ONTOGENETIC SHIFTS AND SYMBIONT SUCCESSION IN A FRESHWATER CLEANING SYMBIOSIS MUTUALISM

A Thesis
by
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Abstract

ONTOGENETIC SHIFTS AND SYMBIONT SUCESSION IN A FRESHWATER CLEANING SYMBIOSIS MUTUALISM. (August 2014)

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Host-symbiont associations and the outcome of symbioses may vary over host ontogeny; however, this has received little attention, especially in systems in which the host is a mobile animal. I examined how changes in crayfish size can influence their associations with ectosymbiotic branchiobdellidan worms and the consequences of these associations for both the host and the symbiont. In the Middle Fork of the New River, the dominant worm species shifts from *Cambarincola philadelphicus* to *Cambarincola ingens* as crayfish (*Cambarus bartonii*) increase in size. I evaluated whether this change in the dominant symbiont had effects on different size classes of crayfish, how the worms responded to different sized crayfish and what mechanisms promoted the shift. In a lab experiment, small and large crayfish were stocked with either *C. philadelphicus* or *C. ingens*. I monitored crayfish growth, worm numbers and worm reproduction. Neither worm species had an effect on small crayfish but both increased the growth of large crayfish. *Cambarincola philadelphicus* were more abundant and had higher cocoon production than *C. ingens* on small crayfish. *Cambarincola philadelphicus* persisted on large crayfish in the absence of *C. ingens*. In contrast, *C. ingens* populations were higher on large crayfish and this species

exhibited higher cocoon production on large hosts. *Cambarincola ingens* were rapidly removed by small crayfish. A subsequent lab experiment demonstrated that intraguild predation by *C. ingens* was responsible for the decline in *C. philadelphicus* on large crayfish. My results demonstrate that the outcome of the crayfish-branchiobdellidan association changes over crayfish ontogeny. Further, host regulation and intraguild predation by *C. ingens* determine ectosymbiont succession in this mutualism. The symbiont succession reported here is similar to that observed on various sessile hosts (e.g., ant succession on tropical trees species) suggesting that such succession may be a general phenomenon in many symbioses regardless of host mobility.

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Dedication

I dedicate this thesis to my loving family.

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Foreword

The research detailed in this thesis will be submitted to *Ecology*, an international peer-reviewed journal owned and published by the Ecological Society of America. The thesis has been prepared according to the guidelines of the journal to which it will be submitted.

INTRODUCTION

Species interactions can change during ontogeny, especially if a species exhibits drastic changes in body size or morphology during its life history (Werner and Gilliam 1984, Thompson 1988). Increases in body size can result in a species changing habitats, interacting with new species and outgrowing predators (Werner and Gilliam 1984). Moreover, increases in size may enable a species to prey on former competitors (i.e., intraguild predation) (Polis et al. 1989, Olson 1996). These ontogenetic niche shifts maximize growth rates by reducing resource overlap and minimizing size-selective predation at particular life stages, both of which affect the survivorship and recruitment of individuals to larger, reproductively active size classes (Werner and Gilliam 1984). Thus, ontogenetic niche shifts have important consequences for population dynamics as well as community structure (Werner and Gilliam 1984, Polis et al. 1989). The effect of size on competitive and predator-prey interactions has been well-documented in freshwater fish, amphibians, and invertebrates (Mittelbach 1988, Osenberg et al. 1988, Werner and Hall 1988, Bergman and Greenberg 1994, Creed 1994, Olson 1996, Rudolf and Rasmussen 2013).

Interactions between hosts and symbionts should also exhibit changes in the nature of the interaction during ontogeny, particularly if the host is relatively long-lived in comparison to the symbiont and increases markedly in size over its lifespan. If the rewards and services provided by partner species in a symbiosis are a function of host size, the net effects of symbiotic interactions on components of partner fitness should change with changes in host

size (Thompson 1988, Bronstein 1994). Further, symbionts may associate with hosts during life stages that provide greater rewards and services to the symbiont. However, if there are multiple symbionts in a community that could utilize a host during a particular life stage then it is possible that dominant symbionts might actually replace subordinate symbiont taxa over time. Competition and predation by dominant symbionts could potentially lead to symbiont succession in which symbionts colonize and become extirpated during particular life stages of the host (Fonseca and Benson 2003). Alternatively, symbiont populations could disappear for other reasons (e.g., disease, active removal by host, changes in environmental conditions) leaving a host open to recolonization by other symbionts (Palmer et al. 2010).

There is limited evidence from symbiotic associations for ontogenetic shifts in dominant symbionts. Similarly, there is little evidence showing that the outcome of an association may change with changes in host size or life stage. Recent evidence from the ant-plant protective mutualisms demonstrates that these associations are not fixed but may change over the life of the host tree (Palmer et al. 2010). Moreover, the outcome of the interactions may change, with some ant species having a positive effect on the host while others have no discernable effect or even a negative effect (Palmer et al. 2010). Additionally, corals may exhibit specificity for different species of obligate algal symbionts during particular life stages (Abrego et al. 2009), and associations with housekeeping crabs may be most beneficial when coral colonies are small (Stewart et al. 2013). Overall, few studies have examined the influence of host size in mutualistic interactions (but see Abrego et al. 2009, Palmer et al. 2010, Yang and Rudolf 2010, Stewart et al. 2013) and there is no evidence of size-dependent outcomes in cleaning symbiosis mutualisms. In this study, I investigated size-specific associations between two species of ectosymbiont branchiobdellidan annelids and

their crayfish host *Cambarus bartonii*. Crayfish exhibit ontogenetic shifts in habitat with changes in body size in order to avoid particular predators (Creed 1994, Englund and Krupa 2000, Flinders and Magoulick 2007). Ontogenetic shifts in branchiobdellidan ectosymbionts of crayfish and their effect on partner fitness have not been demonstrated experimentally.

Branchiobdellidans are small annelids commonly associated with many species of crayfish in the Northern Hemisphere (Gelder 1999). While associations between branchiobdellidans and their crayfish hosts are generally considered to be commensalisms (McManus 1960, Young 1966, Bishop 1968, Jennings and Gelder 1979, Keller 1992) or parasitisms (Grabda and Wierzbicka 1969, Quaglio et al. 2006, Rosewarne et al. 2012), three of these associations have recently been described as cleaning symbiosis mutualisms (Brown et al. 2002, 2012, Lee et al. 2009). Cleaning symbioses involve the removal of ectoparasites, debris and damaged tissues from client organisms by a cleaner organism (Limbaugh 1961, Losey 1972). While engaging in a cleaning symbiosis, cleaners should benefit by obtaining food resources while clients benefit by being relieved of potentially harmful parasites and fouling material (Losey 1972, Arnal et al. 2001). Demonstration of positive effects on client fitness in cleaning symbioses has been difficult (Poulin and Grutter 1996, Weeks 2000, Cheney and Côté 2003, Bshary et al. 2007); however, positive effects on components of client fitness (e.g., growth and survival) have been demonstrated multiple times in the crayfish-branchiobdellidan symbiosis (Brown et al. 2002, 2012, Lee et al. 2009). Unlike other cleaning symbioses, these are long term associations in which the branchiobdellidan worms benefit by using their crayfish host as a site for reproduction and maturation (Young 1966, Brinkhurst and Gelder 2001, Creed et al., in press). Grazing by branchiobdellidans may

benefit crayfish by removing epibionts and fouling material from the gill filaments, thereby facilitating gas exchange and ammonia excretion across gill epithelia (Brown et al. 2002).

