

The dilution effect in a freshwater mutualism

by

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## Abstract

The dilution effect describes the effect of host diversity on parasite abundance—with greater host diversity, parasite levels per host are predicted to be lower, due to several possible mechanisms including a higher probability of parasites encountering non-competent hosts. No research has examined whether host dilution affects the prevalence of mutualistic symbionts on their hosts. Using the crayfish-branchiobdellidan worm mutualism, I performed an experiment investigating the effect of host density (1 vs 2 *Cambarus chasmodactylus*) and host diversity (1 *C. chasmodactylus* paired with 1 *O. cristavarius*) on branchiobdellidan (*Cambarincola ingens*) abundance. Worm numbers decreased over time on *C. chasmodactylus* alone and in the treatment in which it was paired with *O. cristavarius*, the latter being a poor host for *C. ingens*. Worm numbers declined the least in the 2 *C. chasmodactylus* treatment. While increased host diversity may reduce levels of parasitism (a positive effect of increased diversity) it could have negative effects on mutualistic symbionts depending on the quality of alternative hosts.

## Introduction

The dilution effect hypothesis describes the effect of host diversity on parasite abundance—with greater host diversity, parasite levels per host are predicted to be lower on competent hosts (Keesing, Holt & Ostfeld 2006, Johnson et al. 2013, Civitello et al. 2015, Huang et al. 2016). There are several potential mechanisms for the dilution effect, one of which is that in more diverse systems, parasites are more likely to encounter non-competent (unsuitable) hosts in or on which they are unable to proliferate (Civitello et al. 2015). Keesing, Holt and Ostfeld (2006) refer to this mechanism as encounter reduction. Other mechanisms include reduced probability of transmission success, decreased density of competent hosts, increasing recovery rate, and increased death rate of infected individuals (Keesing, Holt & Ostfeld 2006).

Because pathogens often infect multiple host species with varying levels of competence, the composition of an ecological community can influence disease risk, a phenomenon known as “community competence” (Johnson et al. 2013). If increased species richness introduces more non-competent hosts into an assemblage, there could be a dilution effect. In contrast, if it results in more competent hosts entering the system, 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 the disease is vector-borne or directly transmitted (Huang et al. 2016). Especially in vector-borne diseases, prevalence and abundance of infection may respond differently to species diversity (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 has been interpreted as a health benefit of biodiversity and an argument for diversity conservation efforts (Civitello et al. 2015, Huang et al. 2016).

Much empirical support has been produced for the dilution effect in host-parasite 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 (Ostfeld & Keesing 2012, Johnson et al. 2013, Huang et al. 2016). Huang et al.'s (2016) review concluded that most empirical studies to date support a negative competence-extinction relationship. One potential mechanism behind the negative competence-extinction relationship is that the energy costs associated with defenses may lead to a trade-off with resilience (Johnson et al. 2013). However, this relationship is still controversial (Huang et al. 2016). In addition to *Ribeiroia ondatrae*, dilution effects have been observed in numerous parasite systems, such as Lyme disease (Ostfeld & Keesing 2000, LoGiudice et al. 2003) and West Nile virus (Swaddle & Calos 2008, Allan et al. 2009), among others. A meta-analysis by Civitello et al. (2015) found a significantly negative relationship between host richness and parasite abundance, even when controlling for parasite type, lifestyle, functional group, specialization and study type. Like Johnson et al. (2013), they found that the frequency of focal host species was more important than their density for reducing parasite abundance. They then compared the dilution effect seen in parasites to that with herbivores and plant diversity described by the associational resistance hypothesis. They found similar statistical strength

between the two, suggesting that dilution effects can also exist for other types of natural enemies besides parasites.

There appears to be support for the dilution effect hypothesis in both parasitism and herbivory relationships, in which one partner benefits while the other is disadvantaged. However, does the dilution effect apply to other ecological relationships, such as mutualisms?

Ectosymbiotic mutualists depend on dispersal between hosts to reduce resource competition and to increase their fitness (Skelton, Creed & Brown 2015). If the dilution effect works similarly in a mutualism as it does in parasitism, higher levels of biodiversity may end up harming one or both partners by reducing the number of competent hosts available for ectosymbiont dispersal, leading to decreasing ectosymbiont populations and therefore decreasing beneficial services for the host.

