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Keegan McCaffrey

University of Colorado Boulder

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Patterns of Multi-Symbiont Community Interactions in California

Freshwater Snails

By

Keegan McCaffrey

Ecology and Evolutionary Biology, University of Colorado at Boulder

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Thesis Advisor:

Dr. Pieter Johnson, Department of Ecology and Evolutionary Biology

Defense Committee:

Dr. Pieter Johnson, Department of Ecology and Evolutionary Biology

Dr. Barbara Demmig-Adams, Department of Ecology and Evolutionary Biology

Dr. Suzanne Nelson, Department of Integrative Physiology

Abstract

Virtually all organisms function as hosts for a variety of symbionts that can interact and form complex communities. Recently, research has begun to highlight the influence that symbiont communities can have on human and animal health through multi-symbiont interactions. Here, we examine symbiont community patterns both by host species and specific symbiont interactions in California freshwater snails. Specifically we explore two questions. First, what are the broad patterns observed among five commonly occurring snail species (*Helisoma trivolvis*, *Physa* spp., *Gyraulus* spp., *Lymnaea columella*, and *Radix auricularia*)? Second, what are the dynamics between the symbiont annelid *Chaetogaster limnaei limnaei* and larval trematode infections? We sampled and necropsied 12,713 snail hosts from Contra Costa, Alameda, and Santa Clara counties in California and found that among wetlands, symbiont communities varied significantly between snail species. The prevalence of symbionts and the richness of symbiont communities were both positively correlated to the abundance of each snail host species across the landscape. Within individual snail hosts, larval trematode infection and *C. l. limnaei* abundance correlated positively, with ~30% more *C. l. limnaei* in trematode infected hosts as compared to uninfected snails. This relationship, however, was variable among trematode species, suggesting that the underlying mechanism of interaction may be a combination of preferred predation by the annelid worm and other less direct interactions. This study presents evidence that links patterns of host availability to symbiont community richness and prevalence as well as potential interactions among symbionts, stressing the importance of considering multi-host and multi-symbiont communities when studying community interactions.

Introduction

Despite a rich history of research and recent exposure by world leaders and the media, global biodiversity decline remains a major concern for the scientific community (Balvanera et al. 2006, Carpenter et al. 2009). Changes in biodiversity affect a variety of ecosystem processes, such as nutrient cycling and disease transmission which, in turn, influence social issues like food security and human and animal health (Butchart et al. 2010, Cardinale et al. 2012). As human-catalyzed biodiversity loss continues to alter ecosystems, several biases within our knowledge of biodiversity have emerged (Hortal et al. 2008). The Millennium Ecosystem Assessment (2005) underlined that, historically, most efforts to catalogue the variety of life on earth have been focused heavily on plants, mammals, birds, and reptiles. The richness and diversity of other organisms like fungi, bacteria, insects, and flatworms (helminthes), remains largely unknown. One possible reason for this lack of clarity is that many of these organisms live as symbionts: organisms that utilize another organism as a host. This use of a host causes symbionts to be more difficult to observe than free-living species. The task of classifying symbiont biodiversity is daunting; by some predictions there are 300,000 species of vertebrate parasites (a type of symbiont) alone (Dobson et al. 2008) and others estimate that about 50% of all species are parasitic (Toft, 1986). Not only is symbiont diversity poorly understood, we are just beginning to understand the importance of symbiont interactions within hosts.

The consequences of symbiont biodiversity loss will largely depend on what ecological interactions are also lost when a symbiont species goes extinct. Symbionts are well known for interacting with their hosts, but can also interact with other symbionts and free-living organisms in an ecosystem (Bush and Holmes 1986, Fernandez et al. 1991, Ibrahim 2007, Mieog et al. 2009). Recently, there has been a growing appreciation for the importance of symbiont

interactions within an individual host and how these interactions form unique symbiont communities (Dale and Moran 2006). These communities tend to be complex because multiple symbionts often inhabit a single host, proximity and shared reliance on that host is a potential catalyst for multi-symbiont interactions. Associations within a symbiont community can have important effects on host disease patterns, like virulence (severity) and transmission (communicability) (Graham 2008, Johnson et al. 2012). For example, Rodgers et al. (2005) experimentally demonstrated a decrease in the prevalence (commonness) of *Schistosoma mansoni* in aquatic snails (*Biomphalaria glabrata*) when the latter were co-infected with the commensal annelid *Chaetogaster limnaei limnaei*. This small-scale interaction has potential for real-world consequences on human health because *S. mansoni* infects more than 80 million people worldwide (Crompton 1999), causing intestinal schistosomiasis, which is known for its symptoms of anemia, malnutrition, and learning disabilities (King 2005). Thus far, most studies focus on interactions between two or, at most, three specific symbionts, and rarely have complete inventory of symbiont organisms living within a studies' host species.

It is inherently difficult to draw conclusions about symbiont communities because there are a number of variables that structure community interactions (Johnson and Buller 2011). Symbionts determined to be correlated through observation could be interacting in three ways. First, directly within a host through predation or mechanical facilitation (Bandilla et al. 2006). Second, indirectly within a host by competing for limited host resources (Ishii et al. 2002, Hardin 1960), or by altering host immune responses (Su et al. 2005). Third, indirectly through site level co-existence by altering host behavior (Daly and Johnson 2010). Some of the most thoroughly explored symbiont-interaction studies focus on parasite antagonism in where the presence of one parasite reduces the success of another. In the same system described above, Sandland et al.