The crayfish-branchiobdellidan association can shift from mutualism to parasitism as a function of abiotic and biotic factors (Lee et al. 2009, Brown et al. 2012). Previous studies have demonstrated that the positive effects of worms on crayfish growth and survival are mediated by worm density and environmental context (Brown et al. 2002, 2012, Lee et al. 2009). Worms are maintained at beneficial densities through crayfish regulation (i.e., directed grooming) (Farrell et al. 2014), the extent of which is influenced by crayfish size (Skelton et al. 2014). Moreover, the degree of environmental fouling influences the amount of crayfish regulation as well as worm population size (Thomas et al. 2013). Small crayfish grow rapidly and molt frequently which may minimize any potential benefits worms might provide. Accordingly, the outcome of the association may not be a mutualism until crayfish growth rate slows and molts are less frequent. Further, with increasing size crayfish may become more tolerant of worms (Skelton et al. 2014). Nonetheless, the effect of branchiobdellidans on growth of different size classes of crayfish hosts has not been determined experimentally. Moreover, the effects of crayfish size on the fitness of different branchiobdellidan species have not been examined. Given the ease of manipulation, the crayfish-branchiobdellidan association is a model system for evaluating how partner size affects populations of both the host and symbiont. Further, these associations are interesting in that the interaction involves a mobile host with a relatively sedentary symbiont.

In the Middle Fork of the New River in North Carolina, USA, preliminary surveys suggested that size-specific associations between the crayfish *Cambarus bartonii* and two species of branchiobdellidan, *Cambarincola philadelphicus* and *Cambarincola ingens*, may

occur. *Cambarincola philadelphicus* appeared to be the dominant worm species on small size classes of *C. bartonii* while *C. ingens* dominated larger crayfish. I conducted an extensive field survey to better quantify worm species abundance on different size classes of *C. bartonii*. These surveys were completed at three sites on the Middle Fork during early and late summer in order to discern any spatial or temporal effects. I then assessed the effects of *C. philadelphicus* and *C. ingens,* separately, on growth of small and large size classes of *C. bartonii* in a laboratory experiment. Finally, I conducted an additional experiment to determine the relative importance of crayfish regulation or interactions with *C. ingens* as the mechanism for replacement of *C. philadelphicus* on larger *C. bartonii*. I hypothesized that both worm species would have no effect on small crayfish growth (i.e., the associations with small crayfish are commensalisms) and, in accordance with preliminary survey data, small *C. bartonii* would be more tolerant of *C. philadelphicus* than *C. ingens.* I predicted that *C. philadelphicus* would remain a commensal as crayfish increased in size. Alternatively, I predicted that the late colonizing *C. ingens* would have positive effects on large crayfish growth and survival (i.e., the large *C. bartonii*-*C. ingens* association is a cleaning symbiosis mutualism) as *C. ingens* is engaged in a cleaning symbiosis with *Cambarus chasmodactylus,* a co-occurring crayfish species (Brown et al. 2002, 2012). Moreover, large *C. bartonii* would be more tolerant of *C. ingens* at this size class and provide a more suitable habitat for these worms. Finally, I predicted that *C. ingens* would replace *C. philadelphicus* on large *C. bartonii* through predation as this large species of worm has been observed consuming other branchiobdellidans.

METHODS

Survey sites and study animals

Crayfish and their associated worms were collected at three sites on the Middle Fork of the New River (Watauga County, North Carolina, USA). The Middle Fork is a third-order stream that flows northward from Blowing Rock, NC, to the South Fork of the New River in Boone, NC. The three sampling sites are as follows: Jordan Cook (MFJC, 81^{o}39'27.907"W, 36^{o}11'27.056"N), Tweetsie (MFT, 81^{o}38'49.374"W, 36^{o}9'57.727"N) and Mustard Seed (MFMS, 81°64'66.91"W, 36°15'28.54"N). I used an additional site (Bench; MFB, 81^{o}38'44.083"W, 36^{o}9'32.08"N) for the collection of experimental crayfish, hence MFB is not included in survey data analysis. The substrate at each survey site consisted of boulders and cobbles with areas of sand and gravel. Sections of the stream bank at all sites consisted of emergent vegetation. The MFJC, MFB and MFMS sites exhibited relatively higher canopy cover than MFT.

Cambarus bartonii (Fabricius) are common crayfish throughout headwater streams of eastern North America (Hobbs 1972). These crayfish are dominant in first- and second-order tributaries and frequently co-occur with the New River crayfish, *Cambarus chasmodactylus*, in the Middle Fork of the New River (Fortino and Creed 2007). *Cambarus bartonii* are typically found underneath cobbles or in the vegetation along stream margins (RPC, pers. obs.). These crayfish function as ecosystem engineers (Creed and Reed 2004) and may host several species of branchiobdellidans (Hobbs et al. 1967, MJT, pers. obs.).

Branchiobdellidan worms commonly associate with crayfish throughout the Northern Hemisphere (Gelder 1999). Branchiobdellidans are considered obligate ectosymbionts in that they require a live crustacean host for cocoon deposition (Young 1966, Brinkhurst and Gelder 2001, Creed et al., in press). Various branchiobdellidan species have been described as commensals (McManus 1960, Young 1966, Bishop 1968, Jennings and Gelder 1979, Keller 1992), parasites (Grabda and Wierzbicka 1969, Quaglio et al. 2006, Rosewarne et al. 2012) and mutualists (Brown et al. 2002, 2012, Lee et al. 2009, Creed et al., in press) of crayfish. In the Middle Fork watershed, *C. bartonii* may host up to six species of branchiobdellidan including *Bdellodrilus illuminatus* (Moore), *Cambarincola fallax* (Hoffman), *Cambarincola ingens* (Hoffman), *Cambarincola philadelphicus* (Leidy), *Pterodrilus alcicornus* (Moore) and *Xironogiton instabilius* (Moore) (MJT, pers. obs.). *Cambarincola ingens* is engaged in a cleaning symbiosis mutualism with the co-occurring crayfish *C. chasmodactylus* (Brown et al. 2002, 2012, Creed et al., in press).

Crayfish and branchiobdellidan surveys

I conducted two separate field surveys in June and September 2013 to determine the relative abundance of branchiobdellidan species on different size classes of *C. bartonii*. Surveys were conducted along a longitudinal gradient to assess whether there were spatial patterns in worm presence and abundance. I collected the majority of *C. bartonii* from the vegetation along stream margins. Crayfish were captured by disturbing instream vegetation as well as lifting cobbles and boulders and allowing individuals to be swept into a net downstream. I collected crayfish from three size classes based on total carapace length (TCL): small (10-19 mm TCL), medium (20-29 mm TCL) and large (30-39 mm TCL).

Crayfish < 10 mm TCL did not appear to host worms and were not included in the survey. Crayfish were preserved individually in jars or vials of 70% ethanol.

Following capture, I identified and counted worms present on each crayfish in the laboratory. Crayfish carapaces were also removed to collect any worms that were present in the gill chamber. For each worm species at each survey site, worm data were analyzed across crayfish size classes with a one-way analysis of variance (ANOVA). A Kruskal-Wallis non-parametric ANOVA on ranks was used due to numerous zero values for worm species present in certain size classes of crayfish. To examine significant differences among size classes, all pairwise comparisons for the small, medium and large size class were analyzed using Dunn's method. Because worm abundance was similar at all three sites, data were pooled prior to analysis.

