research was provided by the National Science Foundation (DEB-0949823 to Robert Creed and DEB-0949780 to Bryan Brown), the North Carolina Association of Environmental Professionals, and from the Appalachian State University Office of Student Research, Graduate Student Association Senate, and Cratis D. Williams Graduate School.

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CHAPTER 1

GENERAL INTRODUCTION

Interspecific symbiotic associations are widespread and occur in freshwater, marine, and terrestrial environments. These interactions include parasitism, commensalism, and mutualism, which occur along a continuum and may shift in response to environmental factors (Ewald 1987; Bronstein 1994). Cleaning symbioses are specialized interspecific interactions in which a cleaner organism removes ectoparasites, bacteria, diseased tissue, food particles, and other material from the host, or client (Feder 1966; Losey 1972). To date, cleaning symbioses have been observed in many environments, including both aquatic and terrestrial ecosystems. However, many so-called cleaning symbioses are supported only by anecdotal observations of presumed cleaning behaviors (Amadon 1967; MacFarland & Reeder 1974; Christain 1980; Keyes 1982; Margulis 1987; Peres 1996; Grossman et al. 2006; Sazima & Grossman 2006). While cleaning symbioses are often considered mutualisms, experimental evidence showing direct fitness benefits to either partner is rare (Poulin & Grutter 1996). In systems where formal assessments and experiments have been conducted, results have shown that the nature of the interaction may be mutualistic, commensalistic, or even parasitic, indicating that cleaning symbioses, like many interspecific interactions, are complex interactions whose benefits can vary in different contexts (Ewald 1987; Bronstein 1994; Leung & Poulin 2008).

While presumed cleaning symbioses have been recorded in taxa as diverse as oxpeckers gleaning ticks from African ungulates and isopods cleaning epibionts from sea grasses (Attwell 1966; Hay et al. 2004), the most studied cleaning symbiosis occurs between cleaner and client fishes on coral reefs (Poulin & Grutter 1996). Observations in both the eastern Pacific and Atlantic/Caribbean have found that client fish appear to go to specific cleaning stations, where cleaner and client engage in a brief interaction during which the cleaner fish picks ectoparasites and necrotic tissue from the client body (Feder 1966). The first evidence suggesting that cleaning was a mutualism was provided by Limbaugh (1961), who observed that after removing cleaner fishes and shrimps from an isolated reef in the Bahamas, fish diversity declined and the remaining fish appeared to be less healthy. However, successive cleaner-removal experiments conducted in natural systems failed to show consistent reductions in parasite loads, leading to the conclusion that client fish do not appear to suffer reduced fitness in the absence of cleaners (Poulin & Grutter 1996).

Research on coral reef systems has shown that cleaner fish remove parasites from client fish (Grutter 1996, 1999; Arnal et al. 2001; Cheney & Côté 2003). Cleaner fish also tend to prefer parasitized fish to unparasitized clients (Arnal et al. 2001). However, it is worth questioning whether the observed reductions in parasite load are physiologically relevant. For example, Grutter (1999) found that fish deprived of cleaners could have up to 3.5 times more gnathiid parasites, but this represented a reduction from a mean of ~0.7 to ~0.2 gnathiids per fish. While such results may be statistically significant, they are likely not physiologically significant. In addition, the fitness cost of parasite loads is unclear, as the effects of parasites vary by parasite type and host fish (Grutter 1996).

Despite the abundance of observation and experimentation, whether the cleaner-client fish interaction is indeed a mutualism has yet to be shown definitively (Poulin & Grutter 1996; Cheney & Côté 2003, 2005). By and large, experiments have not provided direct evidence that being cleaned improves the fitness of the client, via improvements in survival, growth, or reproduction (but see Bshary et al. 2007). Indeed, some observations and experiments indicate that cleaner fish will remove client scales and mucus, as these materials provide high-quality nutrition compared to ectoparasites (Gorlick 1980; Grutter 1997). For example, Grutter and Bshary (2003) found that in free choice experiments, at least one cleaner wrasse, *Labroides dimidiatus* (VALENCIENNES 1839), fed preferentially on mucus from parrotfish clients over gnathiid isopods, the clients' most abundant ectoparasites. Feeding on fish tissues could shift the interaction toward parasitism, since the removal of scales and mucus exacts a cost on the client fish (Gorlick 1980). Despite such shortcomings in research to demonstrate a concrete benefit of cleaning for client fish (Poulin & Grutter 1996; Cheney & Côté 2003), many remain adamant that the interaction is a mutualism. However, without direct evidence that cleaner fish improve the fitness of their clients, referring to such interactions as textbook examples of mutualism is overreaching (Bshary et al. 2007).

A freshwater cleaning symbiosis was recently discovered between crayfish and branchiobdellid worms (Brown et al. 2002). While some researchers have suggested that branchiobdellid worms have a commensal relationship with their crayfish host (Goodnight 1941; Jennings & Gelder 1979), experimental tests of these interactions are few. Keller (1992) examined the effect of a branchiobdellid, *Cambarincola fallax* (HOFFMAN 1963), on growth and stamina in the crayfish *Orconectes rusticus* (GIRARD 1852). Finding no negative

effect of the worms on crayfish growth or physical condition, he concluded that the association was a commensalism (Keller 1992). Brown et al. (2002) found that a branchiobdellid, *Cambarincola ingens* (HOFFMAN 1963), may be engaged in a cleaning mutualism with the New River crayfish, *Cambarus chasmodactylus* (JAMES 1966), in that the worms appear to remove fouling epibionts and particulate matter from the gill chamber. They determined that the presence of intermediate densities of *C. ingens* increased growth rates in *C. chasmodactylus* while simultaneously reducing mortality of the crayfish host (Brown et al. 2002). Brown et al. (2002) and Lee et al. (2009) suggested that the outcome of the interaction between crayfish and branchiobdellids appears to be context dependent, whereby environmental conditions that promote the growth of fouling epibionts may shift the relationship from commensal to mutualistic. Recent field experiments demonstrated that worms can benefit crayfish in natural environments (Brown et al. 2012). These experiments also demonstrated that worm density can affect the outcome of the relationship, with high worm densities resulting in damage to crayfish gill filaments as worms become food-limited (Brown et al. 2012).

While the interaction may be facultative for the crayfish, it appears to be an obligate interaction for the worms. *C. ingens* spend their entire life on their crayfish host and appear to lay eggs only on live crayfish (Creed et al., in prep). Because the worm depends on the host for the proliferation of its offspring, fatally harming the host would be selected against, and cooperation by the worm is favored (Bull & Rice 1991).

To date, the crayfish/branchiobdellid system is the only cleaning symbiosis to show direct fitness benefits of cleaning and being cleaned, via increased crayfish growth and survival, and success in worm reproduction. However, many questions remain as to the

mechanisms that drive the interaction. The chapters that follow detail further investigations into aspects of the relationship between branchiobdellid worms and their crayfish hosts.

CHAPTER 2

ABSTRACT

SYMBIONT DISTRIBUTION DURING HOST REPRODUCTION:
SHIFTS IN THE LOCATION AND ABUNDANCE OF ECTOSYMBIOTIC WORMS ON REPRODUCTIVE FEMALE CRAYFISH

Mutualisms provide net benefits to both species involved by improving the fitness of both partners. Determining whether cleaning symbioses are true mutualisms is limited in many systems by a lack of evidence of such fitness benefits, i.e., improvements in survival, growth, and reproduction. The ectosymbiont branchiobdellid *Cambarincola ingens* has been shown to increase growth and survival of the crayfish *Cambarus chasmodactylus*, but influences of the worm on crayfish reproduction have not been previously evaluated. As a first step in addressing this question, I conducted field surveys to quantify branchiobdellid loads on female *C. chasmodactylus* in different stages of reproduction. Reproductive female crayfish had significantly fewer total worms compared to non-reproductive female crayfish due to a significant reduction in large worms. The distribution of worms also varied between crayfish groups, with reproductive females having a greater proportion of worms on the lateral body surfaces, compared to a predominance of worms on ventral surfaces in non-reproductive crayfish. Cleaning behaviors by the female crayfish prior to egg extrusion appear to drive the reduction in worms, limiting potential direct effects of the worms on crayfish reproduction.

INTRODUCTION

Interspecific symbioses are widespread and occur in freshwater, marine, and terrestrial environments (Trench 1993; Poulin & Grutter 1996; Usher et al. 2007). These interactions include parasitism, commensalism, and mutualism, and the outcome of the interaction between the two species involved can shift in response to changes in symbiont density or environmental conditions (Ewald 1987; Bronstein 1994; Lee et al. 2009; Brown et al. 2012). Measures of partner fitness can be used to assess costs and benefits of symbioses, and include measurements of survival, growth, and reproduction (Boucher et al. 1982). When both partners obtain positive values for one or more components of fitness, the symbiosis is considered a mutualism (Boucher et al. 1982; Cushman & Beattie 1991).

Cleaning symbioses are interactions in which cleaner species remove bacteria, parasites, or other epibionts and detritus from the client organism, and have been considered textbook examples of mutualism (Losey 1979; Poulin & Grutter 1996; Brown et al. 2002; Bshary et al. 2007). While presumed cleaning symbioses occur in taxa as diverse as oxpeckers gleaning ticks from African ungulates and isopods cleaning epibionts from sea grasses (Attwell 1966; Hay et al. 2004), to date, a substantial portion of the literature on cleaning symbioses has focused on interactions between cleaner and client fishes on coral reefs (Limbaugh 1961; Poulin & Grutter 1996; Cheney & Côté 2003). In this system, cleaner fish have been shown to reduce ectoparasite loads on client fishes (Grutter 1999; Cheney &

Côté 2001), and may promote client survival by reducing stress (Bshary et al. 2007). However, experimental evidence that cleaning improves the fitness of clients is lacking (Poulin & Grutter 1996; Cheney & Côté 2003; Bshary et al. 2007).

Crayfish and branchiobdellid worms (Annelida: Branchiobdellida) also engage in cleaning symbioses (Jennings & Gelder 1979; Brown et al. 2002, 2012; Lee et al. 2009). The New River crayfish *Cambarus chasmodactylus* (JAMES 1966) and an ectosymbiotic branchiobdellid, *Cambarincola ingens* (HOFFMAN 1963), engage in an interaction in which the worms remove particulate matter and fouling epibionts from the crayfish exoskeleton including the gills (Brown et al. 2002). Experimental evidence indicates that the worms have direct positive effects on the crayfish by increasing growth and survival. In a lab experiment, the presence of *C. ingens* increased growth rates of *C. chasmodactylus* while simultaneously reducing mortality of the crayfish (Brown et al. 2002). Positive effects of worms on crayfish growth have also been reported from field experiments (Brown et al. 2012). Lee et al. (2009) also reported positive effects of branchiobdellids on host crayfish growth. However, it remains to be seen whether there is a relationship between worm load and crayfish reproduction. Here, I present results from field surveys that quantify worm presence on reproductive female crayfish. Survey results show reduced worm numbers and altered worm distributions on female *C. chasmodactylus* that are carrying eggs (in berry) or carrying newly hatched young.

METHODS

Field Surveys

During June, July, and August 2011, I conducted field surveys of *Cambarus chasmodactylus* and symbiotic *Cambarincola ingens* at four locations in the New River watershed in Watauga County, NC. I collected crayfish and worms in three third-order tributaries of the South Fork of the New River: Meat Camp Creek, Howard's Creek, and the Middle Fork of the New River. I also collected crayfish and worms in the South Fork of the New River, a fourth-order stream.

Crayfish were captured by lifting boulders from the stream bed and flushing out resident crayfish into dip nets. Exposed individuals on the stream bed were also captured with hand nets. After capture, crayfish were placed in individual, lidded plastic containers filled with stream water to prevent possible transfer of worms between crayfish. Each crayfish was examined on site using a 10x OptiVisor binocular headband magnifier (Donegan Optical Company, Lenexa, Kansas). For each crayfish, I recorded its sex and carapace length (CL), the location and number of large and small branchiobdellids (Table 1), and the location and number of branchiobdellid eggs. Female crayfish were also examined for the presence of attached eggs, i.e., if they were ovigerous, or if they were carrying recently hatched young. Crayfish were then released.

I compared worm and egg numbers as well as worm locations between female crayfish that were either ovigerous or lacking eggs. Females were excluded from analysis if

their carapace length was less than that of the smallest ovigerous female (32mm CL) or if they had recently molted. The analysis included 18 females without eggs or attached young, 7 ovigerous females, and 3 bearing recently hatched young crayfish. The carapace lengths of these female crayfish ranged from 32-45 mm.

Data Analysis

I compared the number and location of large, small, and total branchiobdellids as well as the number of branchiobdellid eggs found on ovigerous (n=7), young-bearing (n=3), and non-reproductive (n=18) females. One-way ANOVA was used to assess differences in worm number and worm location between ovigerous, young-bearing, and non-reproductive females, and chi-square analysis was used to determine if there were significant differences in the distribution of worms on the crayfish.