One well-studied mutualism is between crayfish and a group of ectosymbiotic worms called branchiobdellidans (Brown et al. 2012, Skelton et al. 2013). This symbiosis is a useful model for studying symbioses because both the hosts and the branchiobdellidans are widespread throughout the Northern hemisphere, can be easily kept in laboratory aquaria or used as subjects in field experiments, and the branchiobdellidans are easy to manipulate and monitor during experiments (Skelton et al. 2013). Some branchiobdellidans are mutualistic associates of crayfish (Brown, Creed & Dobson 2002, Brown et al. 2012, Lee, Kim & Choe 2009, Thomas et al. 2016), while other species appear to be commensals and some are parasitic.

In my study I used the New River crayfish *Cambarus chasmodactylus* as the competent host species. The branchiobdellidan we used was *Cambarincola ingens*, an obligate mutualist that feeds on algae and other microorganisms it encounters on the exoskeleton of the crayfish including the gills (Brown et al. 2012, Creed et al 2015). This interaction has been seen to boost

host fitness, probably by increasing gas exchange and ammonia excretion capabilities across the crayfish gill epithelium (Brown, Creed & Dobson 2002, Brown et al. 2012, Creed & Brown unpublished data). The crayfish *Orconectes cristavarius* occurs sympatrically with *C. chasmodactylus* in parts of the New River drainage, but is a non-competent host for *C. ingens* (Brown & Creed 2004, Farrell, Creed & Brown 2014). Farrell, Creed and Brown (2014) observed that both species of crayfish can moderate the abundance of worms by grooming, but *O. cristavarius* increased grooming in response to the presence of only one worm whereas *C. chasmodactylus* only increased grooming in response to ten worms. In two field experiments, *C. chasmodactylus* with intermediate levels of associated *C. ingens* received a fitness benefit, growing larger than crayfish with no worm partners. However, this relationship shifted from mutualism to parasitism at high densities, with the worms consuming the gill tissues of their hosts instead of cleaning them (Brown et al. 2012).

A range of factors prompts branchiobdellidans to disperse from one host to another, including competition for prime microhabitats (Skelton, Creed & Brown 2015). Dispersal to competent hosts means that the worms can improve their access to food and prime cocoon-laying locations and increase their populations. However, if worms disperse to non-competent hosts, they are likely to be lost from the system through host grooming and populations will decrease. If the crayfish-branchiobdellidan system shows host dilution effects, we would expect for *C. chasmodactylus* co-occurring with *O. cristavarius* to have decreasing worm populations over time and lower reproductive rates than *C. chasmodactylus* co-occurring with other *C. chasmodactylus*. In this case, host diversity may have negative fitness impacts on branchiobdellidans.

## Methods

*Cambarus chasmodactylus* and *C. ingens* were collected from the Middle Fork of the New River in August of 2016. *Orconectes cristavarius* individuals 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 minutes to kill any remaining worms and cocoons. Single *C. chasmodactylus* in the Cc alone treatment and donor *C. chasmodactylus* were each stocked with 4 large and 2 medium *C. ingens* on the dorsal carapace. *Cambarus chasmodactylus* and *O. 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. Treatments were set up as follows: Cc alone contained a single *C. chasmodactylus*, Cc/Cc contained a *C. chasmodactylus* donor with a *C. chasmodactylus* receiver, and Cc/Oc contained a single *C. chasmodactylus* donor with an *O. cristavarius* receiver. Crayfish were matched by size for each row of aquaria to standardize host area and to reduce the risk of cannibalism. These three treatments were placed in a row on wire shelves in separate adjacent aquaria and replicated six times. Locations of treatments within rows and crayfish to treatments were randomized. Lights were set on a 14:10 light/dark schedule and the ambient water temperature was held between 22-23°C during the experiment. Crayfish were fed two shrimp pellets every other day.

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 underneath a dissecting microscope. The dorsal and ventral surface of each crayfish and all appendages were checked for worms and cocoons and their locations were recorded. The experiment was terminated after 34 days due to loss of replicates.

