Modification of host behavior and transmission in the acanthocephalan Acanthocephalus dirus: effects of development, intraspecific conflict, and host sex

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Modification of host behavior and transmission in the acanthocephalan *Acanthocephalus dirus*: effects of development, intraspecific conflict, and host sex

A Thesis Presented in Partial Fulfillment of the Requirements for the Degree

of Master of Science

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ABSTRACT

Parasites are organisms that live on or in another in order to survive. In some cases, parasites require more than one host to complete their life cycle and rely on a predation event for transmission to the next host. Inside the host, the parasite must access host resources to grow and develop from the non-infective to infective stages. At the infective stage, the parasite is able to survive within the definitive host. Development to this stage has been correlated with changes in antipredatory behaviors, body size and color, and reproduction of intermediate hosts in ways that may increase predation by definitive hosts. However, these changes may not always be adaptive manipulation by the parasite, and may be the result of pathological responses to infection, or a counter-adaptation of the host. If host modification is adaptive for the parasite, then the timing of these changes should occur when the parasite is capable of surviving in the definitive host.

Acanthocephalan parasites have been commonly associated with changes in host phenotype after infection. Early summer, the parasite *Acanthocephalus dirus* infects the stream-dwelling isopod *Caecidotea intermedius* as its intermediate host. Throughout the fall and winter, the parasite develops from the non-infective (acanthella) to the infective stage (cystacanth) inside the isopod. During this time, changes in isopod refuge use behavior, activity, body color, and mating responses have been associated with infection by *A. dirus*. In the spring, parasites are then transmitted through predation to definitive hosts, green sunfish (*Lepomis cyanellus*) and creek chub (*Semotilus atromaculatus*), where they can complete their life cycle. The relative synchrony of this system may give insight into host manipulation as an adaptive strategy for parasites.

This was one of few studies that evaluated the relationship between *A. dirus* and its hosts using a field-based approach. The isopod and fish surveys were conducted every month for 12
months, between 2016 and 2017. Isopods were collected from a stream in Lake County, Illinois, and information about refuge use, host sex, body size, and infection status were recorded. Individuals of *A. dirus* were recovered from isopods and measured, sexed, and developmental stage was determined. Fish hosts were collected using seine nets, and were later measured and examined for parasites. Isopod and parasite data were later combined with data from previous surveys.

I examined pre-existing differences in refuge use behavior by comparing refuge use in infected and uninfected isopods during early stages of infection. I found that pre-existing differences were present but they were unlikely to influence behavior patterns seen during late-stage infection.

I assessed the relationship of parasite development as a predictor of refuge use, and found that the pattern of refuge use modification was sex-specific. In males, parasite development was related to changes in refuge use behavior, which indicated that development to the cystacanth stage was associated with timing of modification. Thus, changes in refuge use appeared to be adaptive manipulation by parasites in male hosts. In females, parasite development was not related to changes in refuge use behavior, which indicated that development to the cystacanth stage was not related to changes in refuge use behavior. It is possible that size constraints present only in female isopods affect the timing of refuge use modification, so that parasite development is indirectly related to changes in host behavior. However, this may still be explained by other hypotheses.

One potential explanation for sex-specific differences is that female isopods are naturally smaller than males and may allocate energy towards reproduction over growth. Thus, parasites within females may be more constrained than in male hosts and may need to modify host size
before growth and development can occur. Consistent with this explanation, I found that infected females were larger than uninfected females, and this was not due to pre-existing differences in size. I also found that infected males were larger than uninfected males, but this may be explained by pre-existing differences in size. However, analysis of parasite growth showed no effect of host sex on parasite body size.

I also examined the potential for conflict between infective and non-infective parasites within hosts, and this was also sex-specific during the time period examined. In females, there was no difference in the level of refuge use behavior between acanthellae-only and cystacanth-only infections, and thus, there was no potential for conflict. In males, there was a potential for conflict, and mixed-stage infections indicated that non-infective parasites may sabotage manipulative effects of mature parasites when sharing a host.

