

Towards an understanding of symbiont natural history through studies of  
crayfish and their annelid associates

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**Abstract**

Crayfish throughout North America, Europe, and Asia host assemblages of obligate ectosymbiotic annelid worms called branchiobdellidans. The work presented here is a detailed experimental and observational study of the ecological interactions between crayfish and their worms. In a comprehensive literature review, I show that branchiobdellidans have complex and context-dependent effects on their hosts, serving as both beneficial cleaners and tissue-consuming parasites. Using a field survey and laboratory experiments, I provide novel evidence for age-specific resistance as an adaptation to maximize life-long benefits of a mutualism. Specifically, I show that *Cambarus* crayfish display a consistent ontogenetic shift in resistance to the colonization of branchiobdellidans and this shift likely reflects underlying changes in the costs and benefits of symbiosis. I then show that this change in host resistance creates predictable patterns of symbiont diversity and composition throughout host ontogeny. Host resistance limits within-host symbiont communities to a few weakly interacting species, whereas relaxed resistance leads to more diverse symbiont communities that have strong interactions among symbiont taxa. Thus, host resistance has direct effects on within-host symbiont community structure by selectively filtering colonizing species, and indirect effects by moderating the strength of interactions among symbionts. Lastly, in a detailed study of the worm *Cambarincola ingens*, I depict a symbiont dispersal strategy that yields highly predictable transmission dynamics during pairwise host-host encounters and shows that variation in transmission dynamics can be explained by the fitness outcomes for dispersing symbionts. Field observations

revealed that worm reproduction is contingent on host size and intraspecific competition for preferred microhabitats. Using a predictive model that assumes transmission of symbionts only when current conditions yield fitness below a minimum threshold, I was able to predict individual transmission events much more accurately than a comparable null model that assumed a fixed probability of transmission. My work provides empirical support for the emerging trend among researchers that advocates the adaptation of general ecological frameworks to understand symbiont population structure and diversity, but my work also emphasizes the value of detailed natural history studies to uncover system-specific ecological and co-evolutionary processes such as partner control mechanisms, symbiont microhabitat selections, and symbiont dispersal strategies.

## **Dedication**

To my father Rodney G. Skelton for inspiring a life-long love of the natural world and an appreciation for the simple things that make life good.

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**Chapter 1. Servants, scoundrels, and hitchhikers: Current understanding of the complex interactions between crayfish and their ectosymbiotic worms (Branchiobdellida)**

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ABSTRACT

Astacoidean crayfishes serve as hosts to obligate ectosymbiotic annelids called branchiobdellidans. Branchiobdellidans can act either as mutualistic cleaners or as ectoparasites and can have strong effects on crayfish growth and survivorship. This potentially vital aspect of crayfish biology has gone largely unexplored until recently. We reviewed the current state of knowledge regarding this symbiosis and examined factors that contribute to variability in the effects of branchiobdellidans on crayfish. We show that branchiobdellidans affect crayfish in various ways depending on branchiobdellidan species, abundance, and ecological context. We also discuss evidence for regulatory controls that crayfish exert over their symbionts and symbiont–host preferences. Last, we evaluate the utility and challenges of using the crayfish–branchiobdellidan association as a model system for ecological and evolutionary research and point to promising areas for future study. Further investigations of the complex interactions between crayfish and their ectosymbionts will greatly advance the field of crayfish biology and offer many exciting opportunities for the study of symbioses.

**Key words:** symbiosis, mutualisms, cleaning symbiosis, context dependence, partner choice, partner regulation.

## **Introduction**

Crayfish are well known inhabitants of streams, rivers, lakes, and ponds, and have drawn the attention of many aquatic biologists for several important reasons. Crayfish are keystone consumers that have strong effects on plant and animal community structure through direct and indirect trophic interactions (Hart 1992, Creed 1994, Momot 1995, Charlebois and Lamberti 1996, Parkyn et al. 1997). Many species are ecosystem engineers that entrain sediments while feeding and excavating burrows and alter in-stream organic-matter processing by shredding coarse particulate organic matter (Parkyn et al. 1997, Statzner et al. 2000, Creed and Reed 2004, Usio and Townsend 2004, Zhang et al. 2004, Brown and Lawson 2010), particularly highly recalcitrant material, such as *Rhododendron* sp. (Huryn and Wallace 1987, Schofield et al. 2001). Moreover, several invasive species of crayfish threaten biodiversity in ecosystems around the globe (Gherardi 2007), and many more crayfish species are threatened by extinction from multiple anthropogenic factors (Taylor et al. 2007). Thus, crayfish have been the focus of a large body of ecological and organismal research.

An underappreciated aspect of crayfish biology is that the crayfish body is not simply a single organism, but rather a complex consortium of microbial and metazoan taxa. The crayfish exoskeleton can host a wide diversity of organisms including bacteria, clusters of stalked ciliates, sessile rotifers, annelid worms, flatworms, and other crustaceans (review by Edgerton et al. 2002). Many of these organisms may be only

incidental associates, but others are obligate ectosymbionts (Hobbs et al. 1967, Gelder and Rowe 1988, Gelder 2010). Branchiobdellidans, members of an order of clitellate annelids, are obligate ectosymbionts primarily of astacoidean crayfishes. This association is common across much of the Holarctic, including the Euro-Mediterranean region, East Asia, and North and Central America (Gelder 1999b, Fard and Gelder 2011).

Historically, branchiobdellidans were considered ectocommensals that had no significant effects on their hosts (e.g., McManus 1960, Young 1966, Bishop 1968), although some were described as parasites with negative host effects (Holt 1963, Hobbs et al. 1967, Grabda and Wierzbicka 1969). More recent empirical studies have shown that crayfish and branchiobdellidans maintain a complex and variable association that potentially benefits both the host and its symbiotic worms (Brown et al. 2002, 2012, Brown and Creed 2004, Lee et al. 2009).

Branchiobdellidans benefit from their associations with crayfish in a variety of ways. They graze on material, such as diatoms, bacteria, and protozoans, that accumulate on their host's exoskeleton or in the host gill chamber (Jennings and Gelder 1979, Gale and Proctor 2009), and a small number of species may consume host tissue (Grabda and Wierzbicka 1969). Most branchiobdellidans obtain their nutrition from material acquired on the exoskeleton of the host, but individuals also consume algae and metazoans from nearby substrate (Jennings and Gelder 1979, Gelder 2010) and, consequently, can survive long periods without a host, at least in vitro (Penn 1959, Young 1966). Despite their ability to survive separated from their host, branchiobdellidans are generally considered obligate ectosymbionts because, to the best of our knowledge, they reproduce only on a live crustacean (Young 1966, Gelder 2010; see Woodhead 1950 for a possible exception),

and therefore, are reproductively dependent on their hosts. Current knowledge of branchiobdellidan reproductive biology is summarized in Gelder (2010). Only one account has been published of a possibly “free-living” branchiobdellidan, but it seems probable that this observation was of worms that were separated from their hosts before collection (Holt 1973a).

Historically, the effects of branchiobdellidans on their hosts received little attention, and claims of commensal or parasitic interactions were largely speculative (e.g., McManus 1960, Young 1966, Hobbs et al. 1967, Hobbs and Lodge 2010). Recent experimental work has demonstrated that branchiobdellidans may exert both positive and negative effects on their hosts and, in some cases, affect crayfish growth and survivorship (Brown et al. 2002, 2012, Lee et al. 2009). Therefore, this complex interaction is a potentially important aspect of crayfish biology. Because of the influential role that crayfish play in aquatic systems, branchiobdellidans may have indirect effects on local communities and ecosystem processes via their direct effects on crayfish. Field and laboratory experiments have provided methods for manipulative experiments using the crayfish–branchiobdellidan association and have demonstrated utility of this symbiosis for addressing more general ecological and evolutionary questions (Brown et al. 2002, 2012, Brown and Creed 2004, Lee et al. 2009). Thus, the objectives of our contribution are to: 1) bring attention to the crayfish–branchiobdellidan association as an important yet understudied facet of crayfish biology and ecology and 2) illustrate the utility of the crayfish–branchiobdellidan association as a model system for addressing general ecological and evolutionary questions. We summarize the current state of knowledge of the crayfish–branchiobdellidan association and briefly discuss on-going research efforts

and promising future directions for work using the crayfish–branchiobdellidan association as a model system for testing theory concerning the ecology, evolution, and maintenance of symbiotic interactions.

### **Pairwise Interactions between Crayfish and Branchiobdellidans**

Early researchers studying branchiobdellidans assumed most species were commensals of crayfish (e.g., Goodnight 1941, McManus 1960, Young 1966, Bishop 1968), though some species were thought to be parasites largely because of their propensity for inhabiting the host’s gill chamber (Holt 1963, Hobbs et al. 1967). In the first attempt to demonstrate parasitism in branchiobdellidans, Grabda and Wierzbicka (1969) used radioactive tracers to confirm that one gill-chamber-inhabiting species (*Branchiobdella hexadonta*) consumed tissues or hemolymph from their host. More recent observational studies have revealed significant correlations between the presence/abundance of gill-chamber-inhabiting branchiobdellidans and the number of visible scars (melanization) on the hosts’ gills (Quaglio et al. 2006, Rosewarne et al. 2012). Such studies suggest that some branchiobdellidan taxa may consume host tissues and can cause visible damage, but they do not necessarily demonstrate that the interaction is parasitic based on net outcomes for the species involved (sensu Bronstein 1994). Moreover, these studies did not account for other effects of branchiobdellidans on their host, including possible positive effects that could effectively offset the negative effects of parasitism.

Experiments designed to assess the net effect of branchiobdellidans on aspects of host fitness have produced conclusions ranging from net-positive to net-negative

outcomes. The branchiobdellidan *Cambarincola fallax* did not affect the growth, molting frequency, or stamina of *Orconectes rusticus* during a 115-d laboratory experiment, results leading the author to conclude that the association between these taxa is commensal (Keller 1992). Conversely, a similar experiment demonstrated significant positive effects of the branchiobdellidan *Cambarincola ingens* on growth rate and survival of the host crayfish *Cambarus chasmodactylus* (Brown et al. 2002). The positive effects of *C. ingens* on their hosts were attributed to a cleaning service because *C. ingens* removed potentially fouling organic materials that had collected in the host's gill chamber (Brown et al. 2002). Consequently, Brown et al. (2002) concluded that the association between *C. ingens* and *C. chasmodactylus* is probably a cleaning-symbiosis mutualism similar to the long known and familiar associations among tropical coral reef fishes (e.g., Limbaugh 1961, Trivers 1971, Losey 1972, Losey and Margules 1974, Grutter 1999). Together, the results of Keller (1992) and Brown et al. (2002) suggest that the effects of branchiobdellidans on their hosts may vary among hosts or branchiobdellidan species.

The net effects of branchiobdellidans on a crayfish also may depend on the abundance or density of branchiobdellidans on an individual host. In field experiments involving 2 single-species pairs of crayfish and branchiobdellidans in 2 different watersheds, the net effect shifted from positive to negative with increasing initial branchiobdellidan densities (Brown et al. 2012). Individual crayfish (*C. chasmodactylus* and *Cambarus chaugaensis*) that were exposed to low to intermediate branchiobdellidan densities (*C. ingens* and *Xironodrillus* sp., respectively) had significantly higher growth rates than crayfish with no branchiobdellidans (Brown et al. 2012). However, crayfish

that were exposed to high densities of branchiobdellidans consistently showed reduced growth rates compared to no-worm controls (Brown et al. 2012; Fig. 1). During these experiments, branchiobdellidan density and the number of scars on crayfish gills were strongly positively correlated (Brown et al. 2012), suggesting the interaction shifted from mutualism to parasitism at high branchiobdellidan densities as the negative effects of increasing gill damage outweighed the positive effects of cleaning (Brown et al. 2012). Branchiobdellidan densities vary considerably among locations and branchiobdellidan species. In the experiments mentioned above, 12 worms/host was considered high because it was greater than the typical densities observed for those species at those locations. However, crayfish in some populations regularly host hundreds of branchiobdellidans (BWW, JS, personal observation).

In addition to species-specific and demographic variability, the outcomes of symbiotic interactions are often influenced by the biotic and abiotic factors that form the environmental context of the interaction (Bronstein 1994, Thompson 2005). Recent experimental evidence suggests that context probably is an important determinant of the net effect of branchiobdellidans on their hosts. In laboratory experiments, the Korean crayfish (*Cambaroides similis*) experienced increased growth in the presence of multiple species of branchiobdellidans when conditions favored the growth of microbial biofilms (Lee et al. 2009). However, under conditions that reduced microbial growth, the same suite of worms had no measurable effect on host growth (Lee et al. 2009). These results are congruent with conclusions of Brown et al. (2002) that the positive effects of branchiobdellidans on their host result from removal of epibiotic material and demonstrate the importance of context in determining the net outcome for the host.

## **Spatiotemporal Variability in Branchiobdellidan Assemblages**

Species composition, abundance, and diversity of branchiobdellidans vary across spatial and temporal scales. Approximately 150 species representing 21 genera have been described from North and Central America, Europe, and eastern Asia (Gelder et al. 2002), but taxonomic diversity is unevenly distributed across these regions. Surveys and synopses have encompassed broad geographic areas of North and Central America including the Canadian Prairie Provinces (Williams et al. 2009), Mesoamerica (Holt 1973b), and the west coast of North America (Holt 1974, 1977, 1981) and more limited areas including New England (Gelder et al. 2001), the Mountain Lake region of northwestern Virginia (Hobbs et al. 1967), and Great Smoky Mountains National Park (Gelder and Williams 2011). Each of these studies provides a glimpse of the natural variability in branchiobdellidan species composition across a wide range of spatial extent. However, branchiobdellidan diversity and abundance across space, time, and environmental conditions are poorly understood. Authors of early ecological studies described how branchiobdellidan abundance varied as a function of season (Young 1966, Koepp and Schlueter 1977), water temperature, and O<sub>2</sub> tension (Berry and Holt 1959), but the roles of environment, space, and time are only now being explored in combination (DeWitt et al. 2013). Branchiobdellidan diversity is either unknown or underestimated in many regions because the life history of individual species and their sensitivity to environmental conditions are rarely assessed quantitatively. Measuring how branchiobdellidans vary spatiotemporally and across environmental conditions is an important step to elucidating implications of branchiobdellidan diversity for crayfish

populations.

Branchiobdellidans display spatial patterns in microhabitat selection on their hosts' bodies. Some species, including the wide-spread *Bdellodrilus illuminatus*, are found almost exclusively within the crayfish gill chamber (Holt 1963, Hobbs et al. 1967, Gelder and Williams 2011). Others, such as most species of the genera *Xironogiton*, *Ankyrodrilus*, and *Xironodrilus*, show preferences for the chelae and anterior walking legs and share a dorsoventrally flattened morphology that probably is an adaptation to shear stresses experienced at these locations (Fig. 2; Hobbs et al. 1967, Gelder and Williams 2011). Other species are less specific in their microhabitat selection and are found attached at many locations on their hosts (see Gelder and Williams 2011 for descriptions of the microhabitat selection of several wide-spread and localized species). Researchers who have examined the effects of branchiobdellidans on their hosts have focused mainly on species that inhabit the gill chamber and adjacent areas (Brown et al. 2002, 2012, Quaglio et al. 2006, Rosewarne et al. 2012), and the potential importance of other species is generally unknown. The evolutionary and potential coevolutionary forces that have shaped microhabitat selection in branchiobdellidans and the implications of microhabitat selection for the host remain an unexplored opportunity for future research.

### **Host Specificity, Preference, and Regulation**

Studies of the biogeography of branchiobdellidans and their hosts indicate that branchiobdellidans are not strictly host specific. More than twice as many described species of crayfish exist in North America (~382; Crandall and Buhay 2008) as there are branchiobdellidan species world-wide (~150; Gelder et al. 2002), although many

undiscovered or undescribed cryptic branchiobdellidan species certainly exist (Williams et al. 2013). Moreover, a single branchiobdellidan species may be found on multiple host species at a site, and a single crayfish may host several species of branchiobdellidans (e.g., Hobbs et al. 1967, Gelder 1999b, Gelder and Williams 2011). The geographic ranges of branchiobdellidans do not typically reflect the distribution of any particular host species (Holt 1969, Gelder 1999b, Gelder and Williams 2011; but see Füreder et al. 2009), and some species have spread to new host species or, along with their host, successfully invaded novel environments (Gelder 1999a, Gelder et al. 2002, 2012, Quaglio et al. 2006). Branchiobdellidans clearly are not host species-specific *sensu stricto*, but evidence exists that species in this association are not perfectly interchangeable.

Recent observational and experimental work suggests that crayfish species vary in their suitability as branchiobdellidan hosts and that some branchiobdellidans prefer particular host species (Brown and Creed 2004, Gelder and Williams 2011, Farrell et al. in press). The crayfish *C. chasmodactylus* and *Orconectes cristavarius* co-occur in the South Fork of the New River in western North Carolina. The branchiobdellidan *C. ingens* occurs with much greater frequency and abundance on *C. chasmodactylus* than on *O. cristavarius*, even after accounting for differences in host size and despite the greater abundance of *O. cristavarius* (Brown and Creed 2004). In a host-choice experiment, *C. ingens* was 2.5× more likely to colonize *C. chasmodactylus* when given a choice between the 2 host species (Brown and Creed 2004).

Differences in the abundance of *C. ingens* on *C. chasmodactylus* and *O. cristavarius* may reflect more than just the preferences of the worms. Crayfish are

covered with mechanoreceptors that enable them to detect the presence and location of branchiobdellidans on their exoskeletons (Gelder 2010). Recent experimental work suggests that crayfish use grooming behaviors to actively regulate branchiobdellidan density and that regulation intensity may vary with crayfish species and local environmental conditions (Thomas et al. 2013, Farrell et al. in press). When branchiobdellidan densities on *C. chasmodactylus* were experimentally manipulated to levels  $\sim 2\times$  those typically observed in situ, the crayfish rapidly groomed branchiobdellidans from their exoskeletons using the fingers and dactyls of their anterior walking legs (Farrell et al. in press). Moreover, not all worms were removed and the densities of branchiobdellidans remaining were similar to the in situ densities that yielded a net positive outcome for *C. chasmodactylus* in previous studies (Brown et al. 2002, 2012). Conversely, introduction of just 1 worm initiated strong and directed grooming behaviors in *O. cristavarius* (Farrell et al. in press), a result suggesting that this species is intolerant of *C. ingens* and that potentially explains *C. ingens* preference for *C. chasmodactylus* as a host. A disparity between *Cambarus* and *Orconectes* as branchiobdellidan hosts also was observed by Gelder and Williams (2011), who noted that branchiobdellidan richness in Great Smoky Mountains National Park was typically greater on *Cambarus* than on *Orconectes*. Thus, for a particular branchiobdellidan species, not all crayfish species are equally suitable hosts.

Crayfish may adjust regulatory grooming behaviors to match environmental context and concomitant changes in the potential benefits of being cleaned or costs of being parasitized. In environments with low levels of epibiotic fouling, crayfish probably experience less benefit from being cleaned and could actually be more susceptible to

parasitism if limited epibiotic resources cause a compensatory shift in branchiobdellidan feeding behavior toward increased parasitism (Brown et al. 2012, Thomas et al. 2013). In a laboratory experiment, Thomas et al. (2013) demonstrated that regulatory grooming of *C. chasmodactylus* changed with conditions that promoted either high or low levels of epibiotic fouling of the crayfish exoskeleton and gills. They observed a higher rate of branchiobdellidan removal by *C. chasmodactylus* under low-fouling conditions, suggesting that crayfish modify their worm loads to match changes in the potential costs and benefits of symbiosis.

### **Branchiobdellidan Taxonomy and Phylogenetics**

The crayfish–branchiobdellidan association presents many opportunities for advancing our knowledge of crayfish biology and may provide a useful model system for testing ecological and evolutionary theory, but recent and ongoing shifts in our understanding of branchiobdellidan taxonomy and phylogenetics pose potential challenges to researchers working with this system. Early understanding of branchiobdellidan phylogeny was based primarily on intuition (Holt 1968, 1986) and reflected taxonomy at the time. Later attempts at phylogenetic reconstruction using morphological characters (Gelder and Brinkhurst 1990), spermatological characters (Cardini et al. 2000, Cardini and Ferraguti 2004), and molecular markers (Gelder and Siddall 2001) resulted in low resolution and low node support among most examined taxa. A recent molecular phylogenetic study focused on phylogenetic relationships among North American branchiobdellidans recovered strong support for clustering that is not fully consistent with current taxonomy (Williams et al. 2013). These results provide a

base from which to examine the relative importance of morphological characters currently used to designate taxonomic and systematic rankings within the Branchiobdellida. However, molecular data suggest that in some cases phylogenetically disparate species exhibit a high degree of morphological similarity (Williams et al. 2013). As a result, accurate identification of branchiobdellidans requires care and expertise. Much of the recent work on branchiobdellidan evolution and ecology has taken advantage of live microscopy techniques (described in Gelder 2010) that enhance examination of subtle morphological differences among species. Efforts are currently underway to create a comprehensive interactive on-line key to facilitate identification of branchiobdellidans with live and fixed specimens to make studying them more tractable for biologists with limited familiarity with branchiobdellidan taxonomy (BWW, unpublished data).

### **Utility of the Crayfish–Branchiobdellidan Symbiosis as a Model System**

The evolution of mutualistic interactions has fascinated theoreticians since Darwin (1859). Early researchers realized that the evolution of these associations had to be explained in terms of individual fitness (Hamilton 1964a, b), and researchers using purely theoretical approaches have demonstrated quantitatively that mutualisms may be evolutionarily stable strategies (e.g., Trivers 1971, Axelrod and Hamilton 1981, Nowak and May 1992, Noë and Hammerstein 1994, Doebeli and Knowlton 1998, Nowak and Sigmund 1998). Theoretical approaches are essential for explaining the evolution of species interactions in terms of individual fitness, but they necessarily simplify organisms and their interactions. Such simplifications prevent assessments of many important and

multifaceted details that stem from organismal diversity (Herre et al. 1999). Therefore, empiricists must verify theory by testing specific hypotheses about the mechanisms that shape real symbiotic interactions.

One of the best known and most fruitful model systems for studying symbiotic interactions among animals is the cleaning symbioses among tropical coral reef fishes. Multiple cleaner species provide a service to clients by feeding on ectoparasites from the clients bodies. This interaction may be mutualistic because the client is relieved of detrimental parasites and the cleaner receives a meal (Limbaugh 1961). However, participants also may enhance individual fitness in other, nonmutual ways. First, clients may increase their benefit by consuming cleaners after they have performed their cleaning service (Trivers 1971), but cleaners are rarely consumed even when entering the mouths of clients they clean. Second, cleaners could increase their benefit by consuming client tissues in addition to ectoparasites and may actually prefer the latter (Grutter and Bshary 2003). Indeed, cleaner fish may effectively become parasitic when ectoparasite abundances are low (Losey 1972, Cheney and Cote 2005), a situation that demonstrates the influence of local context on the outcome of the interaction.

Empirical work on cleaning symbioses among tropical fishes has elucidated multiple mechanistic explanations for their evolutionary stability. Clients can influence cleaner behavior through punishment (Bshary and Grutter 2002, Grutter and Bshary 2003) and can minimize parasitic attacks by selectively interacting with cooperators (Noë and Hammerstein 1994, Bshary and Noë 2003). Moreover, clients can limit the temptation for cleaners to defect by regulating the duration of cleaning interactions (Johnstone 2002). These empirical explorations of specific aspects of cleaning

interactions and their underlying mechanisms demonstrate that mutualisms may be very tenuous and complicated associations affected by local biotic and abiotic contexts and maintained by numerous complex feedbacks between and among partners.

The association between crayfish and branchiobdellidans offers an opportunity to corroborate and expand on the theoretical and empirical advances gained from studying other mutualistic interactions. Like in coral reef systems, experimental work with crayfish and branchiobdellidans has demonstrated variable (Lee et al. 2009, Brown et al. 2012) and context-dependent (Lee et al. 2009) effects of cleaners on their hosts, and complex species-specific control behaviors that may limit overexploitation (Farrell et al. in press). Differences between crayfish–branchiobdellidan systems and tropical cleaner–fish systems provide an opportunity to test the generality of theory derived largely from coral reef work and to evaluate further necessary constraints for the evolution of cleaning-symbiosis mutualisms.