To examine how host composition affected the transfer of symbionts, we used a mixed effects linear model to examine transfer rate of symbionts between hosts with the `lme()` function in the `nlme()` package in the R programming environment (R Core Team 2016). In this model, host composition and time were fixed effects while controlling for the random effect of experimental unit, i.e., tank. Model effects were estimated using the method described in Laird and Ware (1982).

## Results

There were significant effects of treatment and day on total worms remaining in the aquaria as well as a significant treatment x day interaction (Figure 1, Table 1). 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/Oc treatments by day 20, and remained higher for the remainder of the experiment (Figure 1, Table 1). The mean 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 2). The mean number of worms on the *C. chasmodactylus* donors co-occurring with *O. 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 1 and 3). Worms were observed on the *O. cristavarius* receiver on two occasions. The number of worms recovered from the *C. chasmodactylus* receiver



was significantly higher than the number recovered from the *O. cristavarius* receiver (Table 2, Figure 2).

Thirteen of the eighteen *C. chasmodactylus* molted, mostly between days 10 and 20 of the experiment. This is reflected in the decline of the worms in the Cc alone and Cc/Oc 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 crayfish hosts but there was no clear effect of treatment on cocoon numbers (Figure 3).

### **Discussion**

A dilution effect was observed in this experiment. In the higher host diversity treatment (Cc/Oc), worm populations decreased over time while worm populations in the low diversity treatment (Cc/Cc) remained relatively stable. Worms dispersed from the *C. chasmodactylus* donor to the *C. chasmodactylus* receiver in the Cc/Cc treatment and were thereby able to colonize new, competent hosts. Worms were observed briefly on the *O. cristavarius* receiver in the Cc/Oc treatment on two occasions, showing that worms did disperse to this non-competent host but were quickly lost from the system, most likely as a result of host grooming (Farrell, Creed & Brown 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 Cc alone treatment.

Of the eighteen *C. chasmodactylus* participating in this experiment, thirteen 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 process appears to have led to loss of worms in this experiment when the worms did not transfer back to the newly molted crayfish. However, since crayfish often consume most of their shed exoskeleton (Creed, personal

observation), this provided an opportunity for post-molt contact in which worms were sometimes able to regain their positions on their host. The worms appeared to have a higher chance of survival during the molt of their initial host when there were two competent hosts in an aquarium. Since 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 being picked back up by another competent host. In contrast, worm populations in the Cc/Oc treatment and the Cc alone treatment decreased substantially during molting of the *C. chasmodactylus* host. These two treatments exhibited similar reductions in worm populations during host molting, which indicates that there was no benefit for the worms of having a non-competent host present during the donor's molt.

In addition to branchiobdellidan retention, I tracked worm cocoon production over time. I hypothesized that worm populations would have higher growth rates in the Cc/Cc treatment than the Cc/Oc treatment, and that the Cc alone treatment would have reproductive rates somewhere in the middle. However, cocoon production was suppressed prior to the molting period in all treatments and was variable following it. For the majority of our experiment, *C. ingens* exhibited lower than usual reproductive rates (Creed, personal observation). If this experiment is repeated, is run for a longer period and does not involve a molt, clearer trends may be distinguished between treatments as branchiobdellidans should be able to reproduce normally.

If a similar 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 results in a higher abundance of non-competent hosts in a community, branchiobdellidans may be more likely to disperse to a non-competent host on which they cannot survive. This depends partially on whether and how well branchiobdellidans can discriminate between potential hosts.

*Cambarincola ingens* has been shown to preferentially colonize *C. chasmodactylus* over *O. cristavarius* (Brown & Creed 2004), though the mechanisms for this discrimination are unknown. If this system adheres to the negative competence-extinction relationship, the more competent species of crayfish should be more common in less diverse assemblages. In this case, worms would be more likely to encounter competent hosts for dispersal and have higher fitness, even through disturbances like molting. The crayfish hosts could also be negatively affected by the dilution effect through receiving less gill-cleaning benefits from their ectosymbionts if branchiobdellidan abundance declines in communities with higher host diversity.