(2007) demonstrated that co-infection of *S. mansoni*-positive snails with *Echinostoma caproni* (a complex-lifecycle trematode) reduced pathology and prevalence within the snail host as compared to snails solely infected with *S. mansoni*. However, two parasite-in-host interactions may be an oversimplification of what occurs at the symbiont community level because it's likely that one or more such interactions are co-occurring within any given symbiont community (Pedersen and Fenton 2007). Looking at symbiont interactions from this more broad perspective have led to interesting insights on how symbionts can influence host disease. One major hypothesis linking biodiversity to disease is the 'dilution effect' which proposes that an increase in parasite richness (number of different parasites species) will increase cross-parasitic species competition. This, in turn, reduces the success of the most virulent parasites. Johnson and Hoverman (2012), using lab and field data, demonstrated an intra-host dilution effect by showing that an increase in parasite richness was correlated with a decrease in overall infection success, including infection by the most virulent parasites. For these reason it is crucial to include all organisms inhabiting a studies' host when studying symbiont communities.

Freshwater pond snails are an excellent system for studying symbiont interactions at both individual and community levels. Aquatic gastropods serve as obligatory first hosts for a range of trematode parasites and are considered a keystone species to freshwater ecosystems (Esch, Curtis, and Barger 2001). The trematode lifecycle begins as the parasite egg enters water and a miracidium hatches to find a mollusk host. Upon infection, trematode rediae or sporocysts develop in the snail host's gonad, causing pathology that can lead to castration and even death of the host (Sousa 1983). The trematode then produces motile cercariae, which swim through the water column looking for a second intermediate host that can be, depending on the trematode, a variety of fishes, amphibians, or invertebrates. The final stage of the trematode lifecycle occurs

when the second intermediate host is consumed by a vertebrate definitive host, where the adult parasite develops and reproduces. Because trematodes have long been a focus in parasitology, extensive identification manuals are readily available (see Yamaguti 1971 or Schell 1985). Additionally, lentic ecosystems are well delineated from one another, facilitating sampling of multiple replicate ponds across a landscape.

The snails featured in this study have long been known to host a variety of other symbiotic organisms, one example is *C. l. limnaei*. These annelid worms, which live under the mantle, can then feed on various small organisms like rotifers, algae, and trematode cercariae. With these feeding habits in mind, *C. l. limnaei* offers an interesting case study for symbiont interactions because of its high probability of interacting directly with other symbionts. Other known snail symbionts include insect larvae (Chironomidae), the parasitic nematode *Daubaylia potomaca*, and the leech *Helobdella punctato-lineata* (Hugh 1971, Prat et al. 2004). One study by Zimmermen et al. (2011b) examined the relationship between *C. l. limnaei*, trematode infection, and *D. potomaca*. From their data, the authors postulated that *C. l. limnaei* indirectly increases nematode presence by down-regulating the presence of trematode infections, which were negatively associated with the nematode through apparent competition. While this study highlights interesting patterns derived from snails in a single pond, other organisms or interactions may be at play in the mechanisms underlying these relationships.

Given the need to better understand both symbiont community diversity and potential interactions among symbionts, we analyzed data collected through comprehensive necropsies of field-caught host specimens. This study asks two specific questions. First (Aim 1), how do symbiont communities differ among freshwater snail species? We address this question by testing whether symbiont community richness and total symbiont prevalence varied among snail

species which, based on past research, might differ by host body size, local host abundance or life history characteristic (Blower and Roughgarden 1988). Second (Aim 2), what patterns of co-occurrence are observable between trematode infections and populations of the symbiont annelid *C. l. limnaei* in *Helisoma trivolvis* snail hosts? We choose to examine this specific example because trematodes and *C. l. limnaei* were the most common symbionts observed in *H. trivolvis*, which was the most common snail host at our field sites. Additionally, *C. l. limnaei* predation upon trematode cercariae has been suggested as a potential device against human and animal disease (Michelson 1964, Fried et al. 2008, and Ibrahim 2006, 2007). However, these studies have been limited to single pond systems and rarely look at their relationship at a regional level or assessed the patterns of co-occurrence among multiple species of trematodes.

Materials and Methods

Field Surveys

From May to August 2013, snails were collected from 101 freshwater systems across three counties in California: Contra Costa, Alameda, and Santa Clara. The majority of these freshwater systems are artificial ponds located on public lands. Each wetland was sampled two times over the summer to account for seasonality of parasite infections, with the first round of sampling occurring from 9 May to 3 July, 2013. During these dates, we completed ten haphazard dip net collections around the perimeter of each wetland to assess pond biodiversity and collect 50 individuals from each snail species present. All organisms caught in the dip nets were identified, quantified, and snails were stored in chilled water for necropsy at a later date. In ponds with small snail populations collecting 50 individuals from each snail species was not possible using only the standardized dip net sweeps so we allocated an additional three person-

hours per site to maximize the number of snails collected. To prevent disease and symbiont transfer between ponds all equipment was soaked in 10% bleach after each pond visit.

