

The dilution effect in a freshwater mutualism: Impacts of introduced host species on native symbionts

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Abstract

The dilution effect was originally proposed to describe the negative effect of increased host diversity on parasite or pathogen abundance; with greater host diversity, parasite or pathogen levels per host are predicted to be lower due to a higher probability of dispersing parasites or pathogens encountering noncompetent hosts. Dilution effects could occur in many mutualisms if dispersing symbionts encounter hosts that vary in their competence. Introduced, non-native hosts can change the community competence of a local group of host species. Crayfish introductions are occurring world-wide and these introductions are likely disrupting native crayfish-symbiont systems. Branchiobdellidan symbionts declined on native *Cambarus* crayfish co-occurring with non-native *Faxonius* crayfish in the New River watershed, USA. We performed an experiment investigating the effect of host density (1 vs. 2 native hosts) and host diversity (1 native host and 1 introduced host) on branchiobdellidan abundance. The introduced *Faxonius cristavarius* is a noncompetent host for these worms. Six *C. ingens* were stocked on a *Cambarus chasmodactylus* in each treatment and worm numbers were followed over 34 days. Worm numbers decreased over time on *C. chasmodactylus* alone and in the treatment in which a *C. chasmodactylus* was paired with an *F. cristavarius*. Worm numbers remained highest in the 2 *C. chasmodactylus* treatment. There was no effect of host diversity on worm reproduction. Crayfish invasions may have negative effects on mutualistic symbionts depending on the competence of introduced hosts. Loss of native symbionts is one of the potential hidden, negative effects of invasions on native freshwater diversity.

KEYWORDS

biodiversity, branchiobdellidan, community competence, crayfish, symbiosis

1 | INTRODUCTION

The dilution effect hypothesis was originally proposed to describe the effect of host diversity on parasite and pathogen abundance; with increasing host diversity, parasite or pathogen levels per host tend to

be lower (Civitello et al., 2015; Huang, Van Langeveide, Estrada-Peña, Suzán, & De Boer, 2016; Johnson, Preston, Hoverman, & Richgels, 2013; Keesing, Holt, & Ostfeld, 2006). There are several potential mechanisms for the dilution effect, one of which is that in more diverse communities, parasites or pathogens are more likely to

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encounter hosts in or on which they are unable to proliferate (Civitello et al., 2015). Keesing et al. (2006) refer to this mechanism as encounter reduction. Other mechanisms include the reduced probability of transmission success, increasing recovery rate, and increased death rate of infected individuals (Keesing et al., 2006).

Central to discussions of dilution effects is the concept of host competence (Stewart Merrill & Johnson, 2020). Host competence is generally defined as the ability of hosts to produce more parasites or pathogens that can be transmitted to new hosts (Barron, Gervasi, Pruitt, & Martin, 2015; Martin, Burgan, Adelman, & Gervasi, 2016). However, various factors contribute to competence including the ability of parasites or pathogens to encounter hosts, their abilities to successfully develop in or on a host and, finally, to be transmitted to other hosts (Barron et al., 2015; Stewart Merrill & Johnson, 2020). Further, hosts can resist infections or physically remove parasites or vector species which further influences host competence (Stewart Merrill & Johnson, 2020). All together, these factors influence whether parasites and pathogens can successfully colonize, reproduce, and disperse, and, thus, the degree of host competence.

Because parasites and pathogens often infect multiple host species with varying levels of competence, the composition of an ecological community can influence parasite or disease risk, a phenomenon known as community competence (Johnson et al., 2013). If increased species richness results in more low- or noncompetent hosts in a community, there could be a dilution effect. In contrast, if additional competent hosts enter a community, parasite or disease amplification could result. Other factors modify the influence of community structure on dilution or amplification, such as the amount of contact between potential hosts (Johnson et al., 2013) and whether a parasite or pathogen is vector-borne or directly transmitted (Huang et al., 2016). Especially in vector-borne diseases, prevalence and abundance of infection may respond differently to changes in community competence (Huang et al., 2016). In addition, landscape-level factors such as habitat fragmentation may influence the disease-diversity relationship (Huang et al., 2016). The dilution effect with respect to the prevalence of parasites and pathogens has been interpreted as a benefit of biodiversity and an argument for the conservation of biological diversity.