We present 5 reasons for using the crayfish–branchiobdellidan association as a model system for empirical symbiosis research: 1) *Distribution*. The crayfish–branchiobdellidan association is common throughout many regions of the Holarctic, making it an accessible system to researchers in several countries. 2) *Ease of experimental manipulations*. The number of branchiobdellidans on individual crayfish can be easily manipulated to establish specific initial worm densities. Methods for these manipulations can be found in Brown et al. (2002) and have been validated by multiple published studies (Brown and Creed 2004, Lee et al. 2009, Brown et al. 2012). 3) *Ease of husbandry*. Both crayfish and branchiobdellidans can be maintained in the laboratory with minimal equipment. Successful experiments have been conducted in small aerated

plastic or glass bowls or aquaria (Keller 1992, Brown et al. 2002, Brown and Creed 2004, Lee et al. 2009). Moreover, branchiobdellidans can be stored without a host for long periods in shallow dishes of stream water with no apparent ill effects (Woodhead 1950, Penn 1959, Young 1966). 4) *Amenability of field experimental methods*. Flow-through stream enclosures have been used in field experiments to determine symbiont effects in the context of natural systems (Fig. 3; Brown et al. 2012). To the best of our knowledge, branchiobdellidans transfer only through direct contact between hosts, so maintaining experimentally established densities is a straightforward process of keeping enclosed crayfish separated from naturally-occurring crayfish using either fine-mesh (McManus 1960) or enclosures featuring double walls (Brown et al. 2012). 5) *Nonintrusive data collection*. Several experiments conducted thus far on crayfish and their branchiobdellidan associates have used nondestructive repeated-measures data collection to assess crayfish growth and epibiont population dynamics (Brown et al. 2002, 2012, Lee et al. 2009, Thomas et al. 2013). Repeated-measures data collection increases statistical power for assessing treatment effects and allows assessment of changes in effects over time (Potvin et al. 1990). We acknowledge that nonintrusive data collection may not be an option for all species because of wide variation in size, microhabitat, and visibility of branchiobdellidans.

### **Continuing and Future Work**

Our perception of symbioses has changed from that of interactions with exclusively positive or negative outcomes to a more realistic perspective of interactions with variable outcomes (Ewald 1987, Bronstein 1994). Moreover, appreciation is

growing among ecologists of the importance of both positive and negative outcomes in structuring natural communities and modulating ecosystem processes (Bertness and Callaway 1994, Callaway 1995, Bertness and Leonard 1997, Bruno et al. 2003). Crayfish are highly influential in aquatic systems as ecosystem engineers and keystone species (Creed 1994, Creed and Reed 2004, Usio and Townsend 2004, Brown and Lawson 2010). Ongoing research is being done to explore the variable effects of branchiobdellidans on crayfish and the indirect effects that this association may have on aquatic communities and ecosystems. This body of work combines recent theoretical advances in our understanding of symbioses to illustrate how variability in species interactions can lead to variable effects that radiate beyond the focal species throughout natural communities and ecosystems.

Mutualists can have the potential to act as parasites, but coevolved controls can prevent overexploitation that leads to parasitism and potentially could stabilize mutualisms through evolutionary time (Trivers 1971, Axelrod and Hamilton 1981, Bull and Rice 1991, Pellmyr and Huth 1994, Johnstone 2002, Bshary and Bronstein 2011, Kiers et al. 2011). Ongoing work is being done to examine behavioral responses of crayfish to branchiobdellidans under multiple contexts and to document age- and species-specific host responses that may resist overexploitation by epibionts and maximize the life-long net benefit of symbiosis. This work is improving our understanding of how positive interactions are maintained by describing how control mechanisms and changes in ecological context and species ontogeny permit mutually beneficial interactions over a broad range of conditions and developmental stages.

The relationship between branchiobdellidans and crayfish has been studied

primarily in surface-water environments, but most crayfish have the ability to burrow and may spend part or all of their life cycle underground (Hobbs 1981). Branchiobdellidans are associated with some burrowing crayfishes (e.g., *Cambarus carolinus*, *Cambarus diogenes*, and *Procambarus clarkii*; Hobbs et al. 1967, Holt and Opell 1993), but the extent of the associations under these conditions is poorly understood, and important basic questions about burrowing crayfish–branchiobdellidan symbioses remain unanswered. The physicochemical disparity between fully aquatic and semiterrestrial environments offers a valuable opportunity to investigate how costs and benefits associated with cleaning symbioses vary among hosts with different life-history strategies or among hosts that move between vastly different habitats within a lifetime. Ongoing research in replicate burrowing chambers (Stoeckel et al. 2011) is beginning to address questions pertaining to burrowing crayfish and their symbionts.

Research has provided a foundation for understanding of the ecology and evolution of the crayfish–branchiobdellidan association, but what we know is dwarfed by what we do not know. Systematic sampling in understudied or unsampled areas is needed to increase our knowledge of branchiobdellidan diversity and distribution and to inform studies assessing evolutionary and ecological patterns. In addition, work evaluating concordance among different data sets (e.g. morphological, molecular), and thus, species concepts, is needed to infer the evolutionary history of the Branchiobdellida. Basic life-history characteristics and the physiological constraints of most branchiobdellidan species remain undescribed. The effects that branchiobdellidans have on crayfish have been elucidated for only a handful of species, and the implications of variability in local branchiobdellidan diversity and interspecific interactions among branchiobdellidans are

still unknown. Exploring how ecological and evolutionary forces have shaped patterns in branchiobdellidan diversity and the consequences of those patterns for crayfish and surrounding communities is an exciting and promising avenue for future research. Furthermore, understanding how anthropogenic disturbances, such as species introductions and habitat alteration, alter the symbiotic interaction between crayfish and branchiobdellidans will help inform efforts to protect biodiversity and ecosystem integrity in the many places that crayfish live.

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## Figure Captions

Fig. 1.1

Results of 3 *in situ* experiments assessing the effect of variable initial branchiobdellidans density (worms/host) on crayfish growth. Two experiments were done on the South Fork of the New River near Boone, North Carolina (NC), USA with the crayfish *Cambarus chasmodactylus* and the branchiobdellidan *Cambarincola ingens* over 71 d during the summer 2008 (filled circles) and 103 d during the summer of 2010 (open circles). A 3<sup>rd</sup> experiment was done over 81 d during summer 2010 in Walldrop Stone Creek near Clemson, South Carolina (SC), USA, with the crayfish *Cambarus chaugaensis* and branchiobdellidans of the genus *Xironodrilus* (open triangles). Plots show total % growth relativized to controls (no worms) vs initial branchiobdellidan density (worm treatments). For each treatment, total growth was relativized by subtracting the mean total growth of the control group in each experiment. In all 3 experiments, low to intermediate initial branchiobdellidan densities had significant positive effects, but high densities marginally reduced growth (Brown et al. 2012).

Fig 1.2

The branchiobdellidan *Xironogiton instabilis* on the chela of the crayfish *Cambarus bartonii* from Little Stoney Creek, northwest VA, USA. *Xironogiton* displays a dorso-ventrally flattened morphology typical of branchiobdellidan species that specialize on crayfish chelae (photo credit: J. Skelton).

Fig 1.3

Flow-through enclosure/exclosures used in field experiments to test for effects of branchiobdellid worms on host crayfish growth (Brown et al. 2012). Flow-through design maintains natural stream conditions while double-walled barriers prevent exchange of branchiobdellidans between enclosed and free-living crayfish (photo credit: B. L. Brown).

Figure 1.1 Complex effects of branchiobdellidan abundance on crayfish growth

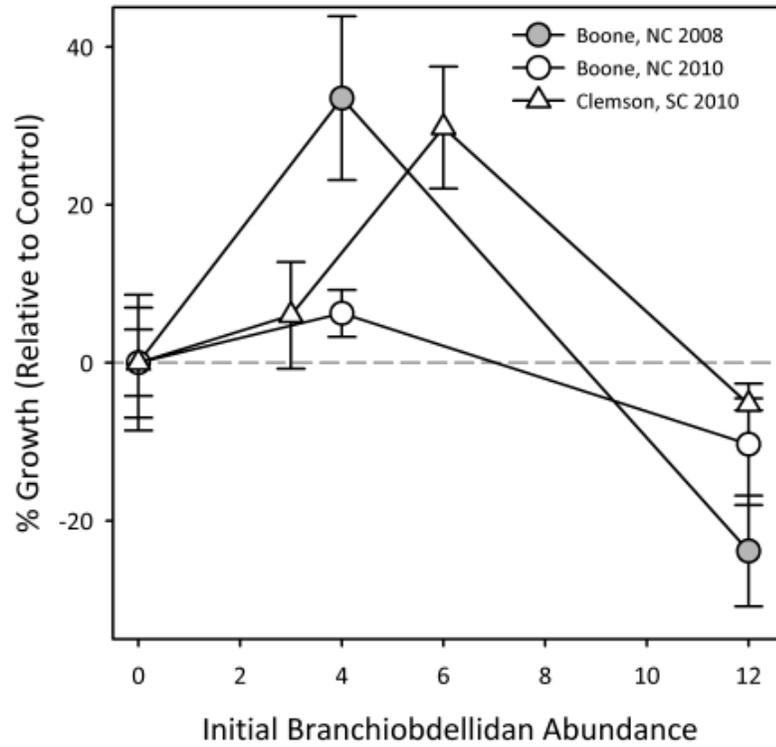


Figure 1.2 *Xironogiton instabilis* attached to the claw of *C. bartonii* from Little Stoney Creek, southwestern VA.

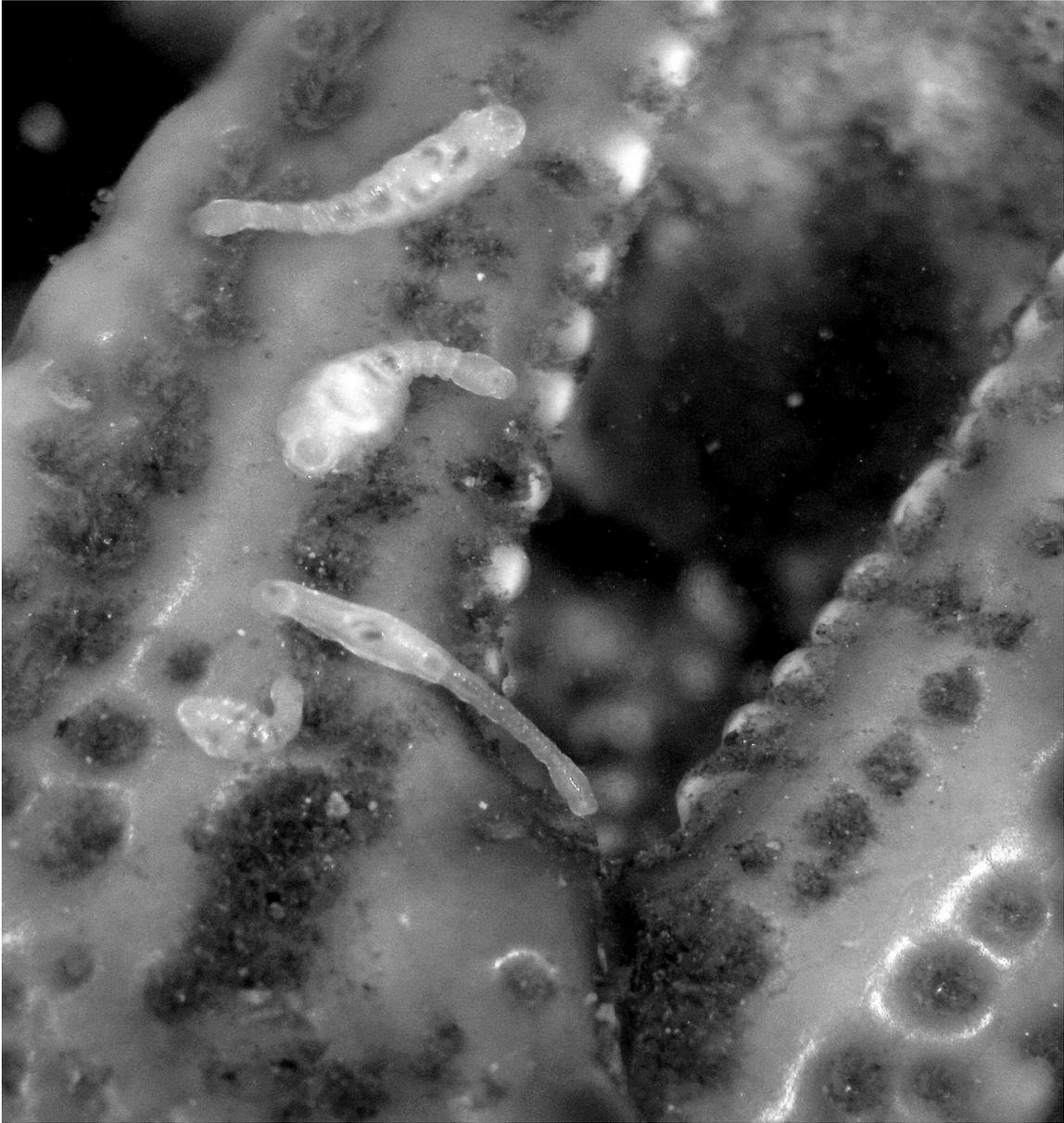


Figure 1.3 Flow-through enclosures used in crayfish/branchiobdellidan field experiments



## Chapter 2. Ontogenetic shift in host tolerance controls initiation of a cleaning symbiosis

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### ABSTRACT

When the interests of mutualists are not perfectly aligned, control mechanisms that modulate interactions can maintain mutually beneficial outcomes and stabilize mutualisms over evolutionary time. However, the costs and benefits of symbiosis often change with ontogeny and whether control mechanisms are adjusted to reflect ontogenetic changes is largely unknown. We examined the recently described cleaning symbiosis between crayfish (*Cambarus chaugaensis*) and ectosymbiotic annelids (*Xironodrilus appalachius*) for evidence of ontogenetic changes in symbiont control. *Xironodrilus appalachius* provide a beneficial cleaning service to *C. chaugaensis* by removing epibiotic accumulations from the gills, but also incur costs via density-dependent facultative parasitism of gill tissue. A series of laboratory experiments using crayfish from three size (age) – classes demonstrated that crayfish use grooming to limit cleaner density and grooming effects on cleaners varied with crayfish age. Small crayfish quickly removed essentially all of their cleaners. Intermediate crayfish removed most of their cleaners, but some cleaners persisted at a location apparently inaccessible to grooming and far from the gill chamber. Large crayfish removed a smaller proportion of cleaners and cleaners were allowed access to the gill chamber, thus initiating the cleaning symbiosis. Cleaner removal was not dependent on cleaner density, suggesting that

crayfish do not regulate cleaners to a specific density. Experimental results were corroborated by patterns observed during a field survey. We argue decreased cleaner removal and relaxed control of cleaner attachment sites corresponds to ontogenetic changes in the costs and benefits of symbiosis. This study integrates two major theoretical perspectives from ecological literature; control mechanisms and ontogenetic shifts, and illustrates how changes in control mechanisms with ontogeny may favor life-long positive outcomes of symbiosis. Ontogenetic shifts in the costs and benefits of symbiosis may be common; therefore future theoretical and empirical studies of symbioses should incorporate both perspectives.

**Key-words:** altruism, coevolution, life-history, parasitism, reciprocal exploitation

## **Introduction**

Mutualisms are pervasive, affecting most species either directly or indirectly (Herre et al. 1999). Although prevalent in nature, the evolution and long-term maintenance of mutualistic interactions may seem paradoxical because natural selection should favor individuals that cheat or overexploit their partners to gain increased benefits at the expense of their partners and non-cheating conspecifics (Axelrod and Hamilton 1981, Trivers 1971). Adaptive response among mutualists that limit cheating and overexploitation and/or favor beneficial interactions (“control mechanisms”) offer an explanation. Control mechanisms such as punishment, rewards, selectively choosing partners or dictating interaction duration allow a wide diversity of disparate species to maintain mutually beneficial interactions (Bshary and Bronstein 2011, Johnstone and Bshary 2002). Thus, the evolutionary stability of mutualisms can be preserved by adaptive responses between partners that prevent over-exploitation and a resultant

transition towards parasitism (Bshary and Bronstein 2011, Bull and Rice 1991, Johnstone and Bshary 2008, Kiers, et al. 2011, Pellmyr and Huth 1994).

Changes in the age-specific biology of an organism add another layer of complexity to symbiotic interactions. An interaction may be more or less advantageous at one life-history stage and inconsequential or even disadvantageous at another (Palmer, et al. 2010, Yang and Rudolf 2010, Yule, et al. 2013). Consequently, the life-long effects of symbiosis on fitness may integrate variable effects experienced throughout ontogeny (Yule, et al. 2013). It follows that species could maximize the life-long benefit of an interaction by adjusting control mechanisms to reflect ontogenetic changes in the costs and benefits of an interaction, but this potentially crucial facet of symbioses has yet to be thoroughly evaluated. We examined the recently described cleaning symbiosis between the crayfish *Cambarus chaugaensis* Prins and Hobbs and an ectosymbiotic annelid (Branchiobdellida; *Xironodrilus appalachius* Goodnight) (Brown, et al. 2012) for evidence of ontogenetic changes in a control mechanism that limits overexploitation.

Branchiobdellidan annelids are ectosymbionts of freshwater crustaceans, and almost exclusively of crayfish (Gelder 2010). They graze on bacteria, algae, fungi and other organisms and materials that collect on the exoskeleton and gills of their hosts (Brown, et al. 2002, Gelder 2010, Weigl 1994). Branchiobdellidans can have positive effects on the host growth rate and survivorship by grazing potentially harmful epibiotic material from the surfaces of their host, especially the gills (Brown, et al. 2002, Brown, et al. 2012, Lee, et al. 2009). However, crayfish do not always benefit from interactions with branchiobdellidans. Some branchiobdellidan species may actually be obligate gill parasites (e.g. Grabda and Wierzbicka 1969, Hobbs, et al. 1967, Quaglio, et al. 2006,

Rosewarne, et al. 2012), whereas other branchiobdellidan species, including known cleaners, are facultative parasites (Brown, et al. 2012). Parasitic attacks on crayfish gills increase with branchiobdellidan density (Brown, et al. 2012, Rosewarne, et al. 2012). Consequently, the negative effects of parasitic attacks under very high cleaner density can outweigh the positive effects of cleaning, resulting in a density-dependent shift from mutualism to parasitism (Brown, et al. 2012).

The relationship between crayfish and their cleaners may also change as crayfish age and concomitantly the potential benefit of being cleaned increases. We hypothesized that crayfish regulate cleaner density to yield a net positive outcome, and that the density to which cleaners are maintained changes with crayfish age. Crayfish rid their respiratory surfaces of epibiotic material by molting (Bauer 1998), time spent between molts (inter-molt) increases with age, and therefore accumulations of epibiotic material also increases with age (Bauer 1998, St John 1976). Increasing epibiotic accumulations presumably results in a greater potential benefit from cleaning services and a density-dependent shift from net positive to net negative effects of branchiobdellidans likely occurs at higher densities on older hosts, whereas young crayfish likely experience little or no benefit at any cleaner density. Crayfish use the opposing finger and dactyl of their walking legs to groom debris and epibionts from their exoskeleton (Farrell, et al. In press, Jones and Lester 1996), and on several occasions we (JS and BLB) have observed crayfish removing and subsequently consuming individual branchiobdellidans from their carapaces. We conducted a series of laboratory experiments and a field survey to assess the following questions: 1) Does crayfish grooming to significantly influence cleaner

density? 2) If crayfish influence cleaner density by grooming, are cleaners regulated to a particular density and does that density increase with crayfish age?

Understanding the ecological and evolutionary forces that maintain mutualisms has been a core objective of organismal biology since Darwin (1859)). Our work builds on long-standing frameworks that explain the maintenance of mutualisms through control mechanisms. We show that incorporating ontogenetic behavioral changes can expand our understanding of how control mechanisms promote mutually beneficial outcomes of symbiosis. The perspective of controls in symbioses has an established history in ecological literature (e.g. Bull and Rice 1991, Pellmyr and Huth 1994), but the importance of ontogeny in dictating the costs and benefits of symbiosis is a newly emerging perspective (Palmer, et al. 2010, Yang and Rudolf 2010, Yule, et al. 2013). We have synthesized the long-standing controls perspective, with the emerging ontogeny perspective to show how changing controls with ontogeny may promote life-long benefits of symbiosis and therefore our work provides a new understanding of how mutualisms are maintained.

## **Methods**

### *Study site*

All crayfish and branchiobdellidans used in this study were collected from Walldrop Stone Creek (WSC), near Clemson SC USA. WSC is a low order perennial tributary ( $\approx 2$  m wetted width) of the Savannah River system that runs 1.2 km through a mostly closed hardwood canopy to its confluence with the Lake Hartwell impoundment. Although at least two species of branchiobdellidans occur in WSC, only used the cleaner *X. appalachius* in grooming experiments because this species was previously

demonstrated to have measurable positive and negative density-dependent effects on crayfish growth in WSC (Brown, et al. 2012).

#### *Handling and aquarium setup*

Crayfish were collected by hand, measured by carapace length (CL) in the field, and transported back to the laboratory contained individually in plastic bags filled with stream water packed into plastic coolers. Large *X. appalachius* (>3 mm) were carefully removed from the crayfish using fine-tipped forceps under a dissecting microscope and kept in a small dish of aerated stream water for later use. All crayfish were submerged in a 10% MgCl<sub>2</sub> hexahydrate solution for 5 minutes, a procedure that effectively kills any remaining branchiobdellidans and their eggs, but causes no noticeable harm to the crayfish (Brown, et al. 2002). Crayfish were then placed individually into 24 glass aquaria (38 L) filled to one third of total volume with aged and aerated tap water and were housed in the Aquatic Animal Research Laboratory (AARL) at Clemson University, Clemson SC. Aquaria contained natural substrate from WSC, which consisted primarily of sand and fine gravel, and several small cobbles. Aeration was supplied by a single airstone, temperature was maintained at 18 – 20 °C to reflect temperatures of WSC, and standard fluorescent ceiling fixtures provided light for fourteen hours per day. We replaced 50% of the water in each aquarium weekly and crayfish were fed 2 – 3 commercial shrimp pellets (Omega One Shrimp Pellets, Omega Sea, Alaska, USA) twice weekly. Crayfish were given one week to acclimate to aquaria and recover from dactyl ablation before cleaner treatments were applied. After acclimation, cleaners were placed on the dorsal aspect of the hosts' carapaces and successful attachment of all cleaners was confirmed before crayfish were returned to their aquaria.

### *Grooming experiments*

We conducted a series of laboratory experiments using hosts from 3 size-classes to assess grooming as an age-specific control in the cleaning symbiosis between crayfish (*C. chaugaensis*) and branchiobdellidans (*X. appalachius*). Treatment groups in which the dactyls of the crayfish walking legs (pereopods 1 and 2) were ablated were compared to control groups which had their dactyls intact and we interpreted positive effects of dactyl ablation on branchiobdellidan density (all else being equal) as an indication of a negative effect of grooming on branchiobdellidan density. To determine if crayfish regulate their cleaners to a targeted density and if the targeted density changes with ontogeny, we compared grooming effects of three crayfish size-classes at multiple initial cleaner densities. We supposed that if cleaner density is actively regulated by the host, then densities observed under natural conditions should reflect the targeted density of each host. Furthermore, if crayfish regulate cleaner density then crayfish exposed to densities higher than the targeted density should reduce cleaner loads to the targeted densities. Therefore, cleaner density treatments were scaled for host size-class to reflect normal and higher than normal densities observed in the field. For each size-class, the “normal density” treatment was defined as the mean number of mature *X. appalachius* found on crayfish of that size-class at the time of collection. Mean field densities were determined for each size-class by counting the number of cleaners present on crayfish used in each experiment at the time of collection. High density was defined as the normal density plus two standard deviations, and very high was defined as the normal density plus four standard deviations. Small and large size-classes were exposed to “normal” and “high” cleaner density. The intermediate size-class received an additional “very high”

density treatment. The decision to include a third density treatment level in this size-class but not the others was based on the availability of appropriately sized *C. chaugaensis* and *X. appalachius*. This yielded 2 and 7 cleaners for the smallest size-class; 5, 10, and 15 cleaners for the intermediate size-class; and 20 and 40 cleaners for the largest size-class. Our first experiment began May 11, 2011 and included 24 crayfish from the smallest cohort present in WSC at that time (15–18 mm CL), hereafter referred to as “small” crayfish. The second experiment began June 6, 2011 and included 24 crayfish that were 18–23 mm CL and are referred to hereafter as “medium” crayfish. Our final experiment began July 4, 2011 and included 16 “large” crayfish (27–35 mm CL). The number of experimental units in the large size-class was limited by the number of *X. appalachius* that could be collected from WSC in a timely manner

Each experiment lasted approximately 3 weeks (21 d, 21 d, and 18 d for small, intermediate and large hosts respectively), during which we periodically counted the number of cleaners remaining and their locations on their hosts. Cleaners were located by placing crayfish in a small beaker of water and systematically examining all visible surfaces with a 5× magnification hand lens. This approach permitted careful inspection of all the external aspects of the crayfish while minimizing disturbance to the crayfish and their cleaners. Cleaners were occasionally dislodged from their host while being retrieved from the experimental chambers and were counted as present on the host, but their location was recorded as unknown and not included in analyses of attachment sites. The large size-class experiment was terminated on day 18 due to a failure of the laboratory climate control system.

### *Data analysis*

For each crayfish size-class experiment, we tested for effects of dactyl ablation and initial cleaner density on the proportion of cleaners remaining on their hosts through time (persistence) and all possible interaction terms using repeated measures analysis of variance (RMANOVA, `aov()` function in R base package; Team 2010). Additionally, we used a two-way ANOVA (`aov()` function in R base package; Team 2010) to test for the effects of dactyl ablation and initial cleaner density on the proportion of cleaners remaining at each day of data collection, for each experiment. All proportional data were normalized prior to analysis using an arcsine square root transformation. We also tested for effects of host size-class, dactyl ablation, and initial cleaner density on the proportion of cleaners present at all locations on the hosts' bodies using permutational multivariate ANOVA conducted on a Euclidean distance matrix (PERMANOVA, `adonis()` function in `vegan` package [2.0-0], R; Oksanen, et al. 2011).