The second round of sampling occurred from 7 July to 13 August, 2013. Again, 50 snails from each species at a site were collected by dip net for symbiont community assessment. Due to normal environmental conditions in California, during this round some sample sites went dry and were excluded from the sampling routine. During both rounds of sampling, *H. trivolvis* snails greater than five millimeters in shell length were preferentially collected because trematodes are highly unlikely to infect snails smaller than this critical value (Richgels, unpublished) and smaller snails were therefore excluded from the data.

Snail Necropsies

Snail processing and necropsies for both sampling events occurred at Blue Oak Ranch Reserve in San Jose, California. Snail processing methods followed procedures outlined in Richgels et al. (2013). First, all snail species from each site were stored separately upon returning from the field. Then all *H. trivolvis* snails were checked for infections via “shedding”. The shedding process began by placing individual *H. trivolvis* into 40-ml centrifuge tubes with about 30 ml of store-bought artisan well water. The snails were left in their tubes for 24 hrs and checked every 12 hrs for infection by visually examining the water column for released cercariae. If snails were infected, the released cercariae were identified by key morphological characters established by Yamaguti (1971) and Schell (1985), as observed through an Olympus compound microscope (Olympus Corporation, Tokyo). Infected snails were then inspected visually for presence of *C. l. limnaei* and other organisms co-inhabiting the snails. All *H. trivolvis* individuals that did not shed cercariae, along with all other species of snails collected for each site, were necropsied on at our field-station. Necropsies consisted of lightly cracking the

outer shell with pliers and teasing apart the tissue to examine visually for trematodes and other endosymbionts using an Olympus stereomicroscope. Parasitic infection was identified using methods described above and trematode tissue was vouchered in 70% ethanol for further genetic analysis. Prior to necropsy, snails were measured using Fisher Scientific digital calipers. All other symbionts found living in or on the snails were identified and quantified. Because of their high concentration within some snail hosts, *C. l. limnaei* were counted by scraping under the hood of the snail with forceps into a necropsy tray and then counted under the stereomicroscope.

Analysis

We analyzed host species effect on symbiont communities by examining patterns of average site-level symbiont prevalence (aggregated across symbionts) and community richness. We first used linear regressions to examine these as a function of snail host species relative abundance (percentage of sites that supported each snail species). We then ran the same models again, but switched the predictor variable to average snail size (mm, calculated for the species as a whole across all sites) to assess whether patterns were driven by snail size or snail relative abundance.

To test whether trematode infection was related to the presence of *C. l. limnaei* in *H. trivolvis* snails, we first used a generalized linear model with trematode site-level presence (binary) as a binomial response with *C. l. limnaei* presence (binary) and snail size as fixed effects. We narrowed this analysis to sites with at least 25 snails necropsied to account for sample size biases. This analysis explored whether sites that supported trematode infection were also more or less likely to support *C. l. limnaei* at the site-level. Next, among wetlands that supported the annelid, we further examined the relationship between the *C. l. limnaei* and trematode infection on the individual snail level. Here, we constructed a generalized linear mixed

effects model using the glmmADMB (R Core Development team 2008) package with *C. l. limnaei* count (intensity) as a negative binomial response variable as a function of trematode infection (binary) and snail size fixed effects. To account for non-independence of snails from the same wetland, we nested snails within sites as a random factor in this model. To help us decide whether to leave snail size in our model as a fixed effect, Akaike's information criterion (AIC) was used to select for models with the best fit for our data (see Zuur et al. 2009). By using the lowest possible AIC score we were able to ensure that our model was of the best quality between the complexity and goodness of fit in competing model scenarios.

Results

Aim 1: Snail Symbiont Communities

We sampled 101 snail-inhabited freshwater sites in the California Bay Area. Within these sites, we examined a total of 12,713 individual snails of the species *Helisoma trivolvis*, *Physa* spp., *Gyraulus* spp., *Lymnaea columella*, and *Radix auricularia*. Of these hosts, *H. trivolvis* was the most common species across the landscape, occurring in a total of 85 wetlands, from which we collected 5,579 individual specimens, followed closely by *Physa* spp. (multiple species suspected), which occurred in 80 of our 101 sites and 5,249 individuals collected. We observed a large decrease in occurrence between the former two species and our next three snail species *Gyraulus* spp., *L. columella*, and *R. auricularia*, which were observed at 26, 19, and 11 sites respectively (Fig. 1).