There is considerable empirical support for the dilution effect in host-parasite and host-pathogen systems. Johnson et al. (2013) found decreased transmission success of the multi-host trematode *Ribeiroia ondatrae* from infected snails to their typical amphibian host with increased amphibian richness in observational studies as well as both laboratory and field experiments. Their results showed that with increased host richness, community competence decreased, resulting in an overall decrease in successful transmission. In addition, they found that the most competent hosts were the most common species, providing support for the negative competence-extinction relationship, in which low diversity assemblages often contain higher proportions of highly competent hosts (Huang et al., 2016; Johnson et al., 2013; Ostfeld & Keesing, 2012). Huang et al.'s (2016) review concluded that most empirical studies to date support a negative competence-extinction relationship. In addition to *Ribeiroia ondatrae*,

dilution effects have been observed in various parasite and pathogen systems, such as Lyme disease (LoGiudice, Ostfeld, Schmidt, & Keesing, 2003; Ostfeld & Keesing, 2000) and West Nile virus (Allan et al., 2009; Swaddle & Calos, 2008), among others. A meta-analysis by Civitello et al. (2015) found a significant negative relationship between host richness and parasite abundance, even when controlling for parasite type, life history, functional group, specialization, and study type. Like Johnson et al. (2013), Civitello et al. (2015) found that the frequency of focal host species was more important than their density for reducing parasite abundance.

While there is ample evidence that dilution effects can influence the prevalence of organisms with negative effects on their hosts such as parasitic symbionts and pathogens, few studies have evaluated whether dilution effects influence the abundance and diversity of mutualistic symbionts, that is, symbionts that benefit their hosts (Bell, 2018). Mutualistic ectosymbionts often depend on dispersal between hosts to reduce resource competition and improve their fitness (Gruffydd, 1965; Skelton, Creed, & Brown, 2015). If the dilution effect works similarly in mutualistic symbioses as it does in parasitic ones, then higher levels of host diversity could end up harming one or both partners by reducing the number of competent hosts available for ectosymbionts, leading to decreasing symbiont populations and therefore decreasing beneficial services for the host. The introduction of new hosts into a community is one way that such a host dilution effect could occur in mutualistic symbioses.

One well-studied freshwater mutualism occurs between crayfish and some species of ectosymbiotic worms called branchiobdellidans (Ames, Helms, & Stoeckel, 2015; Brown, Creed, & Dobson, 2002; Brown, Creed, Skelton, Rollins, & Farrell, 2012; Creed, Skelton, Farrell, & Brown, 2021; Lee, Kim, & Choe, 2009; Thomas, Creed, Skelton, & Brown, 2016). In these mutualisms, the worms benefit from a relatively safe habitat with resources, and a site for reproduction (Creed, Lomonaco, Thomas, Meeks, & Brown, 2015). The crayfish benefit by having their gills cleaned of epibionts and detritus which results in increased host survival and growth (Brown et al., 2002, 2012; Creed et al., 2021). This host-symbiont system is a useful model for studying symbioses because both the crayfish hosts and the branchiobdellidans are widespread throughout many regions of the world, both taxa can be easily kept in laboratory aquaria or used as subjects in field experiments, and the worms are easy to manipulate and monitor during experiments (Creed et al., 2021; Skelton et al., 2013). It is also a good system for studying invasion-related dilution effects as crayfish have been introduced into waterways around the world where they can interact with native crayfish hosts and their symbionts (Gherardi, 2006; Hobbs III, Jass, & Huner, 1989; Twardochleb, Olden, & Larson, 2013).

In the New River in Virginia and North Carolina, USA, several native crayfish species in the genus *Cambarus* serve as hosts for various branchiobdellidan species (Brown et al., 2012; Skelton, Doak, Leonard, Creed, & Brown, 2016; Thomas et al., 2016). Although branchiobdellidans do not appear to have host species specificity, worm community composition does change significantly with changes in host species composition suggesting that worm species may

experience varied success on different hosts (Skelton, Creed, Landler, Geyer, & Brown, 2016). *Cambarus appalachiensis* and *C. bartonii* may host 4–5 species of branchiobdellidan while *C. chasmodactylus* generally hosts 1–2 species of worm (Brown & Creed, 2004; Skelton, Creed, et al., 2016; Thomas et al., 2016). The New River is now home to some introduced crayfish species including *Faxonius virilis* and *F. cristavarius*, and the latter is now widely distributed throughout much of the watershed in North Carolina and Virginia. *Faxonius cristavarius* is a noncompetent host for branchiobdellidans (Bell, 2018; Farrell, Creed, & Brown, 2014). Farrell et al. (2014) observed that both native *Cambarus* and introduced *Faxonius* crayfish can moderate the abundance of worms by grooming, but *F. cristavarius* increased grooming in response to the presence of only one worm whereas *C. chasmodactylus* increased grooming only in response to ten worms.