### *Field survey*

Crayfish of all sizes were collected from the entire length of WSC throughout June and July of 2010 to determine relationships between crayfish size and cleaner density and attachment sites. Crayfish were collected by hand and by dip net and transported individually in plastic bags filled with stream water. After sex and species identification, all crayfish were inspected live under low-magnification dissecting microscopes for the presence and location of branchiobdellidans. Due to the difficulty in determining the identity of branchiobdellidans still on their host, we did not distinguish branchiobdellidan species in these surveys. A subsequent collection of 493 branchiobdellidans from WSC (collected November 2012) recovered two sympatric

species, *Cambarincola philadelphicus* Leidy in addition to *X. appalachius*. The smaller *Cambarincola philadelphicus* constituted an average of 84% of the branchiobdellidans on each crayfish and *X. appalachius* constituted 16% ( $\pm 12.8\%$  std ). Species identifications were made using a combination of live examination under high power magnification (125 – 400 $\times$ ) and differential interference contrast (DIC), as well as examinations of cleared and permanently mounted individuals (for description of methods see Gelder 2010). Identifications follow the keys and descriptions of (Ellis 1919, Goodnight 1943, Hoffman 1963, Holt and Opell 1993).

## **Results**

### *Grooming experiments*

Cleaners declined rapidly from small crayfish control groups and by day 7 almost no cleaners remained on un-ablated hosts (Fig. 1a). In contrast, ablated crayfish retained nearly half of their cleaners through the duration of the experiment resulting in a significant effect of ablation on the transformed proportion of cleaners remaining (Table 1). Controls and ablated crayfish were significantly different by day 7, and remained so until the conclusion of the experiment (Table 2.3). Initial cleaner density did not have a significant main effect, but there was a significant interaction between ablation and initial cleaner density, particularly on day 1, but this interactive effect disappeared by day 3.

Similar to the small host experiment, dactyl ablation significantly increased the proportion of cleaners that remained on medium hosts over the course of the experiment (Fig. 1b; Table 1). In contrast to small hosts, un-ablated medium hosts retained a considerable proportion of their cleaners for the duration of the experiment and the

majority of cleaner loss occurred during the first 24 hours. However, ablation effects were not significant until day 7 because of high within-group variability during days 1 and 3. There was no effect of initial cleaner density on the proportion of cleaners that remained on medium hosts at any day.

In contrast to the small and medium size-classes, large crayfish showed no significant main effects of dactyl ablation on cleaner persistence over the duration of the experiment. There was however, a significant interaction between ablation and time, indicating that cleaners on controls were declining through time at a rate greater than amputees, resulting in a significant effect of ablation on the proportion of cleaners remaining at the final day of observation (Two-way ANOVA;  $F_{1,12} = 6.222$ ,  $p = 0.028$ ), but on no previous days. This slow decline resulted in a significant effect of ablation on the final day of observation only, according to a two-way ANOVA (Fig 1; Table 2.3). Similar to other size-classes, there were no effects of initial density on cleaner persistence on large hosts (Table 1).

Cleaner attachment sites were largely restricted to one location on small and medium hosts. In both cases cleaners were observed almost exclusively on the dorsal aspect of the host's carapace (Fig. 2a,b). In contrast, cleaners placed on large hosts occupied a greater variety of locations with the highest proportions occurring at the bases of the walking legs and relatively few occupying the dorsal aspect of the carapace (Fig. 2c). PERMANOVA (9,999 permutations) showed a highly significant effect of host size-class ( $pseudo-F_{(2,168)} = 89.95$ ,  $r^2 = 0.49$ ,  $P = 0.0001$ ) on the distribution of cleaners. There was also a significant, but weak effect of initial density ( $pseudo-F_{(2,168)} = 5.082$ ,  $r^2 = 0.03$ ,  $P = 0.0015$ ). Our initial model found no significant effects of dactyl ablation

(*pseudo-F*<sub>(1,156)</sub> = 2.894,  $r^2 = 0.008$ ,  $P = 0.06$ ) or day (*pseudo-F*<sub>(5,156)</sub> = 1.862,  $r^2 = 0.03$ ,  $P = 0.06$ ) and therefore these predictors were excluded from the final analysis.

### Survey

*Cambarus chaugaensis* was the dominant crayfish species observed in WSC. A few *Cambarus asperimanus* Faxon, *Cambarus bartonii* Fabricius, and *Procambarus clarkii* Girard were also collected but not included in the survey. We examined a total of 213 *C. chaugaensis* (99 males, 114 females) that ranged in size from 14.24 to 44.10 mm CL (1.07 – 28.06 g BWM) and observed a total of 747 branchiobdellidans. There was a strong positive linear relationship between crayfish mass and the total number of branchiobdellidans on each crayfish ( $F = 164.1$ ,  $P \ll 0.001$ , *adj. r*<sup>2</sup> = 0.43; Fig. 3). Host sex was not a significant predictor of branchiobdellidan density and was therefore excluded from the linear model ( $t = 0.926$ ,  $P = 0.36$ ). An analysis of a subsequent sample in which branchiobdellidans were identified to species revealed that *X. appalachius* and *C. philadelphicus* showed similar positive linear relationships with host mass ( $F = 4.768$ ,  $P < 0.05$ , *adj. r*<sup>2</sup> = 0.22 and  $F = 15.34$ ,  $P < 0.005$ , *adj. r*<sup>2</sup> = 0.52, respectively). Also, the relative abundance of the two symbiont species did not change with host mass ( $F = 0.847$ ,  $P = 0.38$ ,  $r^2 = 0.066$ ; Fig. 2.4). There were obvious differences in the most frequently used attachment sites of branchiobdellidans among host size-classes observed from the field (Fig. 3). Similar to experimental results, the largest proportion of branchiobdellidans inhabiting small and medium crayfish was found on the carapace. Branchiobdellidans attached to large crayfish occupied more locations overall. Approximately one fifth (20%) of branchiobdellidans observed on large crayfish in the field were found at the opening of the gill chamber, attached to the proximal portions of

the walking legs. Conversely, no branchiobdellidans were observed attached to the walking legs of small or medium crayfish.

## **Discussion**

The results of our experiments demonstrate that crayfish effectively use the dactyls of their walking legs to remove ectosymbiotic cleaners through grooming. Contrary to our predictions, we did not find evidence of a density-dependent grooming response. Instead, young crayfish were entirely intolerant towards cleaners, whereas older crayfish were more tolerant, irrespective of cleaner density. Additionally we found that cleaners have host-size specific behavioral responses which allow them to occupy young and intolerant hosts by occupying an area apparently inaccessible to grooming. Since branchiobdellidans are thought to benefit their hosts by cleaning the respiratory surfaces, the restricted distribution of cleaners on intolerant hosts precludes any benefit to the host. Therefore, host tolerance dictates the ontogenetic stage at which a mutualistic association occurs. Our view is supported by several lines of evidence from our experiments and survey data.

In all 3 experiments, more cleaners persisted on crayfish with ablated dactyls than on controls indicating that the dactyls are used to remove cleaners. This result supports previous speculation that crayfish can detect branchiobdellidans by way of mechanoreceptors spread across their exoskeletons (Gelder 2010), and corroborate the findings of previous work on another group of crayfish ectosymbionts, the temnocephalidans (Platyhelminthes), which replace branchiobdellidans as crayfish ectosymbionts throughout Southeast Asia and Australia (Gelder 1999). In an experiment that was methodologically similar to the one presented in this paper, Jones and Lester

(1996) demonstrated that the crayfish *Charex quadricarnatus* von Martens uses its chelipeds and pereopods to remove temnocephalidans which results in a large and significant reduction of overall temnocephalidan densities. Clearly, crayfish are capable of detecting and removing their ectosymbiotic cleaners by grooming, thus the possibility that crayfish control cleaner density by removing excess cleaners is plausible.

Crayfish showed age-specific responses to the introduction of cleaners that indicates an ontogenetic shift towards tolerance of cleaners. Small and medium crayfish removed most or all cleaners at all experimental cleaner densities. After 7 days, only a single cleaner remained on small crayfish with intact dactyls, whereas small crayfish with ablated dactyls retained ~40% of their cleaners to the conclusion of the experiment. Therefore, we concluded that small crayfish use the dactyls of the walking legs to remove cleaners and can effectively remove all cleaners. Dactyl ablation of medium crayfish also significantly increased cleaner persistence, though interestingly ~25% of cleaners persisted on controls for the duration of the experiment, regardless of initial cleaner density. We also observed some cleaner removal by large hosts, as evidenced by a slow decline in cleaners on control animals, but not on ablated animals. However, the difference between controls and ablated crayfish was only significant on the final day of the experiment. These results suggest that even large hosts remove some cleaners, but this response is weaker than in small and medium crayfish which are wholly intolerant of cleaners and quickly removed all or most cleaners introduced to them. One possible explanation is that slow and continual removal of cleaners offsets cleaner reproduction and colonization to maintain cleaner densities at beneficial levels (i.e. Brown et al. 2012), however this speculation requires experimental verification.

One explanation for increased cleaner persistence on large crayfish is that large crayfish are simply unable to detect cleaners or lack the dexterity to remove them. Experimental work on another crayfish species suggests that this explanation is unlikely. The crayfish *Orconectes cristivarius* occurs in sympatry with the New River crayfish (*Cambarus chasmodactylus*) and its ectosymbiotic cleaner *Cambarincola ingens* in the New River, near Boone, NC (Brown and Creed 2004). While adult New River crayfish often host dozens of cleaners, *O. cristivarius* typically host few if any (Brown and Creed 2004). Recent work demonstrated that even large *O. cristivarius* respond to the introduction of a single branchiobdellidan by quickly removing it, in contrast to similarly sized *C. chasmodactylus* (Farrell, et al. In press). Instead, adult *O. cristivarius* may rely on antimicrobial properties of their hemolymph to resist epibiotic accumulations (Farrell, et al. In press). This result demonstrates that large crayfish can both detect and remove branchiobdellidans, and strongly suggests host tolerance as an explanation for the proliferation of cleaners on the adults of some, but not all crayfish species (but see Brown and Creed 2004 for another possibility).

Additionally, the possibility that the observed change in host response was driven by temporal changes in environmental covariates unrelated to ontogeny requires discussion. Size-class experiments were conducted in sequence in order to capture the ontogenetic change of host response in a single cohort (small and intermediate size-classes). We are confident that our results reflect an ontogenetic shift in host tolerance rather than temporal environmental covariates for the following reasons: 1) All experiments were conducted within the summer months of 2011 to minimize temporal variation in environmental covariates, 2) experiments were conducted under constant

controlled laboratory conditions (similar temperature, water conditions, photoperiod, etc.) and crayfish were given a week to acclimate to laboratory conditions, and 3) our experimental data are supported by field observations from a smaller time period (June and July) in the previous year (2010).

Based on the hypothesis that crayfish regulate cleaners to some targeted density, we predicted that they would remove cleaners only when supplied with cleaners in excess of size-specific densities observed in the field, and that the final density of all un-ablated hosts would converge on field densities. Contrary to our predictions, initial cleaner density did not affect the proportion of cleaners that persisted on hosts of any size-class. Thus our results do not support the hypothesis that grooming is used to maintain a targeted density of cleaners, at least for the species examined here. Rather young crayfish are intolerant of cleaners at any density, and become more tolerant with age. However, we must address two points that appear to be inconsistent with this interpretation: 1) that ~25% cleaners remained on medium crayfish with intact dactyls for the entire experiment, and 2) that large crayfish with intact dactyls removed some of their cleaners. These inconsistencies can be reconciled with our interpretation of an ontogenetic switch in tolerance towards cleaners by considering host-age specific behavioral responses of the cleaners.

Just as crayfish showed an ontogenetic shift in their tolerance towards cleaners, the cleaners too displayed behavioral responses specific to host age. The large majority of cleaners placed on small and medium hosts remained attached to the dorsal-most aspect of the hosts' carapaces. Dactyl ablation had no detectable effect on the distributions of cleaners across their hosts' bodies and therefore the restricted

distributions of cleaners on small and medium hosts did not arise simply as the result of the crayfish removing any cleaners that strayed from the dorsal aspect of the carapace. Instead, the restricted distribution of cleaners on small and medium hosts indicates a strong affinity for a particular attachment site. Unlike small and medium hosts, cleaners occupied a variety of locations on large hosts. Perhaps the strong affinity of cleaners for dorsal carapace attachment sites on small and medium hosts is an adaptive response to strong selective forces imposed by young crayfish that rapidly remove branchiobdellidans from easily accessible areas. However, our experimental data suggest that this strategy may only be effective once crayfish reach a particular size. Cleaners were unable to persist on small hosts with intact dactyls, indicating that small crayfish can effectively groom cleaners from the dorsal portion of their carapace. Conversely, ~25% of cleaners persisted on medium crayfish with intact dactyls for the duration of the experiment. Furthermore, cleaner removal on medium hosts occurred almost exclusively during the first 24 hrs of the experiment, indicating that the initial colonization time is critical to cleaner persistence on medium hosts, but once cleaners have attached to the dorsal refuge they are safe from removal. Combined, the results from the small and medium crayfish experiments suggest that cleaners seek refuge from grooming on young hosts by attaching to the dorsal aspect of the carapace, but this strategy may be ineffective on the smallest crayfish.

Host age-specific distributional patterns of cleaners during our experiments were generally consistent with patterns observed in the field. Similar to experimental results, the largest fraction of cleaners observed from the field were found on the dorsal carapace, and cleaners occupied a greater diversity of locations on larger hosts. Therefore the

distributional patterns observed during laboratory experiments were not an artifact of experimental conditions. Thus, at some point during host ontogeny, cleaners leave the sanctuary of their refuge to occupy preferable locations at the continued, yet reduced peril of being removed. By selecting an attachment site that is inaccessible to grooming on young hosts, cleaners may be able to colonize crayfish earlier yielding an advantage over conspecifics competing for limited space on hosts.

Changing cleaner attachment sites through host ontogeny have important implications for the outcome of the association for the host. Branchiobdellidans are thought to benefit their hosts by cleaning potentially harmful epibiotic material from crayfish respiratory surfaces (Brown, et al. 2002 Brown, et al. 2012, Lee, et al. 2009). Furthermore, previously reported negative effects of cleaners on crayfish were associated with damage inflicted to the gills under high cleaner densities (Brown, et al. 2012). In our experiments, cleaner distribution was almost exclusively limited to the dorsal-most aspect of the carapace on small and medium crayfish, making the gills inaccessible to cleaners and precluding both known costs and benefits of symbiosis. In contrast, cleaners on large crayfish were found frequently in areas proximate to the gill chamber (e.g. proximal portions of the walking legs) under both field and experimental conditions. Therefore, crayfish may dictate the initiation of the cleaning symbiosis not only by removing cleaners, but also by permitting cleaners access to vital areas.

Can the observed ontogenetic shift in host tolerance to cleaners be related to changing potential costs and benefits of symbiosis? Although crayfish possess structures known to effectively relieve gills of large fouling particles (setobranchs), molting of the gill cuticle is the only known means by which crayfish rid themselves of gill fouling

micro-epibionts (Bauer 1998, but see Farrell, et al. In press for another possibility). Molt frequency declines with crayfish age and small juvenile crayfish may undergo many closely spaced molts during their first year, whereas adults may only molt once or twice per year (St John 1976). Therefore, the potential for accumulation of epibiotic microorganisms is greater on adults than juveniles and the potential benefit of cleaning is presumably greater for older hosts. Additionally, low availability of epibiotic material on young crayfish may increase facultative parasitism of cleaners as they switch to feeding on host tissue in response to decreases in resource availability, as has been observed with increased cleaner densities (Brown, et al. 2012) and decreased epibiotic resources (Cheney and Cote 2005).

We argue that the ontogenetic shift from intolerance to tolerance corresponds with a threshold stage in ontogeny at which the benefits of symbiosis outweigh the costs. Ontogenetic change in a control mechanism is not entirely without precedent. Previous work on cleaning symbioses among fishes of the tropical coral reefs provides some evidence of behavioral controls that are modulated with age-specific changes in costs and benefits of symbiosis. Like branchiobdellidans, cleaner fish can have a positive effect on clients by removing and consuming harmful epibionts (Clague, et al. 2011, Grutter 1999, Limbaugh 1961) but often parasitize their clients by feeding on client tissues and mucus (Gorlick 1980, Grutter and Bshary 2003). Client fish use multiple controls to obtain the benefits of being cleaned while limiting parasitic attacks (Bshary and Grutter 2002, Bshary and Grutter 2005, Bshary and Noë 2003, Bshary and Schäffer 2002, Johnstone and Bshary 2002, Johnstone and Bshary 2008). For instance, client fish reduce parasitism by controlling cleaning duration, as the likelihood of parasitism by cleaner fish

increases with cleaning duration (Johnstone and Bshary 2002). Additionally, cleaner fish consume more client tissue when ectoparasites are scarce, suggesting increased parasitism in response to decreasing resources (Cheney and Cote 2005). Client fish extend the duration of encounters as they grow because larger clients carry higher parasite loads and thus the incurred benefit of being cleaned persists longer (Johnstone and Bshary 2002). We argue that the crayfish/branchiobdellidan cleaning symbiosis is similar insofar as young crayfish receive little to no benefit from being cleaned and therefore avoid or greatly reduce their contact with cleaners by simply removing them or restricting their distribution to areas inaccessible to grooming. However, when increased inter-molt periods lead to increased benefits of cleaning, resulting a net positive outcome of symbiosis, cleaners are allowed to remain and permitted access to vital areas such as the opening of the gill chamber.

Strong positive relationships between branchiobdellidan density and host size have been reported here and elsewhere for several species of crayfish and branchiobdellidans (Bishop 1968, Brown and Creed 2004, Keller 1992, Mc Manus 1960, Young 1966). This seemingly ubiquitous pattern may be at least partly explained by age-specific differences in host control. Although other explanations for this pattern have been proposed, including resource limitation and disturbance associated with host molts (Bishop 1968, Mc Manus 1960, Young 1966), these speculations have not been experimentally verified. In our experiments, small and medium crayfish removed most of their cleaners within the first 24 hours and small crayfish had removed essentially all cleaners within one week. The rapid removal of cleaners placed on small crayfish suggests that host control is a stronger driver than molting or resource limitation, at least

on very young crayfish. Conversely, a recent study of the crayfish *Astacus leptodactylus* Eschsholtz and their branchiobdellidan associates (*Branchiobdella kozarovi* Subchev) concluded that temperature was a stronger driver of branchiobdellidan density than host size (DeWitt, et al. 2012). In our survey, all samples were collected within a short time period (~2 months) and therefore we cannot address the potential influence of varying environmental temperature. However, we have provided very strong evidence for a relationship between host size and branchiobdellidan density and demonstrated age-specific grooming behaviors as a likely contributing force for that relationship for *X. appalachius*. Additionally, we found similar relationships between host size and cleaner abundance for both species of cleaners found in WSC and the relative abundance of these species did not change over host ontogeny. This finding suggests that both *X. appalachius* and *C. philadelphicus* are under similar age-specific host control. There remains the enticing possibility that environmental factors such as temperature and host characteristics, such as age-specific tolerances, interact to create complex temporal and spatial patterns in branchiobdellidan abundance.

Our understanding of positive species interactions lags behind negative interactions such as predation and competition, despite being of similar importance to community structure and ecological processes (e.g. Bertness and Callaway 1994, Bertness and Leonard 1997, Bruno, et al. 2003, Callaway 1995). This lag may be attributed to the inherent complexity and variability of such interactions. Because the net outcomes of potentially positive interactions often vary from positive to negative with changes in context and ontogeny, mutualisms and other positive interactions are often a moving target for researchers studying the outcomes and implications of species

interactions. Improving our understanding of the role of mutualisms in community processes requires a better conceptualization of the internal and external factors that erode and promote reciprocally positive interactions among organisms. The work presented here suggests that organisms engaged in mutualisms can maintain reciprocally beneficial outcomes by exerting controls that change with ontogeny and concomitant changes in potential costs and benefits. This example highlights the potential for integrating ontogeny and control mechanisms to achieve a more complete picture of mutualisms and how they are maintained. Future efforts to specifically characterize and quantify age-specific costs and benefits of symbiosis are needed and will contribute greatly to our understanding of the role ontogeny that plays in the evolution of stabilizing control mechanisms between mutualists.

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## Figure and Table Captions

### Figure 2.1

Persistence of the branchiobdellidan *Xironodrilus appalachius* on the crayfish *Cambarus chaugaensis* in laboratory experiments conducted using three size-classes of crayfish; small at 15-18 mm carapace length (CL), medium at 18-22 mm CL, and large crayfish at 27-35 mm CL. Two treatments were crossed in a full factorial design to assess the removal of *X. appalachius* by the host under varying *X. appalachius* density. Hosts in “ablated” groups (open symbols) had their dactyls removed from the first and second walking legs whereas “control” groups (shaded symbols) were left with dactyl intact. Density levels refer to the initial abundance of *X. appalachius* at the beginning of each experiment and are relative to observed field density for each size-class; ○ = normal, □ = high, and △ = very high). Symbols represent mean proportion of *X. appalachius* remaining on each host within each treatment combination  $\pm$  1SE. Asterisk (\*) indicates significant effect ( $p \leq 0.05$ ) of dactyl ablation for each day (Two-way ANOVA, ablation and initial cleaner density as factors and normalized proportion of cleaners remaining as the response). There were no significant effects of initial cleaner density for any day.

### Figure 2.2

Proportional distribution of *Xironodrilus appalachius* on different attachment sites of the crayfish body (see Table 2 for attachment site descriptions) from all three experiments; small, medium, and large crayfish. Bars indicate the mean ( $\pm$ 1SE) proportion of *X. appalachius* found on each host at each attachment site and include observations from all sampling dates. Hosts which had no *X. appalachius* remaining were excluded. *X.*

*appalachius* on small (a) and medium (b) hosts were largely restricted to the dorsal aspect of the host's carapace (shown as DC), but frequently occupied several locations on large hosts (c). We detected no significant effect of ablation on the distribution of *X. appalachius* on any size-class of host (PERMANOVA).

### Figure 2.3

Distribution and density of branchiobdellidans on *Cambarus chaugaensis* in Walldrop Stone Creek from field survey data collected during June and July of 2010. (*Upper*) Pie chart slices represent the proportion of total branchiobdellidans observed within each size-class at each attachment site on the host. Divisions among size-classes are based on size (CL) and same as divisions made for the grooming experiments to aid in comparisons. The largest portion of branchiobdellidans occupied the carapace on small and medium hosts. (*Lower*) Number of branchiobdellidans observed versus host blotted wet mass (g). Symbols are color-coded to reflect size-classes. "Unclassified" represents crayfish that did not fall into any of the size-classes used in grooming experiments. Dotted line represents best fit linear model. Note that  $x$  - axis is log scaled for clarity.

### Figure 2.4

Changes in the abundance of two branchiobdellidan species in Walldrop Stone creek with host age. Absolute abundance of *X. appalachius* and *C. philadelphicus* as a function of crayfish mass (left panel). Both branchiobdellidan species showed similar significant increases in abundance with host mass. The relative abundance of each species did not change with host mass (right panel).

Table 2.1

Results of three repeated measures ANOVA's testing for effects of ablation, initial density, and sampling day on the proportion of *Xironodrilus appalachius* remaining on crayfish during three experiments using small, medium and large crayfish.

Table 2.2

Descriptions and abbreviations of attachment sites occupied by *Xironodrilus appalachius* on the host *Cambarus chaugaensis* during grooming experiments.

Table 2.3

Results of two by two factorial ANOVA's testing for effects of crayfish dactyl ablation and initial cleaner density at each sampling day on the proportion of *Xironodrilus appalachius* remaining on crayfish during three experiments using small, medium and large crayfish. Bold font indicates significant  $p$  – values ( $\alpha = 0.05$ ).