RESULTS

Worm Number

I captured 61 female *Cambarus chasmodactylus*, of which 7 were ovigerous and 3 were carrying recently hatched young. There were no significant differences between the numbers of worms or worm eggs present on ovigerous female crayfish and those bearing recently hatched young, so these two groups were pooled for subsequent analyses. The two groups analyzed were classified as reproductive (n=10) and non-reproductive (n=18) crayfish. The mean carapace length of reproductive females did not differ from that of non-reproductive females ($F_{1,26}$=2.128, p=0.157). Remaining females were not analyzed as they were smaller than 32mm CL.

Reproductive females carried significantly fewer total worms than non-reproductive females ($F_{1,26}$=8.107, p=0.008). While the number of small worms was similar for all females (Fig. 1, $F_{1,26}$=1.393, p=0.249), the number of large worms was significantly lower on ovigerous female crayfish (Fig. 1, $F_{1,26}$=8.495, p=0.007). Non-reproductive females had an average (mean±SE) of 4.4±0.6 large worms per crayfish, while reproductive females had an average of 1.8±0.5 large worms. Branchiobdellid eggs were only found on non-reproductive female *C. chasmodactylus* (Fig. 1).

Worm Location

The distribution of worms on the crayfish varied as a function of crayfish reproductive status. Large worms were distributed unevenly on both reproductive and non-reproductive crayfish. In non-reproductive crayfish, more large worms were found on the ventral surface than expected, while in reproductive crayfish, more worms than expected were found on the lateral surfaces ($\chi^2_{2\ df}$ =17.761, p<0.001). For small worms, I found similar patterns of worm distribution. In non-reproductive crayfish, more small worms were found on the ventral surface than expected, while more small worms than expected were found on the lateral surfaces of reproductive crayfish ($\chi^2_{2\ df}$ =12.672, p=0.002).

When comparing the two groups of crayfish, non-reproductive females had significantly more large worms on the ventral surface (Fig. 2A, $F_{1,26}$=10.635, p=0.003), while reproductive females had more large worms on the lateral surfaces (Fig. 2A, $F_{1,26}$=28.813, p<0.001). Reproductive females also had more small worms on the lateral surface (Fig. 2B, $F_{1,26}$=18.486, p<0.001) and dorsal surface (Fig. 2B, $F_{1,26}$=7.169, p=0.013) than non-reproductive females. Worm eggs were found predominantly on the ventral surface of non-reproductive female crayfish, and were absent from reproductive females (Fig. 2C).

DISCUSSION

My survey data indicate that the nature of the symbiosis between branchiobdellids and female crayfish may vary depending on the reproductive state of the host. The total number of worms on ovigerous female crayfish as well as those with recently hatched young was reduced compared to non-reproductive females of similar size, and was driven by a significant reduction in the number of large worms. Furthermore, no branchiobdellid eggs were found on any of the female crayfish that were in berry or were carrying recently hatched young. We do not believe that the observed reduction in worms was due to the crayfish molting just prior to egg extrusion, as the crayfish body surfaces were moderately fouled with sediment and detritus, which is indicative of crayfish intermolt stages. In addition, others have observed that the spring molt of reproducing female Cambaridae, including *Orconectes immunis* (HAGEN 1870) and *Orconectes propinquus* (HAGEN 1852), is delayed compared to males and non-reproducing females (Van Deventer 1937; Tack 1941; Scudamore 1948). We predict instead that grooming behaviors prior to egg extrusion drove the observed reduction in branchiobdellids.

Previous studies on the natural history of various crayfish taxa reported that females thoroughly clean the ventral surface of their exoskeletons 4-5 days prior to egg extrusion (Andrews 1904; Tack 1941). Andrews (1904) observed that female *Orconectes limosus* (RAFINESQUE 1817) performed a thorough cleaning of the ventral abdomen using the small walking legs to remove particulates and brush dirt from both the abdomen and pleopods, onto

which the female crayfish later attaches her eggs. It is likely that such intensive grooming behaviors could dislodge branchiobdellids and their eggs from the ventral surface of the crayfish abdomen prior to egg-extrusion and during the period that the females are in berry.

Cleaning behaviors by the female crayfish continue to discourage worm presence on the ventral surface of the abdomen following egg extrusion. After the eggs are extruded and attached, females beat the pleopods to ensure water circulation around the egg mass and later, the young crayfish, which prevents embryo fouling and death (Bauer 1989). Such frequent disturbances likely caused the continued reduction in large worms and eggs found on the abdomens of female crayfish carrying recently hatched young.

Worms that remained on female crayfish carrying eggs or young were in different locations than those found on non-reproductive crayfish. The observed changes in worm location between non-reproductive individuals and females either in berry or carrying young indicate that worms may be moving in response to the disturbance generated by the crayfish as they clean their abdomens. Previous surveys in this system found that nearly 50% of branchiobdellids were located on the ventral surface of the abdomen or on the underside of the cephalothorax (Brown et al. 2002). In my surveys, this held true for males and non-reproductive females, but not reproductive females, where the majority of large and small worms were found on the lateral surfaces, specifically the margins of the carapace. The observed changes in the spatial distribution of remaining worms on females in berry or carrying recently hatched young suggests that it may not be beneficial for the crayfish to have worms on the abdomen during reproductive periods.

The presence of intermediate densities of branchiobdellids has been shown to directly improve crayfish growth and survival (Brown et al. 2002, 2012). If the continued presence

of worms during crayfish reproduction also directly improved reproductive output, we would expect worm numbers and distributions to be consistent among non-reproductive and reproductive female crayfish. While this study suggests that the worms are not having a direct positive effect on crayfish reproduction, there is evidence that worms have positive indirect effects on crayfish reproduction. Female crayfish must reach a minimum size prior to laying eggs, though the minimum size varies between taxa (Jezerinac et al. 1995). A survey of *Procambarus clarkii* (GIRARD 1852) found that females as small as 31mm CL had mature eggs in the ovaries, but the smallest ovigerous *P. clarkii* was 35mm CL (Penn 1943). *Cambarus robustus* (GIRARD 1852) females became ovigerous at an average carapace length of 29.7mm, which was estimated to be the crayfish's third summer (Corey 1990). In a lab experiment, Brown et al. (2002) found that the presence of worms increases *C. chasmodactylus* growth rates. Successive field experiments to test this hypothesis have confirmed that intermediate densities of branchiobdellids increase crayfish growth rates compared to crayfish without worms (Brown et al. 2012). This suggests that worm-laden female *C. chasmodactylus* should reach reproductive size sooner than those without worms.

Studies across multiple crayfish taxa indicate that the number of eggs extruded by a female crayfish is proportional to carapace length (Penn 1943; Corey 1990; Jezerinac et al. 1995). Since the presence of branchiobdellids causes *C. chasmodactylus* to grow faster, a crayfish of a given age would be larger, and thus produce more eggs, if branchiobdellids had been present.

The cleaning behaviors typical of female crayfish prior to egg extrusion likely drive the reduction in large worms seen in my surveys. However, since the number of small worms does not change, these individuals probably mature into large worms once the crayfish

reproductive period is over. While survey data alone prevent us from determining whether branchiobdellids directly affect crayfish reproduction, the fact that large worms are removed and the absence of worm eggs suggests that, at least for this period, the worms do not benefit the crayfish. It is possible, though, that worms still enter the crayfish gill chamber from their lateral locations on the carapace, clean the gills and influence gas exchange and ammonia excretion thus influencing survival of reproductive females. Experiments will need to be conducted to determine whether or not worms have positive or negative effects on females during this period.

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Table 1. Regions of crayfish body surface. Crayfish body surfaces were classed into ventral, lateral, and dorsal regions to track worm and worm egg locations.

Area	Included body surfaces
Ventral	Ventral surface of telson & uropods
	Ventral surface of abdomen
	Ventral surface of cephalothorax
	Genital pore & gonopods
	Cephalic area, including all mouth parts
Lateral	Margins of carapace
	Walking legs & chelipeds
Dorsal	Dorsal surface of telson & uropods
	Dorsal surface of abdomen
	Carapace
	Rostrum, including around eyes & antennae/antennules

FIGURE LEGENDS

Fig. 1. Counts of large worms, small worms, worm eggs on female crayfish. Bars represent mean+1SE. ** denotes $p<0.01$.

Fig. 2. Spatial distribution of A) large worms, B) small worms, and C) worm eggs on female crayfish. Bars represent mean+1SE. ** denotes $p<0.01$, *** denotes $p<0.001$.

Fig. 1.

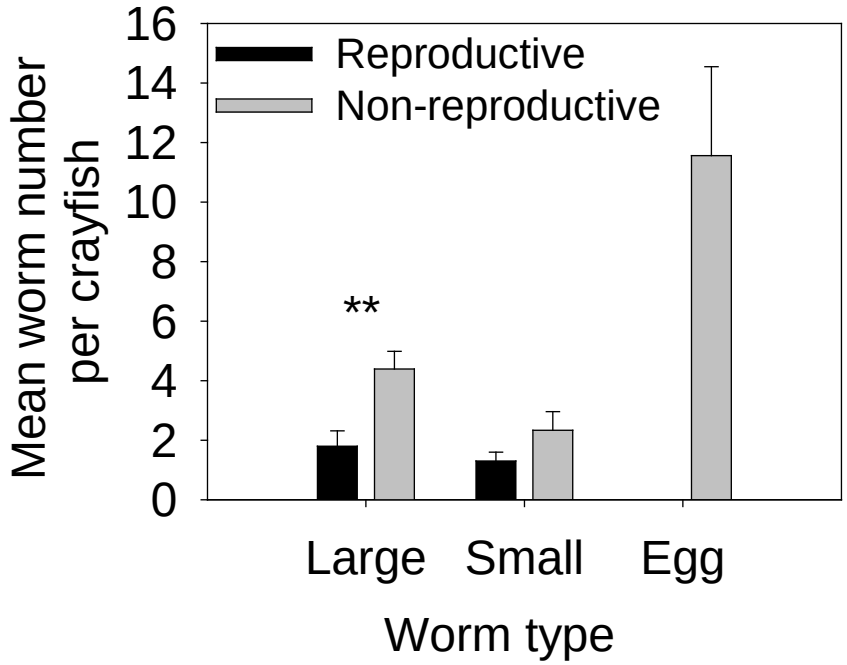
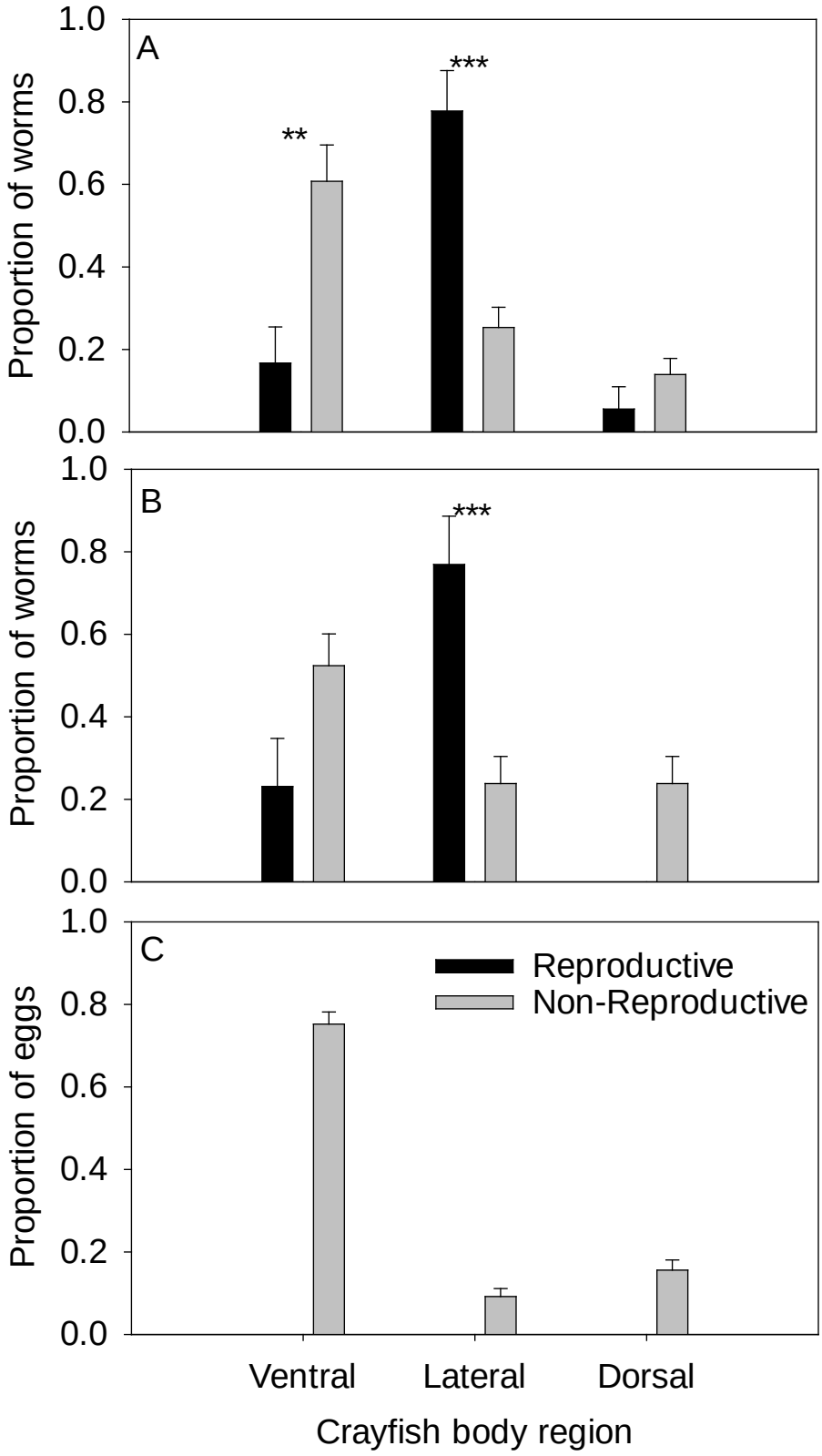

Mean worm number per crayfish
16
14
12
10
8
6
4
2
0
Reproductive
Non-reproductive
**
Large
Small
Egg
Worm type

Fig. 2.