Crayfish size by branchiobdellidan species experiment

This experiment was designed to determine the effects of *C. ingens* and *C. philadelphicus*, separately, on the growth of two sizes of *C. bartonii*. I conducted this experiment in laboratory aquaria from July through October 2013. I collected crayfish from the MFB site on July 1, 2013 and sorted individuals based on size. Mean (± 1 SE) TCL of experimental crayfish in the small and large size classes were 21.1 ± 0.1 mm and 31.7 ± 0.3 mm, respectively. In the laboratory, I identified *C. ingens* and *C. philadelphicus* on crayfish, then mature and juvenile worms of each species were coaxed from the exoskeleton using a fine-tipped probe. Worms were designated as mature if they were markedly larger (≥3 mm and ≥6 mm in length for *C. philadelphicus* and *C. ingens*, respectively) and in close proximity to cocoons on the crayfish exoskeleton or if developing cocoons were visible within their bodies. Juvenile and mature worms of each species were placed separately into

dishes of river water for later restocking. The remaining worms and cocoons were killed by placing the crayfish in a 10% magnesium chloride ($MgCl_2$) hexahydrate bath for 5 minutes (Brown et al. 2002). Following the $MgCl_2$ bath, dead worms and cocoons were manually removed from the exoskeleton and crayfish were allowed to rest in a container of stream water for several minutes.

Each experimental crayfish within the small (S) or large (L) size class was randomly assigned to *C. ingens* (CI), *C. philadelphicus* (CP) or no worm (0W) treatments. Each treatment included both male and female crayfish. I stocked crayfish within each worm treatment by placing five worms on the dorsal carapace. Worm treatments consisted of three reproductively mature worms and two juvenile worms. Stocking density was determined using survey data at MFB where mean worm density of *C. bartonii* (inclusive of all size classes) was 5.3 ± 0.7 worms per crayfish. I then crossed crayfish size with worm treatment to produce the following six treatments (N = 6 replicates per treatment): small crayfish with *C. ingens* (S-CI); small crayfish with *C. philadelphicus* (S-CP); small crayfish with no worms (S-0W); large crayfish with *C. ingens* (L-CI); large crayfish with *C. philadelphicus* (L-CP); and large crayfish with no worms (L-0W).

Crayfish were assigned to individual aquaria (38 L, 51 x 25 x 31 cm) and blocked by location and treatment using a randomized complete block design. Experimental aquaria contained aquarium aggregate (~1 L) mixed with stream sediments (~1 L of sand and organic matter) as well as five cobbles collected from the Middle Fork. Aquaria were filled with ~28.5 L of stream water and were constantly aerated. Water temperature was maintained at ~16˚C and laboratory lighting was adjusted throughout the experiment to mimic light and dark cycles of the season. Crayfish were fed two shrimp pellets three times weekly. Aquaria

water (~9.5 L) was drained and replaced every other week to reduce the accumulation of ammonia waste.

I recorded crayfish blotted wet mass (BWM) at the start of the experiment and every two weeks thereafter for 12 weeks. To determine BWM, individual crayfish were blotted with paper towels for 1 minute and then weighed. I then calculated crayfish percent growth for each replicate after weighing. At the conclusion of the experiment, arcsine-transformed percent growth data were analyzed using repeated measures analysis of variance (rmANOVA) in R (version 3.0.1; R Project for Statistical Computing, Vienna, Austria). Due to unsynchronized molting, replication was reduced on certain dates for each treatment throughout the 12 week period, therefore, rmANOVA growth data were analyzed on days when all replicates were present in the small (days 0, 42 and 84) and large (days 0, 14, 42, 70 and 84) crayfish treatments. For consistency, graphical representation of crayfish growth in the small size class treatments utilize the same sampling days as in the large size class treatments. All crayfish in the large size class molted once during the experiment except for one crayfish in the L-CP treatment that did not molt; this replicate was determined to be an outlier using a Dixon's test and was removed from the analysis. All crayfish within the small size class molted twice during the experiment except for one crayfish in the S-0W treatment that only molted once; this replicate was retained in the analysis as it was not an outlier. Further, final growth data (day 84; arcsine-transformed) for the small and large size class treatments were analyzed by an ANOVA with orthogonal contrasts. This approach allowed me to determine significant differences between worm and no worm treatments (CP and CI vs. 0W) and between worm treatments (CP vs. CI) on the final date.

I also counted mature and juvenile worms and cocoon production on each crayfish weekly for 12 weeks. Worm and cocoon location were noted to determine distribution patterns. The number of worms and cocoons on crayfish were analyzed using rmANOVA for the entire 12 week period using R. In the analysis, a generalized linear mixed effects model was fit to all response variables except for large worms which were fit with a linear mixed effects model. The generalized model allowed me to specify the underlying distribution of the data in order to produce a better model fit. A negative binomial or Poisson distribution was used for the generalized models (all models except for large worms) based on residual plots that varied per response variable. The R function for generalized models is the *glmer()* and the *glmer.nb()*, the latter for negative binomial distributions, found in the *lme4* package. The R function for linear mixed models is the *lme()* found in the *nlme* package. The same model was used for all variables (with the exception of underlying distribution): *Response = Size x Species + Day + (Day|EU)*. This model allowed me to examine direct effects plus interactions for *Size* and *Species*, the direct effect of *Day* and crayfish (*EU*) as a random variable nested in *Day*. In addition to the rmANOVA, a two-way ANOVA was used to analyze all response variables on days 28, 56 and 84.

On day 84, the experiment was terminated and experimental crayfish were preserved in 70% ethanol after weighing. I removed crayfish carapaces to obtain final worm counts and to examine possible gill damage by worms. Gill scarring was quantified by counting the melanized spots and tips on gill filaments of each podobranch. Gill scarring data were analyzed using a one-way ANOVA.

During the experiment, crayfish mortality was addressed by replacing dead individuals with a crayfish of similar TCL. All replacements were stocked with worms,

placed in clean aquaria and examined weekly for worms and cocoons as well as weighed biweekly during the 12 week period. Mortalities occurred in the S-0W (N = 2), the S-CP (N = 2) and the L-0W (N = 1) treatments.

Branchiobdellidan interaction experiment

This experiment sought to determine the mechanism underlying the ontogenetic succession in dominant worm species on large *C. bartonii*. The experiment was designed to determine the relative importance of crayfish regulation and predation by *C. ingens* as the mechanism for the replacement of *C. philadelphicus* on larger *C. bartonii*. The worm interaction experiment was conducted in laboratory aquaria from July through August 2013. I collected large *C. bartonii* (ranging from 32 to 36 mm TCL) from the MFB and MFT sites on July 17 and August 12, 2013 to conduct two separate trials of the experiment. Worms were identified as *C. ingens* or *C. philadelphicus* on crayfish, then mature and juvenile worms of each species were coaxed from crayfish and placed separately into dishes of river water for restocking. Worms and cocoons were manually removed and additional cocoons and worms were killed using the $MgCl_2$ bath as described above.

Experimental crayfish were paired based on similar TCL and each crayfish within the pair was randomly assigned to the interaction (INT) or control (C-CP) treatment (N = 8 pairs). Crayfish in the C-CP treatment were stocked with 10 *C. philadelphicus* (four mature and six juvenile worms) on the dorsal carapace. Using a substitutive design to maintain similar worm densities, crayfish within the INT treatment were stocked by introducing two mature and three juvenile *C. philadelphicus* then two mature and three juvenile *C. ingens* (N = 10 total worms) on the dorsal carapace. By introducing *C. ingens* after *C. philadelphicus*, I attempted to simulate the late colonization by *C. ingens* on experimental *C. bartonii*.

Crayfish were randomly assigned to individual aquaria and blocked by size. Aquaria contained ~19 L of stream water and three small cobbles. I did not include additional substrate so that aquaria could be examined for any worms that may have emigrated from experimental crayfish. I followed the same aeration, temperature and lighting protocols as described in the previous experiment. Crayfish were fed two shrimp pellets every other day.

I examined crayfish for the presence and absence of worm species after nine days (a pilot experiment had previously demonstrated that nine days was sufficient for experimentation). Worm numbers and cocoons of each species were quantified and recorded for location on crayfish. Additionally, the cobbles and the aquaria were carefully examined to determine if worms had emigrated from the crayfish. I then determined the percent remaining worms for each species on experimental crayfish. Percent data were arcsine-transformed and analyzed using a two-way ANOVA to test for treatment and block (=pair) effects. Analyses compared percent remaining *C. philadelphicus* and *C. ingens* in the INT treatment, separately (due to lack of independence), to percent remaining worms in the control treatment.