Figure 2.1 Persistence of *X. appalachius* during a laboratory experiment

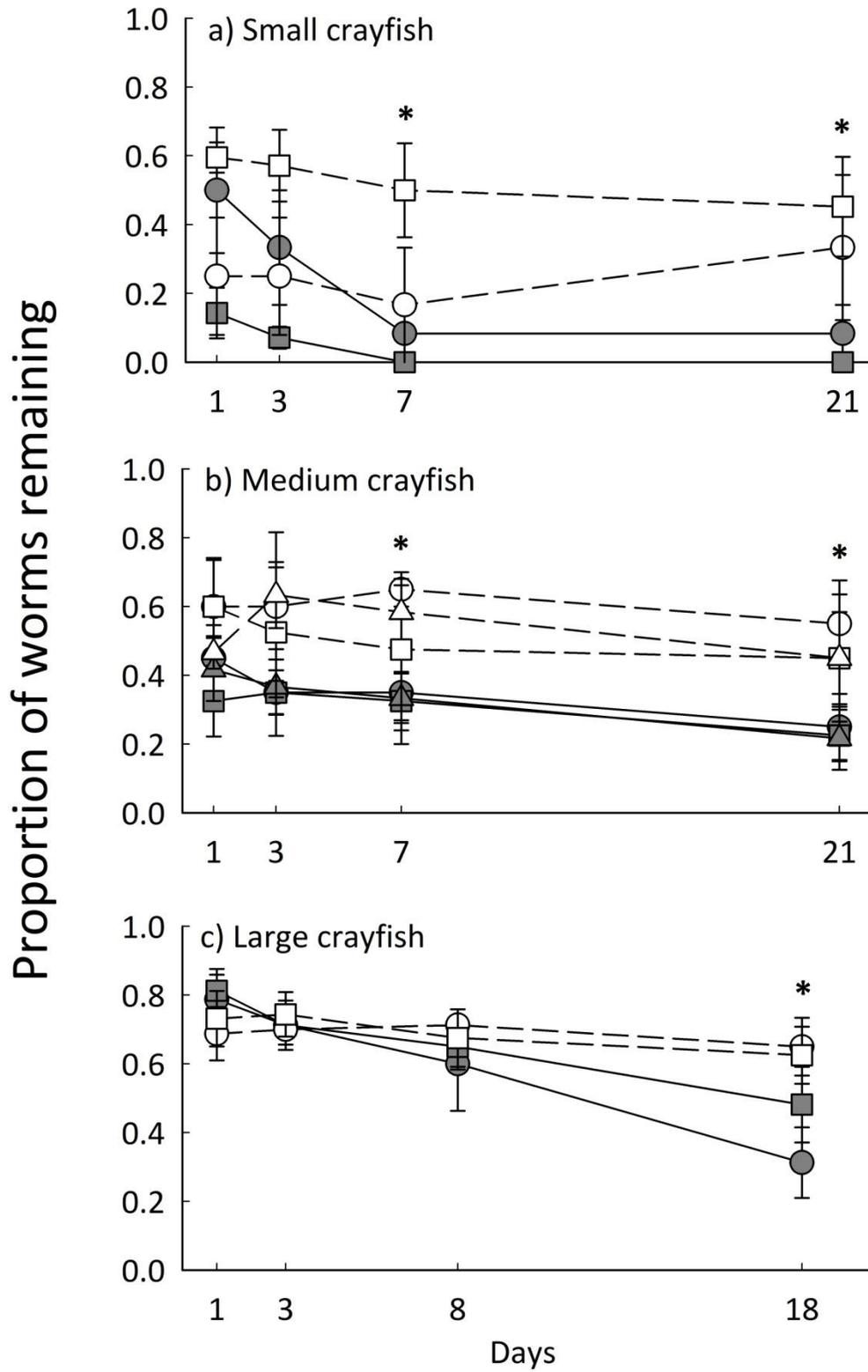


Figure 2.2 Attachment site use of *X. appalachius* during laboratory experiment

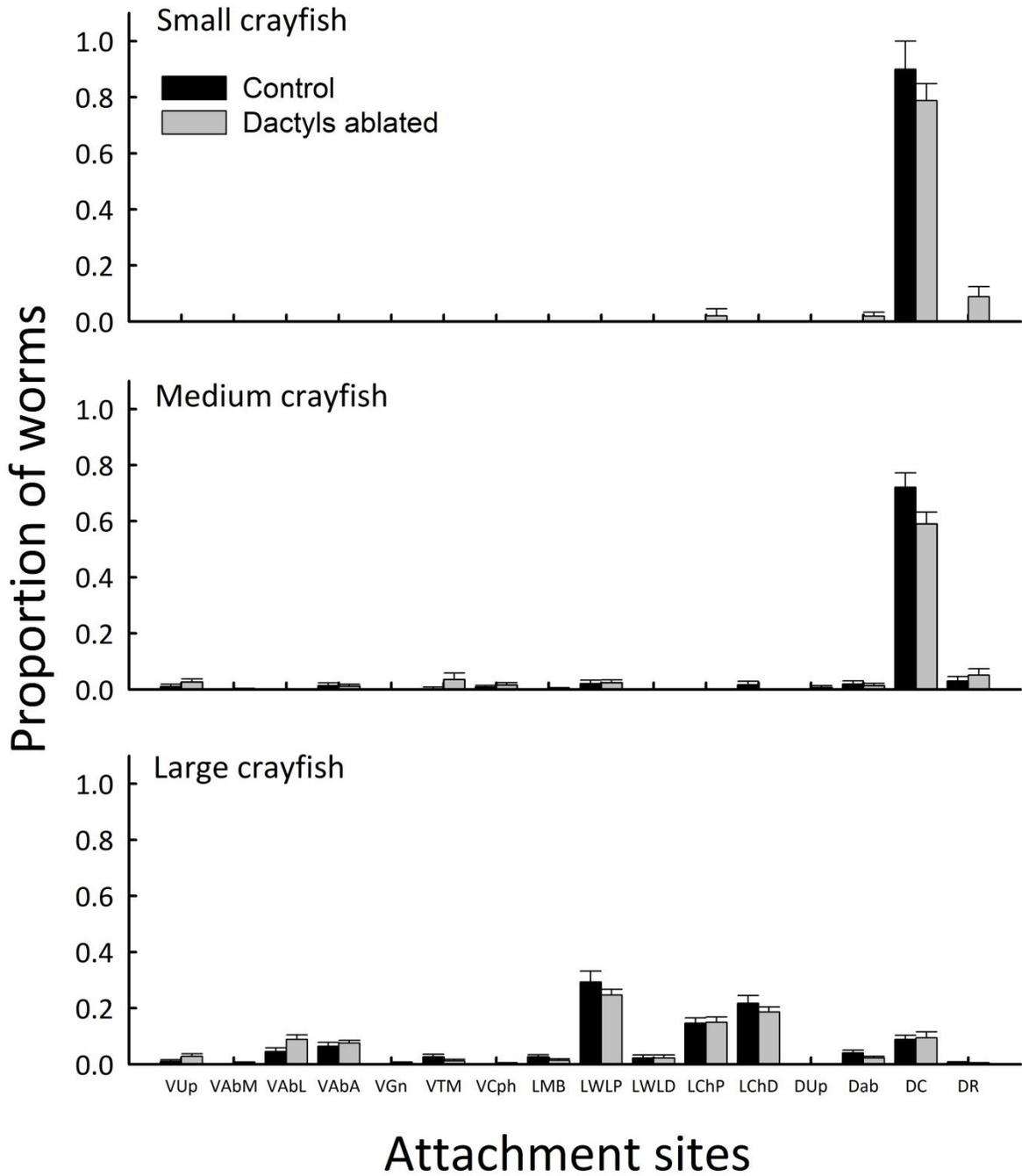


Figure 2.3 Field observations of *X. appalachius* attachment site use and abundance in relation to host size

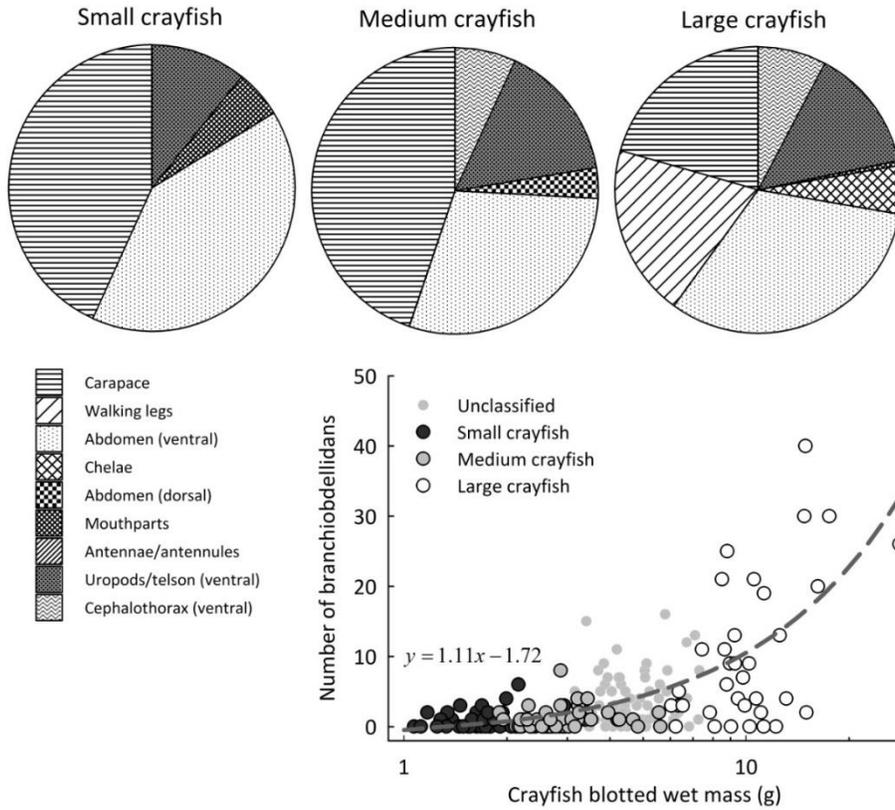


Figure 2.4 Absolute and relative abundances of *X. appalachius* and *C. philadelphicus* in relation to host size

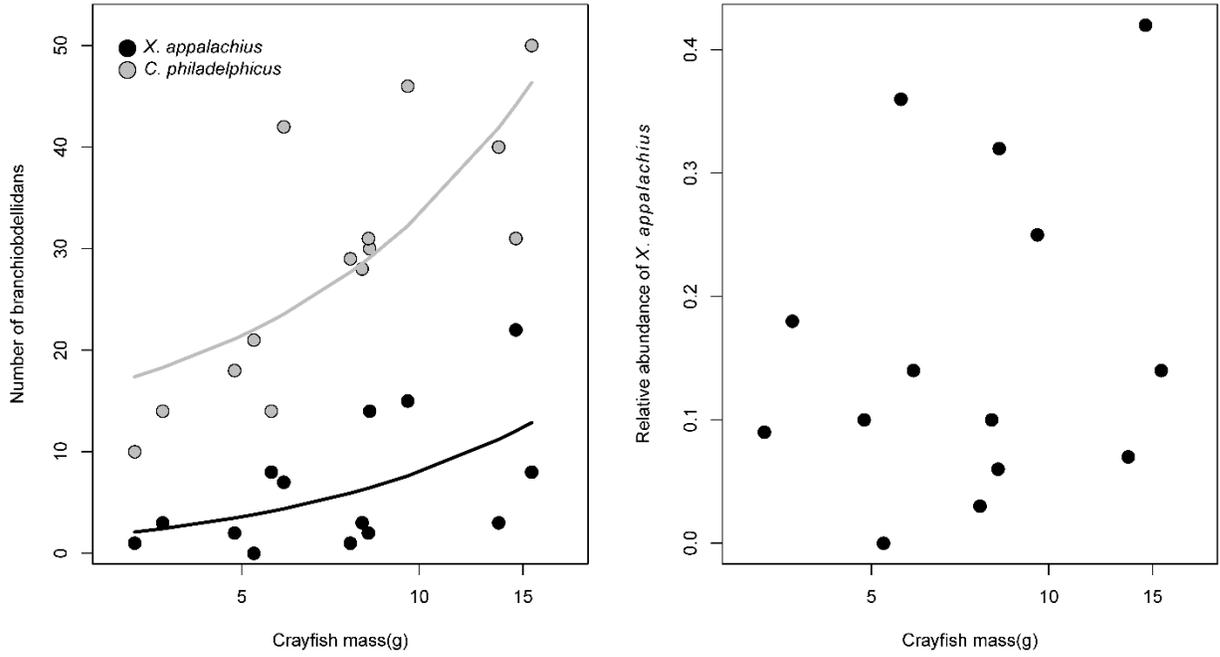


Table 2.1 Repeated measures ANOVA's for laboratory experiment

Host size-class	Source	d.f.	MS	<i>F</i>	<i>P</i>
Small crayfish	<b>Ablation</b>	<b>1</b>	<b>3.011</b>	<b>4.409</b>	<b>0.049</b>
	Density	1	0.151	0.221	0.643
	<b>Density × Ablation</b>	<b>1</b>	<b>3.011</b>	<b>4.409</b>	<b>0.049</b>
	Error Between	20	0.683		
	<b>Day</b>	<b>1</b>	<b>0.568</b>	<b>5.694</b>	<b>0.020</b>
	Day × Ablation	1	0.532	5.335	0.024
	Day × Density	1	0.002	0.002	0.963
	Day × Ablation × Density	1	0.341	3.424	0.069
	Error Within	68	0.100		
Medium crayfish	<b>Ablation</b>	<b>1</b>	<b>1.874</b>	<b>5.994</b>	<b>0.024</b>
	Density	1	0.013	0.040	0.843
	Density × Ablation	1	0.027	0.085	0.774
	Error Between	20	0.315		
	<b>Day</b>	<b>1</b>	<b>0.366</b>	<b>13.162</b>	<b>&lt;0.001</b>
	Day × Ablation	1	0.053	1.917	0.171
	Day × Density	1	0.007	0.247	0.621
	Day × Ablation × Density	1	0.001	0.002	0.962
	Error Within	68	0.028		
Large crayfish	Ablation	1	0.041	0.428	0.525
	Density	1	0.015	0.155	0.701
	Density × Ablation	1	0.007	0.075	0.788
	Error Between	12	0.096		
	<b>Day</b>	<b>1</b>	<b>0.691</b>	<b>48.576</b>	<b>&lt;0.001</b>
	<b>Day × Ablation</b>	<b>1</b>	<b>0.311</b>	<b>21.834</b>	<b>&lt;0.001</b>
	Day × Density	1	0.009	0.645	0.426
	Day × Ablation × Density	1	0.053	3.706	0.061
	Error Within	44	0.014		

Table 2.2 Descriptions of branchiobdellidan attachment sites

Attachment Site	Description
<i>Ventral</i>	
VUp	Ventral aspect of the uropods and telson
VAbM	Ventral aspect of the abdomen, attached mesial to the swimmeretes
VAbL	Ventral aspect of the abdomen, attached to lateral margins of abdominal tergites or swimmerets not including VAbA and VGn
VAbA	Ventral aspect of abdomen, attached to or near lateral margins of first abdominal tergite
VGn	Genital opening of females or the gonopods of males
<i>Lateral</i>	
LMB	Branchiostegite within 1 cleaner body length of the lateral margin
LWLP	Coxa, basis, and ischium of walking legs
LWLD	Walking legs not including LWLP
LChP	Coxa, basis, ischium and merus of chelipeds
LChD	Chelipeds and chelae not including LChP
<i>Dorsal</i>	
DUp	Dorsal aspect of uropods and telson
Dab	Dorsal aspect of abdomen
DC	Dorsal aspect of carapace, not including LMB and DR
DR	Rostrum

Table 2.3 Two by two factorial ANOVA's for each day and host size class in laboratory experiment.

Size-class	Day	Source	d.f.	MS	<i>F</i>	<i>p</i>
Small	1	Ablation	1	0.0680	0.261	0.615
		Density	1	0.0005	0.002	0.967
		Interaction	1	1.4948	5.746	<b>0.026</b>
		Residuals	20	0.2602		
	3	Ablation	1	0.5040	1.982	0.175
		Density	1	0.0496	0.195	0.664
		Interaction	1	1.0621	4.176	0.054
		Residuals	20	0.2543		
	7	Ablation	1	1.2624	6.527	<b>0.019</b>
		Density	1	0.2326	1.202	0.286
		Interaction	1	0.6447	3.333	0.083
		Residuals	20	0.1934		
	21	Ablation	1	1.8942	7.294	<b>0.014</b>
		Density	1	0.0088	0.034	0.856
		Interaction	1	0.1717	0.661	0.426
		Residuals	20	0.2597		

Table 2.3 continued

Size-class	Day	Source	d.f.	MS	<i>F</i>	<i>p</i>
Medium	1	Ablation	1	0.2255	3.485	0.077
		Density	1	0.0300	0.464	0.504
		Interaction	1	0.0110	0.170	0.684
		Residuals	20	0.0647		
	3	Ablation	1	0.4871	3.386	0.081
		Density	1	0.0136	0.094	0.762
		Interaction	1	0.0014	0.010	0.923
		Residuals	20	0.1438		
	7	Ablation	1	0.5022	5.501	<b>0.029</b>
		Density	1	0.0004	0.0004	0.949
		Interaction	1	0.0131	0.144	0.709
		Residuals	20	0.0913		
	21	Ablation	1	0.7330	6.892	<b>0.016</b>
		Density	1	0.0224	0.211	0.651
		Interaction	1	0.0049	0.046	0.832
		Residuals	20	0.1064		

Table 2.3 continued

Size-class	Day	Source	d.f.	MS	<i>F</i>	<i>p</i>
Large	1	Ablation	1	0.0686	1.593	0.231
		Density	1	0.0013	0.030	0.866
		Interaction	1	0.0033	0.077	0.787
		Residuals	12	0.0430		
	3	Ablation	1	0.0001	0.008	0.932
		Density	1	0.0025	0.145	0.710
		Interaction	1	0.0037	0.220	0.647
		Residuals	12	0.0170		
	8	Ablation	1	0.0147	0.370	0.554
		Density	1	0.0002	0.001	0.982
		Interaction	1	0.0044	0.111	0.744
		Residuals	12	0.0397		
	18	Ablation	1	0.2865	6.22	<b>0.028</b>
		Density	1	0.0267	0.581	0.461
		Interaction	1	0.0496	1.077	0.320
		Residuals	12	0.0460		

### **Chapter 3. Host resistance sets the rules for symbiont community assembly**

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#### **ABSTRACT**

Diverse internal and external symbiont communities affect nearly all facets of animal biology and contribute greatly to biodiversity world-wide. Symbiont diversity is influenced by host resistance to colonization and by within-host interactions among symbiont taxa. Here we show that these processes combine during symbiont community assembly, and host resistance can modulate the influence of symbiont interactions. A survey of crayfish and their annelid symbionts (Branchiobdellida) revealed patterns of symbiont abundance, diversity, and species composition through host ontogeny. We show that these patterns correspond with an ontogenetic shift in host resistance. Young hosts use a directed grooming behavior to resist colonization of symbionts. Grooming prevents colonization of some symbiont taxa and thus acts as a species filter to early colonization. Older hosts do not resist colonization and consequently host more symbiont individuals and species. Using experimentally reconstructed symbiont communities, we show interactions among symbiont taxa are prevalent in diverse symbiont communities of older hosts, and weak or absent in young resistant hosts. Thus, host resistance directly influences crayfish symbiont communities by filtering species, and indirectly by moderating symbiont-symbiont interactions. Our results demonstrate that host resistance

and interactions among symbionts combine to shape patterns of symbiont diversity within a host population.

**Keywords:** symbiosis, mutualisms, parasites, ontogeny, crayfish, succession

## **Introduction**

Most animals host diverse communities of internal and external symbionts which may include beneficial mutualists, innocuous commensals, detrimental parasites, and disease causing pathogens. There is an increasing realization that within-host interactions among symbionts and complex host-symbiont feedbacks are fundamental to an understanding of symbiont diversity and evolution (Råberg et al. 2006, Telfer et al. 2010, Ulrich and Schmid-Hempel 2012). As a consequence of these potentially interacting mechanisms, symbionts display complex patterns of diversity that cannot be understood from the perspective of multiple independent pair-wise interactions (Holmes and Price 1986, Esch et al. 1990, Poulin 2001, Rigaud et al. 2010, Ulrich and Schmid-Hempel 2012, Miller and Moran 2014). This complexity has likely been the cause of widespread disagreement across studies seeking to explain observed patterns of symbiont diversity (e.g. reviews in: Sousa 1994, Bush et al. 1997, Poulin 2001, Poulin 2007). Recently, several authors have made compelling cases for implicating a within-host community ecology framework to understand deterministic outcomes of parasite co-infection (Pedersen and Fenton 2007, Graham 2008) and a similar approach should be equally valuable for non-parasitic symbionts. Disentangling the complex and often interacting within-host mechanisms that underpin patterns of within-host symbiont diversity requires

experimental studies (Johnson and Buller 2010, Rigaud et al. 2010), but experimental evidence is currently limited. In this study we examine how two such mechanisms combine to create deterministic patterns of symbiont diversity throughout the lives of their hosts. We provide observational and experimental evidence to show how host resistance and interactions among symbionts lead to explainable patterns of symbiont diversity in the cleaning symbiosis between crayfish and branchiobdellidans annelids.

Hosts often directly influence the symbiont colonization process and this influence can be highly variable across and within systems. For instance, when symbiosis offers a fitness advantage, selection favors hosts that allow or actively facilitate colonization of symbionts (e.g., Van Rhijn and Vanderleyden 1995, Nyholm et al. 2000). Conversely, when symbiosis is detrimental to the host (i.e. parasitism), selection can lead to co-evolutionary arms races between hosts which try to resist colonization, and parasites which try to circumvent host resistance (Van Valen 1974, Ebert and Hamilton 1996). While these direct effects on the colonization of symbiont taxa are relatively straight-forward, the influence of the host on symbiont colonization dynamics may lead to more complex indirect influences over the processes that shape diverse within-host communities of symbionts, a level of ecological organization termed the *symbiont infracommunity* (*sensu* Bush et al. 1997) .

Parasitologists have long recognized the potential importance of symbiont interactions in structuring infracommunities (Sousa 1994, Poulin 2007, Johnson and Buller 2010, Ulrich and Schmid-Hempel 2012) and several authors have attempted to formalize a conceptual framework for understanding the circumstances that lead to strong interactions among parasitic symbionts (Holmes 1973, Rohde 1979, Holmes and Price

1986, Esch et al. 1990). A summary of this work described parasite infracommunities as lying on a continuum with “isolationist communities” at one end which are composed of weakly interacting or non-interacting symbiont species. At the other end of the continuum are “interactive communities” which are composed of more strongly interacting symbiont species (Sousa 1994). Symbiont communities are predicted to be interactive when colonization rates are relatively high and within-host communities approach saturation. Isolationist communities are predicted when colonization is low and within host communities do not approach saturation (Holmes and Price 1986, Bush et al. 1997). However, attempts to empirically verify the predictions of this framework have yielded very mixed results, often requiring ad hoc explanations, and causing the authors of two comprehensive reviews of the subject to conclude that the importance of parasite-parasite interactions is obfuscated by too many confounding factors and must be considered on a case-by-case basis (Sousa 1994, Poulin 2001). We argue that explicitly considering variation in host resistance to colonizing symbionts could account for much of this observed variability in the strength of symbiont interactions among symbiont infracommunities and help advance a more generalized understanding of infracommunity assembly processes.

General ecological theory predicts that higher patch colonization rates result in higher local diversity, more niche overlap among species, and, consequently stronger interspecific interactions (Cornell and Lawton 1992). By extension of general theory, we hypothesize that host resistance to symbiont colonization affects symbiont community assembly in multiple ways. First, resistance acts as a barrier to colonization that restricts symbiont infracommunities to few specialized taxa. As an indirect effect of reduced

colonization, host resistance decreases the strength and frequency of interspecific interactions within the symbiont infracommunity, yielding more isolationist infracommunities. Conversely, relaxed host resistance results in higher colonization rates and infracommunity diversity, and consequently more interactive symbiont infracommunities.

We tested our hypothesis in a combined observational and experimental study of the cleaning symbiosis between crayfish and a multi-species assemblage of ectosymbiotic annelid worms (Annelida: Branchiobdellida). We examined the relationships among host resistance, symbiont colonization, and symbiont-symbiont interactions across an ontogenetic shift in host resistance. We conducted a field survey of the crayfish *Cambarus sciotensis* and their ectosymbionts to identify patterns of symbiont diversity and composition through host ontogeny. We then used laboratory behavioral assays to demonstrate an ontogenetic shift in host resistance to several branchiobdellidan species. We experimentally assessed the effects of host age and host resistance on the successful colonization each symbiont species and experimentally assessed numerical and functional responses to interspecific interactions among infracommunities typical of young hosts and those typical of old hosts. Our findings demonstrate that nonspecific host resistance acts as a strong species filter to colonizing symbionts and lead to isolationist infracommunities, whereas relaxed resistance in older hosts leads to more diverse and more interactive infracommunities.

## Methods

### *Study system*

Crayfish throughout Europe, North America, and Asia host multi-species assemblages of obligate ectosymbiotic worms (Annelida: Branchiobdellida; Gelder 2010). An individual crayfish can host several hundred worms, belonging to multiple genera and species (Skelton et al. 2013). Some worms provide a beneficial cleaning service by consuming potentially harmful epibiotic accumulations from their host (Brown et al. 2002, Lee et al. 2009). However, branchiobdellidans may also be commensals (Keller 1992) or facultative parasites which feed on host tissues (Brown et al. 2012). Previous work has shown that young *Cambarus* crayfish resist colonization by actively removing and often consuming colonizing symbionts, whereas older crayfish do so infrequently, and argue that this ontogenetic change in behavior is an adaptive response to changing costs and benefits of symbiosis (Skelton et al. 2014).