A
B
C
**
***
***
Proportion of worms
Proportion of worms
Proportion of eggs
1.0
0.8
0.6
0.4
0.2
0.0
Reproductive
Non-Reproductive
Ventral
Lateral
Dorsal
Crayfish body region

CHAPTER 3

ABSTRACT

PREVENTING OVEREXPLOITATION IN A MUTUALISM: PARTNER CONTROL IN THE CRAYFISH-BRANCHIOBDELLID SYMBIOSIS

Interspecific mutualisms require benefits received to exceed costs incurred for both partners, and if costs outweigh benefits for either partner, overexploitation occurs. Partner control by one or both participants can help prevent such overexploitation. In a symbiosis between crayfish and branchiobdellid worms, the worms can improve crayfish survival and growth by removing fouling material from the gills. However, overexploitation by the worms through damage to crayfish gill filaments is also possible. Here, I used behavioral observations to assess how partner control affects worm density on crayfish by documenting crayfish grooming behaviors and relating them to worm retention on the crayfish. I found that crayfish can actively reduce worm densities through worm-directed grooming behaviors. The proportion of total grooming directed at the worm differed between co-existing crayfish species, and also as a function of how many worms were present on the crayfish. *Orconectes cristavarius* removed a single stocked branchiobdellid more often than did *Cambarus chasmodactylus*, which corresponds to observed differences in worm density on these crayfish species in the field. I also assessed a possible explanatory factor in worm association patterns of the two crayfish species; the propensity for crayfish hemolymph to kill bacteria. *O. cristavarius* hemolymph inhibited test bacteria more effectively than did *C.*

chasmodactylus hemolymph. I conclude that crayfish use partner control behaviors to maintain worm densities at levels where gills are cleaned while minimizing the potential for gill damage, and that these levels may vary between crayfish species.

INTRODUCTION

Cleaning symbioses involve a cleaner organism removing bacteria, parasites, or other epibionts and detritus from a client. These interactions have been identified for both aquatic and terrestrial taxa (Attwell 1966; Losey 1972; Poulin and Grutter 1996; Brown et al. 2002, 2012). Cleaning symbioses are often considered textbook examples of mutualism since cleaners receive nourishment and clients should benefit from reduced parasite loads and the removal of diseased or necrotic tissue (Bshary et al. 2007). Despite these potential benefits, cleaning symbioses are rarely benign interactions. Cheating has been identified in multiple cleaning symbioses when cleaners remove client tissues along with epibionts and fouling material (Weeks 1999; Bshary and Grutter 2002; Hay et al. 2004). Partner control behaviors can help prevent the symbiosis from shifting from mutualism to parasitism through preemptive prevention of overexploitation or punishment of cheating partners (Bshary and Grutter 2002; Johnstone and Bshary 2002; Soares et al. 2008).

Crayfish and branchiobdellid worms (Annelida: Branchiobdellidae) are involved in cleaning symbioses (Brown et al. 2002, 2012; Lee et al. 2009). Brown et al. (2002) found that the branchiobdellid *Cambarincola ingens* engages in a cleaning symbiosis with the New River crayfish, *Cambarus chasmodactylus*, in which the worms remove fouling epibionts and particulate matter from the crayfish exoskeleton and gills. In a laboratory experiment, the presence of *C. ingens* increased growth rates in *C. chasmodactylus* while simultaneously

reducing crayfish mortality (Brown et al. 2002). Field experiments have also found positive effects of intermediate densities of worms on crayfish growth (Brown et al. 2012).

Surveys of *C. chasmodactylus* indicate that worm abundance is strongly correlated with crayfish carapace length (CL), with larger crayfish harboring more branchiobdellids, as has been seen on other species of crayfish that host branchiobdellids (Young 1966; Brown and Creed 2004). When crayfish were stocked with worms at densities above the observed field levels, the worms reduced crayfish growth compared to crayfish without worms (Brown et al. 2012). This result suggests that the crayfish-worm interaction may shift from a mutualism when worms are at intermediate densities to a parasitism at high worm densities. In such situations, partner control by the crayfish would be expected, so that worm densities remain below those at which the host is harmed.

The crayfish *Orconectes cristavarius* co-occurs with *C. chasmodactylus* in much of the New River, NC (Helms and Creed 2005; Fortino and Creed 2007), but rarely harbors *C. ingens* or any other branchiobdellids or branchiobdellid eggs (Brown and Creed 2004). Field surveys in the summers of 2010 and 2011 found that branchiobdellids were present on only 10 of 171 *O. cristavarius* examined (5.8%). Of those individuals with worms, all but two crayfish hosted a single worm. No branchiobdellid eggs were observed on *O. cristavarius*. In a laboratory experiment, *C. ingens* preferentially colonized *C. chasmodactylus* rather than *O. cristavarius*, even when worms were collected from *O. cristavarius* in the field (Brown and Creed 2004). Brown and Creed (2004) hypothesized that these differences in worm preference could be due to differences in crayfish behaviors and activity levels, as *O. cristavarius* tend to be more active than *C. chasmodactylus*. Thus worms may choose to

colonize a host on which they are more likely to derive benefits and/or not be lost as a result of crayfish activity (Brown and Creed 2004).

While the worms may play a role in choosing their host, the crayfish may also be engaged in maintaining ectosymbiont populations (Gelder and Smith 1987). If this is the case, differences in worm numbers between *C. chasmodactylus* and *O. cristavarius* may be due to the crayfish exerting different levels of partner control on the branchiobdellids. Here, I present the results of two laboratory experiments that examined crayfish responses to branchiobdellid worms, with the goal of determining whether crayfish actively regulate branchiobdellid populations. In the first experiment, *C. chasmodactylus* was stocked with 10 large branchiobdellids to determine whether grooming behaviors change when worms are present at densities well above those observed in the field. In the second experiment, grooming behaviors of *O. cristavarius* and *C. chasmodactylus* were observed in response to the presence of a single, large branchiobdellid. A single large worm was used since this represents an increase over the mean number of worms found on *O. cristavarius* (mean ± 1 SE = 0.07 ± 0.02, n = 171). A third experiment was then conducted to determine whether crayfish hemolymph is able to inhibit the growth of bacteria. If circulating hemolymph contains compounds that inhibit bacterial growth, it could limit bacterial gill fouling and thus minimize potential benefits of cleaning by the worms. In this way, I assessed whether antibacterial properties of crayfish hemolymph may be responsible for differences in worm-directed grooming behavior between *O. cristavarius* and *C. chasmodactylus* observed in experiment two.

METHODS

Specimen collection for behavioral observations

Orconectes cristavarius and *C. chasmodactylus* were collected during July and August 2010 from the South Fork and Middle Fork of the New River in Watauga County, North Carolina, USA. I captured 17 *C. chasmodactylus* (CL 24-39 mm) for the 10-worm experiment, and an additional 12 individuals of each crayfish species for the single worm experiment (*O. cristavarius* CL 24-43 mm, *C. chasmodactylus* CL 23-39 mm).

Cambarincola ingens were collected from *C. chasmodactylus* captured in the Middle Fork of the New River. Probes were used to coax the worms from the crayfish, and the worms were placed in a glass dish containing stream water for holding. After removal of large branchiobdellids, all crayfish were immersed for 5 minutes in a 10% solution of magnesium chloride hexahydrate to kill any unseen worms and worm eggs (Brinkhurst and Gelder 2001; Brown et al. 2002).

Behavioral observations: 10-worm *C. chasmodactylus*

In this experiment, 10 worms were placed on *C. chasmodactylus* to assess grooming behaviors and potential partner control. During the observations, crayfish were individually isolated in 38 L aquaria containing 19 L of clear, aerated stream water. Crayfish were placed in aquaria for 30 minutes prior to observation to acclimate to the aquarium environment. After the rest period, each crayfish was stocked with 10 large *C. ingens*, 5 each on the dorsal

carapace and ventral abdomen. After placement, each crayfish was held in a shallow pan of water to ensure that the worms had firmly attached to the exoskeleton. The crayfish was then returned to its aquarium, whereupon the 30-minute behavioral observation period commenced.

During the observation period, I recorded behaviors in the following categories: feeding, locomotion, general grooming, and directed grooming (Table 1). I noted the time that each behavior occurred, along with any details about the behavior, including which legs were involved in general and directed grooming. I watched only one crayfish at a time.

After 30 minutes, the crayfish were examined under a dissecting microscope to confirm the location of the worms. Each crayfish was then returned to its aquarium, given a brick for refuge, two shrimp pellets, and left overnight. I checked each crayfish after 24, 48, and 72 hours to ascertain worm retention. Worm-directed grooming was analyzed using both one-way analysis of variance, using crayfish sex as the test factor, and linear regression, with crayfish carapace length as the independent variable and worm-directed scratches and grabs as the dependent variable. Worm removal after 30 minutes and worm retention after 72 hours were each analyzed using one-way analysis of variance, using crayfish sex as the test factor.

Behavioral observations: *O. cristavarius* vs. *C. chasmodactylus*

These observations used a similar protocol as the experiment detailed above, but aimed to assess differences in grooming behavior between *O. cristavarius* and *C. chasmodactylus* in response to a single worm. Since previous research documented differences in behavior between the two crayfish species (Fortino and Creed 2007), I performed two sets of behavioral observations for each crayfish, corresponding to before and

after branchiobdellid placement. The first no-worm observation period was to ascertain background levels of grooming, feeding, and escape behaviors for each individual. This time also allowed the crayfish to acclimate to the aquarium.

A single branchiobdellid worm was then placed on each crayfish, with half of the crayfish having the worm placed on the dorsal carapace and half on the ventral abdomen. After confirming firm attachment of the worm to the exoskeleton, each crayfish was returned to its aquarium, whereupon the second 30-minute observation period commenced. The same behavior classifications were used as in the 10-worm *C. chasmodactylus* observation (Table 1). If worm removal was observed prior to the end of the 30-minute session, observation ceased. All other crayfish were checked under a dissecting microscope after 30 minutes to confirm the worm location, and 24 hours post-observation to record worm retention. Data were analyzed using one-way analysis of variance, with crayfish species as the test factor.

Hemolymph antimicrobial assay

I performed a radial diffusion assay to assess whether the antibacterial properties of hemolymph differed between *O. cristavarius* and *C. chasmodactylus*. Crayfish were captured using dip nets, and held in water-filled 5-gallon buckets prior to hemolymph extraction. Hemolymph samples were collected from each crayfish using a 25 gauge, 1 mL sterile syringe. The unscleretized basal joint of the first walking leg was swabbed with alcohol and up to 0.5 mL of hemolymph was extracted from the joint and injected into a sterile microcentrifuge tube. Crayfish were then held in a separate water-filled bucket to recover prior to release. A total of 26 *O. cristavarius* and 17 *C. chasmodactylus* were collected from the South Fork of the New River during two sampling dates in August 2011.

In the lab, whole hemolymph was assayed against a representative Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacterium, as well as against an unknown bacterium isolated from the gills of *C. chasmodactylus*. *S. aureus* and *E. coli* were grown from pure culture in Luria-Bertani (LB) broth, while the gill bacterium was cultured in LB broth and isolated using serial dilution on LB agar plates. The isolated colonies were then resuspended in LB broth. Fresh cultures of each bacterium were prepared 12 hours prior to use to ensure bacterial cells in mid-logarithmic phase.

To perform the radial diffusion assay for each hemolymph sample, I plated and spread 100 µL of each bacterial culture onto individual sterile LB agar plates. Plates were allowed to sit for 5 minutes to ensure complete absorption of culture broth. Three sterile 6 mm blank discs were then placed on each agar plate using flame-sterilized forceps and pressed gently to ensure complete contact with the agar surface. A crayfish hemolymph sample was then vortexed for 5 seconds and a 25 µL aliquot of whole hemolymph was pipetted onto each blank disc. Hemolymph from each crayfish was plated individually. Control plates were prepared as above, using either 95% ethanol or 1X phosphate buffered saline in place of crayfish hemolymph. Agar plates were inverted and incubated at 20°C for 24 hours.

After 24 hours, I measured the diameter of the microbial growth inhibition zone that developed around each disc. Each agar plate was photographed using a high-resolution digital camera (Canon EOS Rebel T1i) mounted on a dissecting microscope (Meiji Techno RZ). Inhibition diameters were then measured from the photographs using iSolution software (Image & Microscope Technology Inc.). Inhibition of each test microbe was analyzed using one-way analysis of variance, with crayfish species as the test factor.