RESULTS

Crayfish and branchiobdellidan surveys

A total of 121 crayfish and 669 worms were collected from the Middle Fork in the early and late surveys. In both surveys at all three sites, *C. philadelphicus* and *C. ingens* were the dominant worm species found on the external surfaces of all *C. bartonii* size classes and accounted for 94.1% of external worms in early summer and 92.5% in late summer. In the early summer survey, the number of *C. philadelphicus* ($H = 10.309$, $P = 0.006$) and *C. ingens* ($H = 31.692$, $P < 0.001$) differed significantly across crayfish size classes (Fig. 2a). There were significantly more *C. philadelphicus* on medium crayfish than on small crayfish ($Q = 2.994$, $P < 0.05$). There were significantly more *C. ingens* on the medium ($Q = 3.869$, $P < 0.05$) and large ($Q = 4.399$, $P < 0.05$) crayfish than on small crayfish. In the late summer survey, *C. philadelphicus* ($H = 22.611$, $P < 0.001$) and *C. ingens* ($H = 28.080$, $P < 0.001$) numbers again differed significantly across crayfish size classes (Fig. 1b). There were significantly more *C. philadelphicus* on small ($Q = 3.855$, $P < 0.05$) and medium ($Q = 4.524$, $P < 0.05$) crayfish than on large crayfish. Alternatively, there were significantly less *C. ingens* on small ($Q = 4.820$, $P < 0.05$) and medium ($Q = 4.060$, $P < 0.05$) crayfish than on large crayfish. Both surveys exhibited a similar pattern of *C. philadelphicus* dominating the small and medium size classes of *C. bartonii* while *C. ingens* dominated the large size class.

Crayfish size by branchiobdellidan species experiment

There was no significant effect of worm species on small crayfish growth throughout the experiment ($F_{2,15} = 0.032$, $P = 0.968$) (Fig. 2a). Further, percent change in BWM of small crayfish did not differ significantly on day 84 ($F_{2,15} = 0.081$, $P = 0.922$). However, there was a significant overall effect of worms ($F_{2,11} = 4.234$, $P = 0.043$) and a significant time effect ($F_{1,39} = 206.993$, $P < 0.001$) on large crayfish growth (Fig. 2b). Crayfish in both the L-CP and L-CI treatment exhibited higher percent change in BWM than crayfish in the L-0W treatment. Crayfish growth in the L-CP and L-CI treatments diverged from L-0W by day 42 and remained higher for the remainder to the experiment. Interestingly, there was little variability in growth in both the L-CP and L-CI treatments on days 70 and 84 while the L-0W treatment exhibited greater variability on these dates. There was a significant overall worm effect on large crayfish growth for day 84 ($F_{2,15} = 4.62$, $P = 0.027$) with significantly greater crayfish growth in worm treatments ($F_{1,15} = 9.24$, $P = 0.008$). Crayfish growth did not differ significantly between the L-CP and L-CI treatments on day 84 ($F_{1,15} = 0.002$, $P = 0.965$).

Crayfish size affected the number of mature worms present on crayfish throughout the experiment ($t = -5.893$, $P < 0.001$) (Fig. 3a). There was also a significant crayfish size x worm species effect ($t = 3.315$, $P = 0.004$) for mature worm numbers over time. Mature worms were rapidly removed by crayfish in the S-CI treatment from the onset of the experiment and no mature worms remained on these crayfish by day 28. On day 28, there were significantly more mature worms in the L-CI ($q = 6.746$, $P < 0.001$) and S-CP treatments ($q = 3.572$, $P = 0.020$) than the S-CI treatment. The number of mature worms did not differ significantly between the L-CI and L-CP treatments as well as the L-CP and S-CP

treatments despite the decline of mature worms in both the L-CP and S-CP treatments. This trend continued through day 56, yet at this point the surviving juvenile worms in the S-CI treatment had matured and did not differ significantly from the number of mature worms in the S-CP treatment. On day 84, there were significantly more mature worms in the L-CI treatment than the L-CP ($q = 3.459$, $P = 0.024$) and S-CI ($q = 6.053$, $P < 0.001$) treatments. Mature worm numbers were higher in the L-CP treatment than the S-CP treatment as well as the S-CP treatment than the S-CI treatment, though these values did not differ significantly.

Crayfish size ($z = -6.890$, $P < 0.001$) and a crayfish size x worm interaction ($z = 4.841$, $P < 0.001$) also affected total cocoon production by mature worms throughout the experiment (Fig. 4a). Cocoon production was higher in the L-CI and L-CP treatments earlier in the experiment while it remained lower in the S-CP treatment and was absent in the S-CI treatment. The rapid removal of mature worms appeared to affect cocoon production in the S-CI treatment. Interestingly, the surviving S-CI mature worms did not deposit cocoons during days 0-21. Starting on day 56, the L-CP treatment exhibited a decline in total cocoon production and was significantly lower than the L-CI treatment ($q = 3.861$, $P = 0.013$). On day 56, total cocoon numbers did not differ significantly between the L-CP and S-CP treatments and the S-CP and S-CI treatments. At this point in time the two surviving juvenile worms on one S-CI replicate had matured and began to deposit cocoons. Further, single mature worms present on two S-CI crayfish never deposited cocoons. On day 84, total cocoon numbers converged in the L-CP, S-CP and S-CI treatments. There were significantly more cocoons produced in the L-CI treatment than the L-CP ($q = 4.889$, $P = 0.003$) and S-CI treatment ($q = 5.496$, $P = 0.001$) and cocoon number did not differ significantly between S-CI and S-CP.

Similar to total cocoon production, there was a significant crayfish size (t = -5.139, P < 0.001) and crayfish size x worm species interaction (t = 3.035, P = 0.007) on per capita cocoon production by mature worms over time (Fig. 4b). Per capita cocoon production did not differ significantly between the L-CI, L-CP and S-CP treatments until day 56. On day 56, L-CP and S-CP exhibited a decline in per capita cocoon production. Per capita cocoon production was significantly higher in the L-CI treatment than the L-CP treatment by day 56 (q = 3.136, P = 0.038) and remained significantly higher until the conclusion of the experiment (q = 4.054, P = 0.010). On days 56 and 84, per capita cocoon production did not differ significantly between the L-CP and S-CP treatments as well as the S-CP and S-CI treatments. There was no cocoon production in the S-CI treatment until day 49.

Throughout the experiment, juvenile worm numbers were affected by crayfish size (t = -2.703, P = 0.007) as well as time (t = 3.720, P < 0.001) as juveniles hatched from cocoons (Fig. 3b). Cocoon hatching largely occurred after day 28 and juvenile numbers steadily increased in the L-CI and L-CP treatments. By day 56, the number of juveniles did not differ significantly between the L-CI and L-CP treatments as well as the L-CP and S-CP treatments. Interestingly, juvenile numbers did not increase in the S-CP treatment until day 56 despite the considerable amount of cocoon deposition by mature worms prior to this point in time. Juvenile numbers remained low in the S-CI treatment due to delayed cocoon deposition. By day 84, juvenile worm numbers had diverged in the L-CI and L-CP treatment with significantly more juveniles in the L-CI treatment (q = 5.183, P = 0.002). The number of juveniles did not differ significantly between the L-CP and S-CP treatment at the conclusion of the experiment. Although cocoons had been deposited towards the end of the experiment in the S-CI treatment, there were no juveniles born.