Predictable ontogenetic variation in the response of crayfish to colonizing symbionts provides an opportunity to test our hypothesis without the confounding variation of among system comparisons that have been lamented in previous attempts at cross-system syntheses of infracommunity processes (Sousa 1994, Poulin 2001). Furthermore, the crayfish-branchiobdellidan system is amenable to field and laboratory experimentation because the worms are external symbionts which can be easily manipulated and observed non-destructively (Skelton et al. 2013) and they do not have complex multi-host life cycles typical of many parasite systems which often limits realistic experimentation (Sousa 1994, Poulin 2001). We predicted that successful colonization of branchiobdellidans on young crayfish will be limited by host resistance to

relatively few taxa that are less susceptible to host resistance, and consequently interspecific symbiont interactions on young crayfish will be weak. Conversely, colonization of older crayfish will not be limited by host resistance, leading to more diverse symbiont infracommunities and stronger interspecific symbiont interactions

### *Field survey*

Our study was conducted in the Mountain Lake region of Southwest Virginia. This region supports populations of several native crayfish and at least 12 branchiobdellidan species (Hobbs et al. 1967). Individual crayfish host as many as several hundred branchiobdellidans, belonging to 8 or more species and several genera (Hobbs et al. 1967, Gelder and Williams 2011, Skelton et al. 2013). We collected crayfish and their symbionts from a third order tributary of the New River near Newport Virginia, USA (37.301939, -80.487472; lat/long in DDD) during February of 2012. Crayfish identification followed the keys of Hobbs et al. (1967), and branchiobdellidan identification followed the keys of Hobbs et al. (1967) and Holt and Opell (1993). To assess changes in symbiont abundance and diversity through host ontogeny, we modeled the total number of symbionts and symbiont diversity (Simpson Index) as a function of host size (carapace length [CL] in mm). Carapace length was used instead of age because it is readily measurable, it provides a reasonable proxy of age, and it is more reliable than mass for predicting age of temperate crayfish (Belchier et al. 1998). We explored ontogenetic changes in symbiont composition through ontogeny by regressing principal coordinate ordination (PCo) axes of symbiont infra-communities against host CL. Finally, we modeled the relative abundance of each symbiont species as a function of host CL to identify species-specific changes in symbiont community through ontogeny.

### *Host resistance study*

Previous work demonstrated that the crayfish *Cambarus chaugaensis* regulated the abundance of the symbiont *Xironodrilus appalachius* by grooming, and the strength of the grooming response decreased through crayfish ontogeny (Skelton et al. 2014). To examine the species-specific responses of *C. sciotensis* to the multiple branchiobdellidan species present at our study site, we individually examined age-specific grooming responses towards four branchiobdellidan species present at the study site. For each worm species, we tested 24 crayfish (96 in total; size<sub>min</sub> = 12.11 mm CL, size<sub>max</sub> = 43.42 mm CL) with a stratified sampling design to maximize evenness of host size within each group. Crayfish were tested in 8 groups of 12 individuals throughout the summer of 2012 in a randomized testing sequence. Individual crayfish and worms were not used more than once. Animals were collected from the field the day before observation. On the day of collection, worms to be used in behavioral tests were removed from the host and stored in stream water at 5 C. All remaining worms and their eggs were killed by 5 m immersion in 10% MgCl<sub>2</sub> hexahydrate solution (Brown et al. 2002). Crayfish were placed individually in circular (20 cm diameter) transparent plastic dishes filled with 1 L of stream water at 21 C. Dishes with crayfish were kept in the testing area undisturbed for 12 h before testing. All observations were made under dim red light to reduce visibility of the observer to the crayfish. Each test consisted of a 3 min baseline observation to ensure that individuals were not displaying signs of sickness or stress, followed by inoculation of a single worm placed on the dorsal aspect of the host carapace within the areola followed by a 5 min observation of host behavior and quantification of directed grooming responses. Directed grooming responses were defined as using one or more walking legs

to scratch and/or grab at the inoculated worm or its immediate vicinity (~1 worm body length). Similar methodology has been successfully used in previous work to examine host-specific differences in grooming responses and branchiobdellidan abundance (Farrell et al. 2014). A preliminary test conducted by 2 observers were congruent among observers. Subsequent tests were conducted by a single observer.

### *Colonization experiments*

We conducted a series of experiments to determine symbiont species-specific variation in their ability to colonize hosts of varying sizes. Branchiobdellidans are only known to colonize new hosts during direct host/host contact (Mc Manus 1960, Koeppe 1975, Skelton et al. 2013). Consequently, all members of the infracommunity have similar opportunity for colonization in the field. However, successful colonization requires not just opportunity to move to a new host, but also successful establishment. Therefore we used symbiont persistence on a new host over a 1 week period as a proxy for colonization ability. This approach allowed us to keep dispersal constant by applying equal numbers of individual symbionts from all taxa to each host and eliminated potential confounding host preferences of the symbionts. Each experiment included 24 crayfish that were split into two treatment groups; 1) crayfish with ablated dactyls on pereopods 1 - 2, and 2) a reference group with intact dactyls. Dactyl ablation reduces the effectiveness of crayfish grooming in removing ectosymbionts and therefore provides a control group for detecting host grooming effects on symbiont persistence (Skelton et al. 2014). Crayfish from 14.4 mm CL to 43.60 mm CL were selected from the field to create parallel size gradients for each treatment. All worms and their eggs were killed by 5 m immersion in 10% MgCl<sub>2</sub> hexahydrate solution, and then crayfish were then placed

individually in 40 L experimental chambers and given 1 week to acclimate and recover from ablation. After acclimation we applied 6 worms in monoculture to the dorsal aspect of the carapace on each crayfish. Worm treatments were applied in monoculture to avoid confounding effects of inter-specific competition and intra-guild predation (methods in Brown et al. 2002). After 8 days, we sacrificed all animals in 70% ethanol and enumerated remaining worms.

#### *Symbiont interaction experiment*

We used methods similar to the colonization experiment described above to test for numerical and functional responses (*sensu* Sousa 1994) to species interactions among symbiont species typical of young and old hosts. This experiment included two symbiont species identified during the field study as numerically dominant on young hosts (*Cambarincola fallax* and *Pterodrilus alcornis*), and one species typically only found only on older hosts (*Cambarincola ingens*). Worm species were applied separately and in mixed culture combinations at species-specific densities typical to field observations (15 individuals for *Cambarincola fallax* and *P. alcornis*, and 6 individuals for *C. ingens*). Interspecific numerical responses were assessed by comparing symbiont survival and functional responses were assessed by comparing worm microhabitat use over 40 d as a function of the presence/absence of other symbiont species in an incomplete factorial design. Data were collected non-destructively as repeated measures at 6, 24 and 40 d. Three species combinations were used to assess symbiont-symbiont interactions of species typical to young hosts; *C. fallax* only, *P. alcornis* only, and *C. fallax* with *P. alcornis*. To test for interactions among species typically found on older hosts we combined *C. fallax* with *C. ingens*, *P. alcornis* with *C. ingens*, and a combination of all

three species. We had 4 replicates of each unique species combination for a total of 24 experimental units.

### *Data analysis*

We identified ontogenetic patterns of symbiont communities from survey data by examining changes in total symbiont abundance, symbiont infracommunity diversity (Simpson's Index), and species relative abundances across host CL. We used Generalized Additive Modeling (GAM; mgcv package for R v 1.7-11) to regress each measure of symbiont community structure against host CL. We chose GAM over other analytical techniques because, when provided with large sample sizes, it is flexible to complex non-linear responses over a gradient. Because we predicted a sudden change in infracommunity composition coincident with relaxed host grooming, we used piecewise regression and principal coordinates ordination to look for threshold responses in the slope of the relationship between infracommunity composition and host ontogeny. We conducted a principle coordinates ordination (pcoa; ape package for R v 3.0-1) based on a relativized Bray-Curtis dissimilarity matrix of symbiont infra-communities and used piecewise regression to look for a host size (CL) at which the relationship between host CL and infracommunity composition changed significantly (piecewise.linear function; SiZer package for R v 0.1-4) (Toms and Lesperance 2003).

To assess symbiont species-specific differences in the age-specific grooming response of hosts in the host resistance experiment, we used GAM to model the probability of a "directed grooming" response as a function of crayfish size (CL mm) and the categorical factor worm species (with interaction term), assuming a binomial distribution of error. We used a binary response variable defined as the presence or

absence (1/0) of a directed grooming response during the 5 minute response observation rather than count data of the behavioral responses to avoid violations of independence among responses. In the colonization experiment, we assessed species-specific effects of the age-specific host grooming response on symbiont persistence by using generalized linear models (glm function; stats package for R v 2.13.1) to model the probability of each inoculated worm persisting on the host through the 8 day trial as a function of host size and amputation treatment (with interaction term) and a binomial error distribution. Here we used GLM instead of GAM because practical limitations of experimentation limited our sampling effort and GLM is more robust to small sample sizes (Wisz et al. 2008).

Finally, to assess numerical responses of branchiobdellidans to symbiont-symbiont interactions, we used a generalized linear mixed model (GLMM) for each symbiont species (lmer function; lme4 package for R v 0.999375-42) to model the probability of each worm persisting over the 40 d experiment as a function of time and the presence/absence of other species, assuming a binomial error distribution and including individual crayfish as a random effect with random intercept and slope. We used a mixed model formulation to account for random variation in the probability of individual worm persistence among individual hosts. Functional responses of *C. fallax* and *P. alvicornis* to each other and to *C. ingens* were assessed visually by non-metric multidimensional scaling ordination (metaMDS function; vegan package for R v 2.0 – 0) of a Jaccard distance matrix of the multivariate response of number of individuals of the focal species at each attachment site. We tested for statistical significance using permutational multivariate analysis of variance (PERMANOVA; adonis function; vegan

package for R v 2.0 – 0; jaccard distance, 9,999 permutations) to test for effects of the presence/absence of other species on the distribution of the proportions of individuals across all attachment sites with time as a covariate (Skelton et al. 2014). For all GAMs, GLMs, and GLMMs, predictor variables were determined *a priori* based on hypotheses specific to each study. Validation of error distributions and default basis dimensions were assessed visually by scatterplots comparing fitted values to raw data, predicted values and residuals, histograms of residuals, and Q-Q plots (Zuur et al. 2009). In cases of excessive over-dispersion, “quasi” distribution families were used to estimate the over-dispersion parameter from the data.

## Results

### *Field study*

We recovered 3,533 branchiobdellidans belonging to 4 species and 3 genera from 86 *C. sciotensis* at Sinking Creek, Newport VA (Table 1). Total symbiont abundance increased asymptotically with host size. GAM regression of total abundance as a function of host size showed significant effects of host CL ( $X^2 = 534.7$ ,  $p < 0.0001$ ,  $\text{edf} = 8.724$ ,  $n = 86$ ,  $k = 9$ ,  $\text{adj. } R^2 = 0.27$ ). GAM model fit revealed a rapid increase in total symbiont abundance in hosts from ~15 mm CL to ~30 mm CL, but showed no obvious trend beyond ~30 mm CL (Figure 1A). Symbiont diversity also increased asymptotically with host CL ( $F = 31.01$ ,  $p < 0.0001$ ,  $\text{edf} = 2.6$ ,  $n = 86$ ,  $k = 9$ ,  $\text{adj. } R^2 = 0.54$ ; Figure 1B). Piecewise regression of PCo axis scores identified an infracommunity composition threshold change in the ontogenetic dynamics of symbiont composition at 26.88 mm CL for both PCo axis 1 and PCo axis 2. The slope of the relationship between PCo axis 1 and host size changed from -0.040 to -0.002 at 26.88 mm CL. The slope of the relationship

between PCo axis 2 and host size changed from -0.003 to 0.013 at 26.88 mm CL. All symbiont species showed significant changes in relative abundances through host ontogeny (Figure 2). *Cambarincola fallax* was the most abundant species on crayfish smaller than the infracommunity composition threshold value of 26.88 mm CL identified by piecewise regression (shown as vertical dotted reference lines in Figure 2), but the relative abundance of *C. fallax* generally declined with host size ( $F = 8.673$ ,  $p < 0.0001$ ,  $\text{edf} = 3.228$ ,  $n = 86$ ,  $k = 4.785$ ,  $\text{adj. } R^2 = 0.730$ ). The relative abundance of *P. alvicornis* showed a unimodal response to host CL, peaking at  $\sim 30$  mm, just beyond the composition threshold size of 26.88 mm ( $F = 2.559$ ,  $p = 0.038$ ,  $\text{edf} = 3.695$ ,  $n = 86$ ,  $k = 6.176$ ,  $\text{adj. } R^2 = 0.719$ ). Both *C. ingens* ( $X^2 = 37.26$ ,  $p = 0.001$ ,  $\text{edf} = 5.49$ ,  $n = 86$ ,  $k = 1$ ,  $\text{adj. } R^2 = 0.43$ ) and *A. koronaeous* ( $X^2 = 156.1$ ,  $p < 0.001$ ,  $\text{edf} = 4.229$ ,  $n = 86$ ,  $k = 1$ ,  $\text{adj. } R^2 = 0.67$ ) were generally absent on crayfish smaller than the composition threshold size, but increased in relative abundance thereafter.

### *Behavioral study*

Host directed grooming behaviors decreased significantly with host size ( $X^2 = 21.98$ ,  $p = 0.001$ ,  $\text{edf} = 1.1$ ,  $n = 93$ ,  $k = 1$ ,  $\text{adj. } R^2 = 0.40$ ; Figure 3). There were no significant effects of worm species identity on directed grooming response or significant interactions between worm species and host size and therefore worm species was dropped as a fixed factor from all models. There were obvious differences in behavioral responses to branchiobdellidans among crayfish larger and smaller than the infracommunity composition threshold values observed for survey data of worm composition in the field; 60% (27 / 45) of crayfish smaller than the 26.88 mm CL displayed a directed grooming response to the introduction of a worm during the 5 min observation period, whereas 8%

(4 / 48) larger than 26.88 mm CL, and 0% (0 / 29) crayfish larger than 30.03 mm CL displayed a directed grooming response.

#### *Colonization experiment*

Symbionts differed in their ability to successfully colonize hosts of various sizes (Figure 4). Persistence of *C. fallax* was not significantly affected by either host size or dactyl amputation. *Pterodrilus alcicornis* persistence increased with host size, but was unaffected by the dactyl treatment. Persistence of *C. ingens* increased with host size, with dactyl ablation, and there was a significant interaction term. Lastly, persistence of *A. koronaeus* increased with host size, but was unaffected by the dactyl treatment. See supplemental Table S1 for full models.

#### *Symbiont interaction experiment*

Persistence of *C. fallax* was negatively affected by the presence of *C. ingens* and not significantly changed by the presence of *P. alcicornis* (Table 2). Survival of *P. alcicornis* was not significantly affected by the presence of *C. ingens* or *C. fallax*. PERMANOVA of attachment site use revealed a significant effect of *C. ingens* on the site use of *P. alcicornis* ( $F = 2.282, p = 0.049$ ). Most of this effect was evident along the first NMDS axis, which was strongly correlated with use of the lateral aspect of the ventral abdominal surface ( $r = -0.66$ ), and the pleopods ( $r = 0.84$ ), indicating more frequent attachment of *P. alcicornis* to the pleopods and reduced attachment to the ventral abdomen in the presence of *C. ingens*. Site use of *P. alcicornis* was not

significantly affected by the presence of *C. fallax* and site use of *C. fallax* was not significantly affected by the presence of *C. ingens* or *P. alcornis*.

## **Discussion**

We have shown that host resistance and interactions among symbionts can combine to shape symbiont community assembly, and that these processes do not operate independently. Our field survey uncovered a punctuated change in infracommunity diversity, species composition, and population dynamics during crayfish ontogeny. Small, young crayfish (< 27 mm CL) typically hosted only the smallest two species of branchiobdellidans considered in this study, *C. fallax* and *P. alcornis*. However, there was a threshold change in the relationship between crayfish size and symbiont composition that occurred at ~ 30 mm CL, beyond which two larger species, *C. ingens* and *A. koronaeus*, first appeared and subsequently became an increasingly large portion of the symbiont infracommunity. The increasing relative abundance of these later-colonizing large taxa coincided with reduced relative abundance of the earlier-colonizing taxa, resulting in little or no change in total symbiont abundance beyond ~30 mm CL. Diversity also saturated, but at a considerably larger size, correspondent to older crayfish (~40 mm CL), indicating that infracommunity evenness increased beyond the infracommunity composition threshold as early arriving taxa were replaced by later arriving taxa. These punctuated changes in infracommunity structure were tightly correlated with an ontogenetic shift in host resistance to colonizing symbionts. During the resistance experiment, the introduction of a single worm elicited a directed grooming response from the majority of young hosts. This response was rarely observed on crayfish larger than 27 mm, and was never observed in crayfish larger than 31 mm CL. The

observed ontogenetic shift in host resistance was concomitant to punctuated changes in infracommunity structure, both occurring at 27 – 30 mm CL. Thus, the combined results of our host resistance study and field survey provide strong correlational evidence that host resistance alters infracommunity assembly dynamics and suggests that host resistance of young *Cambarus sciotensis* has species-specific negative effects on colonizing symbionts.

Our study sought to link patterns of symbiont infracommunities with experimentally demonstrated causal processes. We conducted the colonization experiment explicitly to verify that host resistance influences symbiont community assembly by having species-specific negative effects on colonizing symbionts and to demonstrate that the ontogenetic shift in host resistance could explain patterns of infracommunity structure observed in the field. Hosts can resist colonization in either of two ways; 1) specific host resistance in which hosts only respond to particular species or 2) non-specific host resistance in which hosts respond similarly to all species. The results of the resistance experiment indicated nonspecific host resistance in our system. Small crayfish displayed a consistent grooming response to all worm species, and large crayfish did not respond to any symbiont species. Although young hosts consistently tried to remove all species of symbionts, some symbiont species were able to avoid removal. *Cambarincola fallax* was able to persist on crayfish of all sizes. This result matches field data showing complete prevalence of *C. fallax* on crayfish of all sizes. Additionally, the colonization experiment demonstrated that *C. ingens* and *A. koronaeus* were unable to persist on young hosts, but were able to persist on older hosts. Again, this result mirrored observations from the field showing few occurrences of these species on small hosts, but

high prevalence on larger hosts. Persistence of *P. alcicornis* also increased significantly with host size, but unlike later arriving species, a considerable fraction of individuals persisted on young hosts. This result was again consistent to field observations in which the prevalence and relative abundance of *P. alcicornis* was intermediate to the early arriving *C. fallax* and the late arrivers, *C. ingens* and *A. koronaeus*.

During the colonization experiment we applied a dactyl ablation treatment to assess effects of host grooming on the persistence of each worm species. Based on field observations of worm abundance and the results of our resistance experiment, we predicted that crayfish grooming was only effective against the colonization of *C. ingens* and *A. koronaeus*. Consistent with predictions, dactyl ablation increased persistence of *C. ingens* on young hosts. Also consistent with predictions, dactyl ablation did not affect persistence of either early arriving species, demonstrating that these taxa are less affected by host grooming than *C. ingens* and providing evidence for species-specific effects of host grooming on symbiont colonization. Unexpectedly, persistence of the late arriving species, *A. koronaeus*, was also unaffected by dactyl ablation. However, *A. koronaeus* is unique among the species considered in this study in that it typically attaches to the distal portions of the host's claws and walking legs (Hobbs et al. 1967), whereas the other species typically attach to the ventral aspects of the cephalothorax and abdomen (Hobbs et al. 1967, Brown et al. 2002). While attached to the claws and legs, *A. koronaeus* is vulnerable to direct removal by host mouthparts in addition to removal via the fingers and dactyls of the walking legs. The absence of a dactyl ablation effect does not suggest a lack of host resistance to *A. koronaeus*, but merely suggests that dactyl ablation was ineffective at preventing removal because of the preferred attachment site of this

particular species. Two lines of evidence support this view. First, *A. koronaeus* was able to persist on large hosts, but not on small hosts. Secondly, during the host resistance experiment we observed similar grooming responses to all species of symbionts, including *A. koronaeus*.

Our findings echo those of previous studies that demonstrated a strong influence of crayfish grooming behavior over ectosymbiont colonization and survival. Farrell et al (2014) conducted a comparative study of two crayfish species, *Orconectes cristavarius* and *Cambarus chasmodactylus*. They demonstrated that *Orconectes* in the field typically hosts far fewer branchiobdellidans (*C. ingens*) than *Cambarus chasmodactylus* and that this disparity could be explained by a stronger grooming response in *Orconectes cristavarius*. Similarly, Skelton et al. (2014) determined that age-specific differences in the grooming response of *Cambarus chaugaensis* could explain ontogenetic patterns in the abundance of the branchiobdellidans *Xironodrilus appalachius* and *Cambarincola philadelphicus*. Furthermore, Jones and Lester (1996) determined that the grooming response of the Australian crayfish *Charex quadricarinatus* was the strongest influence over population size of the ectosymbiotic temnocephalan flatworm *Diceratocephala boschmai*. Therefore, the effects of crayfish grooming responses on ectosymbiont colonization and survival appear to be a wide-spread phenomenon and an important facet of ectosymbiont biology.

Does the relaxation of host resistance lead to more interactive symbiont infracommunities? We predicted that the removal of host resistance as a species filter would result in colonization of more symbiont taxa, and consequently an increased influence of species interactions within the infracommunity. Although increased diversity

alone may suggest a more interactive symbiont infracommunity, this is not always the case, and experimental evidence is typically needed to verify symbiont interactions (Bush et al. 1997). Sousa (1994) identified two “smoking guns” indicative of strong interspecific interactions within infracommunities. First, numerical responses in which the abundance of one species is altered by presence/abundance of another and second, functional responses in which the behavior of one species changes in response to another. In our symbiont interaction experiment, we found no evidence of numerical or functional responses among the species that commonly co-occur on small *C. sciotensis*, suggesting that the infracommunities of small *C. sciotensis* are representative of the isolationist infracommunities described by others (Holmes and Price 1986). However, we identified numerical and functional responses of interspecific interactions among branchiobdellidans which co-occur on large hosts. Intraguild predation and cannibalism have been observed in other branchiobdellidan species (Gale and Proctor 2009), and observed in *C. ingens* (JS, BLB, RPC, personal observation) and thus, the responses we observed were likely the result of direct and indirect intraguild predation effects of the large *C. ingens* on the much smaller *C. fallax* and *P. alcornis*. During our experiment, infection intensity of *C. fallax* was significantly decreased by the presence of *C. ingens* indicating a direct effect of intraguild predation. Alternatively, the number of *P. alcornis* surviving the experiment was unaffected by *C. ingens*, but attachment site use of *P. alcornis* was significantly altered. Both *P. alcornis* and *C. ingens* commonly attach to the ventral surfaces of the host abdomen (Hobbs et al. 1967, Brown et al. 2002). In our study, *P. alcornis* moved in response to *C. ingens* from the ventral surface of the

host abdomen to the pleopods (which extend from the abdomen), demonstrating a functional response of *P. alvicornis* to *C. ingens*.

We found evidence that both interactive and isolationist symbiont infracommunities exist within a single host population because of individual variation in host resistance to symbiont colonization. But why does such variation in resistance among hosts exist? Variation in host qualities and context often determine the costs and benefits of engaging in symbiotic interactions (Ewald 1987, Bronstein 1994, Lee et al. 2009). Even within a single host population, changes in host biology that occur with ontogeny can create variable outcomes in symbiotic interactions among hosts (e.g. Palmer et al. 2010, Yule et al. 2013). Consequently, organisms may maximize lifetime fitness by engaging in symbiotic interactions only at life stages for which it is beneficial to do so, creating a mixed resistant and non-resistant host population (Skelton et al. 2014). Previous work on crayfish and branchiobdellidans have described age-, species-, and context-specific variation in resistance to symbiont colonization and linked this variation to variable outcomes of symbiosis (Thomas et al. 2013, Farrell et al. 2014, Skelton et al. 2014). Based on these previous studies of closely related congeneric crayfish, we predicted that small *C. sciotensis* would resist colonization of branchiobdellidans because the short inter-molt cycles of young crayfish diminish the prospective benefits of hosting cleaners (Skelton et al. 2014). Our predictions were confirmed which suggests that age-specific variation in the outcomes of symbiosis can lead to different types of symbiont infracommunities within a single host population by maintaining variation in host resistance.

Our results could also have implications for understanding the maintenance of diversity and co-existence with symbiont lineages. Symbiont diversity is correlated with host diversity because diverse host communities accommodate symbiont specialization (Hechinger and Lafferty 2005). In this study, variation in host resistance and resultant variation in the processes that structure symbiont infracommunities could underlie symbiont diversification and specialization, and provide opportunities for co-existence. Holmes and Price (1986), predicted that parasites of interactive infracommunities will tend to be more specialized in their fundamental niche than isolationist infracommunities as a result of character displacement, species sorting, and/or niche packing. Our results provide some support for this prediction if host resistance is taken under consideration. The isolationist infracommunities of small crayfish were comprised of species that occupy a large variety of attachment sites (*C. fallax* and *P. alaicornis*; Hobbs et al. 1967), congruent with theory (Holmes and Price 1986, Sousa 1994, Poulin 2001). In contrast, both species of branchiobdellidans found almost exclusively on older crayfish display high microhabitat specialization (Hobbs et al. 1967, Brown et al. 2002). *C. ingens* is largely limited to the ventral surface of the abdomen, and *A. koronaeus* is restricted to the claws and walking legs. Furthermore, we showed that the presence of *C. ingens* influenced the realized spatial niche of *P. alaicornis*.