In total, we found 23 different taxonomic groups of symbionts, which represented larval trematodes, annelids, fungi, larval insects, nematodes, and hirudineas (leeches). The term 'taxonomic groups' is used here because there is a sizeable amount of unknown in determining trematode species visually, and thus our actual diversity of species is likely higher. Eleven of

these symbiont groups appeared to be generalists, occurring in all or most snail species. Examples of generalists included *C. l. limnaei*, digenetic trematodes in the echinostome complex (which include species in the genera *Echinostoma* and *Echinoparaphyrium*), and fungal infections. Concurrently, we found 12 symbionts that occurred predominantly in a single snail species. For example, *H. trivolvis*-specific symbionts included the larval trematodes *Ribeiroia ondatrae* and *Clinostomum* spp. *Helisoma trivolvis*, our most common snail, also had the greatest diversity of observed symbionts with 20 unique taxonomic groups. The frequency at which a snail species occurred across the landscape (relative species abundance) correlated positively with both average symbiont prevalence within host snails (Fig 2A: $r = 0.889$, $P < 0.05$) and symbiont richness within sites (Fig. 2B: $r = 0.961$, $P < 0.01$). For instance, the most common two snails had communities consisting of, on average, about 3.9 symbiont taxonomic groups per site (richness), whereas the least common snails had an average site symbiont community richness of ~ 1.2 . We did not find a similar trend when our predictor variable was altered to average snail host species size on symbiont prevalence or community richness (Fig. 3: $r = -0.014$, $P > 0.5$; $r = 0.1709$, $P > 0.5$, respectively).

Aim 2: Interactions between *C. l. limnaei* and larval trematodes in *H. trivolvis*

Among 89 sites with *Helisoma trivolvis*, we found no significant relationship between the presence of trematode infection (any species) and the presence of *C. l. limnaei* when sample size was taken into account (GLMM, $z = 0.570$, $P > 0.50$). On the other hand, within *H. trivolvis* snails that supported the annelid, the intensity of *C. l. limnaei* per snail was positively related to whether that snail was also infected with a trematode (GLMM, $z = 3.45$, $P < 0.001$, $n = 2235$). Generally, snails that harbored a trematode infection supported $\sim 30\%$ more *C. l. limnaei* (Fig. 5). When we examined this result by individual trematode species, *Halipegus occidualis* and *R.*

ondatrae infections both correlated positively with the number of *C.l. limnaei* per snail (Fig. 6: GLMM, $z = 4.5$, $P \ll 0.001$; $z = 3.65$, $P < 0.01$, respectively), whereas trematodes in the echinostome complex, *Clinostomum* spp., *Alaria* spp., *Allassostomoides* spp., *Cephalogonimus* spp. and *Schistosoma* spp. showed no significant relationship. Snail size (mm) was also a consistently positive predictor of *C.l. limnaei* count ($z = 13.16$, $P \ll 0.001$).

Discussion

Effectively all organisms function as hosts for an assortment of symbionts. Within hosts, symbionts interact and create ecological communities that can have important influences both on other symbionts and hosts through health and fitness (Bush and Holmes 1986, Graham 2008). Here, we explored patterns of symbiont occurrence in a naturally occurring multi-host and multi-symbiont system. The results of this study suggest that symbiont communities are structured both by their hosts and by other symbiont species in real-world systems.

Our first aim was to examine the differences in symbiont communities between snail species observed in California wetlands. Among these sites we found *H. trivolvis*, *Physa* spp., *Gyraulus* spp., *L. columella*, and *R. auricularia*. Of these hosts, both *L. columella* and *R. auricularia* are non-native species while the other, more common snails, are native to California (Cordeiro and Bogan 2012). We found, in total, 23 different taxonomic groups of symbionts, 15 of which were larval trematodes. Many symbionts in this study likely parasitize their snail hosts. For example, the leech *Helobdella punctato-lineata* is a known snail parasite, often seen engorged with the red blood of *H. trivolvis*, but is believed to also prey on other invertebrates (Hugh 1971). Studies on similar snail leeches have shown the leeches are able to kill up to three snails a day and have been suggested as a mitigation tool against trematode disease (Aditya and Raut 2005). Other known parasites included trematode infections, the nematode *Daubaylia*

potomaca (Zimmerman et al. 2011a), and fungal pathogens. In contrast, symbionts such as insect larvae (Chironomidae) and the annelids *C. l. limnaei* and *Tubifex Tubifex*, have a slightly less identifiable relationship with their snail hosts. Chironomidae larvae, which were observed inhabiting the exterior snail shells, have been suggested to use the snails as a means of transportation (Prat et al. 2004). *Chaetogaster limnaei limnaei*, found living on the mantle and under the hood of snails, can have a positive effect on snail health (Michelson 1964, Rodgers et al. 2005). Conversely, high numbers of *C. l. limnaei* have also been correlated to lower host reproduction and growth (Stoll et al. 2013).

We found strong trends linking snail species to attributes of symbiont communities that they hosted. Specifically, the relative abundance of snail host species was strongly correlated to the average symbiont community richness and prevalence. In contrast, symbiont richness and abundance were unrelated to the average size of each snail species, an indication that this relationship is not being driven by life history characteristics of the host snail species. We tested this alternative hypothesis because snail size has previously been linked trematode presence (Blower and Roughgarden 1988), potentially as a proxy for age of the host. Additionally, both *H. trivolvis* and *R. auricularia* have multiyear life spans and occupy opposite ends of our symbiont community richness and prevalence spectrum (Eversole 1978; Cordeiro and Bogan 2012), further leading us to reject host life history as the main driver of these findings. We postulate two possible reasons for the positive relationship between the abundance of the host snails and their symbiont communities: local adaptation and more symbiont habitat. Local adaptation could drive higher symbiont community prevalence and richness by allowing symbionts to preferentially adapt and specialize to the most common snail hosts across the landscape. This would be a beneficial evolutionary strategy for symbionts, especially if they are parasitic, because they