Total worm numbers were affected by crayfish size (t = -4.446, P < 0.001) and there was a crayfish size x worm species interaction (t = 2.166, P = 0.030) over the course of the experiment (Fig. 3c). Total worm numbers were largely influenced by juvenile numbers, hence the similar patterns in these two variables. Due to stable mature worm numbers and increased juvenile numbers in the L-CI treatment, L-CI exhibited significantly higher total worm numbers than the L-CP treatment by day 84 (q = 5.745, P < 0.001). The L-CP treatment experienced a decline in total worm numbers following day 70 and did not differ significantly from the S-CP treatment at the conclusion of the experiment. Total worm numbers remained low in the S-CI treatment and only included stocked juveniles that had matured during the experiment.

Crayfish gill scarring differed significantly among worm treatments on small crayfish ($F_{2,15}$ = 14.407, P < 0.001). Mean (± 1 SE) number of gill scars was 1.83 ± 0.87, 14.67 ± 2.32 and 5.80 ± 2.01 for the S-0W, S-CP and S-CI treatments, respectively. There was significantly higher scarring in the S-CP treatment than the S-0W (q = 7.315, P < 0.001) and S-CI treatments (q = 5.415, P = 0.005). There was a marginally significant different overall effect for gill scarring on large crayfish ($F_{2,15}$ = 3.332, P = 0.063). Mean number of gill scars was 10.67 ± 2.62, 27.33 ± 5.22 and 23.20 ± 6.51 for the L-0W, L-CP and L-CI treatments, respectively. None of these treatments were significantly different from one another.

Branchiobdellidan interaction experiment

The percent *C. philadelphicus* remaining in the INT treatment was significantly lower than that in the C-CP treatment ($F_{1,7}$ = 75.026, P < 0.001) and there was no effect of pairing (Fig. 5). The percent *C. philadelphicus* remaining in the C-CP treatment did not differ significantly from the percent remaining *C. ingens* in the INT treatment ($F_{1,7}$ = 1.934, P =

0.207), again with no effect of pairing (Fig. 5). No worms were recovered from aquaria or the cobbles.

DISCUSSION

I have demonstrated that the ontogenetic succession of symbionts that has been well-documented in associations involving sessile hosts (Fonseca and Benson 2003, Palmer et al. 2010) also occurs in symbioses involving a mobile host. Interestingly, while this succession has fitness consequences for the symbionts, the larger host exhibits relatively greater fitness regardless of symbiont partner. In the Middle Fork of the New River, *C. philadelphicus* was the dominant worm on small crayfish and was replaced by *C. ingens* as crayfish increased in size, regardless of sample period. In the lab experiment, *C. philadelphicus* persisted on both small and large *C. bartonii* in the absence of *C. ingens*. *Cambarincola philadelphicus* only had a positive effect on large crayfish, thus this symbiosis shifted from commensalism to mutualism as *C. bartonii* increased in size. Alternatively, *C. ingens* were quickly removed from small *C. bartonii*, suggesting a possible antagonistic relationship between this species and small crayfish. *Cambarincola ingens* also had a positive effect on large *C. bartonii* and exhibited a similar shift in their impact on host fitness throughout crayfish ontogeny. Additionally, I have demonstrated that intraguild predation by the later-arriving *C. ingens* will cause a shift in the dominant worm taxon on the host. Hence, the ontogenetic transition in the outcome of this symbiont-host interaction involves not only a change in branchiobdellidan impact as a function of host size and growth rate but also a shift in the identity of the symbiotic associate.

In the crayfish size by branchiobdellidan species experiment, worm abundance patterns on small and large crayfish mirrored the patterns I observed in the field. On large crayfish, mature *C. ingens* remained stable throughout the experiment while mature *C. philadelphicus* exhibited a gradual decline. On small crayfish, the number of mature *C. philadelphicus* exhibited a comparable decline while mature *C. ingens* experienced a dramatic reduction by the first sampling date. Previous studies have demonstrated that crayfish can actively remove worms (Farrell et al. 2014) and the extent of removal is a function of crayfish size (i.e., small crayfish are less tolerant of worms) (Skelton et al. 2014). Moreover, worms are removed by crayfish if worms become food limited and commence to feeding on gill tissue (Brown et al. 2012, Thomas et al. 2013). The removal of *C. ingens* suggests that these worms may have been parasitic on small *C. bartonii*. When comparing worm species, *C. ingens* are larger and may require more resources (e.g., food and space), therefore gill parasitism by *C. ingens* likely occurred on smaller crayfish. In addition, the larger body size of *C. ingens* may have increased their detection by the crayfish and made them more vulnerable to removal by small crayfish. In the experiment, the number of mature *C. ingens* eventually increased as stocked, juvenile worms began to mature. It is important to note that the small crayfish were larger later in the experiment, therefore the availability of resources likely increased and enabled these worms to mature.

Both cocoon production by mature worms and juvenile worm abundance were influenced by crayfish size. Interestingly, cocoons were only deposited when two mature individuals were present on these crayfish, suggesting that single worms are incapable of reproduction due to mate limitation despite being hermaphroditic. Per capita cocoon production by mature worms exhibited a similar pattern to total cocoon production though

production rates by *C. philadelphicus* on small crayfish were comparable to both *C. philadelphicus* and *C. ingens* on large crayfish. Juvenile worm numbers were similar on large crayfish, however, these numbers diverged later in the experiment as *C. ingens* juveniles increased and *C. philadelphicus* decreased. On small crayfish, *C. philadelphicus* juveniles did not increase until later in the experiment. Despite delayed cocoon deposition by *C. ingens* on small crayfish and sufficient time for hatching, no juvenile worms were observed in this treatment. The delayed recruitment or absence of juvenile worms may reflect limited resources on small crayfish such that cocoons were not as well provisioned as those on large crayfish or that juvenile populations could not become established. Moreover, limited resources on small crayfish may have resulted in mature worms cannibalizing juveniles. Total worm numbers were also influenced by crayfish size and were largely driven by juveniles born during the experiment. Interestingly, the *C. ingens* present on small crayfish on the final date included only the surviving juveniles stocked at the beginning of the experiment. These juvenile worms may have avoided removal by crayfish as resources were proportionately more abundant given their smaller size, thus they did not resort to consuming gill tissue as was likely with mature worms. Only when their crayfish hosts increased in size did the juvenile *C. ingens* mature, and only when more than one mature worm was present did these worms begin to reproduce.

The reoccurring pattern of greater *C. ingens* fitness on large crayfish and greater *C. philadelphicus* fitness on small crayfish reflects how each worm species differed in their ability to exploit their host at a particular host size. Additionally, the eventual decline in *C. philadelphicus* reproduction on large crayfish may be due to seasonal, natural senescence. *Cambarincola philadelphicus* appear to be the early successional species and their

populations may be more stable early in the season prior to crayfish molting and the onset of the summer growth period. As the season progresses, crayfish increase in size and become a more suitable habitat for *C. ingens*, the late successional species. The temporal separation of peak fitness per worm population may reflect how these species have evolved to reduce niche overlap throughout crayfish ontogeny.

Neither worm species had a discernible effect on small crayfish growth. Similar results have been documented in an earlier study using smaller crayfish (Keller 1992). Small crayfish molted as many as two times during our experiment. I concluded that frequent molting by small crayfish may overwhelm any detectable effects of worms on crayfish growth. As crayfish increase in size, molting may occur only twice a year, thereby gill cleaning by worms would be more beneficial. Gill scarring by *C. philadelphicus* was highest on small crayfish, yet the number of gill scars fell below those observed under mutualistic associations between *C. ingens* and *C. chasmodactylus* (Brown et al. 2012). Gill scarring by *C. ingens* was lower in the experiment due to rapid removal by the crayfish.