RESULTS

10-worm *C. chasmodactylus* experiment

During the 30-minute observations, worm-directed grooming by male crayfish accounted for a larger proportion of total grooming behaviors than did worm-directed grooming by female crayfish (Fig. 1a, $F_{1,15} = 8.816$, $p = 0.010$). When examined by size, smaller (25-29 mm CL) males performed marginally more worm-directed grooming than larger (32-35 mm CL) males (Fig. S1, $F_{1,6} = 4.909$, $p = 0.069$, $r^2 = 0.45$). In contrast, female crayfish showed no relationship between size and worm-directed grooming (Fig. S1, $F_{1,7} = 0.117$, $p = 0.742$, $r^2 = 0.016$) during the first 30 minutes.

Crayfish removed worms during the 30-minute observation period. Overall, males removed more worms than females (Fig. 1b, $F_{1,14} = 12.403$, $p = 0.003$). By 72 hours post-observation, however, there was no longer a difference in the number of worms remaining on male and female crayfish (Fig. 1c, $F_{1,14} = 1.512$, $p = 0.239$). Among females, final worm number was positively correlated with carapace length (Fig. S2, $F_{1,6} = 6.023$, $p = 0.050$, $r^2 = 0.501$). No significant relationship between crayfish size and final worm number was observed for males.

O. cristavarius vs. *C. chasmodactylus* experiment

Orconectes cristavarius and *C. chasmodactylus* responded differently to stocking with a single branchiobdellid. Eight of the 12 observed *O. cristavarius* performed worm-directed grooming during the observation period, while only one *C. chasmodactylus* exhibited worm-directed grooming. The mean (± 1 SE) number of worm-directed grooming behaviors was greater for *O. cristavarius* than *C. chasmodactylus*, with *O. cristavarius* performing an average of 2.00 ± 0.71 worm-directed scratches and grabs, while *C. chasmodactylus* performed 0.08 ± 0.08 ($H_1 = 7.036$, $p = 0.008$). Worm-directed grooming also accounted for a greater proportion of total grooming behaviors in *O. cristavarius* (Fig. 2, *Orc.* vs. 1-worm *Cam.* $F_{1,22} = 9.419$, $p = 0.006$).

Whether or not a worm was removed during the observation period was not affected by initial placement on the dorsal or ventral surface. All worms moved around the dorsal and ventral surfaces of the crayfish during the observation period. During this period, 3 *O. cristavarius* removed and consumed their branchiobdellid, while all *C. chasmodactylus* retained their worm, leading to marginally significant differences in worm retention between the two crayfish species after thirty minutes ($F_{1,22} = 3.667$, $p = 0.069$). Two of the three *O. cristavarius* that consumed their worm had worm-directed grooming rates greater than the average among *O. cristavarius*. After 24 hours, all *C. chasmodactylus* still retained their worm, while two additional *O. cristavarius* had removed their worm.

Hemolymph antimicrobial assay

Whole hemolymph from both *O. cristavarius* and *C. chasmodactylus* inhibited the growth of all three test microbes. For *E. coli*, the size of the microbial inhibition zone was

significantly larger for *O. cristavarius* than for *C. chasmodactylus* (Fig. 3, $F_{1,41} = 22.415$, $p < 0.001$). There was no significant difference in the size of the inhibition zone for *S. aureus* between the two crayfish species (Fig. 3, $F_{1,41} = 1.714$, $p = 0.198$), while the size of the inhibition zone created by *O. cristavarius* tended to be larger than that of *C. chasmodactylus* for the unknown gill bacterium (Fig. 3, $F_{1,41} = 3.557$, $p = 0.066$).

DISCUSSION

Mutualisms involve reciprocal exploitation (Herre et al. 1999), and partner control by one or both partners is necessary to prevent overexploitation. My results demonstrate that in crayfish-branchiobdellid symbioses, crayfish play an active role in managing the abundance of potential worm partners. The amount of partner control observed varied with the number of worms present, which in turn corresponded to worm loads observed on crayfish in the field.

When stocked with a single worm, only one *C. chasmodactylus* exhibited worm-directed grooming behaviors, and none of the *C. chasmodactylus* removed its worm either during the observation period or within 24 hours post-observation. In the field, *C. chasmodactylus* in the size range I observed typically harbor 3 to 10 total branchiobdellids (Brown and Creed 2004). Because the experimental worm density was well below the level observed in the field, antagonistic behaviors were rare, and all observed *C. chasmodactylus* retained their worm during the experiment. However, when *C. chasmodactylus* were stocked above typical field-observed densities of large worms, the amount of grooming directed at the branchiobdellids increased. During the 10-worm observation experiment, worm-directed grooming by males was especially pronounced, and smaller males made more attempts to remove their worms than larger males. This is not unexpected as smaller (25-29 mm CL) crayfish in the field typically host fewer worms than larger (32-35 mm CL) crayfish (Brown and Creed 2004). Increased worm removal behaviors by male crayfish corresponded to

higher rates of worm removal by males during the observation period. However, after 72 hours the number of remaining worms on male *C. chasmodactylus* was not significantly different from that of female crayfish.

Cambarus chasmodactylus benefits from its association with branchiobdellids through increased growth rates and reduced mortality when worms are present at intermediate densities (Brown et al. 2002, 2012). However, high densities of branchiobdellids increase the likelihood that worms could become food-limited and feed on crayfish gill tissue instead of grazing for epibionts and detritus on the gills (Brown et al. 2012). This would drastically increase the costs of the interaction to the crayfish, through loss of hemolymph from worm-bitten gill filaments. Indeed, a highly significant relationship exists between branchiobdellid density and gill scarring on *C. chasmodactylus*, suggesting that at high densities, the worms do feed more heavily on crayfish tissues (Brown et al. 2012). In such instances, the interaction would shift from mutualism toward parasitism (Ewald 1987), as the costs of maintaining high worm densities exceed the benefits received by the crayfish.

The density of worms that can be supported while maintaining net benefits depends on the size of the crayfish (Fig. 4). Field surveys indicate that there is a significant positive relationship between *C. chasmodactylus* carapace length and the number of total branchiobdellids maintained on the crayfish, with worms rarely found on *C. chasmodactylus* below a carapace length of ~20 mm (Brown and Creed 2004). In size ranges where the crayfish does usually host worms, the worms likely act as commensals when fewer than the optimal number are present, as the worms gain food without providing sufficient cleaning to affect crayfish fitness. In contrast, too many worms could lead to parasitism through gill

damage. Active removal of worms by the crayfish can therefore help maintain worm densities at a level where they derive benefits from removal of detritus and epibionts but do not incur costs due to damage of the gill filaments.

The worm densities at which shifts from commensalism to mutualism to parasitism occur are predicted to increase with increases in crayfish body size (Fig. 4). Also note that the sequence of interaction type may differ from the traditional mutualism-commensalism-parasitism transition (Ewald 1987; Bronstein 1994). Low numbers of worms on crayfish may have little if any effect on their crayfish hosts; at these worm densities the interaction may initially be commensalism (Lee et al. 2009). As worm densities approach optimal numbers at which gills are cleaned but little gill damage occurs, the association should shift to a mutualism (Brown et al. 2002, 2012). At higher worm densities, significant gill damage is likely to occur and the association becomes a parasitism (Brown et al. 2012). If crayfish are able to exert some partner control and reduce worm numbers, the association will be a weak parasitism. If crayfish are weakened by environmental stress (e.g., extreme temperature events, pollutants) or disease and are unable to remove worms then the association may shift towards strong parasitism which may lead to the death of the crayfish (Quaglio et al. 2006).

There may be context dependence with respect to the threshold at which the outcome of a cleaning interaction shifts. In the crayfish/branchiobdellid system, the primary drivers of this threshold appear to be gill fouling rate and client size. Lee et al. (2009) observed that in environments with low fouling pressure, branchiobdellid cleaners appear to act as commensals, while mutualistic benefits were obtained by crayfish in high-fouling environments. Evidence from the coral reef cleaner fish system corroborates that the outcome of cleaning is context-dependent, as the costs and benefits of being cleaned can vary as a

function of client parasite loads (Cheney and Côté 2005). In environments where client fish had high ectoparasite loads, cleaner fish consumed primarily parasites, suggesting that the association between cleaners and client fish was a mutualism. Conversely, when clients hosted few ectoparasites, cleaners primarily consumed client mucus and scales and the association was considered to be a parasitism (Cheney and Côté 2005).

As in the crayfish-branchiobdellid interaction, partner control behaviors help limit opportunities for cheating between partners in other cleaning symbioses (Bronstein 1994). Coral reef cleaner fish can readily exploit their interactions with clients by removing mucus and scales from clients rather than feeding strictly on ectoparasites (Gorlick 1980; Grutter 1997; Grutter and Bshary 2003; Cheney and Côté 2005). Client fishes use partner controls to check such costly negative interactions. The client fish may jolt, rapidly moving its body to prevent further nips by the cheating cleaner, or may utilize different cleaning stations in the future to avoid repeated interaction with the cheating cleaner (Bshary and Grutter 2002; Bshary and Schäffer 2002; Grutter and Bshary 2003; Soares et al. 2008). Similarly, oxpeckers often exploit their cleaning interaction by picking at wounds on large grazers to consume host blood and tissues instead of grazing exclusively on ticks (Weeks 1999). If left unchecked, exploitation by cheating partners exacts a fitness cost on the client (Ewald 1987; Bronstein 1994). Partner control behaviors help limit the potential for such overexploitation.

I found that the two co-existing crayfish species differed in their response to presumed cleaning symbionts. While both species of crayfish were able to reduce branchiobdellid abundance, their responses varied with respect to the number of worms that triggered removal behaviors. The proportion of overall body grooming directed at a single worm was significantly greater for *O. cristavarius* than for *C. chasmodactylus* with either

one or 10 stocked worms. In addition, more of the observed *O. cristavarius* engaged in worm-directed grooming, and I directly observed three *O. cristavarius* removing and consuming their worm during the observation period. These observations suggest that the low worm numbers observed on *O. cristavarius* in the field are due to this species actively removing worms.

Since co-existing crayfish that vary in their field loads of ectosymbiont worms also differed in antagonistic behaviors directed toward those worms, it appears that worm removal behaviors may drive worm loads in the field. The observation that wild *O. cristavarius* harbor significantly fewer branchiobdellid worms than *C. chasmodactylus* (Brown and Creed 2004) suggests that these crayfish do not derive the same cleaning benefits from the worms as *C. chasmodactylus*. Indeed, the relative costs and benefits of the crayfish/branchiobdellid symbiosis may vary by crayfish species (Brown and Creed 2004). If the presence of branchiobdellids presents a net cost to *O. cristavarius*, I would expect the crayfish to keep worm densities as low as possible to prevent exploitation by the worms. My behavioral observations support this hypothesis, as *O. cristavarius* responded to the addition of a single branchiobdellid with a dramatic increase in grooming behaviors directed at the worm, and in one-quarter of the observations, the worm was removed by the crayfish within thirty minutes. In contrast, the low level of worm-directed grooming seen by *C. chasmodactylus* with either 1 or 10 branchiobdellids supports previous findings that the worms provide net benefits to the crayfish (Brown et al. 2002, 2012).

The observation that one crayfish client (*C. chasmodactylus*) tolerates cleaning from a semi-permanent cleaner while a co-existing crayfish (*O. cristavarius*) actively discourages it presents an interesting contrast to other documented cleaning symbioses. Oxpeckers remove

ectoparasites and glean blood from a wide variety of African ungulates, including but not limited to buffalos, zebras, and hippos (Attwell 1966; Grobler 1980; Hustler 1987; Koenig 1997). While birds in a particular geographic area may prefer some host species over others (Attwell 1966; Grobler 1980; Hustler 1987; Koenig 1997), oxpeckers are mobile cleaners that opportunistically feed from available clients. Similarly, cleaner fish on coral reefs clean a wide variety of client fish species (Limbaugh 1961; Grutter and Poulin 1998a; Arnal et al. 2001; Soares et al. 2007). While the specific mechanisms driving the preference of clients for particular cleaners and vice versa remain unknown (Losey 1972; Grutter and Poulin 1998b; Arnal et al. 2000; Soares et al. 2007), cleaners and clients engage in a series of repeated transitory encounters, and clients can escape from cheating or exploitative cleaners with relative ease (Bshary and Grutter 2002; Bshary and Schäffer 2002; Soares et al. 2008).

In contrast, the cleaning symbiosis between crayfish and branchiobdellids represents a longer-term interaction between client and cleaner. *Cambarincola ingens* can spend its entire life on the crayfish host and appears to lay eggs only on live crayfish (Creed et al. unpub. data). Because the worm depends on its host for reproduction, harming the host would be selected against, and cooperation by the worm is favored (Bull and Rice 1991). However, a scarcity of food resources, due to environmental conditions or high worm population density on a crayfish, could lead to parasitism via feeding on crayfish gill filaments. Differences in food resources between crayfish hosts may lead to the apparent intolerance of the worms by *O. cristavarius*. A lack of worm food items would increase the risk that worms would parasitize *O. cristavarius*, and in turn drive partner control behaviors that would maintain few or no worms on the body of this host.