would have the best chance of finding a host in any given site. Moreover, by specializing symbionts could overwhelm the snail hosts ability to selectively adapt against any one organism, similar to the Red Queen Effect (Van Valen 1973, Toft and Karter 1990). This hypothesis is also supported by the relatively low prevalence and richness of symbionts found in the two invasive species *L. columella*, and *R. auricularia*, to which native symbionts have likely had less time to adapt. An alternative explanation is that the most abundant snail species offer the most available habitat for symbionts to colonize. We can think of this explanation as similar to the ‘species-area curve’ (Preston 1962) used widely in island and patch ecology. The concept behind species-area curves is that as habitat area increases so does the number of species that are able to colonize that particular piece of habitat.

For our second aim, we focused on potential interactions between *C. l. limnaei* and larval trematodes in *H. trivolvis*. We found no relationship between the annelid and trematode presence at the site-level, for which these groups occurred in 53% and 81% of sampled populations respectively. This suggests that there is no effect of *C. l. limnaei* on the colonization of larval trematodes at the site level, which is perhaps unsurprising given the overall ubiquity of the latter group. Conversely, examining the data from an individual host perspective, we found a positive association between the intensity of *C. l. limnaei* in a snail and whether that host was also infected by larval trematodes. This relationship, however, varied by trematode species. Our model specifically highlighted *H. occidualis* and *R. ondatrae* as being positively associated with the intensity of *C. l. limnaei* in a given host.

One likely explanation for the presence of *H. occidualis* infection being such a strong positive predictor of *C. l. limnaei* intensity is because *H. occidualis* is particularly susceptible to predation by the annelid. This idea was first postulated by Fernandez et al. (1991) who observed

a similar relationship in a single-pond field study in North Carolina, USA. *Halipegus occidualis* produces considerably smaller and less motile cercariae than other trematodes observed, and *C.l. limnaei*, being a gape limited predator, appears to respond positively. The annelid could be responding to this easy meal by either increasing reproduction or colonization. *Ribeiroia ondatrae*, on the other hand, has a relatively large and motile cercariae and, although still possible, is less likely to be a predatory favorite of *C.l. limnaei*. Other possible explanations for *R. ondatrae*'s significantly positive correlation to *C.l. limnaei* infestation intensity could lie in indirect effects between the parasite, annelid, host, and environment. Such effects could manifest as altered host behavior, immune response, or interactions with other organisms. Another reason for variation among trematode species may be sample size, some trematodes like *Clinostomum* spp., which was observed only 12 times in *C.l. limnaei* infested snails, may be susceptible to type two statistical error. Additionally, because our model used snail size as a covariate, it should be noted that these relationship are not likely driven by host size, i.e. large snails being more likely to be infected with trematodes and host more *C.l. limnaei*.

The study adds to the growing body of evidence that symbionts, both parasitic and benign, interact within hosts to form complex communities. We find strong differences among symbiont communities related to their snail host species' abundance across a landscape and not body size. In addition, we provide field-based evidence for a relationship between trematode parasites and *C.l. limnaei*. We found this relationship to be somewhat variable by trematode species and that it is not likely to be driven by colonization. This finding is consistent with the idea that *C.l. limnaei* may prey upon trematodes proposed by Fernandez et al. (1991). However, unlike previous studies, the vast scope of this research suggests that this relationship is even relevant at large spatial scales. As is the difficulty with all field studies, the mechanisms of

interaction between host, community, and individual symbiont are difficult to tease apart.

Consequently, there is a great need for controlled lab experimentation to help elucidate the nature of symbiont community interactions (Pedersen and Fenton, 2006). Through the dedicated study of symbiont communities there is great potential to better understand the biodiversity of an obscure group of organisms, how they interact, and their implications for host health.