Branchiobdellidans routinely disperse from one host to another (Skelton et al., 2015), including from native to introduced hosts or from one introduced host to another (Vedia et al., 2015; Williams & Weaver, 2021). A range of factors likely prompt branchiobdellidans to disperse to another host, including competition for prime microhabitats and resources (Creed & Brown, 2018; Skelton et al., 2015), as well as escape from intraguild predation and cannibalism as larger branchiobdellidans frequently prey upon smaller branchiobdellidans (Skelton, Doak, et al., 2016; Thomas et al., 2016). Dispersal to other competent hosts means that the worms could improve their access to food, space, and mates and increase their fitness (Creed & Brown, 2018; Skelton et al., 2015). However, if worms disperse to noncompetent hosts on which they cannot survive and reproduce, they are likely to be lost from the community and total worm abundance and richness could decrease. If the crayfish-branchiobdellidan system shows host dilution effects when low- or noncompetent hosts invade a community then this loss of symbiont diversity could be an overlooked impact of crayfish invasions. Further, loss of beneficial symbionts could have negative effects on the fitness of native crayfish hosts and reduce their ability to compete with introduced crayfish species, further exacerbating the negative impacts of invasion.

2 | METHODS

2.1 | Survey

From 2011 to 2014, one of the authors (JS) collected and identified 25,688 branchiobdellidans belonging to nine species from six species of crayfish at 24 sampling sites within the Mountain Lake Region of western Virginia. The details of this survey are reported in Skelton, Creed, et al. (2016). In short, crayfish were collected by hand, seines, quadrat samplers, and kick nets and placed individually in 750 mL plastic bags with 70% ethanol for preservation during transport to the laboratory. In the laboratory, each crayfish was identified, sexed, weighed, and measured for total carapace length (TCL). All branchiobdellidans present on each crayfish were collected and identified, yielding worm census data for each crayfish.

We aimed to address two questions by analyzing the survey data. First, we wanted to determine if crayfish in the genus *Faxonius* (syn. *Orconectes*), which had recently invaded the Mountain Lake Region, hosted fewer worms than native crayfish in the genus *Cambarus*. Second, we wanted to determine if native crayfish at sites that had been invaded by *Faxonius* ($n = 7$) hosted fewer worms than native crayfish at un-invaded sites ($n = 17$). We used negative binomial generalized linear models to achieve these goals. We chose negative binomial over Poisson because our response variable (number of worms on each crayfish) was highly over dispersed. For each analysis, we used a “top-down” approach for model selection described in Zuur, Ieno, Walker, Saveliev, and Smith (2009). To determine if native crayfish hosted more worms than non-native crayfish, we started with a model that included crayfish sex, size (TCL in mm), the categorical variable of native versus non-native (equivalent to *Cambarus* versus *Faxonius* for our study area, with the majority [89%] of the *Faxonius* being *F. cristavarius*), and the interaction between size and native/non-native. To determine if native hosts had fewer worms at sites where *Faxonius* had invaded, we created a subset of the survey data to only include native hosts ($n = 597$) and started with a negative binomial general linear model (GLM) that included host size (TCL), sex, whether the crayfish was collected from an invaded or uninvaded area, and the interaction between size and invaded/uninvaded. We used the categorical variable invaded/uninvaded because we did not have sufficient quantitative information to assess non-native host density or relative density. GLMs were implemented using the `glm.nb()` function of the MASS package (Venables & Ripley, 2002) for R version 3.6.0.

2.2 | Experiment

The purpose of the experiment was to assess the impact of crayfish host abundance and diversity on branchiobdellidan abundance. There were three treatments which were: 1) Cc alone which contained a single *C. chasmodactylus* donor, 2) Cc/Cc contained a *C. chasmodactylus* donor with a *C. chasmodactylus* receiver, and 3) Cc/Fc contained a *C. chasmodactylus* donor with an *F. cristavarius* receiver. *Cambarus chasmodactylus* and the branchiobdellidan *Cambarincola ingens* were collected from the Middle Fork of the New River. *Faxonius cristavarius* were collected from the South Fork of the New River. *Cambarincola ingens* were removed from *C. chasmodactylus* and retained before all experimental crayfish were placed in 10% magnesium chloride hexahydrate solution for 5 min to kill any remaining worms and cocoons.

Crayfish sizes (TCLs) for the two species used in the experiment ranged from 30 to 34 mm for *C. chasmodactylus* and 29–33 mm for *F. cristavarius*. Crayfish were matched as closely as possible by TCL for each replicate that contained two crayfish. Single *C. chasmodactylus* (Cc) in the Cc alone treatment and donor *C. chasmodactylus* in the other two treatments were each stocked with 4 large (7–10 mm long) and 2 medium (4–6 mm long) *C. ingens* that were placed on the dorsal carapace. *Cambarus chasmodactylus* and *F. cristavarius* receivers were left worm-free.