Both worm species had positive effects on large *C. bartonii* growth. Hence, I have identified two additional cleaning symbioses mutualisms between crayfish and branchiobdellidans: the *C. bartonii*-*C. philadelphicus* and the *C. bartonii*-*C. ingens* association. It is important to note that these mutualisms only occur when *C. bartonii* are large. Moreover, variability in growth was greater in the control treatment than the *C. philadelphicus* and *C. ingens* treatments towards the end of the experiment. Less variability in the worm treatments suggests that branchiobdellidans may ameliorate any inherent individual variation in growth exhibited by large *C. bartonii*. Gill scarring for worm

treatments on large crayfish tended to be higher relative to controls but also remained below levels that have previously been determined to be mutualistic.

While the association between *C. philadelphicus* and large *C. bartonii* was a mutualism in my lab experiment, this worm species is uncommon on large crayfish in the Middle Fork. In both experiments, *C. philadelphicus* were able to persist on large crayfish in the absence of *C. ingens*. In the worm interaction experiment, when *C. ingens* were introduced, the relative abundance of *C. philadelphicus* declined. Further, I did not recover any *C. philadelphicus* from aquaria walls or cobbles, i.e., the decline of this species was due to consumption by *C. ingens* and not emigration. I speculate that intraguild predation by *C. ingens* is the mechanism for *C. philadelphicus* replacement on large *C. bartonii*. Altogether, partner regulation by small crayfish and intraguild predation by *C. ingens* on large crayfish drive the observed branchiobdellidan succession throughout *C. bartonii* ontogeny.

Previous studies of other mutualisms have demonstrated that symbionts associate with hosts during particular life stages (Madden and Young 1992, Trager and Bruna 2006, Abrego et al. 2009, Palmer et al. 2010). In ant-plant protective mutualisms, changes in plant size affect the nature of the interaction such that resources provided by the plant host (e.g., habitat and food rewards) may be greater at certain life stages and offer different benefits to ant species within the community (Quintero et al. 2013). In my system, I found that worm fitness varied as a function of crayfish size and ontogenetic succession of these symbionts is driven by crayfish regulation and worm intraguild predation. Similarly, ontogenetic succession of ant species occurs throughout plant ontogeny and is a function of ant life history and behavior, resource availability, competition among ants and each species' impact on host fitness (Feldhaar et al. 2003, Fonseca and Benson 2003, Djiéto-Lordon et al. 2004,

Palmer et al. 2010). Additionally, corals can exhibit specificity for certain algal endosymbionts during particular ontogenetic stages and this can lead to an ontogenetic succession in the algal community (Abrego et al. 2009).

In the ant-plant protective mutualisms, ants may vary in the benefits they provide at particular stages of plant ontogeny (Trager and Bruna 2006, Palmer et al. 2010, Quintero et al. 2013). The extent of protection provided by ants also appears to be a function of ant species and herbivore behavior (Madden and Young 1992, Itino and Itioka 2001, Trager and Bruna 2006, Llandres et al. 2010). Similarly in coral associations, housekeeping crabs that prevent sediment accumulation have been demonstrated to offer varying benefits to different size classes of corals (e.g., smaller corals are more prone to sedimentation) (Stewart et al. 2013). The effect of worms in the crayfish-branchiobdellidan association differs from these other mutualisms in that the interactions described here are only beneficial to both partners when crayfish are large.

Symbioses are ubiquitous in ecological communities and can influence community structure and ecosystem function (Boucher et al. 1982). Concurrently, the nature of mutualisms are affected by communities and ecosystems, namely through changes in environmental context, partner density and the species involved (Bronstein 1994), as has been demonstrated in the crayfish-branchiobdellidan associations (Lee et al. 2009, Brown et al. 2012, Thomas et al. 2013, Farrell et al. 2014). My results show that the ontogenetic stage or size of host organisms directly influences the nature of mutualisms. Additionally, I have demonstrated that ontogenetic shifts in partner fitness may lead to symbiont succession, a relatively new area of research (Feldhaar et al. 2003, Fonseca and Benson 2003, Djiéto-Lordon et al. 2004, Palmer et al. 2010). Interestingly, the crayfish-branchiobdellidan

association involves a mobile host with a relatively sedentary symbiont. As a result, the crayfish plays a much more active role not only in regulating worm populations, but also by functioning as a mobile habitat and vessel of worm dispersal. Given that crayfish are critical to the structure and functioning of freshwater communities (Creed 1994, Creed and Reed 2004, Helms and Creed 2005), it is important to assess how size-specific associations may affect each partner's fitness throughout their respective ontogenies, especially if these interactions are common and occur extensively throughout headwater streams.

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FIGURE LEGENDS

Figure 1. Mean (±1 SE) number of total *C. ingens* (CI) and *C. philadelphicus* (CP) on crayfish collected in the Middle Fork of the New River during early (A) and late (B) summer surveys. Crayfish size classes (based on TCL) were small (10-19 mm), medium (20-29 mm) and large (30-39 mm). Both male (M) and female (F) crayfish (code, replicates per treatment) were collected in the early (M, N = 13; F, N = 31) and late (M, N = 28; F, N = 49) summer surveys.

Figure 2. Mean (±1 SE) percent change in blotted wet mass of small (A) and large (B) *C. bartonii* over 84 days. Treatments (code, replicates per treatment) were small crayfish with no worms (S-0W, N = 6); small crayfish with *C. philadelphicus* (S-CP, N = 6); small crayfish with *C. ingens* (S-CI, N = 6); large crayfish with no worms (L-0W, N = 5); large crayfish with *C. philadelphicus* (L-CP, N = 5); and large crayfish with *C. ingens* (L-CI, N = 4).

Figure 3. Mean (±1 SE) number of mature (A), juvenile (B) and total (C) branchiobdellidans on crayfish (N = 6 replicates per treatment) over 84 days. See Figure 2 for treatment abbreviations.

Figure 4. Mean (±1 SE) total number of cocoons (A) and per capita cocoon production (B) by mature branchiobdellidans on crayfish (N = 6 replicates per treatment) over 84 days. See Figure 2 for treatment abbreviations.

Figure 5. Mean (±1 SE) percentage of worms remaining in the control (C-CP; stocked with 10 *C. philadelphicus* only) and interaction (INT; stocked with 5 *C. philadelphicus* and 5 *C. ingens*) treatments (N = 8 replicates per treatment). The INT-CP and INT-CI represent percent *C. philadelphicus* and percent *C. ingens* remaining, respectively, in the INT treatment.