Variation in host resistance could contribute to symbiont species co-existence and diversification by creating trade-offs in life history strategies. In this study, both early arriving symbiont species were the smallest taxa considered; the maximum total length (preserved) of *C. fallax* is ~4mm and that of *P. alaicornis* is ~ 3 mm. Conversely, both species that were unable to colonize small hosts are considerably larger; up to ~ 10 mm.

Indeed the authors have personally experienced the difficulty of removing these smaller taxa with fine forceps, and the relative ease of removing the larger taxa. Being small may offer *C. fallax* and *P. alcicornis* the ability to exploit a greater portion of the host population. However, being large may offer *C. ingens* and *A. koronaeus* the ability to exploit a larger range of food resources, including other branchiobdellidans. Moreover, given the observed negative impacts of *C. ingens* on *C. fallax*, a body size trade-off that permits *C. fallax* to colonize small hosts that are inaccessible to *C. ingens*, clearly benefits the smaller worm species. The ability of these worms to colonize and persist on smaller sized hosts could be essential to the co-existence of these branchiobdellidans, similar to familiar competition-colonization trade-offs (e.g. Levins and Culver 1971, Yu and Wilson 2001). Thus the co-evolutionary history of branchiobdellidans and crayfish which led to age-specific variation in the resistance to symbiont colonization could help explain contemporary diversity in branchiobdellidans life-histories.

Understanding the underlying processes that drive interactions among symbionts has utility beyond explaining observable patterns of symbiont diversity because interactions among symbionts may be an integral facet of symbiosis outcomes. Two prominent symbiotic systems highlight the importance of strong within-host interactions among symbiont taxa. Studies of symbiont infracommunities, including human microbiomes, is one of the fastest growing and transformative fields of contemporary biology (Bäckhed et al. 2005). Microbial infracommunities protect their hosts against pathogenic organisms, and unlock otherwise unattainable resources through complex syntrophic interactions. Protection against pathogens is a direct result of direct competitive interactions that exclude pathogens, and nutritional contributions can arise

from competition for resources and niche partitioning which results in multi-step modifications that eventually provide a host usable resource (Dethlefsen et al. 2006, Dethlefsen et al. 2007). Another example is the obligate mixotrophic association between many reef-building corals host and dinoflagellates of the genus *Symbiodinium*. Within *Symbiodinium*, there exists considerable phylogenetic and functional diversity and changes in environmental conditions revealed strong competitive interactions among clades that create trade-offs between nitrogen acquisition and thermal tolerance (Baker et al. 2013). These competitively mediated trade-offs among clades may allow corals which depend on symbionts to resist negative effects of climate change because thermo-tolerant strains can still provide fixed carbon when thermo-sensitive strains cannot (Stat and Gates 2010, Baker et al. 2013). Thus, interactions among symbionts may directly provide a benefit to the host by limiting colonization of harmful species, or indirectly benefit the host by creating functional diversity within symbiont assemblages.

Adaptation of well-developed paradigms from community ecology promise to advance the study of symbiont infracommunity dynamics by providing conceptual frameworks for understanding the complex processes that shape within- and among- host symbiont diversity (Dethlefsen et al. 2007, Pedersen and Fenton 2007, Graham 2008, Rigaud et al. 2010). Each of these frameworks emphasizes the importance of different processes. Previous authors have likened host populations to island archipelagos and applied Island Biogeography Theory to emphasize the importance of colonization rates in determining infracommunity diversity (Holmes and Price 1986). Others have emphasized within-patch processes such as “top-down” and “bottom-up” controls on diversity (Pedersen and Fenton 2007, Graham 2008). More recently some have suggested the use

of the emerging metacommunity perspective to better understand the relative importance of multi-scale processes on symbiont communities (Mihaljevic 2012). While these approaches hold promise, special considerations may be needed to effectively apply general ecological concepts to symbiotic systems because hosts, unlike habitat patches, have co-evolutionary histories with their symbionts which make them responsive to evolutionary changes and inter-specific variation in symbiont biology. Our results show the processes that structure symbiont communities do not operate independently. Specifically, natural variation in host resistance within a host populations can modulate the influence of interactions among symbionts during symbiont community assembly. We hope that our contribution will inspire researchers of symbiont diversity to incorporate variation in host resistance into conceptual frameworks for understanding the complex and interacting processes that shape symbiont diversity.

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## Figure and table captions

### Figure 3.1

Positive relationships between crayfish size and (A) total branchiobdellidan symbiont abundance and (B) Simpson diversity per crayfish. Solid grey line represents GAM model fits. Circles are observed data.

### Figure 3.2

Relationships between crayfish size and the relative abundances of four co-occurring branchiobdellidan species. Solid line represents GAM model fit of mean relative abundance, and circles represent observed data. Vertical reference line represents the infracommunity composition threshold identified by piecewise regression of PCo axes 1 and 2 at which the slope of the relationship between host size and symbiont community composition changes.

### Figure 3.3

Decreased host resistance to colonization through host ontogeny shown as probability of host grooming response to the introduction of a single branchiobdellidan during 5 min behavioral tests as a function of host size. Solid line represents GAM model fit, dotted lines represent 95% confidence envelope around grooming probability at a given size. Shapes indicate responses to each worm species where 1 = response, and 0 = no response, circles = *C. fallax*, squares = *P. alcicornis*, triangles = *C. ingens*, and diamonds = *A. koronaeus*. Symbols were jittered for clarity. Vertical reference line represents the infracommunity composition threshold size identified by piecewise regression of PCo

axes 1 and 2 from field survey at which the slope of the relationship between host size and symbiont community composition changes.

#### Figure 3.4

Variation in the individual probability of worm persistence over an 8-day colonization experiment as a function of host size and dactyl ablation for four species of branchiobdellidans. Solid lines represent GLM model fits for dactyl-ablated (grey) and control (black) groups for probability of persistence at a given size. Symbols show proportion of colonists present at the conclusion of the experiment. Significant effects determined by GLM of host size (CL), ablation treatment (Abl), and their interaction (Int) are highlighted in bold. Vertical reference line represents the infracommunity composition threshold size identified by piecewise regression of PCo axes 1 and 2 at which the slope of the relationship between host size and symbiont community composition changes.

#### Table 3.1

Prevalence, mean and maximum intensity, and total number of individuals of four species of branchiobdellidans collected from 86 crayfish during field survey.

#### Table 3.2

Results of GLMMs from symbiont interaction experiment showing the effects of presence/absence of additional species (with interaction) and time on persistence of focal species (first column). \*Significance at  $P < 0.05$ , \*\* significance at  $p < 0.001$ .

Table 3.3

GLM analysis of persistence experiments for four branchiobdellidans showing the effects of host size (carapace length) and ablation of host dactyls on the individual probability of worm persistence through an 8 day trial. Pseudo  $R^2$  for each species model was calculated using McFadden's method for null deviances based on 23 *df*, and residual deviances based on 20 *df*.

Figure 3.1 Branchiobdellidan abundance and diversity versus host size

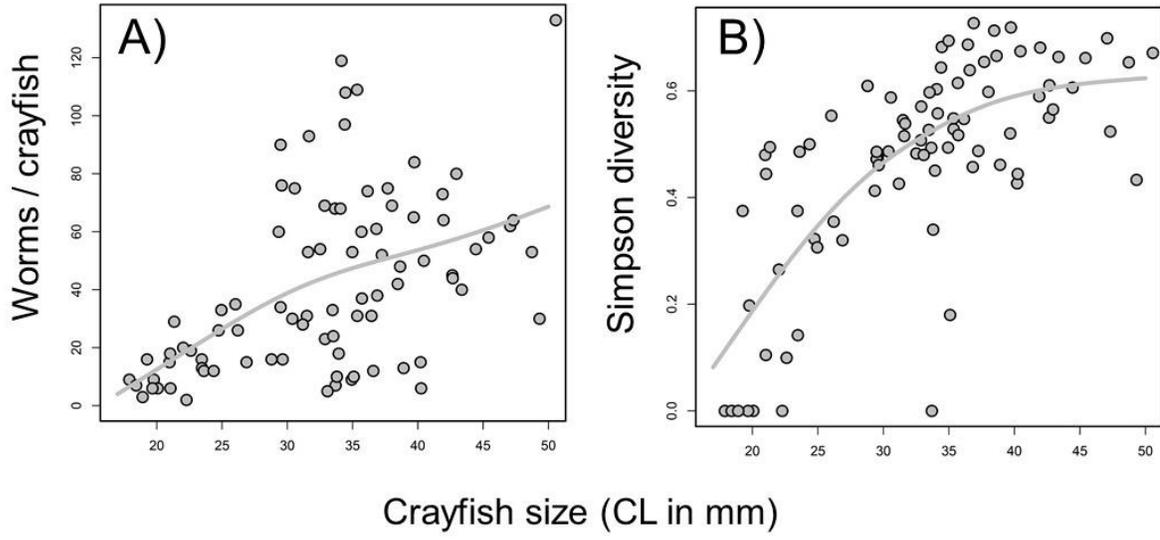


Figure 3.2 Branchiobdellidan community succession through host ontogeny

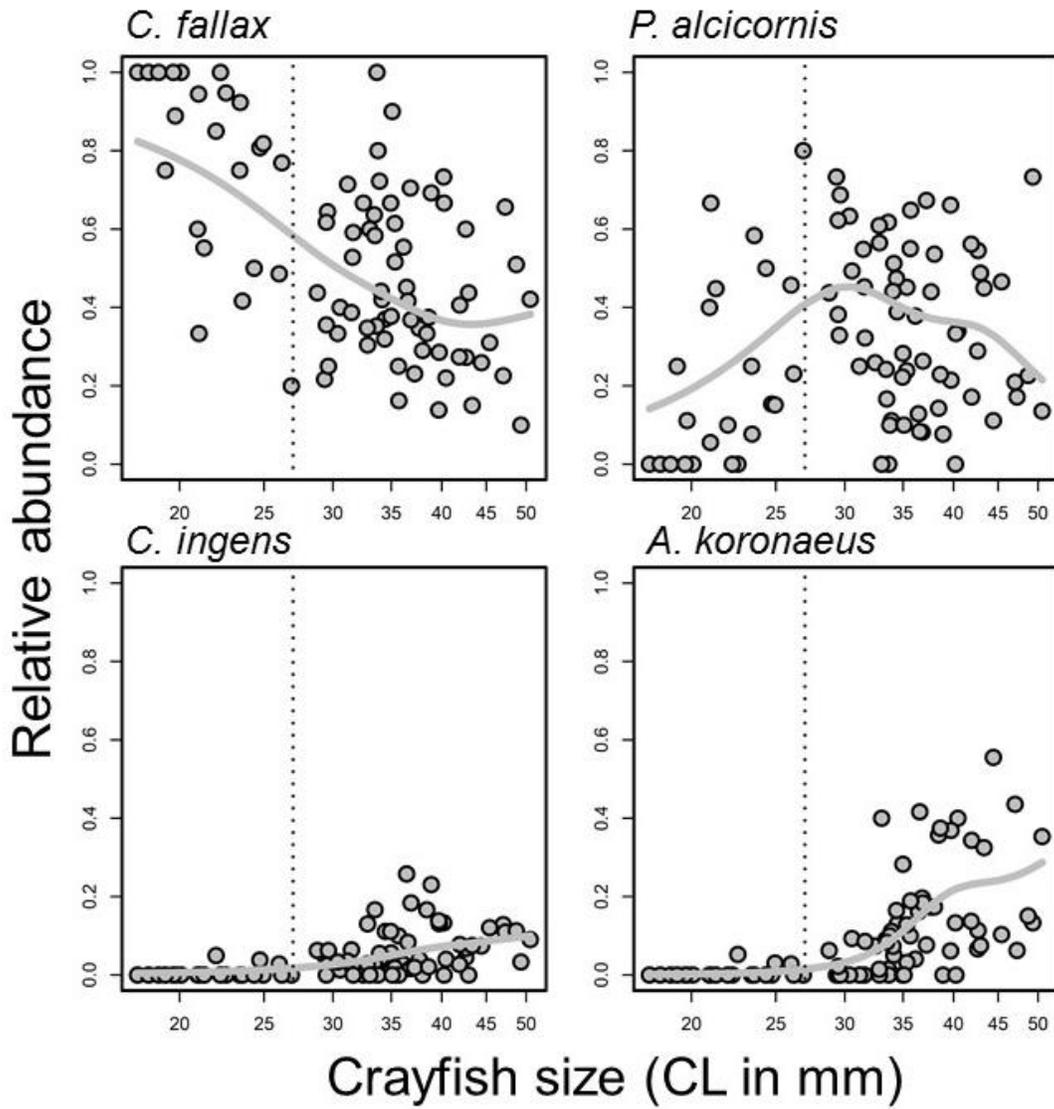


Figure 3.3 Age-specific grooming response of *C. sciotensis* to four branchiobdellidan species

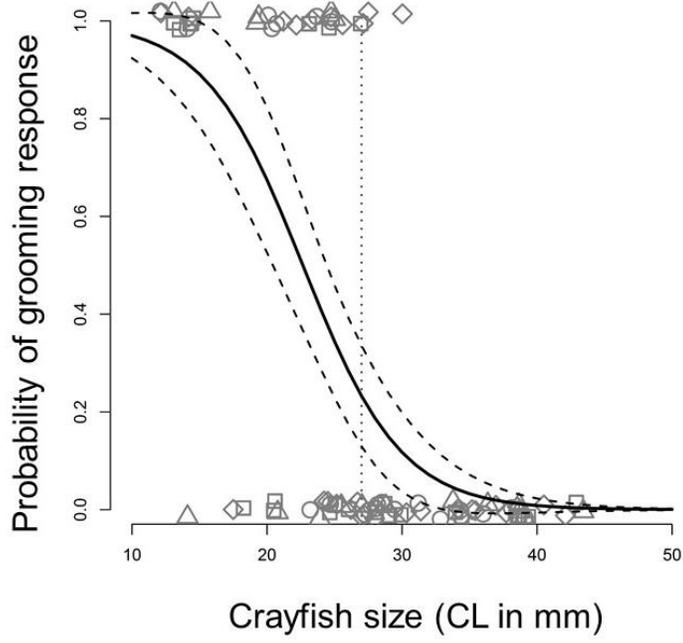


Figure 3.4 Effects of host size and dactly ablation on the persistence of four branchiobdellidan species during laboratory experiment

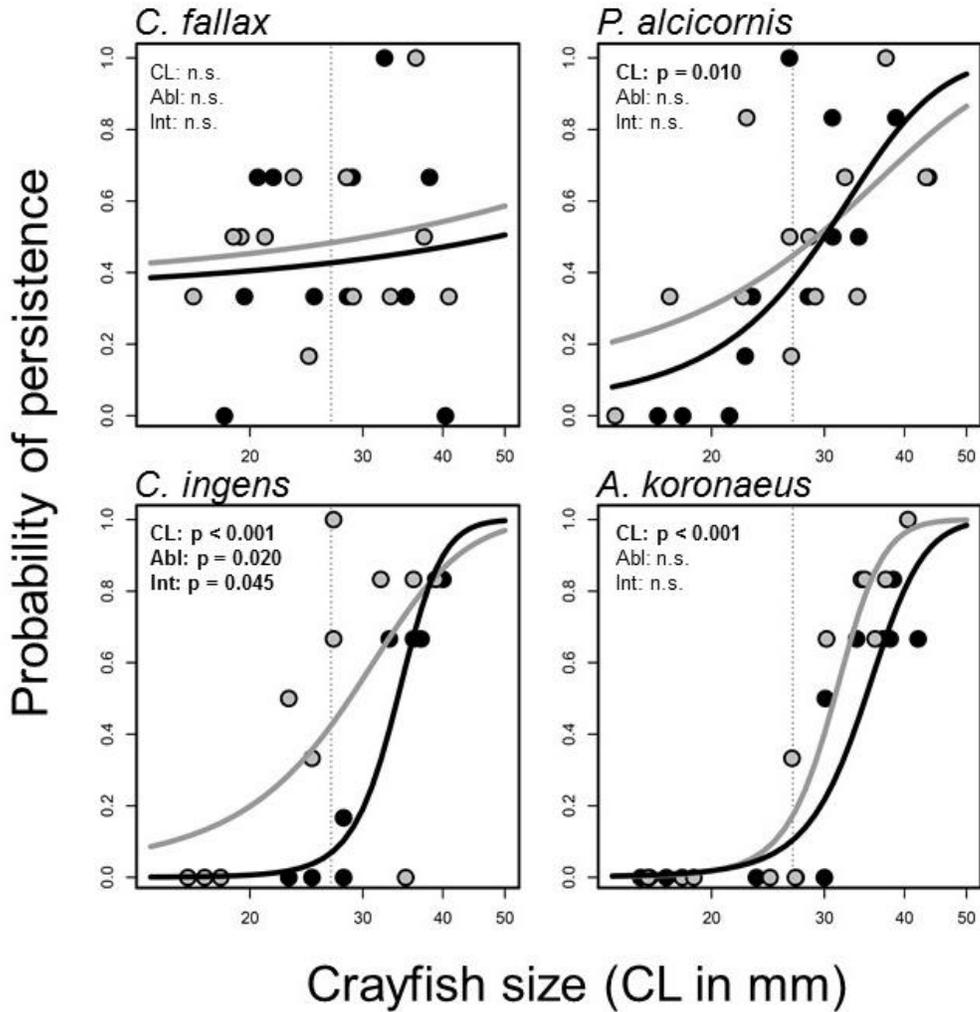


Table 3.1 Summary of branchiobdellidan collections made during field study

Branchiobdellidans	prevalence (% infested)	mean intensity (ind/host $\pm$ SD)	max intensity (ind/host)	total
<i>Ankyrodrilus koronaeus</i>	60	8.88 $\pm$ 9.29	47	462
<i>Cambarincola fallax</i>	100	18.28 $\pm$ 13.70	67	1,572
<i>Cambarincola ingens</i>	62	3.53 $\pm$ 3.08	12	187
<i>Pterodrilus alcicornis</i>	88	17.26 $\pm$ 14.90	61	1,312

Table 3.2 Results of generalized linear mixed models for interactions among branchiobdellidan species

symbiont spp	parameter	estimate	SE	<i>p</i>
<i>C. fallax</i>	intercept	0.445	0.318	0.162
	<i>C. ingens</i>	-0.838	0.374	0.025*
	<i>P. alcicornis</i>	-0.491	0.364	0.178
	Day	-0.041	0.007	< 0.001**
	<i>C. ingens</i> × <i>P. alcicornis</i>	0.616	0.536	0.251
<i>P. alcicornis</i>	intercept	-0.914	0.291	0.001*
	<i>C. ingens</i>	0.127	0.362	0.726
	<i>C. fallax</i>	0.371	0.355	0.296
	Day	-0.053	0.009	< 0.001**
	<i>C. ingens</i> × <i>C. fallax</i>	-0.432	0.509	0.396

Table 3.3 Generalized linear model analyses of persistence experiments

Species model	Coefficient	estimate	SE	$z$	$P$	Pseudo $R^2$
<i>C. fallax</i>	intercept	-0.545	0.869	-0.627	0.531	0.020
	carapace length	0.018	0.031	0.585	0.558	
	ablation	-0.111	1.299	-0.086	0.932	
	carapace length $\times$ ablation	-0.004	0.045	-0.096	0.924	
<i>P. alcornis</i>	intercept	-2.597	0.994	-2.614	0.009	0.387
	carapace length	0.089	0.034	2.590	0.010	
	ablation	-1.982	1.552	-1.277	0.201	
	carapace length $\times$ ablation	0.064	0.054	1.186	0.236	
<i>C. ingens</i>	intercept	-4.634	1.127	-4.113	< 0.001	0.628
	carapace length	0.162	0.040	4.010	< 0.001	
	ablation	-7.675	3.292	2.331	0.019	
	carapace length $\times$ ablation	0.200	0.099	2.005	0.044	
<i>A. koronaeus</i>	intercept	-11.296	2.553	-4.424	< 0.001	0.819
	carapace length	0.363	0.081	4.510	< 0.001	
	ablation	1.914	3.444	0.556	0.578	
	carapace length $\times$ ablation	-0.093	0.105	-0.892	0.372	

## **Chapter 4. A symbiont's dispersal strategy and predictable transmission dynamics**

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### **ABSTRACT**

Dispersal strategies in free-living animals are well-known adaptations to life in heterogeneous landscapes. From a symbiont's perspective, a host population is a heterogeneous patchwork and a symbiont dispersal strategy could improve symbiont fitness and yield predictable transmission dynamics. We provide evidence that variation in host quality and competition among symbionts affect symbiont fitness and cause symbionts to disperse non-randomly. In a symbiosis between crayfish and ectosymbiotic branchiobdellidan worms, symbionts were more likely to disperse to new hosts when current hosts provided a low-fitness environment. Worm reproductive success on wild-caught crayfish was influenced by host size and availability of high quality attachment sites. Based on field data we produced a model to predict transmission events during experimental, pairwise host encounters assuming that individual symbionts would disperse only when conditions on their current host yielded a fitness estimate below a minimum threshold. Our best model included host size and attachment site availability, and correctly predicted transmission in 95% of experimental host encounters and the exact magnitude of transmission in 67%, significantly better than null predictions. Considering the fitness consequences of dispersal will enhance our understanding of

transmission processes and get us closer to accurate predictions of transmission dynamics.

**Keywords:** symbiosis, parasite, mutualism, microhabitat, competition, crayfish

## **Introduction**

Understanding host and symbiont population dynamics requires, perhaps most importantly, an understanding of symbiont transmission processes and the factors that influence individual transmission events (Dwyer and Elkinton 1993, Fenton et al. 2002). However, these processes are probably more complex and nuanced than is generally recognized (Rigaud et al. 2010). Classic and contemporary models of transmission typically focus on factors that operate at the level of the host population and influence the frequency of dispersal opportunities for symbionts, such as contact rates (Kennedy 1990, Guégan and Hugueny 1994, McCallum et al. 2001, Poulin 2001), but do not consider factors that cause individual symbionts to disperse from their current host during host contacts. Theorists and empiricists have devoted considerable effort to exploring individual and population-level variability in host contact rates, but typically consider the probability of transmission during individual host contacts as a fixed rate (see review Begon et al. 2002). However, transmission presents risks as well as rewards to symbionts, and selection should favor symbionts that only disperse when it is likely to increase their fitness. Therefore, considering the factors that influence individual symbiont fitness may help us more accurately predict transmission during host contact events and ultimately host population transmission dynamics.

From a symbiont's perspective, a population or community of potential hosts is a heterogeneous and patchy landscape. Hosts vary in quality across species (Rohde et al.

1995, Brown and Creed 2004, Farrell et al. 2014) and even across individuals within a species (Lie 1973). Even at the within-host level, within-host microhabitats or tissues may vary with respect to the resources they offer, or the risk of mortality within each microhabitat patch (Rohde 1993, Mestre et al. 2011, Skelton et al. 2014). Moreover, each host and each microhabitat offers limited resources that are partitioned among symbionts creating the possibility of strong inter- and intra-specific competition among symbionts sharing a host (Råberg et al. 2006, Ulrich and Schmid-Hempel 2012, Baker et al. 2013). Because hosts are variable in the qualities that directly influence symbiont fitness, it is conceivable that natural selection favors symbionts that move among hosts discriminately, just as free-living organisms display dispersal strategies in response to the local conditions that affect their individual fitness (Bowler and Benton 2005). In free-living organisms, dispersal is influenced by local factors, which are readily perceived by the individual and are linked to the fitness of the individual. For many disparate taxa of free-living organisms, individuals emigrate from local populations in response to high intra-specific population densities, small patch size, and low resource availability (reviewed in Bowler and Benton 2005). By analogy, we predicted that symbiont taxa also display evolved dispersal strategies and that emigration of individual symbionts from their hosts can be predicted by qualities of the host and interactions among other symbionts that influence symbiont fitness.

We examined host size and intraspecific competition among symbionts for attachment sites as predictors of symbiont transmission during pairwise host encounters in the symbiosis between a crayfish (*Cambarus sciotensis*) and an ectosymbiotic annelid worm (*Cambarincola ingens*). We used a field survey to estimate the effects of host

quality and competition for preferred attachment sites on the fitness of individual symbionts. We then used estimates of fitness to make three sets of specific predictions about the frequency and magnitude of transmission events during pairwise host encounters and tested our predictions in a laboratory experiment. We compared our predictions of fitness-based symbiont dispersal to a null model that assumed a constant rate of dispersal and incorporated natural variability in the frequency of dispersal during host contacts. Our results show that variation in individual symbiont transmission events among pairwise host contacts can be explained by fitness advantages of dispersal because symbionts are more likely to disperse to new hosts when intra-symbiont competition and host size create a low-fitness environment on their current host.