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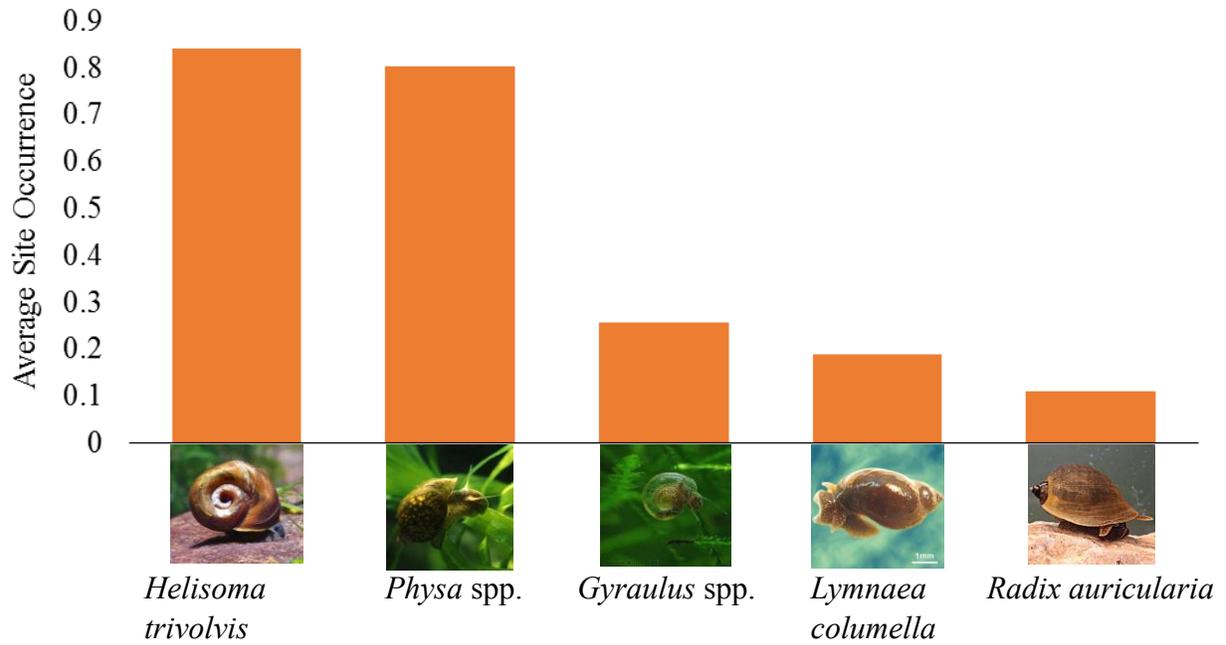


Fig. 1. Average abundance of snail host species across sampled sites. Bars represent total number of sites at which each species occurred divided by the total number of sites sampled.

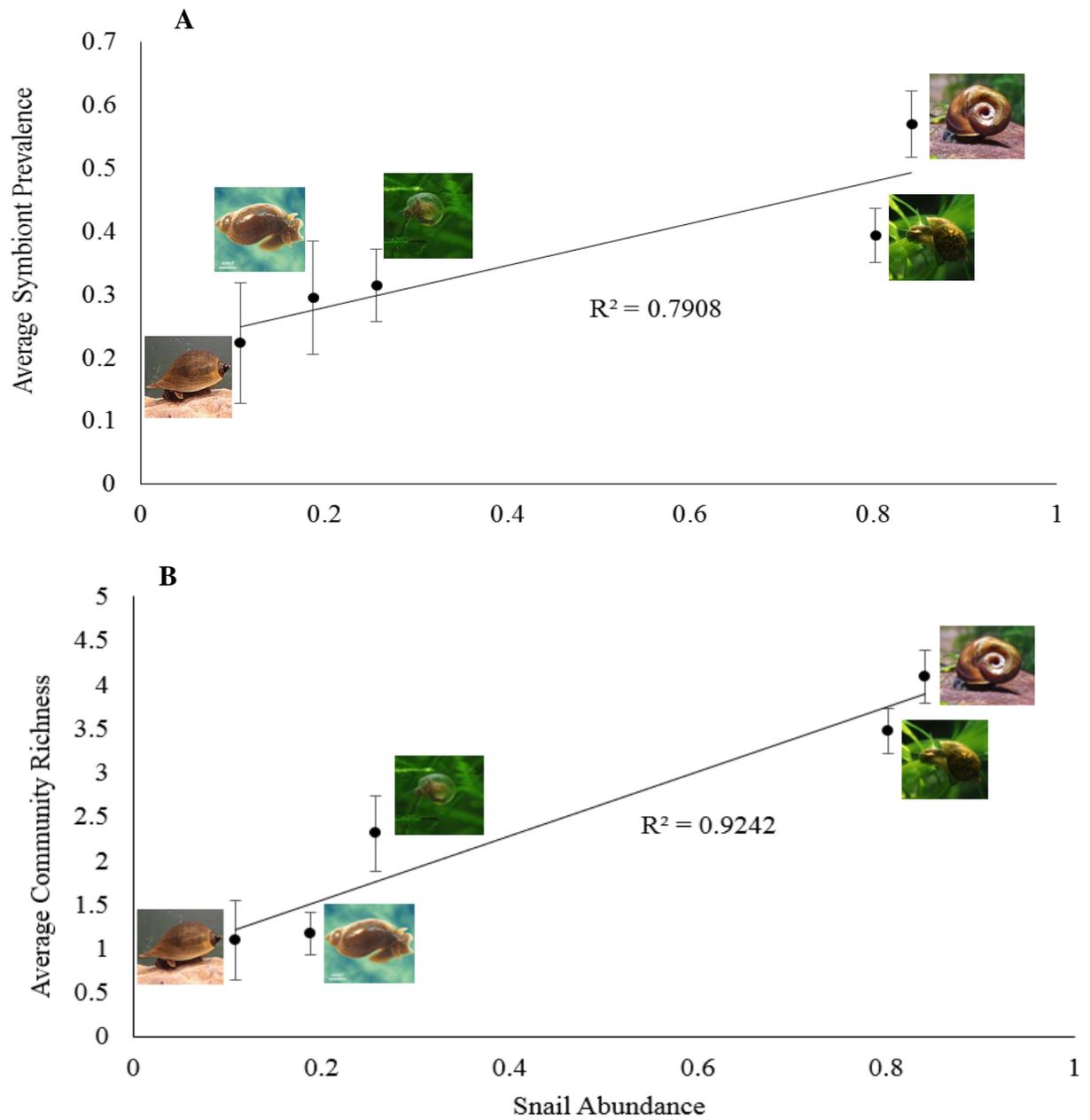


Fig. 2. Average per snail host site-level (A) symbiont prevalence (B) symbiont community richness compared to average host abundance across sites. Error bars are standard error.