Figure 1

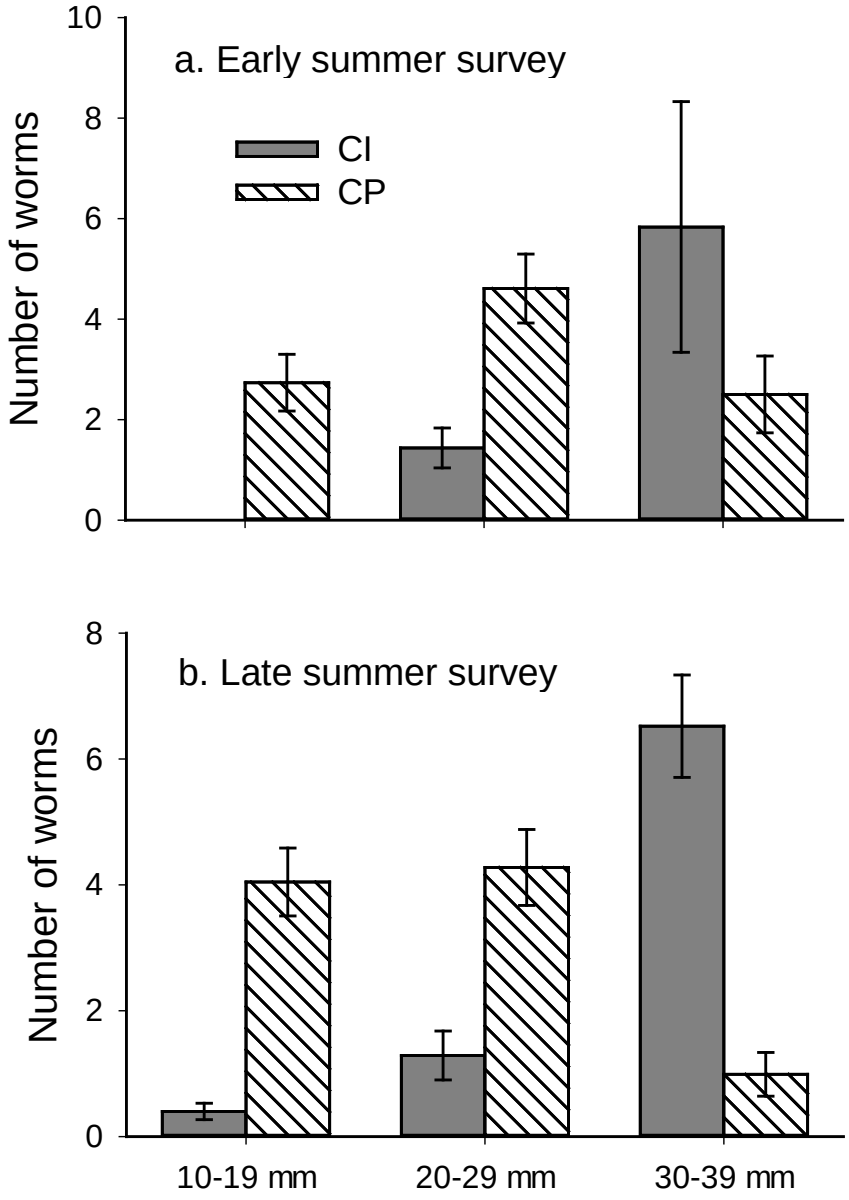

a. Early summer survey
CI
CP
Number of worms
10
8
6
4
2
0
b. Late summer survey
Number of worms
8
6
4
2
0
10-19 mm
20-29 mm
30-39 mm

Figure 2

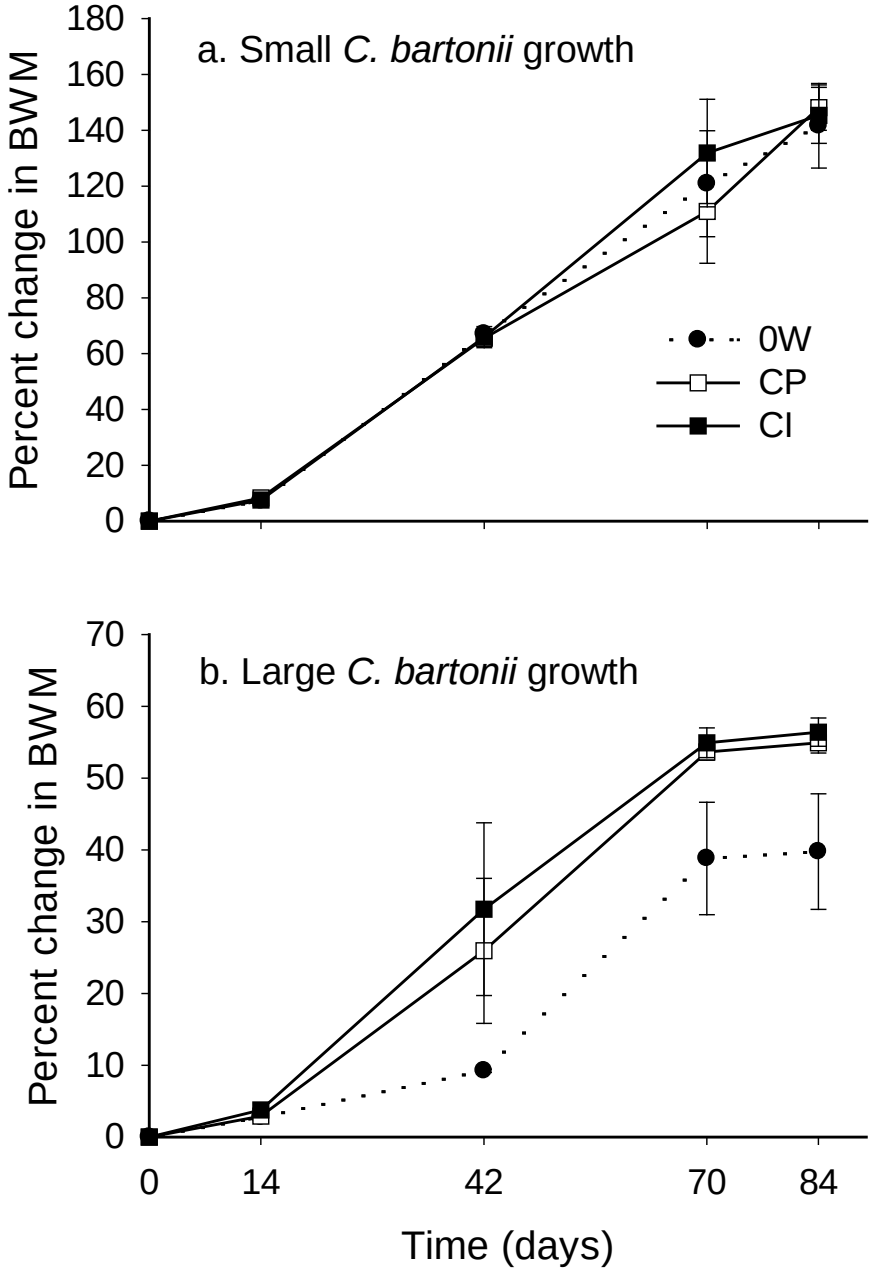

a. Small C. bartonii growth
Percent change in BWM
180
160
140
120
100
80
60
40
20
0
0W
CP
CI
b. Large C. bartonii growth
Percent change in BWM
70
60
50
40
30
20
10
0
0
14
42
70
84
Time (days)