The degree to which *O. cristavarius* and *C. chasmodactylus* responded to worms was likely related to their ability to potentially manage fouling epibionts via their own immune response. Results from the hemolymph antibacterial assays support this hypothesis, and suggest a possible mechanism to explain differences in worm-directed grooming, and in turn worm load, between *O. cristavarius* and *C. chasmodactylus*. Whole hemolymph from *O. cristavarius* was able to inhibit the growth of *E. coli* and the unknown bacterium isolated from crayfish gills more than the hemolymph of *C. chasmodactylus*, suggesting that *Orconectes* may able to more effectively limit the growth of microbes on its gill surfaces. Antibacterial activity has been found in the hemocytes of multiple crustacean species, including crabs, lobsters, and shrimps, and studies have found that crustacean hemocytes are mainly active against Gram-negative bacteria (Bartlett et al. 2002). Indeed, a specific antimicrobial peptide, callinectin, from the blue crab *Callinectes sapidus*, has been shown to have particularly high activity against Gram-negative *E. coli* (Khoo et al. 1999).

Hemolymph assays of the freshwater crayfish *Pacifastacus leniusculus* found that in isolation, the antimicrobial peptide (AMP) astacidin 2 inhibited the growth of a variety of Gram-positive and Gram-negative microbes, including both *E. coli* and *S. aureus* (Jiravanichpaisal et al. 2007). However, the minimum concentration of astacidin 2 needed to inhibit *S. aureus* was twice the concentration needed to inhibit *E. coli* (Jiravanichpaisal et al. 2007). In my assays, the hemolymph from both *O. cristavarius* and *C. chasmodactylus* inhibited *E. coli* better than it did the Gram-positive *S. aureus* or the unknown gill bacterium. While my study did not assess specific AMPs, my results suggest that *O. cristavarius* and *C. chasmodactylus* harbor AMPs or other antimicrobial compounds that have an impact on bacterial growth. The reduced inhibition of *S. aureus* by whole crayfish hemolymph

compared to inhibition of *E. coli* may be due to differences in sensitivity of the microbes to the hemolymph antimicrobial compounds, as was seen with astacidin 2 in *P. leniusculus*.

My results suggest that differences in partner control behaviors drive observed differences in branchiobdellid worm loads on sympatric crayfish species in the field. The results of my hemolymph antimicrobial assays present a possible mechanism to explain differences in the responses of two co-occurring crayfish to cleaning symbionts. The innate immune function of *O. cristavarius* may limit food resources available to the worms on the gills and exoskeleton, lowering the threshold at which the worms would become parasitic, driving the crayfish to rapidly remove colonizing worms. These findings suggest that similar cross-species comparisons may help pinpoint mechanisms that drive the establishment and maintenance of other cleaning mutualisms, and in turn help resolve the issue of why symbioses occur between particular species and not others.

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Table 1: Recorded general and worm-directed grooming behaviors

Behavior	Description
General Grooming	
Scratches	Using walking legs to clean carapace, abdomen, chelae, or other legs
Antennal grooming	Cleaning of antennae and antennules with maxillipeds
Worm-Directed Grooming	
Scratches	Using walking legs to scrape in close proximity to worm
Grabs	Using claw on 1st or 2nd small walking leg to grab at worm

FIGURE LEGENDS

Fig. 1 Worm-directed grooming and worm removal by male and female *Cambarus chasmodactylus* stocked with 10 large branchiobdellids. a) Worm-directed grooming during 30-minute behavioral observation, expressed as percent of total observed grooming. b) Worms removed during initial 30-minute observation period. c) Worms remaining 72 hours after observation. All values expressed as mean ± 1SE, all analyses based on one-way ANOVA.

Fig. 2 Worm-directed grooming as a function of crayfish species and worm number, expressed as percent of total observed grooming (mean ± 1SE). There was a highly significant effect of crayfish species on worm-directed grooming, based on one-way ANOVA ($F_{2,38} = 8.142$, $P = 0.001$). Letters indicate significant differences ($p \leq 0.05$) between groups based on Tukey's post-hoc test.

Fig. 3 Microbial growth inhibition of *E. coli*, *S. aureus*, and the unidentified gill bacterium (Gill bac.) by whole crayfish hemolymph. Inhibition is expressed as the diameter of the inhibition zone (mean ± 1SE) created by hemolymph from *O. cristavarius* and *C. chasmodactylus* for each assay organism. All analyses based on one-way ANOVA.

Fig. 4 Predicted shifts in the relationship between *C. chasmodactylus* and branchiobdellids as a function of crayfish carapace length and worm abundance. The mutualism line intercepts the X-axis to the right of the origin to signify that worms are rarely found on small, young-of-the-year crayfish. Dotted line indicates predicated worm load in the field.

Fig. 1

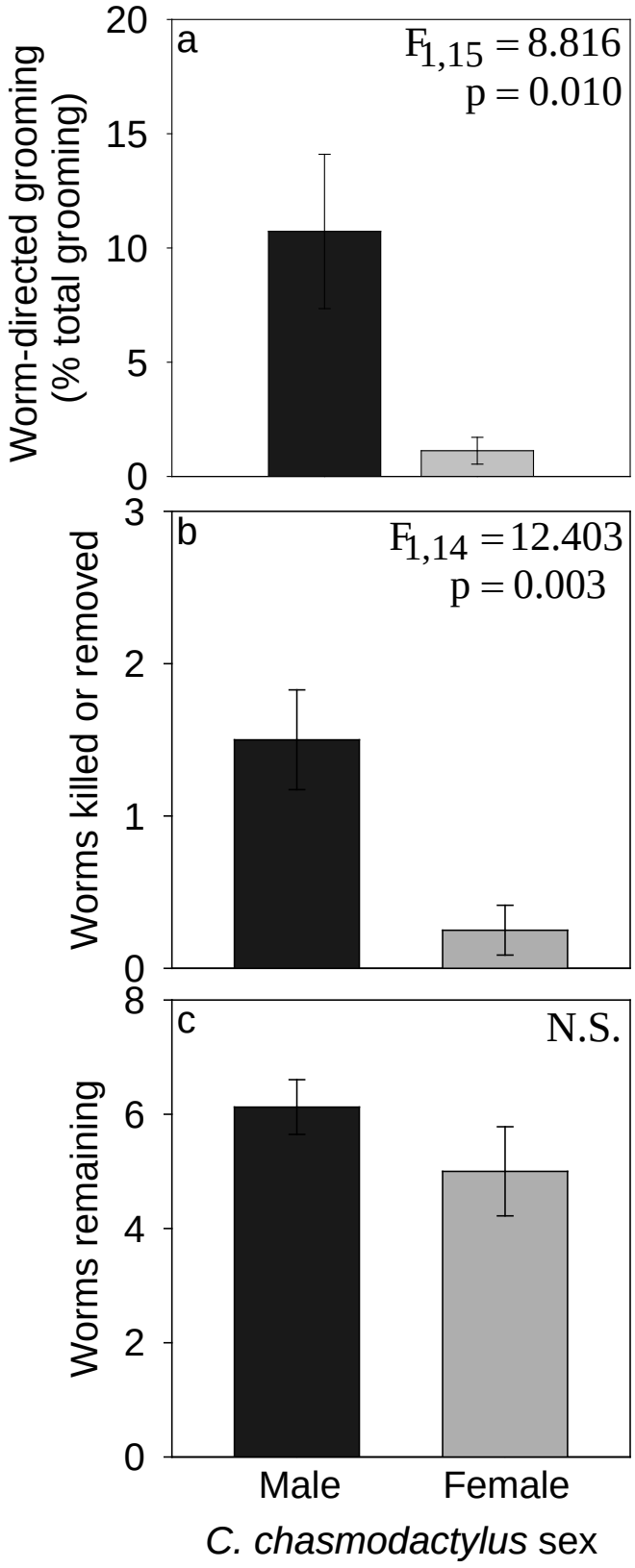

a
$F_{1,15} = 8.816$
$p = 0.010$
Worm-directed grooming
(% total grooming)
20
15
10
5
0
b
$F_{1,14} = 12.403$
$p = 0.003$
Worms killed or removed
3
2
1
0
c
N.S.
Worms remaining
8
6
4
2
0
Male
Female
C. chasmodactylus sex

Fig. 2

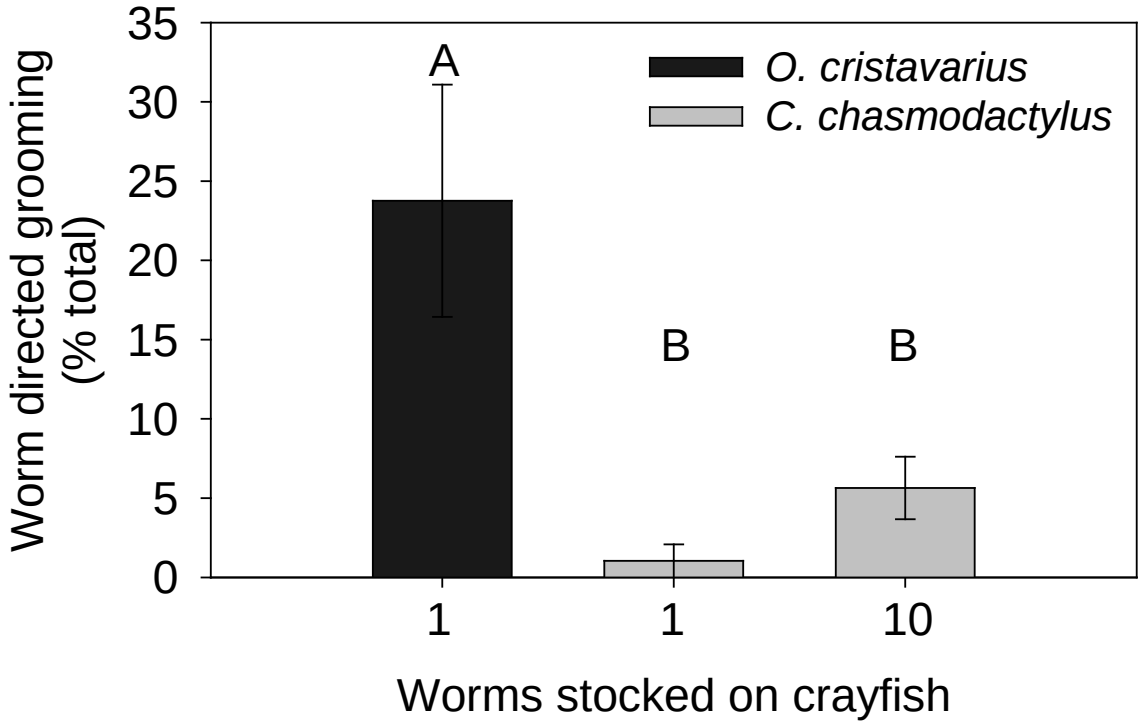

Worm directed grooming
(% total)
35
30
25
20
15
10
5
0
A
B
B
O. cristavarius
C. chasmodactylus
1
1
10
Worms stocked on crayfish