Experimental aquaria (37 L) were filled with water collected from the South Fork of the New River. The bottoms of the aquaria were covered with a layer of aquarium gravel mixed with fine sediments that were also collected from the South Fork of the New River. Flat rocks were used to build two refugia per aquarium. Approximately half of the water in each aquarium was changed weekly to reduce ammonia accumulation. Aquaria for the three treatments were placed three to a shelf on separate shelves of a wire shelf unit. The experimental design was a randomized complete block design and each block was replicated six times. Lights were set on a 14:10 light/dark schedule and the ambient water temperature was held between 22 and 23°C during the experiment. All crayfish were provided two shrimp pellets every other day. The experiment began on August 27, 2016.

On days 3, 6, 10, 13, 20, 27, and 34, worms and cocoons were quantified on each crayfish. Crayfish were placed in a container of water and inspected beneath a dissecting microscope. The dorsal and ventral surface of each crayfish and all appendages were inspected for worms and cocoons and their locations were recorded. The experiment was terminated after 34 days due to the loss of replicates.

We used a mixed effects linear model repeated measures ANOVA (rmANOVA) with the `lme()` function in the `nlme()` package in the R programming environment to examine how host composition affected the total abundance and transfer of branchiobdellidans (R Core Team, 2016). In this model, host composition and time were fixed effects while controlling for the random effect of experimental unit, that is, aquarium. Model effects were estimated using the method described in Laird and Ware (1982). Total worm abundance was the total number of branchiobdellidans recovered from all crayfish in an aquarium. We also compared the number of worms recovered from

C. chasmodactylus versus *F. cristavarius* receivers to assess how the presence of an introduced host could affect total worm numbers.

3 | RESULTS

3.1 | Survey

In the first analysis, the main effects of host size and native/non-native were retained during model selection. The number of worms increased with host size and was significantly higher on native hosts than on non-native hosts (Figure 1a, Table 1). On average, the percentage of worms recovered from non-native crayfish was 28% of that found on native crayfish hosts. In the second analysis, the main effects of host size and whether the crayfish was collected from an invaded versus uninvaded area were retained through model selection. The number of worms on native hosts increased with host size and was significantly lower in areas that had been invaded by non-native crayfish (Figure 1b, Table 1). Native crayfish at invaded sites hosted 34% fewer worms on average than native crayfish at uninvaded sites.

3.2 | Experiment

There were significant effects of treatment and day on total worms remaining in the aquaria as well as a significant treatment \times day interaction (Figure 2, Table 2). The total number of worms on all crayfish hosts in aquaria was significantly higher in the Cc/Cc treatment than in the Cc alone and Cc/Fc treatments by day 20 and remained higher for the remainder of the experiment (Figure 2, Table 2). The mean

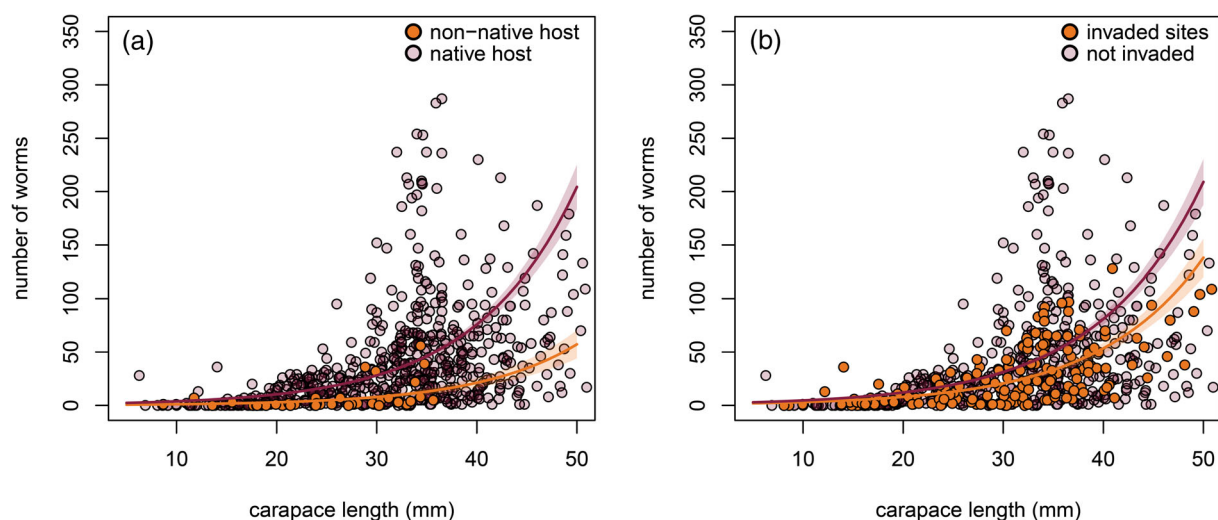


FIGURE 1 Results of the field survey. (a) The total number of branchiobdellidans versus host carapace length for all hosts. Native hosts are shown in maroon, and non-native hosts in orange. (b) The total number of branchiobdellidans versus host carapace length for only native hosts. Native hosts collected from sites at which non-native species were also observed are shown in orange. Native hosts collected from sites in which non-natives were not detected are shown in maroon. In both panels, solid lines show model fit for negative binomial GLM, shading shows standard error