Figure 3

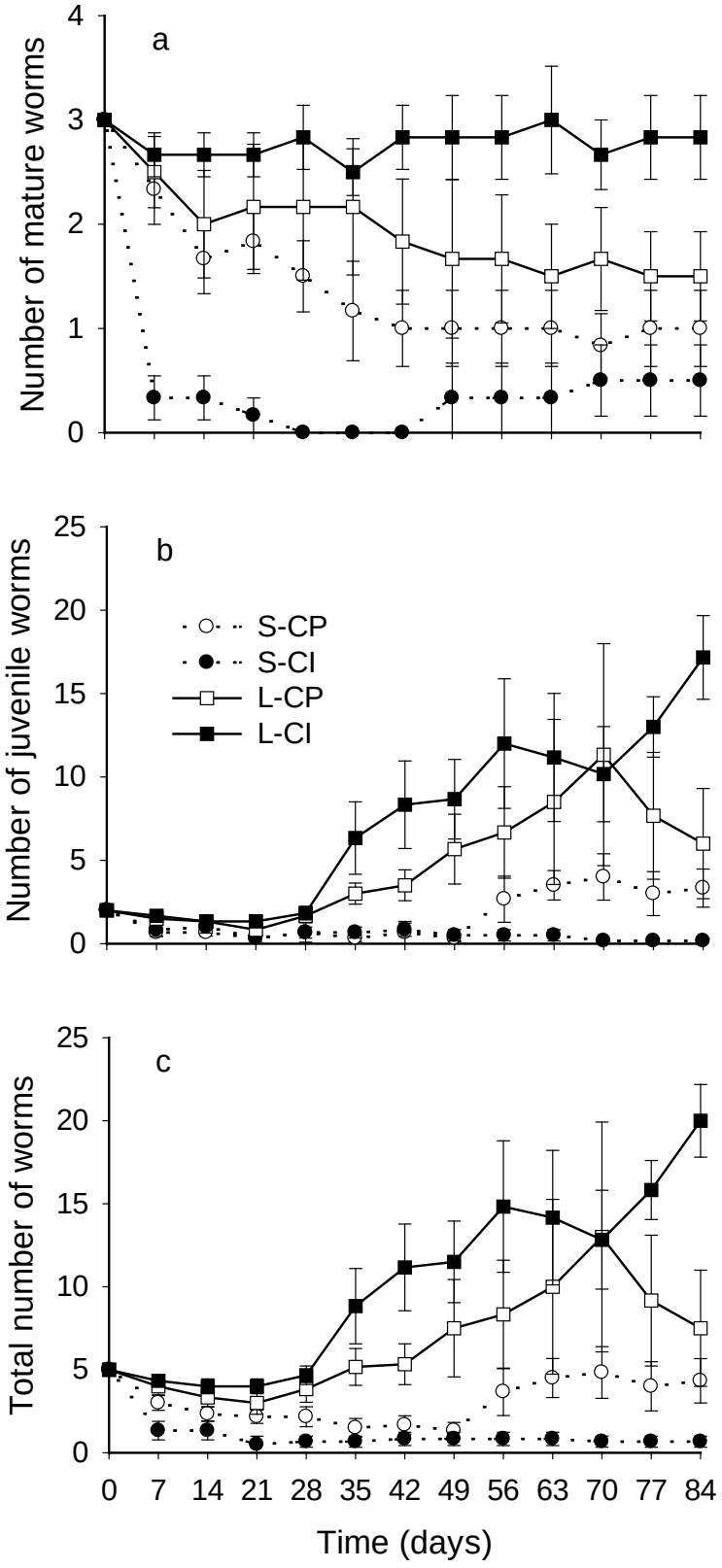

a
Number of mature worms
0
1
2
3
4
b
Number of juvenile worms
S-CP
S-CI
L-CP
L-CI
0
5
10
15
20
25
c
Total number of worms
0
5
10
15
20
25
0 7 14 21 28 35 42 49 56 63 70 77 84
Time (days)

Figure 4

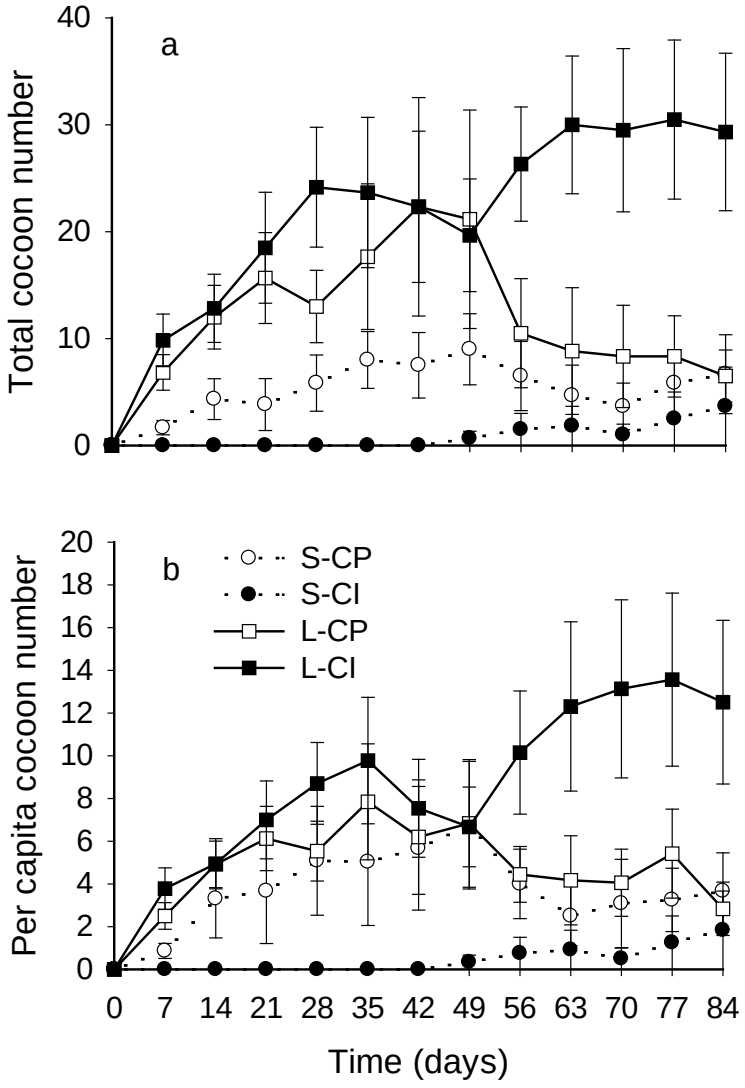
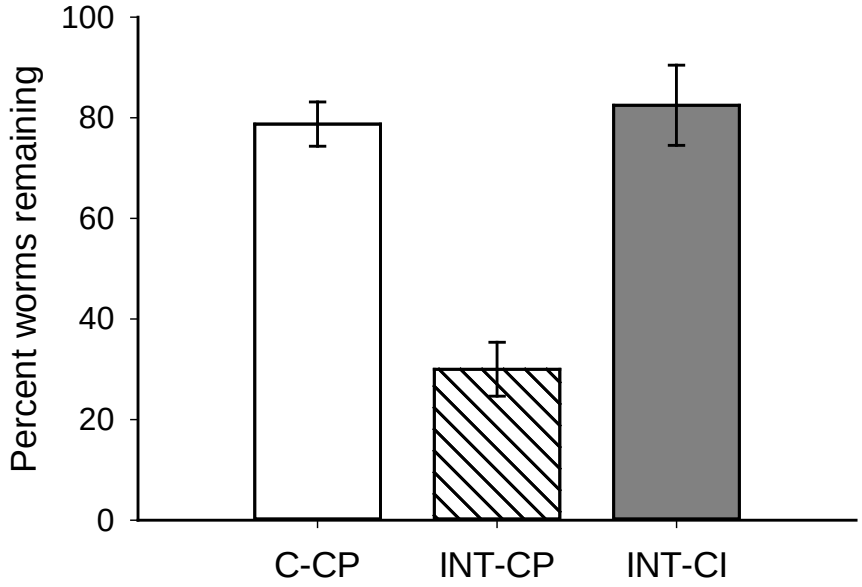

a
Total cocoon number
40
30
20
10
0
b
Per capita cocoon number
20
18
16
14
12
10
8
6
4
2
0
S-CP
S-CI
L-CP
L-CI
0 7 14 21 28 35 42 49 56 63 70 77 84
Time (days)

Figure 5



Vita

Michael J. Thomas was born in Portland, Maine, and grew up with his family in Fayetteville, North Carolina, where he attended grade school at the Fayetteville Academy. Upon graduating high school in 2007, he spent his first year of college at the University of Florida studying a wide range of fields including biology, chemistry and anthropology. In the fall of 2008, Michael transferred to Appalachian State University in Boone, North Carolina, and found his passion for freshwater habitats and ecology. He earned his Bachelor of Science degree in Biology in 2012 with a concentration in Ecology and Environmental Biology and a minor in Sustainable Development. Having completed an honors program with Dr. Robert Creed during his undergraduate career, Michael continued his research in graduate school at Appalachian State University. He earned a Master of Science degree in Ecology and Environmental Biology and a Graduate Certificate in Geographic Information Science in August 2014. Michael's interests are deeply rooted in aquatics and he hopes to pursue a career that contributes to a greater understanding and preservation of our freshwater systems and resources.