Fig. 3

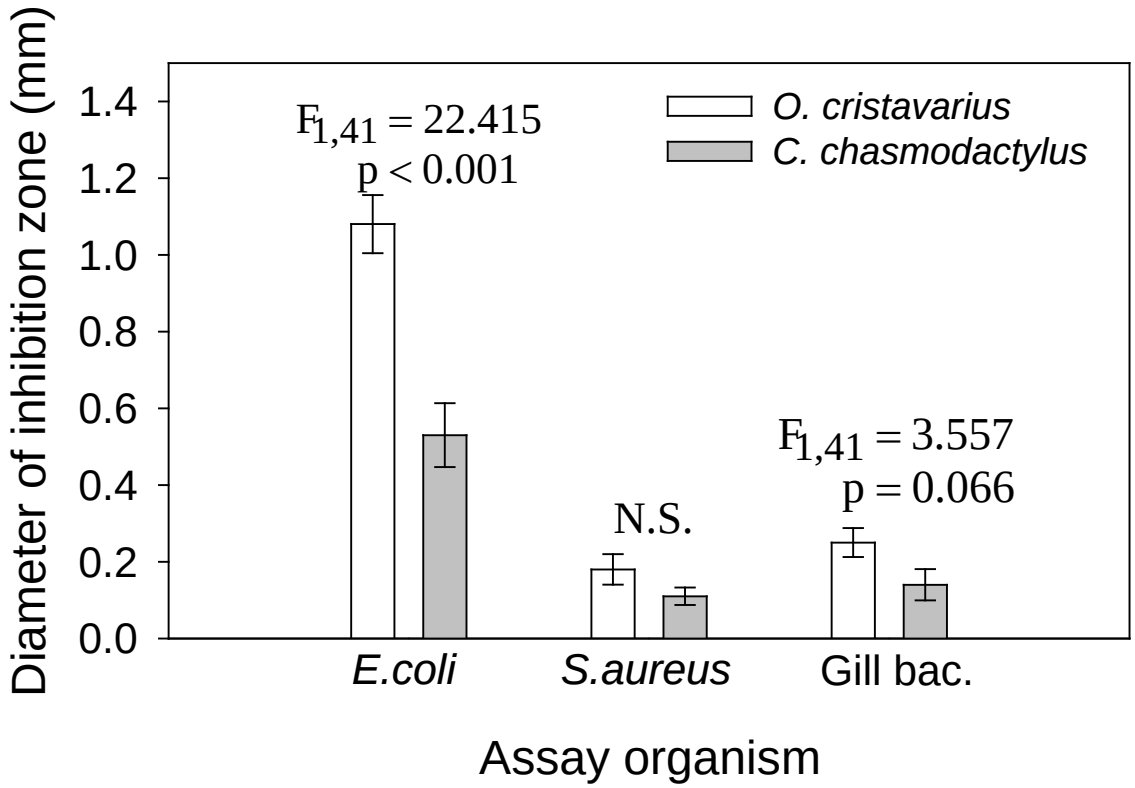
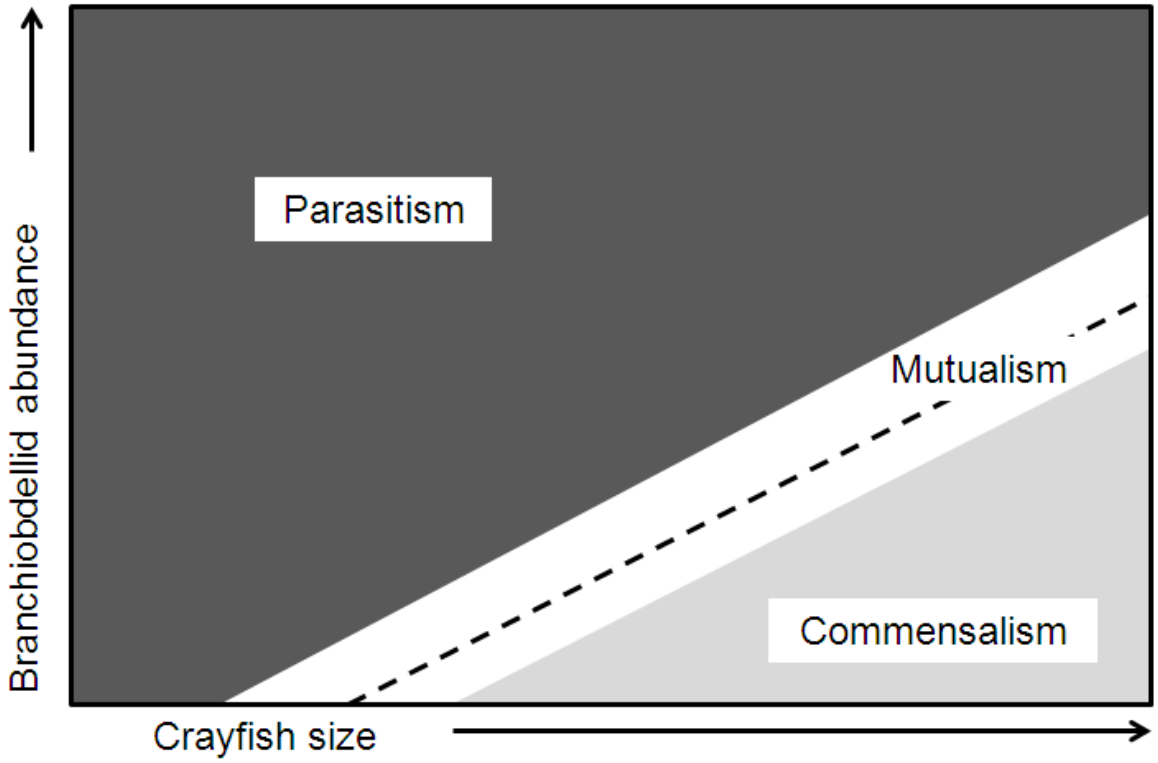

Diameter of inhibition zone (mm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
F1,41 = 22.415
p < 0.001
O. cristavarius
C. chasmodactylus
N.S.
F1,41 = 3.557
p = 0.066
E.coli
S.aureus
Gill bac.
Assay organism

Fig. 4


Parasitism
Mutualism
Commensalism
Branchiobdellid abundance
Crayfish size

CHAPTER 4

ABSTRACT

INFLUENCE OF ENVIRONMENT ON EPIBIOTIC FOULING: DIRECT MEASURES OF MICROBIAL FOULING OF CRAYFISH GILLS

Cleaner organisms benefit their clients by removing fouling material from body surfaces. The benefits derived by clients depend on the amount of fouling they experience, as heavily parasitized or fouled organisms are predicted to experience greater positive effects of being cleaned. In a cleaning symbiosis between crayfish and branchiobdellid worms, environmental conditions, and particularly fouling pressure, appear to influence whether the worms are commensal or mutualistic partners, though direct measures of crayfish fouling have not been made to date. Here, we directly assess differences in microbial fouling of crayfish gills in different environments. We used a laboratory experiment to determine rates of microbial fouling when the source of bacteria was just the water. A field experiment allowed us to assess gill fouling in realistic stream conditions in which crayfish had access to stream sediments. We found that crayfish exposed to stream substrate experienced significantly more microbial gill fouling than those maintained in stream water alone, suggesting that stream sediments provide an important substrate from which microbes contact their crayfish host. Our experiments provide the first direct evidence that environmental conditions drive fouling on crayfish gills, which may in turn dictate the nature of the crayfish client's relationship with branchiobdellid cleaners.

INTRODUCTION

Cleaning symbioses, in which a cleaner organism removes epibiont parasites, detritus, and other fouling material from a client organism, have been documented in a variety of taxa from diverse environments (Attwell 1966; Losey 1972; Poulin & Grutter 1996). In these interactions, the client presumably benefits from the removal of fouling material, while the cleaner gains nutrition from the materials gleaned from the client. Relative benefits can vary with environmental factors that affect fouling pressure, as well as with the density of cleaners (Cheney & Côté 2005; Lee et al. 2009; Brown et al. 2012).

Freshwater crayfish and branchiobdellid worms (Annelida: Branchiobdellidae) engage in cleaning symbioses in which the worms inhabit the crayfish exoskeleton, and remove detritus and fouling epibionts from the crayfish exoskeleton and gills (Brown et al. 2002, 2012; Lee et al. 2009). Environmental factors have been shown to affect the nature of the relationship between crayfish and their branchiobdellid cleaners (Lee et al. 2009). In an environment with low levels of gill fouling, worms had a commensalistic relationship with crayfish, while the greatest benefits of hosting worms were gained by crayfish exposed to high-fouling environments (Lee et al. 2009). While Lee et al. (2009) indicated that fouling pressure may determine the nature of the symbiosis, their experiment lacked direct measures of crayfish fouling in each environment.

Fouling of crayfish body surfaces can affect olfaction, respiration, and the incubation of embryos (Fisher 1977; Bauer 1979, 1989). Epibiotic microbial growth on crustaceans has

been linked to environmental water quality, with nutrient-enriched water increasing the number of fouling epibionts (Fisher 1977), while the enclosed gill chamber, with its rapid respiratory flow, is thought to particularly favor epibiont growth on the crayfish gill surfaces (Bauer 1989). These factors likely contribute to observed differences in the nature of the interaction between crayfish and branchiobdellid worms, as nutrient-enriched waters should favor fouling, which in turn increases potential benefits provided by the worms. However, direct measures of gill fouling are needed to test this hypothesis. Here, we present the results of two experiments designed to assess the amount of microbial gill fouling experienced by crayfish in different environments. A laboratory experiment was conducted to determine fouling levels in a low-fouling environment in the presence and absence of worms. A field enclosure experiment was conducted to assess microbial fouling when crayfish were exposed to stream water and sediments, again in the presence and absence of worms. The results of these experiments provide a direct measure of microbial fouling on crayfish gills, which in turn could explain the shifting nature of crayfish/branchiobdellid symbioses in different environments.

METHODS

Laboratory Experiment

We conducted a laboratory experiment in early autumn 2010 to determine the level of microbial fouling on crayfish gills when crayfish were exposed to only stream water. Study organisms were *Cambarus chasmodactylus* (JAMES 1966) crayfish and their ectosymbiont worms, *Cambarincola ingens* (HOFFMAN 1963). We crossed worm status (present or absent) with sample date (3 weeks or 6 weeks), and sixteen crayfish (30-34 mm carapace length, CL) captured from the South Fork of the New River (Watauga County, NC, USA) were then randomly assigned to one of the four treatments (n=4 for each treatment). Large branchiobdellids were gently removed from all crayfish using a probe and placed in a water-filled glass dish for holding. After worm removal, all crayfish were immersed for 5 minutes in a 10% magnesium chloride hexahydrate solution to kill any unseen worms, as well as kill bacteria present on the gills (Hotchkiss 1923; Brown et al. 2002). We then stocked crayfish in the worm-present treatments with 3 or 4 large branchiobdellids (30-32mm CL crayfish receiving 3 large worms, 33-34mm CL receiving 4 large worms). Crayfish were maintained in 38L aquaria with 19L of aerated stream water collected from the South Fork of the New River by skimming water from the surface to minimize the presence of stream sediments. Crayfish were also provided with a single brick for refuge. Aquaria were arranged in a randomized block design in the laboratory, and exposed to a 14:10 light:dark cycle and a constant water temperature of 22°C. Crayfish were each given two shrimp pellets every other

day, and half the aquarium water was replaced with fresh stream water twice per week to prevent the accumulation of excessive crayfish wastes.

The eight crayfish in the 3-week treatments were sacrificed after 21 days. Prior to removing the carapace, we recorded the number of worms remaining on worm-stocked crayfish. Immediately after carapace removal, podobranch gills from each walking leg were removed and preserved in individual vials of 2.5% glutaraldehyde. One gill from the 5th walking leg was prepared for scanning electron microscopy (SEM) by serial ethanol dehydration followed by critical point drying. Dried gills were immediately sputter coated with a 2 nm layer of gold. Crayfish in the six-week treatments were processed using the same techniques after 41 days, but are not included in this report.

Field Experiment

We conducted a field experiment in the South Fork of the New River in the summer of 2011 to assess microbial fouling of gills from crayfish exposed to stream substrates and associated potential fouling organisms. Ten *C. chasmodactylus* (34-36 mm CL) were selected from crayfish captured in the South Fork on 24 June 2011 and randomly assigned to a worm treatment (present or absent, n=5 crayfish per treatment). We removed large branchiobdellids and then subjected all crayfish to a magnesium chloride bath as described for the laboratory experiment. Crayfish in the worm-present treatment were stocked with three large and three medium branchiobdellids. All crayfish were placed in individual lidded plastic containers filled with stream water for transport back to the field.

Experimental enclosures (Brown et al. 2012) were used to maintain experimental crayfish in realistic field conditions. The experiment ran for 20 days, during which time

water temperatures ranged from 16-24°C. Mean current velocity inside experimental enclosures was not significantly different between the start and conclusion of the experiment (mean±1SE current velocity initial: 10.87±5.15 cm/s, final: 11.32±3.73 cm/s; t_{17}=-0.24, p=0.810). Water depth was higher at experiment initiation (mean±1SE depth initial: 48.1±1.5 cm, final: 41.6±1.5 cm; t_{27}=3.00, p=0.006).

After 20 days, experimental crayfish were captured and returned to the lab in individual lidded plastic containers filled with stream water. Prior to removing the carapace, we recorded the number of worms remaining on worm-stocked crayfish. Crayfish were then sacrificed and their gills prepared for SEM imaging using the same methods described for the laboratory experiment.

Assessment of Fouling and Statistical Analysis

We used scanning electron microscopy to assess microbial fouling of the gill surface. Two haphazardly selected basal filaments on each preserved gill were imaged using an FEI Quanta 200 scanning electron microscope to determine the proportion of the gill filament covered by microbes and detritus. Microbial coverage was estimated from high-resolution SEM micrographs using iSolution measurement software (Image & Microscope Technology Inc.). To compare the microbial coverage of crayfish in the laboratory and field experiments, coverage proportions were submitted to angular transformation to satisfy the one-way ANOVA requirements for equality of variance. One-way ANOVA was then used to compare microbial coverage by experimental environment.

RESULTS

In both the laboratory and field experiments, crayfish with and without worms exhibited similar levels of microbial gill fouling (laboratory experiment: Fig. 1A, $F_{1,4}$=0.20, p=0.678; field experiment: Fig. 1B, $F_{1,7}$=0.39, p=0.552). Crayfish from the two worm treatments were therefore pooled in subsequent analyses for comparisons between laboratory crayfish (n=6) and field crayfish (n=9).

Crayfish in the field environment experienced significantly more microbial fouling than crayfish in the laboratory environment (Fig. 2, transformed data: $F_{1,13}$=63.25, p<0.001). Crayfish in the lab aquaria had very low levels of microbial growth on the examined gill filaments (Fig. 3A), with mean (±1 SE) microbial coverage of 2.14±1.01 percent of the gill surface. In contrast, crayfish exposed to field conditions experienced heavy microbial fouling (Fig. 3B), with a mean (±1 SE) microbial coverage of 58.17±6.89 percent of the gill surface.

Microbes found on crayfish gills from the stream were dominated by an unidentified rod-shaped bacterium, which was often found in large patches on the gill surface (Fig. 3B-F). Additional fouling material seen occasionally on the gills included unidentified filamentous bacteria, amorphous detritus, and particulate matter.