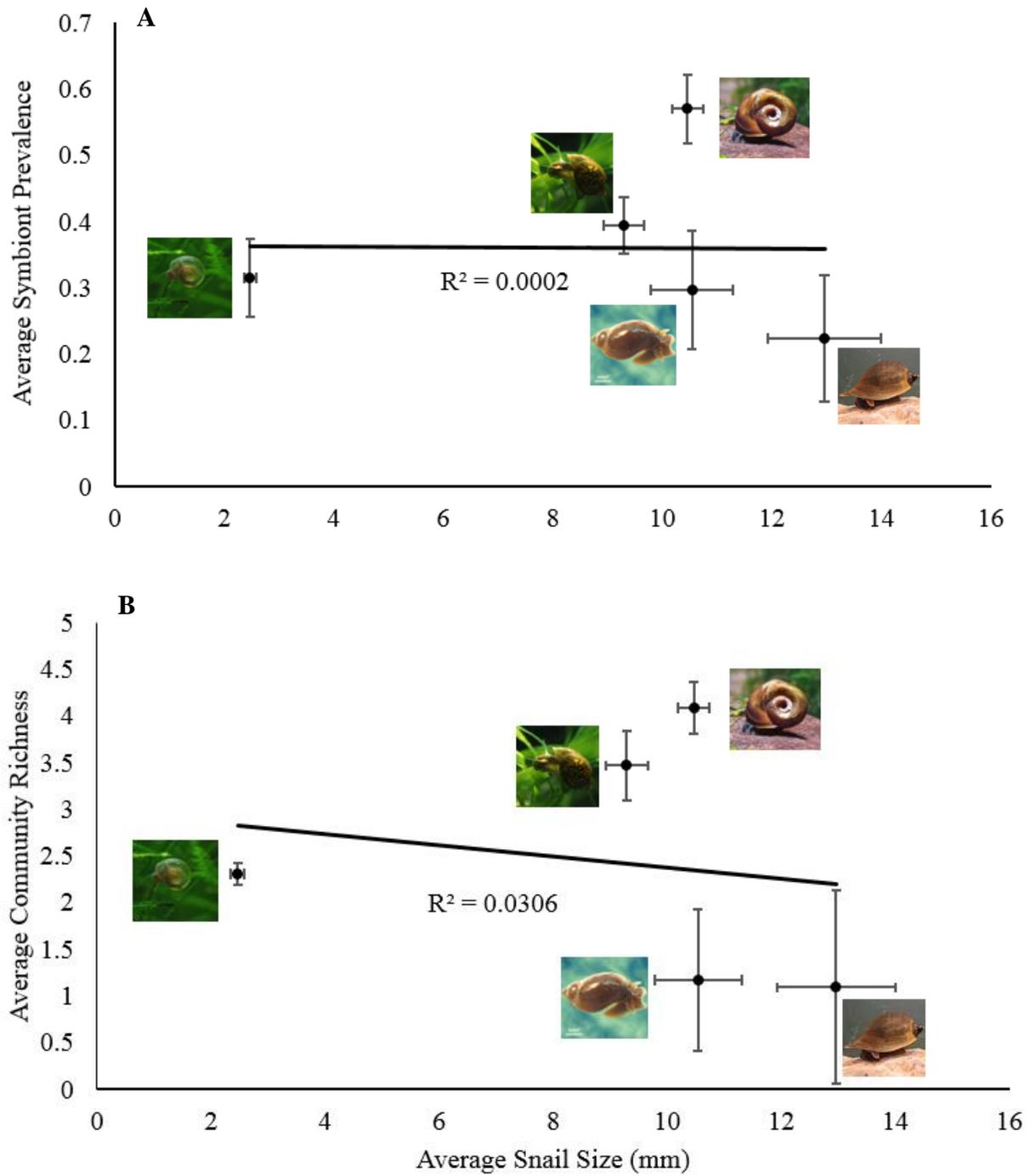


Fig. 3. Average per snail host site-level (A) symbiont prevalence (B) symbiont community richness compared to average host snail size (mm). Error bars are standard error.