However, host diversity may also regulate the proliferation of worms on competent hosts, preventing worm densities from reaching parasitic levels. As Brown et al. (2012) found, crayfish-branchiobdellidan relationships can shift from mutualism to parasitism at high ectosymbiont densities. The 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, maintaining mutualistic conditions and preventing parasitism. Branchiobdellidan dispersal is highly variable and condition-dependent (Skelton et al. 2015). Worms decide whether or not to disperse based on host size and intra-symbiont competition for prime microhabitats, factors which are closely linked to their reproductive success (Skelton et al. 2015). On the other hand, if worms are at high densities on their competent hosts, host dilution may alleviate some of the pressure of excessive worm numbers if some worms disperse to other less suitable hosts and are ultimately lost from the system. As seen in this experiment, a competent host alone and a competent host co-occurring with a non-competent 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 crayfish in nature because crayfish regulate worm levels through grooming behavior (Farrell, Creed & Brown 2014).

Variation in diversity and dominance of crayfish throughout the South Fork of the New River create various conditions for crayfish-branchiobdellidan relationships and potential dilution effects. The distribution of crayfish species in the South Fork watershed is related to stream size. In first- and second-order headwater streams, *Cambarus bartonii*, an even more competent host for branchiobdellidans than *C. chasmodactylus*, is the lone species present (Fortino & Creed 2007). Up to six species of branchiobdellidan have been found on *C. bartonii* (Thomas & Creed, unpublished data). In third-order streams, *C. chasmodactylus* tends to be co-dominant with *C. bartonii* and *O. cristavarius*. In the fourth-order South Fork, *O. cristavarius* is the dominant species (Fortino & Creed 2007). *Cambarus chasmodactylus* adults are still common in this fourth order river, but their young-of-the-year are only found in third-order tributaries (Fortino & Creed 2007). There appears to be a gradient of community competence for branchiobdellidans in the South Fork, with the highest community competence in the smallest tributaries and the lowest community competence in the mainstem of the river. With this pattern of host distribution, branchiobdellidans are predicted to be more abundant and have higher species diversity in smaller tributaries of the South Fork where they have more competent hosts to disperse among, especially during disturbances like molting. In the fourth-order river, a dilution effect would be more likely as more non-competent *O. cristavarius* are present than the competent *C. chasmodactylus*. In addition, there is evidence that *O. cristavarius* is expanding its range in the South Fork watershed, moving into third order tributaries (Creed, personal

observation). This range expansion will most likely continue with climate change as the lower-order tributaries get warmer.

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 South Fork fulfills this prerequisite, with *C. bartonii* the most competent, followed by *C. chasmodactylus* and with *O. cristavarius* as the least competent host. Potential dilution effects between *C. chasmodactylus* and *C. bartonii*, as well as all three species occurring together, should be investigated. Second, low-competence hosts can reduce transmission through various mechanisms. This was seen in this experiment when worms were briefly observed on the *O. cristavarius* receiver in the Cc/Oc treatment and were quickly lost from the system, likely due to removal and consumption by *O. cristavarius*. There is a need for more research on how much and by what other potential mechanisms (besides ingestion) *O. cristavarius* reduces the “transmission” of branchiobdellidans. The third prerequisite is that “competent hosts are relatively resilient to local species loss, whereas low-competence hosts are relatively vulnerable and occur mainly in more diverse communities,” (Huang et al. 2013, p. 1076) also known as the negative competence-extinction relationship. The species distribution in the fourth-order South Fork does not fit with this last prerequisite since *O. cristavarius* is the least competent *and* most abundant. The *C. chasmodactylus* present in the South Fork appear to have migrated to this mainstem habitat from the tributaries (Fortino & Creed 2007). This suggests that this habitat, with its abundant non-competent hosts, may be a sink for worms that originated on crayfish hosts in the tributaries.

The dilution effect with respect to mutualisms is a complex community-level relationship that should be researched further in the crayfish-branchiobdellidan mutualism as well as other

mutualistic systems. If higher host diversity leads to higher abundance of non-competent hosts, this may have a negative effect on symbiont populations and, potentially, the fitness of competent hosts. Though there are many ecological benefits of biodiversity, such as mitigating diseases and parasite abundance (Keesing, Holt & Ostfeld 2006, Johnson et al. 2013, Civitello et al. 2015, Huang et al. 2016), it should not be assumed that higher diversity is always better with respect to symbiont abundance.