DISCUSSION

Previous studies of cleaning symbioses have found that fouling pressure in the environment affects the outcome of the relationship between cleaner and client (Cheney & Côté 2005; Lee et al. 2009). Our experiments provide direct measures of microbial gill fouling on crayfish in high- and low-fouling environments, and indicate that environmental conditions dictate the level of gill fouling. All crayfish exposed to just stream water in the laboratory experiment experienced much lower levels of microbial gill coverage than crayfish in the field experiment. This provides direct evidence that fouling microbes colonize the gills primarily from the substrate.

In our laboratory experiment, crayfish were only exposed to stream water, which does not appear to support substantial numbers of fouling organisms. In the aquatic environment, low nutrient levels are typically unable to support planktonic bacteria, which instead attach to surfaces to gain access to surface-adsorbed nutrients (Geesey & White 1990). Therefore, crayfish in our laboratory experiment likely did not experience high levels of microbial colonization from the water, since microbes in the stream preferentially attach to solid substrates such as sediments. In contrast, crayfish gills from the field experiment were heavily colonized by microbes, which likely came from the stream sediments. The hard exoskeleton of crustaceans provides an ideal substrate for the growth of fouling organisms including bacteria, diatoms, protozoa, and copepods (Fisher 1977; Sawyer et al. 1984), and heavy fouling of the gills is also common (Bauer 1998, 2002).

To cope with fouling and limit its potential negative effects on olfaction, respiration, and the incubation of embryos, crayfish actively engage in body grooming (Fisher 1977; Bauer 1979, 1981, 1989, 2002). While crayfish actively groom their exoskeleton, gill cleaning is passive, with two types of setae involved in crayfish gill cleaning (Bauer 1998). Setobranch setae extend as bundles from their attachment point on crayfish appendages, intertwining among gill filaments (Bauer 1998). As the crayfish moves its legs, the setae are agitated amongst gill filaments, providing an effective means of dislodging particulate material from the gill surfaces (Bauer 1998). However, these setobranch setae are unable to remove attached bacteria and fouling ciliates from the gill surface (Bauer 1998). A second type of setae, the branchiostegal setae, are attached to the inside surface of the carapace, and are presumed to passively clean the lateral sides of podobranch gills as the crayfish moves (Bauer 1998). As with the setobranch setae, contact with branchiostegal setae does not lead to reductions in bacterial fouling on crayfish gills, and gills remain heavily covered with clumps of bacilli (Bauer 1998). In our field experiment, crayfish gills were heavily covered by microbial epibionts, while fouling with sediment and detritus was rare. This confirms that passive setae effectively reduce particulate fouling but cannot eliminate microbial epibionts (Bauer 1998).

While passive grooming is unable to dislodge fouling epibionts from the gill surface, molting provides a means to remove all fouling epibionts from the gills and body surfaces of crustaceans. In environments where fouling pressure is low, periodic molting associated with growth may be adequate to maintain acceptably low fouling levels (Bauer 2002). However, the process of molting is energetically costly and exposes crayfish to an increased risk of mortality (Bauer 1989). It is therefore unlikely that in a high-fouling environment, molting

would be frequent enough to serve as the primary means of gill and body cleaning (Bauer 2002). Engaging in a cleaning symbiosis could thus provide an energetically favorable means of maintaining microbial fouling at levels that do not disrupt respiratory or excretory functions.

Branchiobdellid worms feed on particles inside the crayfish gill chamber (Brown et al. 2002), and as such, likely play a role in limiting or regulating fouling on gill surfaces. In my experiments, I did not find a significant difference in microbial fouling on the gills of crayfish exposed to or deprived of branchiobdellid worms. However, there are several possible explanations for why differences were not observed. First, the selected technique to assess gill fouling may be inadequate to detect differences between worm treatments. In this study, I determined coverage by fouling microbes by examining two filaments from a single podobranch gill. While this method was successful in assessing environmentally-driven differences in fouling, cleaning by the worms may act at a scale not captured in such a snapshot. For example, if the worms remove fouling microbes from arthrobranch gills, my current method would be unable to detect those differences. Second, densities of large worms may not have been high enough to remove fouling bacteria at a detectable rate.

Third, the mechanism by which branchiobdellids benefit their crayfish host has not yet been conclusively established. Branchiobdellids are predicted to remove epibionts and debris from gill epithelia (Jennings & Gelder 1979) and increase gas exchange and ammonia excretion rates (Brown et al. 2002; Creed et al. unpub. data). Laboratory and field experiments have shown that intermediate densities of branchiobdellids improve crayfish survival and growth (Brown et al. 2002, 2012) though the specific mechanism through which the worms improve crayfish fitness is as yet unknown. The lack of significant microbial

reductions in this experiment suggests that the worms may not directly remove microbes from the gill surface. Instead, the worms may provide fitness benefits to the crayfish by removing larger detritus from the gill chamber, improving water flow and thus the efficiency of ammonia excretion and the removal of other metabolic wastes (Brown et al. 2002).

Finally, it is possible that the dominant microbe observed on the gills is a third partner in the crayfish/worm interaction. Symbioses between epibiotic chemoautotrophic bacteria and marine invertebrates are well-documented (Cavanaugh et al. 1981; Jones 1981; Cavanaugh 1994) and in some of these interactions, chemoautotrophic bacteria were found attached to or near the host gills (Gros et al. 2007; Corbari et al. 2008). These symbioses include a vent shrimp that hosts iron-oxidizing chemoautotrophic bacteria in its gill chamber and two genera of marine mussels that host small, rod-shaped sulfur-oxidizing bacteria on their gill surfaces (Gros et al. 2007; Corbari et al. 2008; Duperron et al. 2008). In both of these interactions, the microbes were predicted to be acquired from the environment (Gros et al. 2007; Corbari et al. 2008). Most symbiotic chemoautotrophs to date have been found on marine invertebrates, but that does not preclude the possibility of similar interactions occurring between freshwater species.

Recently, a symbiosis between chemoautotrophic bacteria and a freshwater cave amphipod was discovered in which epibiotic bacteria potentially provide their invertebrate host with a food source as well as protection from environmental sulfide toxicity (Dattagupta et al. 2009). Further, while many symbioses between chemoautotrophic microbes and macroorganisms have been described as two-partner pairwise interactions, microbe/macroorganism symbioses involving three or more partners have also been documented (Currie 2001; Little & Currie 2008; Scott et al. 2008). Thus it is possible that a

tripartite microbe/macroorganism symbiosis occurs between the crayfish, the worm, and the dominant, environmentally-acquired microbe found on crayfish gills. This could help explain why similar levels of microbial fouling were seen in both treatments with and without worms. However, further investigations and molecular analyses are needed to determine the identity and possible ecological role of the dominant microbe seen on the crayfish gills.

These experiments provide direct evidence that environmental conditions affect gill fouling for the crayfish host. Crayfish exposed to just stream water experienced low levels of gill fouling, while crayfish given access to the stream bed in the enclosure experiment experienced heavy gill fouling. Fouling organisms on the gills of the latter crayfish were dominated by a single microbial morphotype. Because fouling in crayfish can only be fully escaped by molting, cleaners such as branchiobdellid worms likely play a role in moderating fouling of the exoskeleton and gill chamber during the crayfish intermolt. We hope that future experiments will be able to effectively and directly quantify differences in fouling between crayfish with and without worms, which in turn could help pinpoint mechanisms through which intermediate worm densities benefit their crayfish hosts.

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FIGURE LEGENDS

Fig. 1. Microbial coverage of crayfish gill filaments expressed as the percent of a haphazardly selected basal filament from 5th podobranch gill that was covered by microbes in crayfish in zero-worm and worm-present treatments (mean+1SE). A) Laboratory experiment with water but no substrate. B) Stream enclosure experiment with exposure to substrate.

Fig. 2. Environmentally-driven differences in microbial coverage of crayfish gill filaments. Lab experiment crayfish were only exposed to water skimmed from the stream surface, while crayfish in the field experiment were exposed to water and stream substrate. Fouling is expressed as the percent of a haphazardly selected basal filament from 5th podobranch gill that was covered by microbes, as assessed using high-resolution SEM micrographs (mean+1SE). *** denotes $p<0.001$

Fig. 3. SEM micrographs of microbes on basal filaments of 5th podobranch gills. A, B) Representative gill filaments from laboratory experiment. Note lack of heavy fouling. A) Arrows indicate particulate material, rest of gill shown is unfouled epithelium. B) Small patch of bacteria indicated by “b”. C-F) Representative gill filaments from field experiment. Note heavy coverage by rod-shaped bacteria. C) Full filament from base of 5th podobranch

gill. Lighter areas represent film of rod-shaped bacteria. Dark areas are unfouled gill epithelium. D) Higher magnification of gill in C. Note much larger bacterial patches “b” relative to area of unfouled epithelium “e” relative to laboratory experiment gills. E, F) Close-up of a bacterial mat from a crayfish in the field experiment. “b” indicates bacterial mat, “e” is unfouled gill epithelium.

Fig. 1.

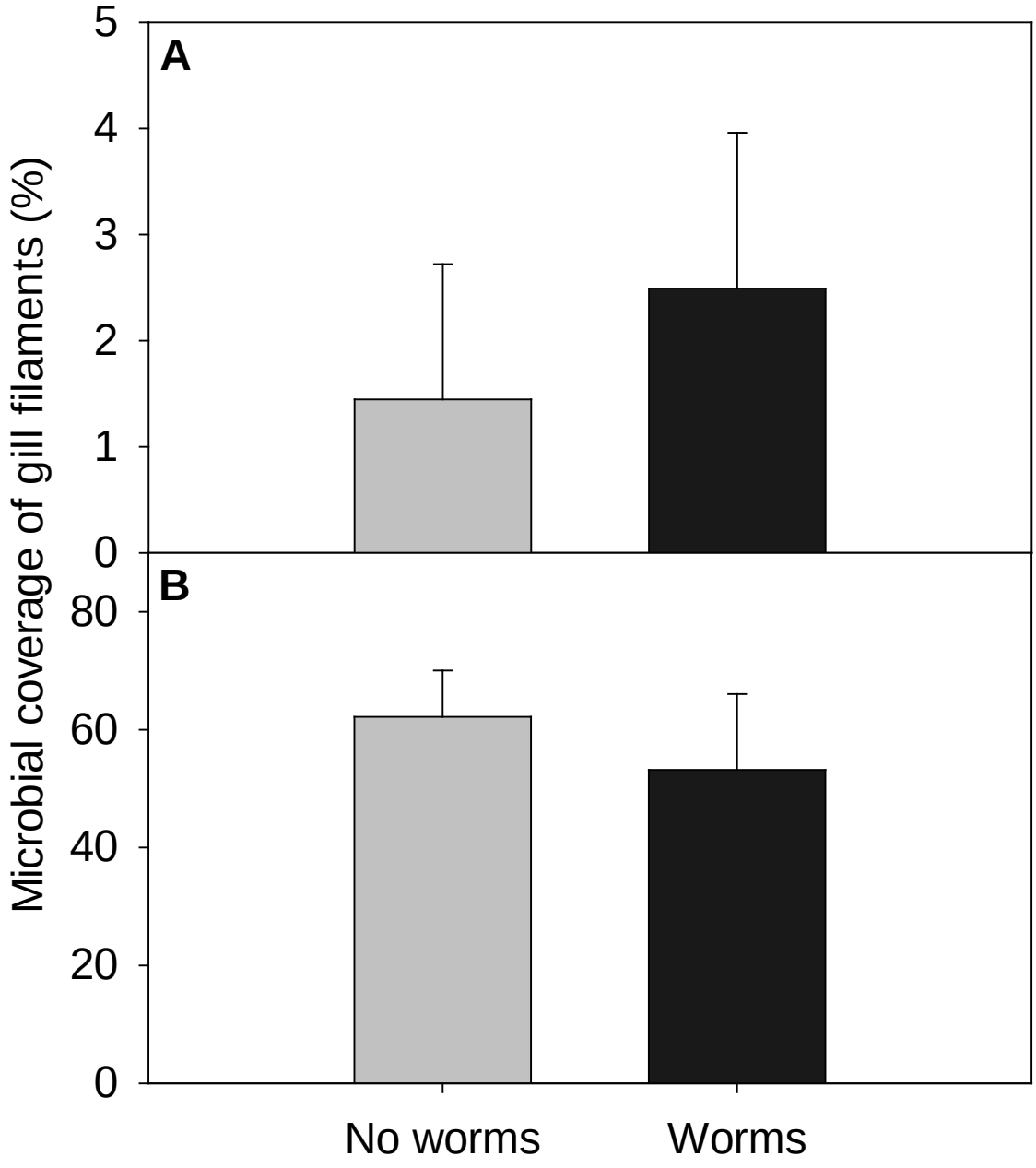

A
B
Microbial coverage of gill filaments (%)
5
4
3
2
1
0
80
60
40
20
0
No worms
Worms

Fig. 2.