TABLE 1 Results of the negative binomial generalized linear models from the analysis of the survey data

| Predictors | Native versus non-native hosts | | | | Invaded versus uninvaded areas | | | |
|-----------------------------|--------------------------------|-----------|-------------|------------------|--------------------------------|-----------|-------------|------------------|
| | Incidence rate ratios | CI | z statistic | p | Incidence rate ratios | CI | z statistic | p |
| (intercept) | 1.43 | 1.04–1.98 | 2.19 | 0.029 | 1.81 | 1.30–2.52 | 3.51 | <0.001 |
| CL | 1.10 | 1.09–1.12 | 19.87 | <0.001 | 1.10 | 1.09–1.11 | 19.06 | <0.001 |
| Non-native | 0.28 | 0.19–0.42 | –6.20 | <0.001 | | | | |
| Invaded | | | | | 0.66 | 0.54–0.80 | –4.17 | <0.001 |
| Observations | 633 | | | | 597 | | | |
| Nagelkerke's R ² | 0.477 | | | | 0.444 | | | |

Note: There was a significant increase in number of worms with crayfish size (TCL) in both models. The first model (left) showed significantly fewer worms on non-native hosts versus native. The second model (right) showed significantly fewer worms on native hosts from invaded areas versus uninvaded areas. p values in bold are significant.

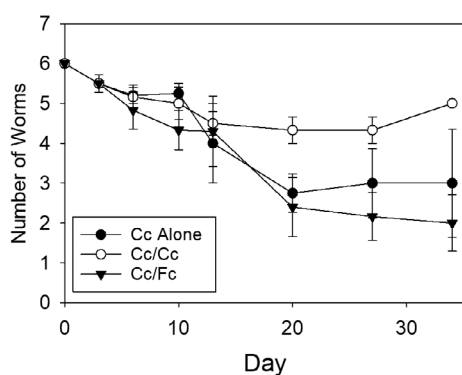


FIGURE 2 Mean (± 1 S.E.) number of *C. ingens* observed on all crayfish in each treatment over 34 days. The treatments were as follows: Cc alone – a single *C. chasmodactylus* stocked with 6 *C. ingens*; Cc/Cc – a *C. chasmodactylus* donor stocked with 6 *C. ingens* in an aquarium with a *C. chasmodactylus* receiver that was not stocked with worms; and Cc/Fc – a *C. chasmodactylus* donor stocked with 6 *C. ingens* in an aquarium with an *F. cristavarius* receiver

TABLE 2 Results of the rmANOVA comparing total worms remaining on crayfish in the following treatments: Cc alone – *C. chasmodactylus* housed alone; Cc/Cc – two *C. chasmodactylus* housed together; and Cc/Fc – a *C. chasmodactylus* housed with an *F. cristavarius*

| Total worms | | | |
|------------------------|--------|-------|---------------|
| Factor | F | Df | p |
| Treatment | 5.11 | 2, 10 | 0.0296 |
| Row | 2.29 | 5, 10 | 0.1236 |
| Day | 106.30 | 1, 98 | 0.0001 |
| Treatment \times day | 4.48 | 2, 98 | 0.0138 |

Note: Data analyzed were the total number of worms recovered from all crayfish in each aquarium. Values in bold are significant.

number of worms on the *C. chasmodactylus* donor in the Cc/Cc treatment decreased for the first 13 days while the mean number of worms on the Cc receiver increased, showing dispersal between the two crayfish (Figure 3a). The mean number of worms on the

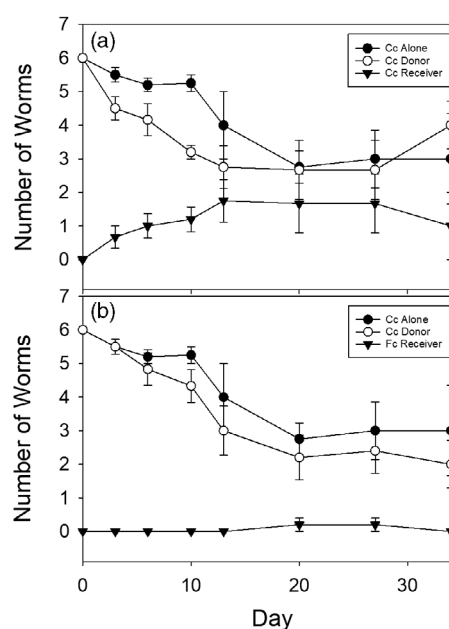


FIGURE 3 Mean (± 1 S.E.) number of worms on crayfish donors and receivers for both the low host diversity (panel A – Cc/Cc) and high host diversity (panel B – Cc/Fc) treatments. Worm numbers from the Cc alone treatment are shown for comparison