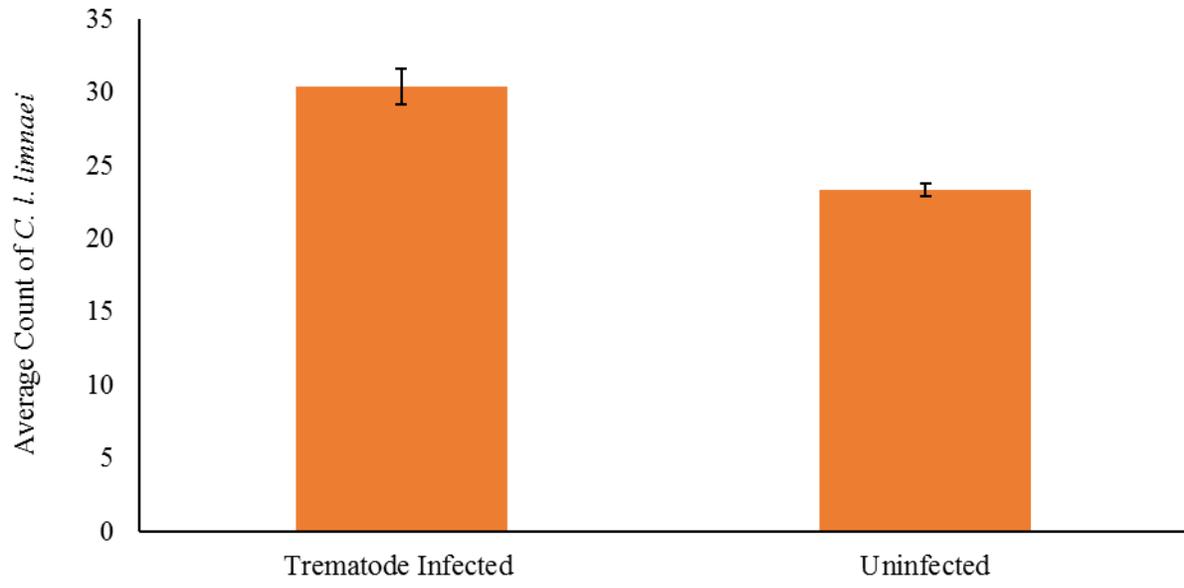


Fig 4. Average effect of host trematode infection status (binary, all species) on *C.l. limnaei* infestation intensity (count of *C.l. limnaei* individuals) in *Helisoma trivolvis*. Bars represent means and error bars are standard error.

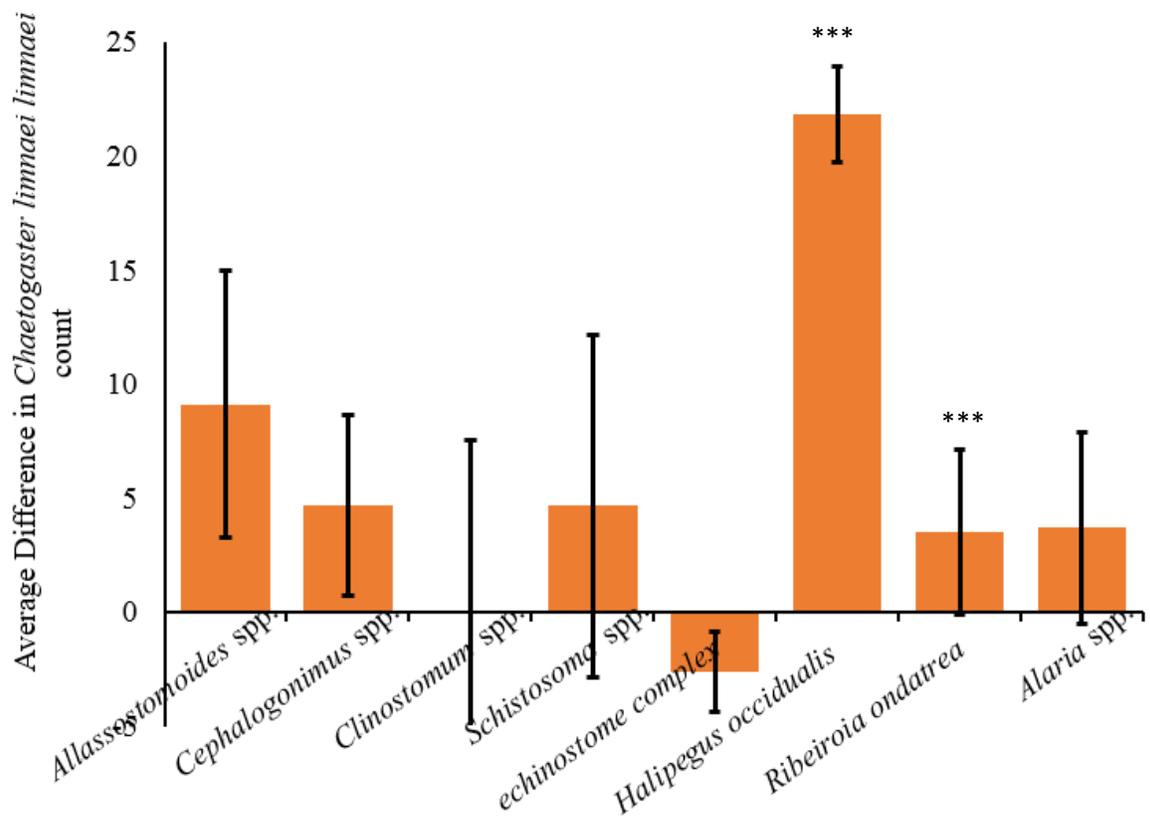


Fig 5. Difference between average overall *C. l. limnaei* intensity in *Helisoma trivolvis* and average intensity in trematode infected snails by trematode species (categorical). (***) Represents statistical significance after controlling for snail host size and random site effects.