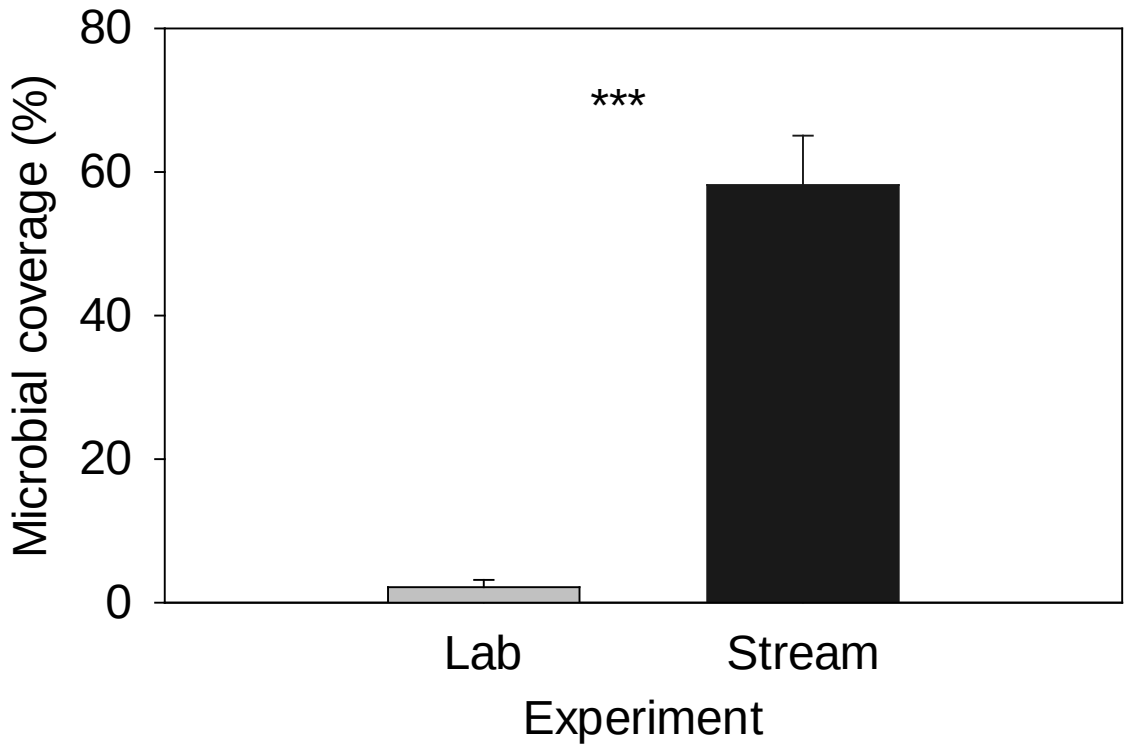
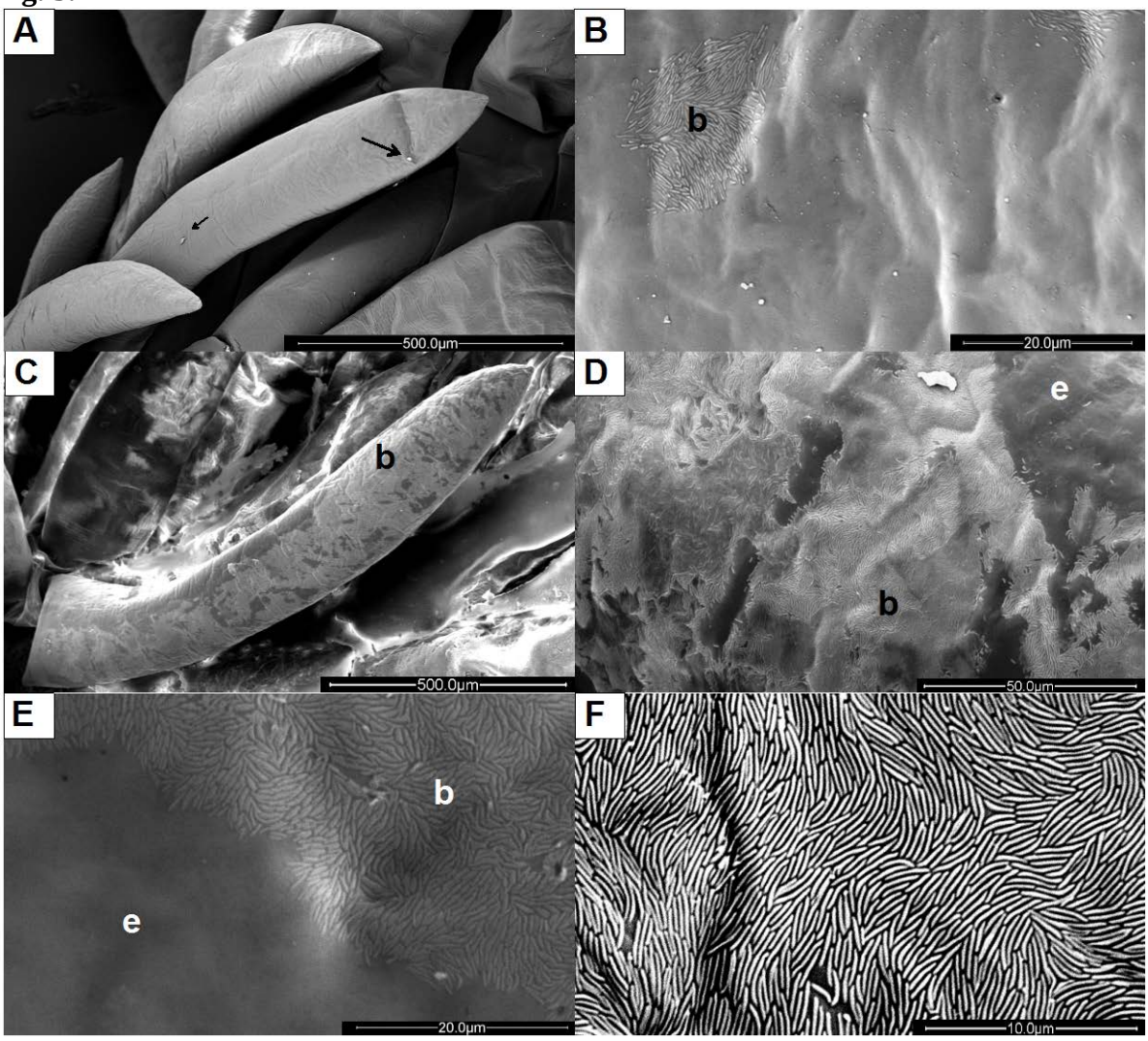


Fig. 3.



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SUPPLEMENTAL FIGURES

Fig. S1. Worm-directed scratches and grabs as a function of crayfish carapace length. Number of worm-directed grooming behaviors was not related to carapace length for female crayfish ($F_{1,7} = 0.117$, $p = 0.742$, $r^2 = 0.016$), but tended to be higher for smaller males than larger males (solid line, $F_{1,6} = 4.909$, $p = 0.069$, $r^2 = 0.45$).

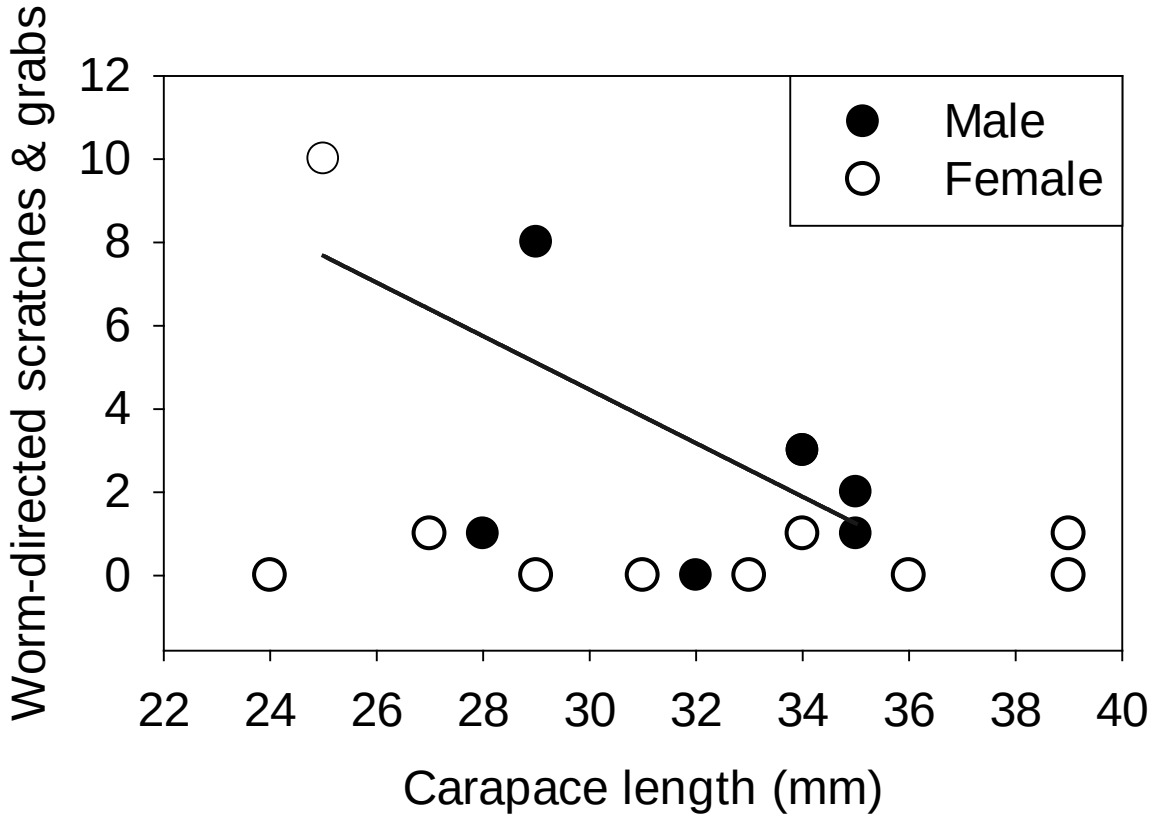
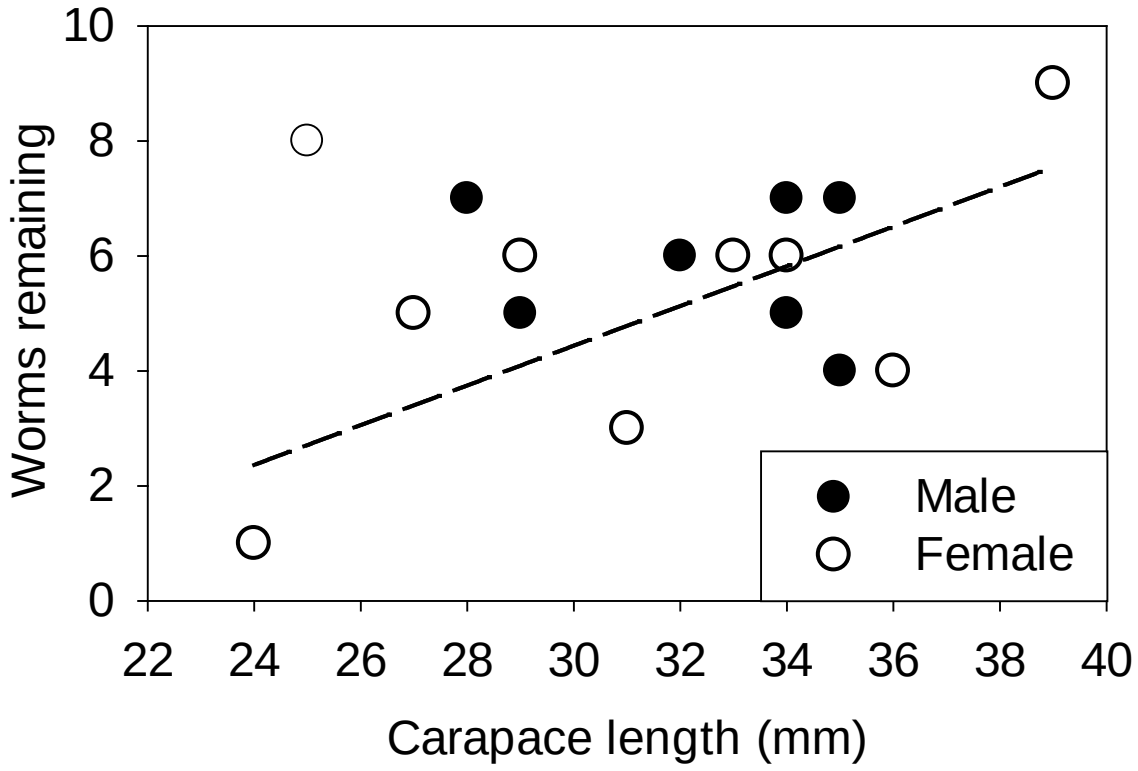


Fig. S2. Final worm number for *C. chasmodactylus* 72 hours post-observation. For female crayfish, final worm number was positively correlated with carapace length (dashed line, $F_{1,6} = 6.023$, $p = 0.050$, $r^2 = 0.501$).



VITA

Kaitlin J. Farrell grew up with her family outside of Charlotte, North Carolina. After graduating from North Mecklenburg High School in 2005, she spent her first year of college at Boston University studying ecology and conservation biology. In the fall of 2006, she transferred to McGill University in Montreal, Quebec, where she earned her Bachelor of Science degree in 2009 with a major in Environmental Biology. She came to Appalachian State University in June 2010, and completed her Master of Science degree in biology in May 2012. Having worked with raptors, bats, tropical stream invertebrates, whooping cranes, and now crayfish, Kaitlin's scientific interests are broad, and she hopes to pursue a career that incorporates her passion for both conservation and environmental education.