C. chasmodactylus donors living with *F. cristavarius* receivers decreased over the course of the experiment and was not significantly different than the mean number of worms in the Cc alone treatment (Figures 2 and 3b). Worms were observed on the *F. cristavarius* receiver on two occasions. The number of worms recovered from the *C. chasmodactylus* receiver was significantly higher than the number recovered from the *F. cristavarius* receiver (Figure 3, Table 3).

Thirteen of the eighteen *C. chasmodactylus* molted, mostly between days 10 and 20 of the experiment. This result is reflected in the decline of the worms in the Cc alone and Cc/Fc treatments. Few cocoons were observed on crayfish prior to the period of molting that began on day 10. Following the molt, cocoons were observed on *C. chasmodactylus* hosts but there was no significant effect of

TABLE 3 Results of the mANOVA comparing worm transfers from *C. chasmodactylus* to another *C. chasmodactylus* in the Cc/Cc treatment compared to an *F. cristavarius* in the Cc/Fc treatment

| Cc/cc versus cc/fc | | | |
|--------------------|-------|-------|---------------|
| Factor | F | Df | p |
| Treatment | 7.82 | 1, 10 | 0.0190 |
| Day | 11.83 | 1, 66 | 0.0010 |
| Treatment × day | 18.78 | 1, 66 | 0.0001 |

Note: Data were worms recovered from the receiver crayfish. Values in bold are significant.

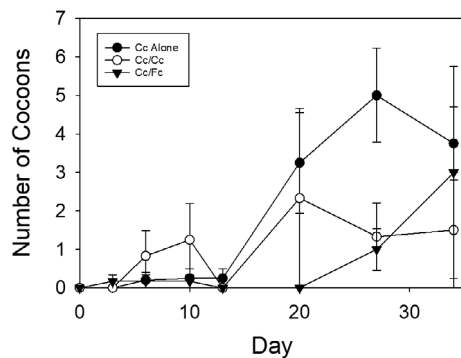


FIGURE 4 Mean (± 1 S.E.) number of cocoons found on *C. chasmodactylus* in all three treatments. No cocoons were found on *F. cristavarius*

treatment on cocoon numbers (Figure 4). No cocoons were observed on *F. cristavarius*.

4 | DISCUSSION

A dilution effect resulting from the introduction of a noncompetent crayfish host was observed in our survey and was confirmed in our experiment. Native crayfish co-occurring with *F. cristavarius* in Virginia streams hosted approximately one third fewer worms than native crayfish that did not co-occur with this introduced crayfish. In our experiment, the abundance of *C. ingens* decreased over time in the treatment containing *F. cristavarius* (Cc/Fc), while worm populations in the low diversity treatment with two native hosts (Cc/Cc) remained relatively stable. Worms dispersed from the *C. chasmodactylus* donor to the *C. chasmodactylus* receiver in the Cc/Cc treatment thus colonizing new competent hosts. Worms were observed briefly on the *F. cristavarius* receiver in the Cc/Fc treatment on two occasions, showing that worms did disperse on to this noncompetent host but were lost from the system before they were able to disperse back on to a *C. chasmodactylus*. This loss of worms was likely due to the strong grooming behavior exhibited by *F. cristavarius* (Farrell et al., 2014). A density effect was also observed, as worm populations in the Cc/Cc treatment were higher and remained more stable than those on the single crayfish in the *C. chasmodactylus* alone treatment.

Several of the *C. chasmodactylus* used in the experiment molted during the 34 day experiment, mostly between days 10 and 20. During ecdysis, some worms stay attached to the old exoskeleton as the crayfish emerges from it. This mechanism appears to have led to loss of worms in this experiment when the worms were unable to transfer back to the newly molted crayfish. However, because crayfish often consume much of their shed exoskeleton (Creed, pers. obs.), some worms had an opportunity of post-molt contact and were able to return to their host. Creed and Brown (2018) documented a 30% loss in worms during molting in a similar experiment. The worms appeared to have a higher chance of surviving during the molt of their initial host when there were two competent hosts in an aquarium. Because usually, only one crayfish was molting at a time, worms were able to either transfer to the non-molting host before or during their host's ecdysis, or if stranded on an old exoskeleton, they had a greater chance of transferring to the other crayfish. In contrast, worm populations in the Cc/Fc treatment and the Cc alone treatment decreased substantially during molting of the *C. chasmodactylus* host. These two treatments exhibited similar responses of worm populations to host molting, which indicates that there was no benefit for the worms when there was a noncompetent host present during the native host's molt.