## **Methods**

### *Study system*

Crayfish throughout North America, Europe, and parts of Asia host multi-species assemblages of obligate ectosymbiotic annelid worms called branchiobdellidans (Gelder 2010). Some of these worms provide a beneficial cleaning service to their hosts, whereas others are parasitic or have no apparent effects on their hosts (reviewed in Skelton et al. 2013). Branchiobdellidans attach to the exoskeletons of their hosts by way of posterior and anterior duo-gland adhesive organs (Weigl 1994). These adhesive organs allow branchiobdellidans to tightly attach to their host, but also allow the worms to easily release their grip and move freely across their host's body. Branchiobdellidans have a simple life-history with no free-living stage, and available evidence indicates that branchiobdellidans require a host to reproduce and require host-host contact for transmission (Young 1966, Skelton et al. 2013).

All organisms included in this study were collected from Sinking Creek in Newport VA, USA, a mid-order tributary of the New River (37°18'07.0"N 80°29'14.9"W; lat/long in DDD). The crayfish fauna at this site was dominated by *Cambarus sciotensis*. Crayfish at this site support populations of 4 species of branchiobdellidans (Hobbs et al. 1967; JS unpub data). In this study we focused on the crayfish *C. sciotensis* and the branchiobdellidan *Cambarincola ingens*. We focus on *Cambarincola ingens* because it is a relatively large and conspicuous branchiobdellidan, which allows for easy and accurate non-destructive detection and enumeration. Previously developed methods allow experimental manipulation of *C. ingens* presence and abundance under field and laboratory conditions (Brown et al. 2002, Skelton et al. 2013, Thomas et al. 2013). Both field and laboratory studies were restricted to adult crayfish and sub-adult crayfish. Previous work has shown that smaller juvenile crayfish will remove branchiobdellidans from their bodies, preventing colonization of worms on young hosts, but this response occurs at a lower frequency in older crayfish (Skelton et al. 2014).

#### *Field study*

We collected 130 crayfish in late November 2013 to identify factors that influence symbiont fitness. Collections were limited to an intensive 2 week period to avoid known confounding effects of seasonal variability on symbiont reproduction and abundance (DeWitt et al. 2012). Crayfish were collected using seines and dip nets, and were placed individually in plastic bags filled with stream water (750 ml Whirlpak) upon collection, and transported to the laboratory in insulated coolers. Under a stereoscopic microscope, we determined the abundance and microhabitat use of all *C. ingens* on each crayfish (described in Table 4.1), and the number of cocoons containing viable eggs associated

with each worm. Branchiobdellidans are simultaneous hermaphrodites that produce egg-containing cocoons that are attached to the host near the symbiont. *C. ingens* are also cannibalistic and are not typically found in close proximity to one another (JS, RPC, BLB *pers. obs.*). Cocoons, therefore, could be reasonably assumed to have been produced by a nearby individual. All data collection took place within several hours of capture to minimize symbiont loss and/or displacement.

#### *Analysis of field data*

We examined three models of individual reproductive success of *C. ingens*, reproduction as a function of: host size (model 1, hereon abbreviated “M1”), microhabitat (“M2”), and host size plus microhabitat (“M3”). Because the number of cocoons observed near each worm may be influenced by other factors such as variation in incubation time, loss of cocoons due to physical disturbance/predation, and occupancy time of individual symbionts at the observed location, we used the binary response variable of cocoon presence/absence to assess individual reproductive success.

Reproductive success was modeled using general linear models (GLM) assuming a binomial distribution of the response variable, with host size as a continuous predictor and attachment site a categorical factor (described in Table 4.1). Observations of worms occupying sites for which reproduction was not observed during the field study were excluded from analysis to avoid zero-inflation of the response variable (Zuur et al. 2009). Analyses were conducted using the `glm` function in the R-base package version 2.15.1 (Team 2010).

We used microhabitat occupancy data from the field study to assess competition for preferred microhabitats. *C. ingens* shows strong preference for particular

microhabitats (Brown et al. 2002) and the number of worms that can occupy a given microhabitat and the number of preferred microhabitats are likely limiting because of physical constraints of space, and strong negative intraspecific interactions such as cannibalism. From these premises, we predicted that only the best microhabitats, which represent a subset of all microhabitats, would be occupied on crayfish with few worms. Further, the remaining and less preferable microhabitats would only be occupied when the number of worms present is greater than the number of worms that can be supported by preferred microhabitats. To test this prediction, we constructed a symbiont presence/absence matrix of attachment sites by individual hosts and looked for a pattern of nestedness in symbiont occupancy of microhabitat. Nestedness was quantified as matrix temperature using the *nestedtemp* function in the R-vegan package 2.0–4. The significance of the observed nestedness metric was assessed by comparing observed matrix temperature to 10,000 randomized matrices with constrained row and column sums using the *oecosim* function in the R-vegan package 2.0–4. This type of null simulation provides the most conservative assessment of significance because it accounts for variation in observed frequency of attachment site use (row sums) and the distribution of symbiont abundance (column sums; Gotelli and Graves 1996, Gotelli 2000).

#### *Transmission experiment*

We experimentally observed transmission dynamics during 24 pairwise host encounters. We collected 48 crayfish and removed all of their worms by 5 min immersion in 10% MgCl<sub>2</sub> hexahydrate solution (Brown et al. 2002). We then ablated the dactyls of the 1<sup>st</sup> and 2<sup>nd</sup> perieopods of all crayfish to limit symbiont removal by the host. This measure was taken because crayfish sometimes remove newly colonizing

branchiobdellidans (Skelton et al. 2014), which could lead to the underestimation of the frequency of transmission events. We also used elastic bands to bind the chelae of the first walking legs to prevent crayfish from killing or dismembering each other. We then inoculated 24 “donor” crayfish, each with 6 adult *C. ingens*. We chose to keep the number of worms applied to donors constant across all experimental units to minimize variability in transmission due to variation in worm density and 6 *C. ingens* per host is typical for adult *Cambarus* hosts in the New River watershed (Brown and Creed 2004, Brown et al. 2012). Each donor was then paired with an un-inoculated “receiver” crayfish and left undisturbed for 16 d in 10 L aquaria. We used a stratified design to pair donors and receivers. Both donors and receivers were classified as either small (< 35 mm CL) or large (>35 mm CL). Our sampling strata consisted of all four pairwise combinations of size classes. Therefore, we had six replicates of each donor-receiver size combination. Aquaria contained natural stream substrate, 2 artificial refugia, and were aerated. The aquaria were maintained at ambient room temperature (~16 °C) and received natural sunlight from nearby windows. Crayfish were fed 3-5 shrimp pellets twice during the experiment. Based on our previous experience (JS, BLB, RPC), 16 d was chosen for experimental duration because it provides ample time for worms to disperse, but is brief enough to preclude worm reproduction that could have obfuscated our measurements of transmission. After 1, 3, 6, and 16 d, we recorded the number of *C. ingens* present at each microhabitat on all crayfish. For each encounter, the presence of *C. ingens* on the receiver signified successful transmission on day 16, and the magnitude of transmission was taken as the number of worms on the receiver.

We used generalized linear mixed models fit by maximum likelihood (glmer function, family = “binomial”, logit link, R-lme4 package version 1.1-7; Bates et al. 2014) to identify the factors that best explained the proportion of worms that were transmitted during experimental encounters. Our initial model included donor size, receiver size, time, and all interaction terms as fixed effects, and individual crayfish as a random effect. Chi square significance tests were used to sequentially remove model terms that did not improve model fit (drop1 function in the R-base package version 2.15.3; Team 2010).

### *Transmission predictions*

We used the GLMs of reproductive success in the field and a logit link to estimate the probability of reproductive success for each microhabitat on each donor crayfish. We then combined these estimates with observations of typical maximum occupancy for each attachment site to make a set of predictions of individual symbiont transmission events for each model of symbiont fitness, hereon abbreviated as “P1”, “P2”, and “P3”, corresponding to fitness models M1, M2, and M3. We made predictions based on each of the three GLMs, rather than just the best model because we did not know if both factors (host size and microhabitat) were perceivable to the symbiont and thus likely to influence dispersal behavior. Consequently, the best model for predicting symbiont dispersal could have been a subset of the best model of reproductive success.

Each set of predictions were made following three rules; 1) symbionts will occupy attachment sites in order of their reproductive fitness values based on GLM estimates from field data, 2) occupancy at each attachment site is limited to the typical maximum number of symbionts found at that attachment site during the field study, 3) symbionts

that are unable to procure an attachment site with an estimated probability of reproductive success greater than a minimum threshold value (minimum acceptable fitness; MAF) will disperse to the alternative host. Competition for preferable microhabitats was implicit in P2 and P3 because microhabitats were filled to maximum occupancy in order of their fitness value and therefore some worms were predicted to experience reduced fitness as a result of conspecifics filling preferable microhabitats. No intra-symbiont competition was implied in P1 because fitness was predicted to be equal across microhabitats and therefore predictions of dispersal were based on host size alone and unrelated to the presence of conspecifics.

We did not have an *a priori* expectation for the exact value of MAF, so MAF was treated as a free parameter and optimized iteratively. The significance of each set of predictions was verified using a conservative null model. For each set of predictions (P1, P2, and P3), we assessed the accuracy of our predictions for all possible values of MAF from 0.001 to 1, at increments of 0.001, by comparing predictions to observed transmission events. For each value of MAF, we calculated a “goodness of fit index” (GFI) to quantify the degree to which experiment-wide predictions deviated from the observed transmission (Equation 1). For each experimental unit  $i$ ,  $Ro$  and  $Rp$  are the observed and predicted number of worms observed on the receiver at the end of the experiment, and  $Total$  is the sum of worms recovered from the donor and the receiver of each tank.

Equation 1

$$GFI = \sum_{i=1}^n \frac{(Ro_i - Rp_i)^2}{Total_i}$$

Thus, GFI equals zero when experiment-wide predictions perfectly match observations, and increases with increasing discrepancy between predictions and observations. To test the statistical significance of each set of predictions, we used a one-tailed test of the observed GFI to a null distribution created by matrix permutations of the observed data; this was done by creating 10,000 randomized matrices in which columns represented donors and receivers, and rows represented experimental units. To minimize the probability of type I error, row sums and column sums were conserved during permutations so that the experiment-wide proportion of worms that dispersed was held constant, as were the number of worms recovered within each experimental unit (Gotelli and Graves 1996, Gotelli 2000). Because the total number of transmission events was conserved among permutations (column sums), this null model served as the expectation under the assumption that transmission rates were equiprobable across all host contacts while incorporating the naturally observed variability in transmission events. Each permutation of the original data set was then used as a null prediction to calculate a GFI score, and GFI scores from all permutations formed the null distribution that we used to assess the one-tailed significance of each set of predictions based on symbiont fitness.

## **Results**

### *Field study*

We observed 300 *C. ingens* and 351 cocoons on 130 crayfish. Symbiont occupancy and reproduction varied across microhabitats (Figure 4.1). The most frequently occupied microhabitat was the anteroventral portion of the abdomen, abbreviated VAbA (69%; as percent of crayfish with worms present at that microhabitat), followed by the lateral margin of the carapace, abbreviated LMB (45.4%), and area

around the genitals, VGn (22.3%). The microhabitats VTM and VAbI were infrequently occupied (17.7% and 13.8%, respectively). Worms attached at VAbA showed the highest frequency of reproductive success (46.7%; as percent of worms present that had cocoons), followed by VGn (22.3%) and LMB (5.1%). Worms attached to VTM and VAbL had no observed reproduction. We also found a strong nested pattern of microhabitat occupancy across hosts. Microhabitats in which reproduction was observed were usually occupied by symbionts, and other sites were typically occupied only when sites with reproduction were also occupied, creating a significantly nested pattern of microhabitat occupancy (observed matrix temperature = 11.455,  $z = -3.248$ ,  $p = 0.0007$ ).

Host size and microhabitat were strong predictors of worm reproductive success, both alone (M1 and M2) and together (M3). All coefficients in all three generalized linear models were highly significant (Table 4.2). AIC indicated that the model that included both microhabitat and host size (M3), was better than the reduced models M1 and M2. A direct comparison of M1 and M2 could not be made using AIC because neither is a nested subset of the other. Generally, the probability of reproductive success increased with host size (M1 and M3), and was highest at attachment site VAbA, followed by VGn, and lastly LMB (M2 and M3).

#### *Transmission experiment*

All crayfish (24/24) and half of their symbionts (77/144) survived the 16 d experiment. There was a considerable exchange of worms from donors to receivers during the first 6 days of the experiment, but little exchange between days 6 and 16 (Figure 4.2). Initially, the majority of worms were present on the lateral margins of the donors' carapaces (LMB; Figure 4.3). Through the course of the experiment, worms

moved from the donors' carapaces to the anteroventral portion of the donor's abdomen (VAbA), or dispersed to the receivers. For both donors and receivers, microhabitat occupancy became increasingly similar to the nested pattern observed in the field.

At the end of the experiment, transmission occurred in 13/21 (61%) of remaining pair-wise encounters. Of the 77 recovered worms, 24 (31%) transferred from the donor to the receiver. Three experimental units for which no worms were recovered were excluded from further analysis. Model selection recovered a model with significant main effects of donor size (estimate = -0.138, se = 0.053,  $z = -2.602$ ,  $p = 0.009$ ) and time (estimate = 0.092, se = 0.027,  $z = 3.477$ ,  $p < 0.001$ ) on the proportion of worms that were transmitted (Table 4.3).

After optimization of MAF, predictions based on host size and competition for attachment sites (P3) were significantly better than null predictions based on fixed transmission rates. For this model, MAF was optimized at 0.061 - 0.068, yielding a significantly lower GFI than null predictions (GFI = 4.33,  $p = 0.0019$ ; Figure 4.4). The optimized model correctly predicted the occurrence of transmission in 20/21 (95 %) of pairwise encounters and exactly predicted the magnitude of transmission (number of worms that switched hosts) in 14/21 (67%) of experimental encounters. Transmission predictions based on fitness models 1 and 2 failed to predict better than null predictions at  $\alpha = 0.05$  for any value of MAF.

## **Discussion**

Our results portray a symbiont dispersal strategy that is related to reproductive success and can be used to predict individual transmission events during host contact. Mixed effects modeling of the transmission experiment identified donor size as a

significant predictor of worm transmission, suggesting that worms were less likely to leave larger hosts. Using estimates of symbiont reproductive success generated from models of field data and hypothesizing that symbionts would only emigrate when conditions on their current host resulted in a fitness environment below a minimum threshold, we were able to predict independent transmission events much more accurately than predictions based on a fixed probability of transmission across all host encounters. The best predictions were based on the symbiont fitness model that included both host size and microhabitat (P3), and by model design, implicitly incorporated intra-symbiont competition for preferred microhabitats. Once we optimized the free parameter for MAF, this model correctly predicted the occurrence of transmission in all but one of experimental host encounters (95%) and correctly predicted the exact number of dispersing symbionts in most encounters (67%). Models based on host size alone, or competition for microhabitat alone, did not predict transmission better than null predictions. This result provides evidence that dispersal of symbionts is not a purely stochastic processes and that the symbiont's decision to disperse is informed by host quality and intra-specific competition with other symbionts for limited host resources.

Symbiont transmission during experimental host encounters was highly variable, but not entirely stochastic. Because we were specifically interested in the factors that cause a symbiont to emigrate, our experiment was explicitly designed to remove barriers to symbiont dispersal by providing long-term (16 d) host-host contact within a confined space (40L aquaria). Throughout the experiment, we observed crayfish in all tanks frequently contacting each other while exploring and often resting in direct contact. We also limited variation in post-dispersal colonization success by ablating host dactlys and

therefore greatly reducing the ability of all hosts to resist colonization (Skelton et al. 2014). Despite these measures, we observed tremendous variation in the transmission dynamics that occur during experimental host-host encounters. The proportion of symbionts within each host encounter that did disperse to the alternative host varied from 0 – 100%, with no detected transmission in a third of host encounters (7/21, 33%). After an initial six days of increase, the number of worms present on the receivers did not vary significantly for the remainder of the experiment. These results confirmed that 16 d was sufficient time for dispersing worms to find their new hosts and demonstrated that even when given full opportunity, symbionts did not always disperse, nor did any constant fraction of the symbiont infrapopulation choose to disperse. The success of our predictive model and the results of GLMM of experimental data both indicate that the variation in transmission among individual host encounters is explainable in terms of individual symbiont fitness.

Positive correlations between host size and symbiont density or biomass are frequently reported, especially in parasite systems (e.g. Mohr 1961, Saad-Fares and Combes 1992, Arneberg et al. 1998, Grutter and Poulin 1998, Poulin 2007). Our field study provided direct evidence of variation in host quality and its effects on individual symbiont reproduction that could explain the observed positive relationship between host size and worm density. Both models of symbiont reproductive success that included host size (M1 and M3) showed a positive relationship between host size and the probability of symbiont reproduction (Figure 4.4, *right panel*), strongly suggesting that host size directly influences individual symbiont fitness. Though this is the first assessment of the factors that influence the reproductive success of individual branchiobdellidans, many

other studies have found positive relationships between crayfish size and branchiobdellidan abundance and explained these relationships in terms of resource availability, molt frequency, host preference, and reduced host resistance (Mc Manus 1960, Young 1966, Bishop 1968, Koepp 1975, Brown and Creed 2004, Skelton et al. 2013, Skelton et al. 2014). Our results suggest that increased reproductive success on large hosts at least partially explains this commonly observed pattern.

Competition and species-specific variation in microhabitat use have been used to explain species co-existence of symbiont taxa in many systems (e.g. Rohde 1993, Mestre et al. 2011). However, intraspecific competition for microhabitats and how that competition influences transmission has not received much attention. We provide evidence of strong intra-specific competition for limited microhabitat resources and a subsequent effect on symbiont transmission. Microhabitat affinities have been described for many internal and external symbionts (Walton and Hobbs Jr 1971, Holmes 1973, Rohde 1979, Holmes and Price 1980, Mestre et al. 2011), as well as several species of branchiobdellidans (Brown et al. 2002, Gelder and Williams 2011, Skelton et al. 2013). In our study, microhabitats in which symbionts were reproductively successful were most frequently occupied whenever symbionts were present on a host (i.e. VAbA, VGn, and LMB; Figure 4.1), whereas microhabitats where reproduction was not observed were typically only occupied when preferable sites were already occupied (i.e. VAbL and VTM), resulting in a nested pattern of microhabitat occupancy (Figure 4.1). Each microhabitat typically supported a limited number of individuals, and that number was related to the physical extent of each microhabitat. For instance, the microhabitat with the highest reproductive success (VAbA) was confined to two (left and right side) small

areas on the ventro-lateral margins of the first abdominal segment and this microhabitat was not found to support more than two symbionts (Figure 4.1). We have frequently observed cannibalism in *C. ingens*, often involving worms of nearly equal size and strongly suspect this tendency as the reason for the apparent maximum occupancy of each microhabitat. Because microhabitats vary in their quality and each can support a finite number of individuals, the average expected fitness of *C. ingens* is reduced with increasing abundance on a host and the potential fitness advantage of dispersing increases.

While most studies relate microhabitat selection to the direct effects on fitness by increasing available resources or limiting mortality (Rohde 1979, Rohde 1993, Mestre et al. 2011), our results suggest that microhabitat selection may also have indirect fitness consequences by facilitating dispersal. Our field study showed that *C. ingens* was more frequently found attached to the lateral aspect of the carapace than to the ventral genital microhabitat. This result was curious because the latter site was found to offer a significantly higher chance of reproductive success. Observations of microhabitat occupancy dynamics during the transmission experiment provide evidence for an explanation. Early in the experiment, most worms were found attached to the donor carapaces. As the number of worms gradually declined in this location, the number of worms present on receivers gradually increased over the first six days. The same general pattern recurred on the receivers as they were colonized by dispersing worms, which were first largely found attached to the carapace and later found elsewhere on the host, principally the ventro-lateral portion of the first abdominal segment. This change in microhabitat occupancy following colonization suggests that the lateral margin of the

carapace may be used as a jumping-off point for dispersing worms. Branchiobdellidans are typically transmitted directly during host-host contact (Mc Manus 1960, Skelton et al. 2013). All other frequently occupied microhabitats were positioned on the ventral portion of the host and may therefore not offer ready access to alternative hosts. Conversely, the lateral margins of the carapace are more exposed and could offer a better position to detect and move to an alternative host.

In summary, our results show that the symbiont *C. ingens* is likely to stay with its host when it is profitable to do so, and leave when it is not. This seemingly simple realization has potentially profound consequences for how we understand transmission dynamics in natural populations. Our understanding of transmission dynamics in host populations has been largely guided by canonical epidemiological models (e.g. May 1977, McCallum et al. 2001). Most of these models predict the rate of infection as a function of the number of susceptible hosts in a population, the rate of contact among hosts, the probability of a contacted host being infected, and the probability of transmission given contact between an infected and susceptible host (Begon et al. 2002). There has been considerable investigation of the factors that cause variation in contact among hosts such as host density (McCallum et al. 2001), spatial properties of the local environment and host population (Fenton et al. 2002), and host interactions such as interference competition among foraging hosts (Civitello et al. 2013). However, the probability of transmission given contact between a susceptible and infected host is typically assumed to be constant (Begon et al. 2002). Our work shows that symbiont transmission can be highly variable even when the opportunity to disperse and the likelihood of successful colonization post-dispersal are held constant. More importantly,

we show that this variation can be explained in terms of fitness outcomes for individual symbionts and knowledge of the factors that influence symbiont fitness can therefore be used to accurately predict individual transmission events during host encounters. Our ability to predict individual transmission events based on factors that were shown to influence symbiont reproductive success in the field strongly suggests that dispersal of some symbionts is non-random and reflects a symbiont dispersal strategy. These results suggest that individual based modeling approaches that consider the distribution of host qualities relevant to symbiont fitness, and consequently symbiont dispersal, may provide new insights to transmission dynamics at the individual host and host population levels. We urge parasite ecologists and scholars of other symbiotic systems to consider the factors that contribute to individual symbiont fitness and how those factors may guide a symbiont's dispersal strategy.

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## Figure and table captions

### Figure 4.1

Field observations of microhabitat occupancy and reproductive success of *C. ingens* observed from 130 crayfish in Sinking Creek, Newport VA. Open circles delineate the five most commonly occupied microhabitats observed during field study (described in Table 4.1), numbers in parentheses indicate the maximum number of worms typically found at each microhabitat. Bars show nested pattern in microhabitat occupancy (rows). Columns represent individual crayfish organized left to right by decreasing total symbiont abundance. Grey bars indicate absence of worms, gold bars indicate presence of non-reproducing worms (no cocoons), and red bars indicate reproducing worms (with cocoons). Microhabitats with observed worm reproduction are typically occupied before other microhabitats.

### Figure 4.2

Transmission dynamics during a 16 d experiment. Symbols show the experiment-wide mean proportion of worms present on donors and receivers, error bars represent  $\pm 1$  standard error. The proportion of worms present on receivers increased for the first 6 days of the experiment and subsequently changed very little.

### Figure 4.3

Microhabitat occupancy and transmission dynamics during transmission experiment. For each matrix, rows represent microhabitat and columns represent individual crayfish. Cell color shows the number of worms present at each microhabitat on each donor (top row of

matrices) and receivers (bottom) at 4 points in time (columns of matrices). The number of worms on donor carapace (LMB) decreased as worms moved to either the donor's abdomen (VAbA) or dispersed to the receiver. Worms colonizing receivers also moved from LMB to VAbA through time.

#### Figure 4.4

Predictive model for individual symbiont dispersal during experimental host encounters. (*left*) Model performance for three competing sets of predictions; P1 = light grey, P2 = dark grey, P3 = black. Horizontal dotted lines represent the lower bounds for one-tailed significance cut-offs for goodness of fit statistic determined by null model permutations ( $\alpha = 0.05$  and  $0.01$ ). Predictions based on the symbiont fitness model that considered host size and microhabitat (P3) predicted transmission significantly better than the null model for most values of MAF between 0.041 and 0.142. \*Optimized value for the assumed minimum predicted probability of reproduction for which symbionts will not disperse (minimum acceptable fitness;  $MAF = 0.061 - 0.068$ , where  $p = 0.0019$ ). (*right*). Graphical representation of the best predictive model. Solid lines show predicted probability of symbiont reproduction as a function of host size (x-axis) and microhabitat. Numbers in parentheses represent the maximum occupancy of each microhabitat. Microhabitats for which symbiont reproduction was not observed are not shown and not included in fitness models. Optimized value of MAF from the best transmission model is shown as horizontal grey dotted line, labeled as MAF\*. Symbionts are predicted to fill microhabitats in the order VAbA, VGn, and LMB. Symbionts that cannot obtain a microhabitat with an estimated probability of reproductive success that is greater than

MAF\* are predicted to disperse during pair-wise host encounters. The displayed mode correctly predicted the occurrence of transmission in 20/21 cases (95%), and exactly predicted the magnitude of transmission in 14/21 cases (67%).

Table 4.1

Descriptions and abbreviations of microhabitat sites of the crayfish body occupied by *C. ingens* during our field survey.