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Table 1. Results of the mixed effects linear model comparing total worms remaining on crayfish in the following treatments: *Cambarus chasmodactylus* alone (Cc alone), *Cambarus chasmodactylus* housed with another *C. chasmodactylus* (Cc/Cc treatment), and *Cambarus chasmodactylus* housed with an *Orconectes cristavarius* (Cc/Oc treatment). Data analyzed were the total number of worms recovered from all crayfish in each aquarium.

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Factor	F	df	<i>p</i>
Treatment	5.11	2, 10	0.0296
Row	2.29	5, 10	0.1236
Day	106.30	1, 98	0.0001
Treatment X Day	4.48	2, 98	0.0138

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Table 2. Results of the mixed effects linear model comparing worm transfers from *Cambarus chasmodactylus* to another *C. chasmodactylus* in the Cc/Cc treatment compared to an *Orconectes cristavarius* in the Cc/Oc treatment. Data were worms recovered from the receiver crayfish in each of the two treatments.

Factor	Treatment			Day			Treatment X Day		
	F	df	<i>p</i>	F	df	<i>p</i>	F	df	<i>p</i>
Cc/Cc vs Cc/Oc	7.82	1,10	0.019	11.83	1,66	0.001	18.78	1,66	0.0001

## Figure Legends

Figure 1. Mean ( $\pm 1$  S.E.) number of *C. ingens* individuals observed on all crayfish in each treatment over 34 days. The treatments are as follows: Cc Alone - a single *C. chasmodactylus* host stocked with 6 *C. ingens* in an aquarium; Cc/Cc - a *C. chasmodactylus* donor stocked with 6 *C. ingens* sharing an aquarium with a *C. chasmodactylus* receiver (not stocked with worms); and Cc/Oc - a *C. chasmodactylus* donor stocked with 6 worms sharing an aquarium with an *O. cristavarius* receiver. The *C. chasmodactylus* alone and each *C. chasmodactylus* donor were initially stocked with 4 large and 2 medium *C. ingens*.

Figure 2. Mean ( $\pm 1$  S.E.) number of worms on crayfish donors and receivers for both the low diversity (A - Cc/Cc) and high diversity (B - Cc/Oc) treatments compared to the *C. chasmodactylus* alone treatment over a 34-day period. A. The mean number of worms on the *C. chasmodactylus* donor and *C. chasmodactylus* receiver B. The mean number of worms on the *C. chasmodactylus* donor and the *O. cristavarius* receiver.

Figure 3. Mean ( $\pm 1$  S.E.) number of cocoons on *C. chasmodactylus* in all three treatments. No cocoons were recovered from *O. cristavarius*.

Figure 1.