Our results, along with those of Farrell (2012), Farrell et al. (2014), and Bell (2018), allow us to assess the competence of *F. cristavarius* as a host for branchiobdellidans relative to the native hosts using the approach outlined by Stewart Merrill and Johnson (2020). The survey results showed that branchiobdellidans are able to colonize this introduced species and persist long enough to be recovered during surveys. It is likely that they do not persist for long given the intense grooming that has been reported for this species by Farrell et al. (2014). In our experiment, we also observed that the worms would colonize *F. cristavarius* but not persist on this host for long. In a more extensive survey of crayfish and branchiobdellidans in the New, James and Roanoke rivers, Bell (2018) found that as the proportion of the crayfish community composed of *F. cristavarius* increased that worm numbers declined significantly. In contrast to the worms on native hosts, worms on *F. cristavarius* did not produce any cocoons in our experiment. Similarly, in a survey of 171 *F. cristavarius* in the South Fork of the New River none were reported to have any branchiobdellidan cocoons (Farrell, 2012). So, while *F. cristavarius* may host worms in the field there does not appear to be any proliferation of branchiobdellidans on this host and, thus, no new worms to be transmitted to other hosts. All these results support the conclusion that *F. cristavarius* is a noncompetent host for branchiobdellidans in the New River.

When a dilution effect occurs in wild populations of crayfish and branchiobdellidans, it may be detrimental to both the ectosymbionts and their hosts. If higher host diversity following a crayfish invasion results in a higher abundance of noncompetent hosts in a community, branchiobdellidans may be more likely to disperse to a noncompetent host on which they cannot survive or reproduce. This outcome depends partially on whether, and how well, branchiobdellidans can discriminate between potential hosts. Brown and Creed (2004)

showed that the branchiobdellidan *C. ingens* was able to discriminate between *C. chasmodactylus* and *F. cristavarius*. However, while *C. ingens* can discriminate between these two hosts, they do not appear to be able to respond quickly enough to disperse off of *F. cristavarius* before being removed or eaten. This conclusion is supported by results from both the survey and the experiment. In both cases, worm abundance on native *Cambarus* crayfish declined when *F. cristavarius* was present. We should note that the ability of most branchiobdellidans to discriminate between hosts has not been determined. Crayfish hosts could also be negatively affected by the dilution effect following invasion by a noncompetent host. This outcome could occur if the abundance of mutualistic worms declines following such an invasion which would result in the native crayfish hosts receiving reduced gill-cleaning benefits from their branchiobdellidans.

Host diversity may also regulate the proliferation of worms on competent hosts, inhibiting the worms from reaching densities at which they can become weakly parasitic. As Brown et al. (2012) reported, crayfish-branchiobdellidan relationships can shift from mutualism to parasitism at high ectosymbiont densities. The host dilution effect could have multiple influences on worm abundance. On the one hand, with a higher abundance of competent hosts and thus less dilution, branchiobdellidans could readily move from host to host which could result in worm densities on any given host remaining at moderate levels. This mechanism could maintain mutualistic conditions and prevent parasitism if worms are able to disperse easily between hosts and therefore not reach high densities on any one crayfish (Skelton et al., 2015), and this is likely what is happening in the uninvaded communities. On the other hand, if worms are at high densities on their competent hosts, dilution effects involving noncompetent hosts may alleviate some of the pressure by drawing some worms away to be ultimately lost from the system. As seen in this experiment, a competent host alone and a competent host co-occurring with a noncompetent host had similar, low worm populations following a molt, which may be beneficial to the competent host if worms are at parasitic levels. However, parasitic levels of worms are rarely found on native crayfish in nature because some crayfish hosts regulate worm levels through grooming behaviors (Farrell et al., 2014).

Variation in diversity and dominance of crayfish throughout the New River watershed creates various conditions for crayfish-branchiobdellidan associations and potential dilution effects. The distribution of crayfish species in the New River watershed is related to stream size (Skelton, Creed, et al., 2016). In first- and second-order, headwater streams in North Carolina, *Cambarus bartonii*, an even more competent host for branchiobdellidans than *C. chasmodactylus*, is the dominant species present. Up to six species of branchiobdellidan have been found on *C. bartonii* (Thomas and Creed, unpublished data). As many as 8 species were found on *Cambarus appalachiensis* in headwater streams during our survey of the Mountain Lake Region. In third-order streams, either *C. chasmodactylus* or *C. appalachiensis* tend to be co-dominant with *C. bartonii*. In mainstem habitats, the introduced *F. cristavarius* is now the dominant species. *Cambarus chasmodactylus* adults are still present in the South Fork of