Table 4.2

Three generalized linear models for the effects of host size and symbiont microhabitat on the probability of symbiont reproduction observed during our field survey. All model terms were highly significant. The model containing both host size and symbiont microhabitat (Model M3) provided the strongest result based on Akaike information criterion (AIC).

Table 4.3

Generalized linear mixed model for the probability of individual worm dispersal. We looked at main effects of donor size and time, and the random effect of individual tank on the proportion of worms that were found on receivers at each date.

Figure 4.1 Microhabitats of *C. ingens*

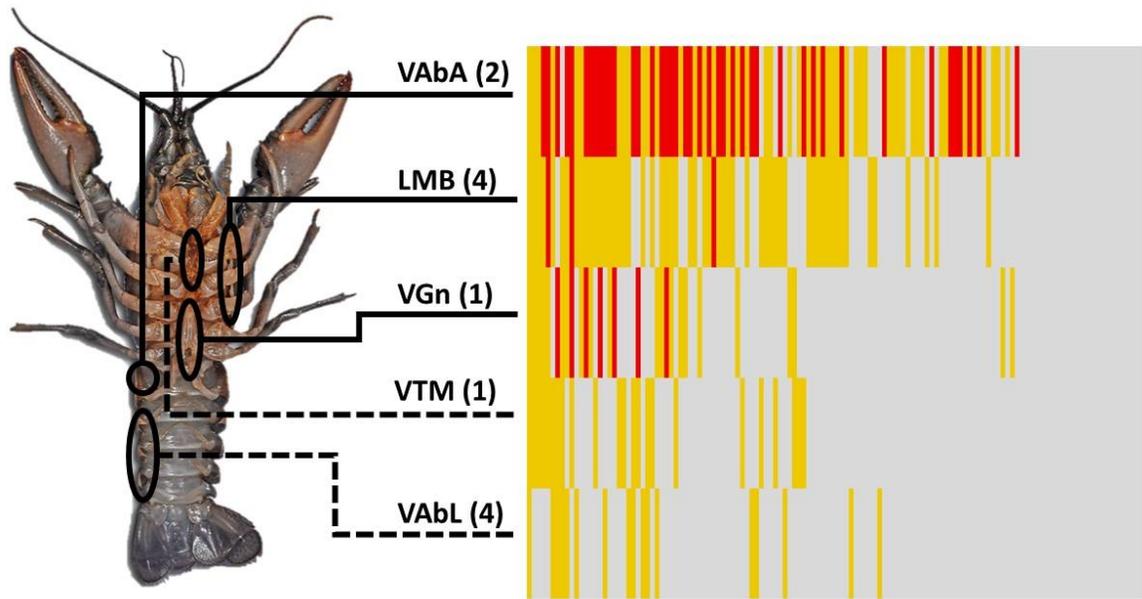


Figure 4.2 Proportion of worms on donors and receivers through time

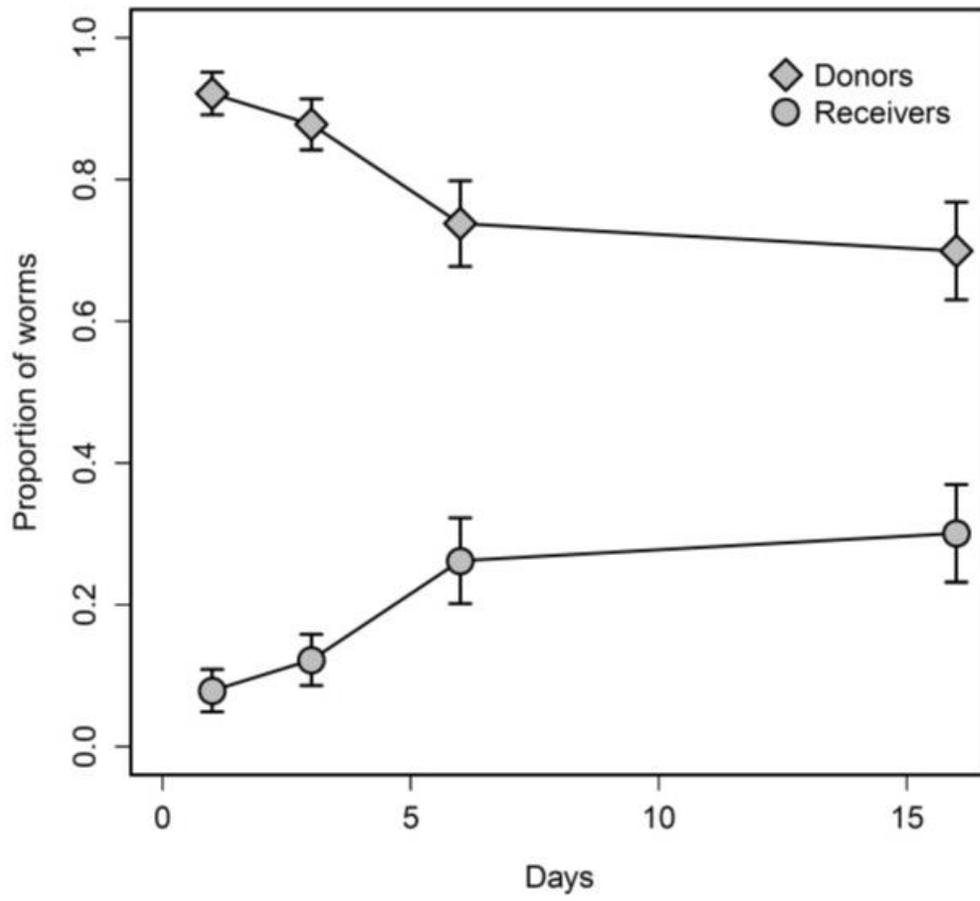


Figure 4.3 Microhabitat use of *C. ingens* during transmission experiment

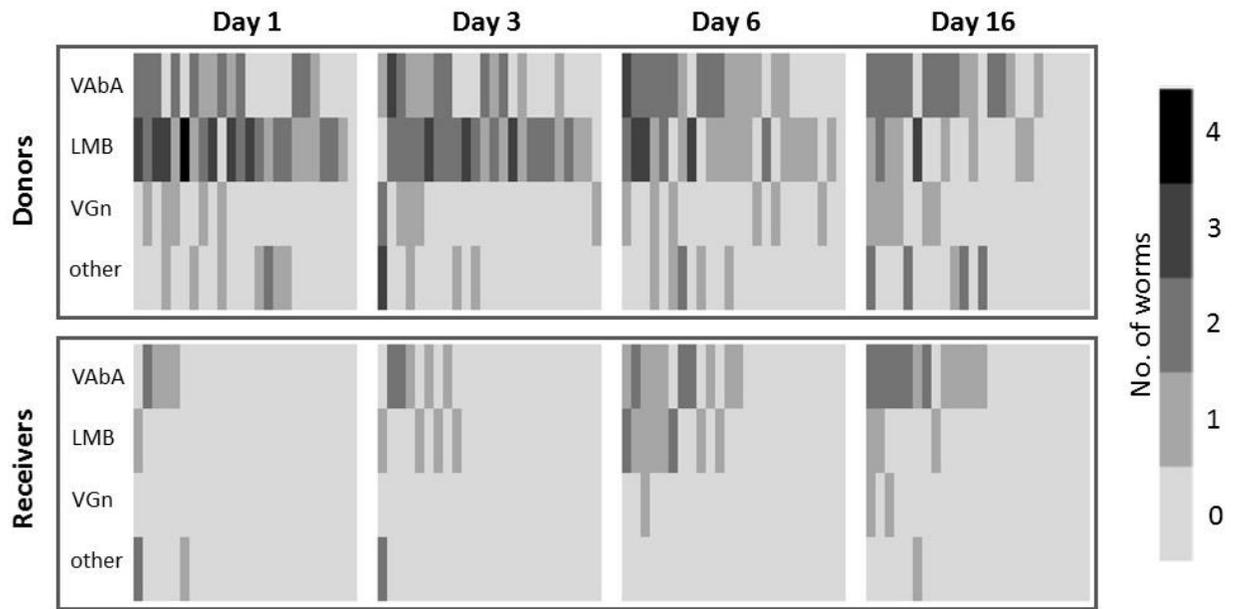


Figure 4.4 Model performance and graphical illustration of the dispersal strategy of *C. ingens*

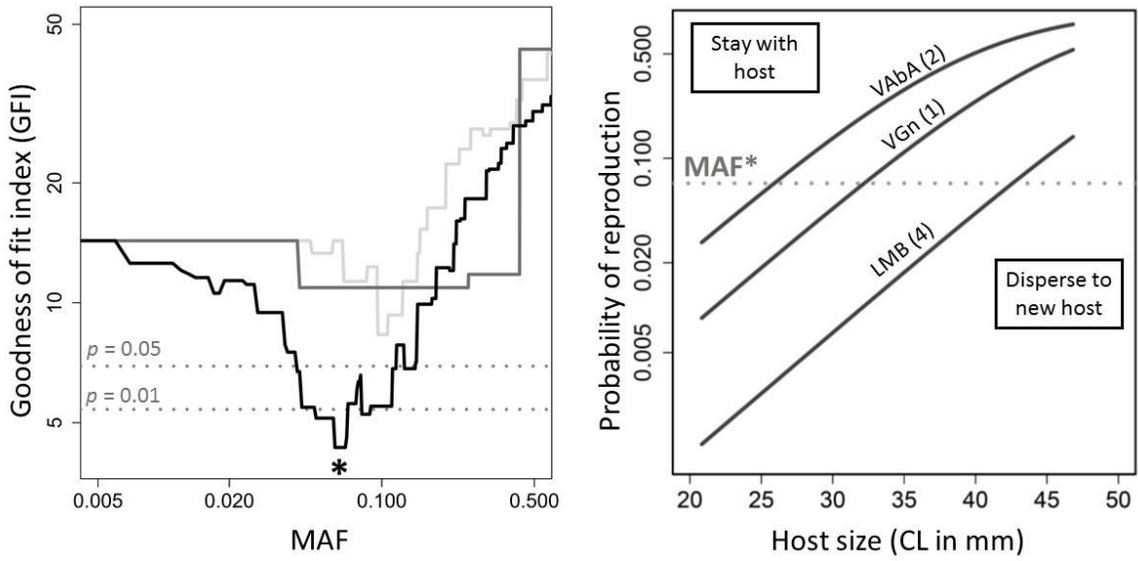


Table 4.1 Descriptions of the commonly used microhabitats of *C. ingens*

Microhabitat	Description
<i>Ventral</i>	
VAbL	Ventral aspect of the abdomen, attached to lateral margins of abdominal tergites or swimmerets not including VAbA and VGn
VAbA	Ventral aspect of abdomen, attached to or near lateral margins of first abdominal tergite
VGn	Genital opening of females or the gonopods of males
VTM	Ventral aspect of the cephalothorax, anterior to VGn and mesial to coxae of walking legs.
<i>Lateral</i>	
LMB	Branchiostegite within 1 worm body length of the lateral margin

Table 4.2 Three generalized linear models for predicting *C. ingens* reproductive success

<i>Model</i>	<i>Coefficient</i>	<i>Estimate</i>	<i>z-value</i>	<i>p</i>	<i>AIC</i>
M1	Intercept	-6.552	-4.679	<b>&lt;0.0001</b>	263.65
	Host size	0.145	4.126	<b>&lt;0.0001</b>	
M2	Intercept	-3.1355	-5.318	<b>&lt;0.0001</b>	244.09
	VAbA	2.848	4.630	<b>&lt;0.0001</b>	
	VGn	2.037	2.777	<b>&lt;0.01</b>	
M3	Intercept	-10.624	-5.786	<b>&lt;0.0001</b>	<b>221.01</b>
	VAbA	3.137	4.921	<b>&lt;0.0001</b>	
	VGn	1.952	2.584	<b>&lt;0.01</b>	
	Host size	0.188	4.483	<b>&lt;0.0001</b>	

Table 4.3 Generalized linear mixed model for the probability of individual worm dispersal during transmission experiment

Random Effects:

Group	Names	Variance	Std. dev.
Tank	intercept	1.661	1.289

Fixed effects:

	Estimate	Std. Error	<i>z</i>	<i>P</i>
Intercept	2.308	1.849	1.248	0.212
Donor size	-0.138	0.053	-2.602	0.009
Days	0.092	0.027	3.477	< 0.001

## Chapter 5: Summary

In December 1958, during his address to the American Society of Naturalists, G. E. Hutchinson succinctly verbalized the question that continues to engage generations of ecologists and natural historians; “Why are there so many kinds of animals?” Hutchinson probably did not realize that most of those “kinds of animals” live as symbionts; organisms that live within or on other kinds of organisms. Because each organism provides a new microcosm to be colonized by a diversity of others, symbiosis creates layers of biological diversity stacked one on top of the other. This phenomenon has not escaped the attention of laypersons or scientists. Two hundred years before Hutchinson, satirist Jonathon Swift acknowledged, “So nat'ralists observe, a flea hath smaller fleas that on him prey; And these have smaller fleas to bite 'em. And so proceeds ad infinitum.” Echoing Swift, preeminent parasitologist P. W. Price proclaimed, “It is clear that parasitism as a way of life is more common than all other feeding strategies combined”. Moreover, the health, nutrition, development, and immune systems of free-living organisms, including humans, are dependent on interactions with mutualistic symbionts. Simultaneously, free-living organisms are threatened by disease, suffering, death, and extinction caused by parasitic symbionts. Therefore, if our goal is to understand the processes that create, shape, and maintain biological diversity, we ought to be squarely fixated on symbionts and the symbiotic life-style. Yet, the study of symbiosis has historically remained a relatively specialized field that has not taken full advantage of the developments in more generalized fields. Improving our currently nascent conception of symbioses is an essential next step towards a more complete

understanding of the biological world and such an improvement has immediate implications for the conservation of biodiversity and human health and welfare.

The study of symbiotic organisms is rich with its own peculiar challenges. Many symbiont taxa, such as microparasites of animal blood and tissue, the endophytic fungi of the rhizosphere, and bacteria and fungi that compose microbiomes in and on organisms are often cryptic and may only be detected and identified using targeted molecular techniques. Moreover, symbiont taxa often have beguiling complex life histories that involve obligate and facultative associations with many hosts, such as many helminthic parasites that plague animal populations. These peculiarities can make realistic experimentation exceedingly difficult or impossible and it is probable that such difficulty is the reason that the study of symbioses has historically remained a specialized subfield of organismal biology and ecology. However, the obscurity of symbiosis is rapidly diminishing as more researchers realize that symbiosis is the rule, not the exception. In the past several years, an increasing number of high profile papers have codified conceptual frameworks to unite the study of symbioses with concepts and theory from more general fields such as community ecology, and more recently metacommunity ecology (e.g. Pedersen and Fenton 2007, Graham 2008, Mihaljevic 2012 ). While these studies make great strides towards a more complete understanding of the symbiotic life-style and the complex and often interacting mechanisms that structure symbiont diversity, the concepts that these contributions describe require empirical and experimental verification, and such verification is not easy.

## **Dissertation Summary**

During my dissertation work I have developed methods for rapidly collecting volumes of field observations from a multi-host and multi-symbiont symbiosis. My collaborators and I have conducted field and laboratory experiments of realistic complexity and circumstance. Over the past five years of studies presented in this dissertation, the symbiosis between crayfish and branchiobdellidans has proven to be an unusually amenable study system for demystifying the symbiotic lifestyle and uncovering the processes that shape symbiont populations and communities. Branchiobdellidans are strictly ectosymbionts and can be easily detected and quantified visually. They have a simple life history that takes place entirely on one host and are therefore amenable to realistic experimentation in the field and laboratory.

The literature review in Chapter 1 sought to solidify the thoughts and observations of 100 years of disparate studies on this system. Although branchiobdellidans have received a modicum of study over the past century, the available literature depicted a diversity of host-symbiont relationships between crayfish and their diminutive annelid hitchhikers. All available evidence indicated that branchiobdellidans are obligate ectosymbionts of freshwater crustacean hosts, and while these hosts are usually crayfish, they may include freshwater shrimp, crabs, and other crustaceans. Branchiobdellidans can be tissue devouring parasites, or host cleaning mutualists, depending on the host and worm species involved, environment, symbiont density, and other factors that define the context of their interactions.

The review in Chapter 1 also summarized what was currently known about branchiobdellidan diversity at scales that range from the individual crayfish body, to

entire continents. While several previous reports addressed branchiobdellidan biogeography in terms of broad to very broad species distributions (e.g. Hobbs et al. 1967, Holt 1973, Gelder and Smith 1987, Gelder 1999, Williams et al. 2009), there was a paucity of study of the proximate ecological processes that influence patterns of branchiobdellidan abundance and diversity. This comprehensive review identified a diversity of opportunities for research in the crayfish branchiobdellidan system. These opportunities included a chance to describe the natural history of a fascinating and little studied group of worms. These opportunities also included gaining new insights towards potentially crucial and overlooked facets of the biology of a group of ecosystem engineering, keystone, sometimes noxious invasive, and other times critically imperiled crayfish species. But more generally, this review highlighted the values of the crayfish-branchiobdellidan system as a model for generating and testing improved conceptual frameworks for the study of the symbiotic life-style.

The interests of symbionts, even those that are typically considered cooperative mutualists, are not always aligned. When the interests of mutualists are misaligned, individuals can exert controls over their partners, which maintain reciprocal positive feedbacks among partners, and can stabilize mutualistic outcomes. In Chapter 2, I use the crayfish branchiobdellidan system to show that partners can adjust such controls to maximize life-long benefits of symbiosis. Previous work demonstrated that some branchiobdellidans can increase crayfish growth (Brown et al. 2002). This effect was hypothesized to be the result of branchiobdellidans consuming potentially harmful epibiotic accumulations from their host's body. However, the worms also incur costs in the form of facultative parasitism and the net outcome of symbiosis for the host

represents a balance between these opposing costs and benefits (Brown et al. 2012). But, the benefits to crayfish are likely to change with ontogeny. Young crayfish grow rapidly and frequently molt and molting relieves the crayfish of all epibiotic fouling. Conversely, older hosts molt infrequently and are constantly challenged by epibiotic fouling. Based on the likely changes in the cost to benefit ratio of symbiosis for the host, I predicted that only older, slow growing crayfish would tolerate the presence of worms. My predictions were supported by a laboratory experiment that demonstrated an age-specific grooming response as a control mechanism in the cleaning symbiosis between crayfish and branchiobdellidans. I showed that small crayfish use the dactyls and fingers of their walking legs to remove colonizing worms from their exoskeletons and older hosts did not. I also found evidence that the worms had an adaptive response to host grooming. Worms that were placed on young hosts utilized microhabitats on the host that were difficult for the host to reach. The experimental results were supported by field observations of changes in branchiobdellidan abundance and microhabitat use through crayfish ontogeny. This paper was the first to describe an ontogenetic shift in host resistance as an adaptation to maximize life-long benefits of a mutualistic symbiosis.

In addition to the insights gained regarding the maintenance of mutualisms, the study described in Chapter 2 had interesting implications for branchiobdellidan community ecology. Several recent studies encouraged using a community ecological framework for understanding how multi-species communities of symbionts assemble on or within their hosts and emphasized the dual roles of host controls and symbiont-symbiont interactions (Pedersen and Fenton 2007, Graham 2008, Mihaljevic 2012). In this system, age-specific variation in the outcomes of symbiosis led to an age-specific

resistance strategy for crayfish. Consequently, crayfish populations were composed of two types of hosts; young resistant hosts, and older non-resistant hosts. For the worms, crayfish populations were heterogeneous archipelagos of island habitats; some with a potentially strong species filter and some without a filter. This natural variation in resistance within host populations presented the opportunity for me to assess how host resistance and interactions among symbiont taxa influence symbiont community assembly processes under realistically complex conditions.

The field study presented in Chapter 3 illustrates predictable branchiobdellidan community assembly through host ontogeny. Branchiobdellidan community assembly occurred in two phases: 1) Early in crayfish ontogeny, host resistance permitted the colonization of only a few worm species. Consequently, the worm communities of young crayfish were typified by low diversity, low density, and weak or entirely absent within-host interactions among symbionts. 2) Later in crayfish ontogeny, host resistance was relaxed and consequently symbiont communities became larger, more diverse, and more interactive. Specifically, the large branchiobdellidan species *C. ingens* could not colonize young hosts. However, once crayfish switched from resistant to non-resistant, *C. ingens* was free to colonize those crayfish and promptly prey upon the much smaller, earlier colonizing branchiobdellidan species. Thus, there appears to be a trade-off for branchiobdellidans, they may be small and colonize resistant hosts but risk being consumed by intra-guild predators, or they may be large and only able to colonize non-resistant hosts, but be able to consume smaller symbiont taxa. I suspect that this host-ontogenetic tradeoff can explain the co-existence of some branchiobdellidan taxa in a

manner similar to Hutchinson's ideas about fugitive species (Hutchinson 1951); in this case the small taxa are fugitives from the intra-guild predators.

The work summarized above brought forth a new understanding of the factors that modulate colonization and species interactions on crayfish host-islands, yet the factors that cause symbionts to disperse were still unknown. From a symbiont's perspective, a host population or community represents a patchy and heterogeneous landscape. A century of work on free-living animals focused has described "dispersal strategies" that organisms use to increase the chances of finding good habitat patches in order to ultimately maximize their individual fitness. Studies of symbionts, however, typically focus on the factors that modulate the opportunities for dispersal, such as contact rates among hosts, but the movement of symbionts from one host to another was typically treated as a purely stochastic process with a fixed probability for a given host population. In the final data chapter of this dissertation, I identified the factors that influence individual fitness of the branchiobdellidan *C. ingens*, and then used fitness information to make predictions about individual dispersal events during experimentally controlled host contacts. I showed that much of the variation in individual fitness was explained by host size and microhabitat. Furthermore, I demonstrated the presence of strong intra-specific competition for space in the best microhabitats. Lastly, I showed that a predictive model based on maximizing individual symbiont fitness predicted symbiont dispersal much better than a comparable null model with an assumed fixed probability of dispersal. This project illustrated a symbiont dispersal strategy and suggests that transmission models could be improved by accounting for explainable variation in transmission dynamics that occur among host contacts.

While the work presented in this dissertation has provided many new insights to branchiobdellidan natural history and in doing so, contributed to our understanding of the symbiont life-style, what we know about these animals remains dwarfed by what we do not know. I will now return to Hutchinson's subtly pointed question (see above). Hutchinson asked "why" there are so many kinds of animals, not *how*. While a *how* question can be answered by demonstrating contemporary mechanisms, such as the work described herein, a *why* question in biology is implicitly one of evolutionary history. In the case of symbioses, if we wish to know why there are so many kinds of symbionts, we must examine their evolution and co-evolution. Hutchinson also asked why there are "so many"; not more, not less, just so many. The next question for branchiobdellidan research is; how do observable ecological processes relate to the evolution and diversification of symbiont lineages? The work presented in this dissertation suggests that relaxed host control in crayfish leads to strong competitive interactions among their symbionts. I have observed over the past five years that those competitive interactions are reflected by a high degree of specialization in which each co-existing worm species specializes on inhabiting a certain part of the crayfish body. Because each worm species specializes on a particular part of the host's body, many worm species can coexist on a single host. How did this diversity come about (the "why" part of Hutchinson's question)? And, what are the limits to this partitioning of space (this is the "so many part")? Future work in this system should try to relate contemporary patterns of resource-use and symbiont competition to inferred phylogenetic history to uncover the ecological and evolutionary processes that sculpt diversity in branchiobdellidans and to help us get closer to an answer for the question "why are there so many kinds of *symbionts*?"

### **A personal comment to the next branchiobdellecologist**

The remainder of this text is devoted to the next ecology student who becomes enthralled with the branchiobdellidans. You are almost certainly on your own, but relax and don't panic. Take a moment to admire a crayfish and its diminutive hitchhikers. Wonder at the aggregations of *Ankyrodrilus* or *Xironogiton*, packed like suckling piglets along the claws of a large *Cambarus*. Exalt while close proximity between two *Cambarincola ingens* elicits a race to see whom will devour whom. Ask yourself why *Pterodrilus* is as "floofy" as the appendages they frequently attach themselves to (adjective borrowed from a personal conversation with B. W. Williams). The charms of your new microcosm will carry you through the woes of taxonomy that you are about to endure. Read the extensive works of M. M. Ellis, R. L. Hoffman, P. C. Holt, S. Gelder, and B. W. Williams. Now go to your microscope. Start with living specimens. The delicate internal structures painstakingly depicted in those volumes are indeed real, and there is probably nothing wrong with your objective lenses or iris diaphragm; also, higher magnification will not help you. If you don't see anything, remove some water from under your coverslip with a bit of tissue, or add some back. Improper water pressure is almost always the problem. If you get a glimpse at the spermiducal gland and prostate, or paired versus single nephridial pores, take a picture because it might be a while before you see it again. Expect that learning to reliably find and identify the defining characteristics of your new subjects will take a while, a long while, and there are no good shortcuts. But also expect to be rewarded for your efforts. The branchiobdellidans are not just fascinating in their own peculiarities, but they provide an unusually large and clear

window to the underpinnings of symbiont ecology and evolution. And, with your new and unique skills, the stories are all yours to tell.

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