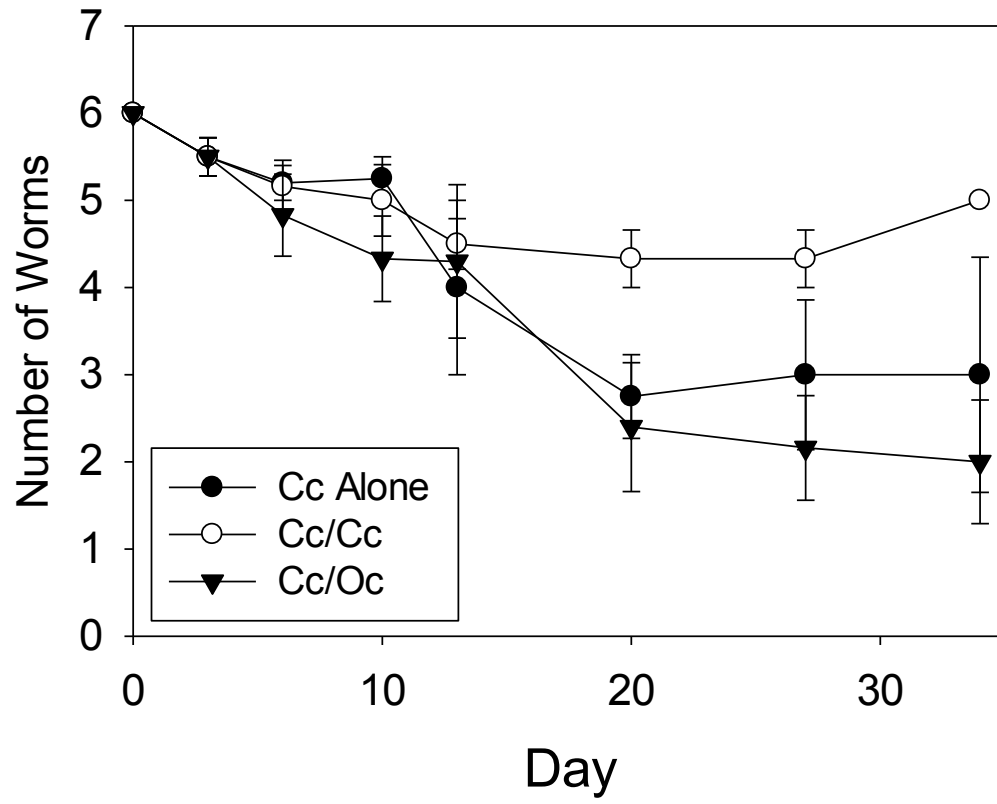


Figure 2.

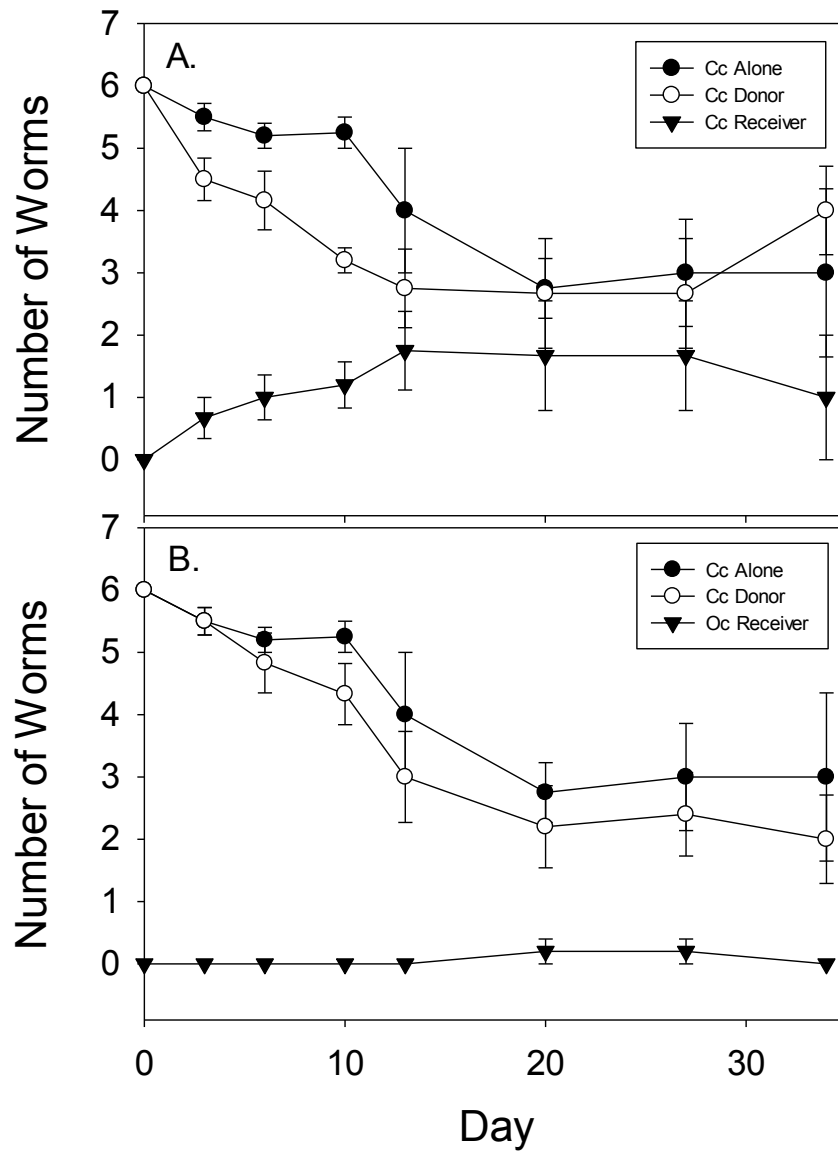


Figure 3.