the New River in North Carolina, but their young-of-the-year are only found in third-order tributaries (Fortino & Creed, 2007). It is not clear if a similar distribution of young and adult *C. appalachiensis* occurs in the New River and its tributaries in Virginia. Thus, there appear to be gradients of community competence for branchiobdellidans in the New River watershed, with the highest community competence in the smaller tributaries and the lowest competence in the mainstem habitats (Skelton, Creed, et al., 2016). With this distribution, branchiobdellidans are predicted to be more abundant and have higher species diversity in smaller tributaries of the New River where they have more competent hosts to disperse among, especially during disturbances like molting. In the mainstem habitats, a negative dilution effect is more likely as the noncompetent *F. cristavarius* are now more abundant than the competent *Cambarus* hosts. In addition, *F. cristavarius* has been expanding its range in the New River for the last 70 years (Bell, 2018, NCWRC, unpublished data; Creed, personal observation). This range expansion has likely contributed to the decline in branchiobdellidan abundance in much of the New River watershed.

Huang et al. (2016) suggest three prerequisites for the generality of the dilution effect hypothesis. First, host species must differ in their level of competence. The crayfish-branchiobdellidan system in the New River fulfills this criterion, with *C. bartonii* and *C. appalachiensis* being the most competent hosts, followed by *C. chasmodactylus* and with *F. cristavarius* being a noncompetent host. Potential dilution effects among the different *Cambarus* species, as well as when all three species occur together, should be investigated. Second, non- or low-competence hosts can reduce transmission through various mechanisms. This outcome occurred in this experiment when worms were briefly observed on the *F. cristavarius* receiver in the Cc/Fc treatment and were quickly lost from the system, likely due to mortality on *F. cristavarius*. The third prerequisite is that competent hosts are more resilient to local species loss than non- or low-competence hosts, also known as the negative competence-extinction relationship. The abundance of crayfish host species in the New River does not fit with this last prerequisite as *F. cristavarius* is a noncompetent host and now the most abundant. Adults of at least one species (*C. chasmodactylus*) are present in the mainstem habitats of the New River but do not appear to be able to successfully reproduce in this habitat and likely migrated to these habitats from nearby tributaries (Fortino & Creed, 2007). This observation suggests that mainstem habitats, with their abundant noncompetent *Faxonius* hosts, may be a sink for worms that originated on crayfish hosts in the tributaries further exacerbating the negative impacts of this host dilution effect.

Our data show a pronounced, hidden effect of crayfish introductions on native symbiont communities. Those charged with preventing and managing crayfish invasions need to be aware of the fact that losses of mutualistic symbionts like branchiobdellidans, which can increase crayfish survival and growth (Brown et al., 2002, 2012), could exacerbate the impacts of invasions. Loss of mutualistic symbionts on native crayfish hosts could weaken their competitive ability against the invading crayfish which could accelerate the rate of decline in the abundance of the native crayfish. This is yet another reason that

crayfish introductions need to be prevented whenever possible. Strict regulations on the importation of crayfish as well as on the capture and transfer of crayfish within and across watersheds could help prevent crayfish invasions and reductions in beneficial symbionts and their native hosts (Taylor, DiStefano, Larson, & Stoeckel, 2019). Sections of the river above small dams that are impassable to introduced crayfish may create reserves for native crayfish and their symbionts (see Gangloff, 2013, Rahel, 2013, and Barnett & Adams, 2021 for additional discussions of this idea). These habitats could be protected by maintaining these small dams although all species potentially influenced by the presence or absence of a given dam need to be considered when deciding whether to maintain or remove a given dam. Alternatively, barriers to the upstream dispersal of invading crayfish that allow fish passage could be built to protect habitats containing native crayfish and their symbionts (Frings et al., 2013; Krieg, King, & Zenker, 2021; Krieg & Zenker, 2020). Regardless of the conservation measures used (Taylor et al., 2019), these measures need to be designed to mitigate negative impacts on both the native crayfish and their symbionts.

The dilution effect with respect to mutualisms is a complex community-level phenomenon that should be researched further in the crayfish-branchiobdellidan mutualism as well as other systems. If higher host diversity as a result of invasions leads to a higher abundance of low- or noncompetent hosts, invasions may have a negative effect on native symbiont populations and, potentially, native host fitness. Alternatively, introductions of more competent hosts could benefit native symbiont communities. Though there are many ecological benefits of biodiversity, such as mitigating diseases and parasite abundance (Civitello et al., 2015; Huang et al., 2016; Johnson et al., 2013; Keesing et al., 2006), it should not be assumed that higher diversity is always better with respect to symbiont abundance.

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DATA AVAILABILITY STATEMENT

Our data are available to other researchers upon request.

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