Lastly, I used measures of behavior modification and prevalence of parasites in fish hosts to determine the overall pattern of modification and transmission. I found that behavior modification was highest in the spring, and that the onset of these changes coincided with the occurrence of parasites in fish hosts. The results indicated that behavior modification is likely an effective strategy for transmission. Further analysis showed that patterns of transmission were also sex-specific. It is likely that male isopods provide conditions for parasites that are unconstrained in energy and space available for growth, and are transmitted to fish sooner in the year relative to females. Thus, parasites infecting fish during the fall months likely originated from a male isopod, and are more likely to have higher fitness payoffs than parasites in females.
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INTRODUCTION

Parasitism is a common relationship in nature where one organism lives on or inside another and benefits from using host resources. Parasites can be found in every ecosystem and as a consequence, every organism is susceptible to infection by a parasite at some stage of its development. The effects of parasites on host physiology, morphology, and behavior has been well documented at the individual level. However, parasites have also been shown to have effects at the population (e.g., reproductive rates; Dobson 1988), community (e.g., predator-prey dynamics; Lafferty 1999), and ecosystem levels (e.g., nutrient cycling; Thomas et al. 1999). The ubiquitous presence of parasites within and across ecosystems allows scientists to answer questions about the natural world through ecological and evolutionary lenses.

All parasites require a host to survive, but differ in the number of hosts and methods of transmission. Parasites may have direct or indirect life cycles. Direct life cycles involve infection of only one host and are typically spread to individuals of the same species (Dobson 1988). In contrast, indirect life cycles require at least two hosts (intermediate and definitive), and in many cases, the parasite relies on a predation event for transmission between hosts (Dobson 1988; Moore 2002). Trophic transmission is a common form of indirect life cycle where the different hosts play important roles at certain stages of development for the parasite (Dobson 1988). In the intermediate host, the parasite will grow and develop necessary structures for successful attachment and reproduction in the definitive host. After the parasite matures and prepares for transmission, the intermediate host may undergo changes in behavior (e.g., mating, taxis, refuge use), physiology (e.g., body color, castration), and other traits (Moore 2002). This phenomenon, known as host modification, has been especially well documented in systems that rely on trophic transmission (e.g., trematodes, Mouritsen 2002; acanthocephalans, Bethel and Holmes 1973) but
has been demonstrated in numerous other host-parasite relationships (Moore 2002; Thomas et al. 2005; Poulin 2010).

The nature of host modification differs across transmission strategies. One example occurs among parasites (parasitoids) that modify their hosts to protect the parasite during its vulnerable pupa stage until they can exit the host (Poulin 2010). Parasites may also modify host behaviors to move hosts to a different habitat. In some cases, this strategy involves hosts relocating to habitats that are unsuitable for themselves but are necessary for parasite reproduction and survival (e.g., terrestrial crickets jumping into water; Thomas et al. 2002b). Hosts can also be modified to increase the likelihood of infecting other individuals. This strategy is most notably carried out by mosquitoes infected by malaria-inducing vector-borne parasites where the parasites are then transmitted when the mosquito feeds on vertebrate hosts (De Moraes et al. 2014). Many other examples include changes in body color (Camp and Huizinga 1979), burrowing behavior (Mouritsen and Poulin 2003), activity (Park and Sparkes 2017), mating success (Sparkes et al. 2004), phototaxis (Bethel and Holmes 1973), and refuge use (Hechtel et al. 1993). All of these examples have been proposed to increase conspicuousness and predation of the intermediate host, and thus transmission of the parasite. In many cases, more than one trait is modified (Benesh et al. 2008; Thomas et al. 2010). These traits together, if correlated, have been proposed to increase conspicuousness of intermediate host more efficiently than a single trait alone. However, a recent study suggests that traits may be modified independently and in a sex-specific manner (Park and Sparkes 2017).

Historically, changes in host phenotype were often considered to be due to adaptive manipulation by the parasite, but other explanations have been proposed since the 1980s and require careful consideration (Poulin 1995, 2010; Thomas et al. 2005). Observed changes may be
a result of: 1) true modification of host phenotype by the parasite for the purpose of increasing parasite fitness, as described above (Poulin 1995, Moore 2002); 2) phenotypic changes that result from a pathological response to infection (e.g., Helluy 2013); or 3) host adaptations that counter infection by parasites (Hart 1990).

It is reasonable to expect that a sick animal will not behave like one that is healthy (Moore 2013). Modification of host phenotype has been associated with interference of host neurochemical pathways, in ways that do not benefit the host or parasite (Moore 2013). Disruption of these pathways may induce sickness behaviors, such as lethargy, while host immune system responds to infection (Dantzer 2001). Thus, differences in behavior between infected and uninfected individuals may be the result of pathological effects of infection.

Changes in host behavior may also be due to a host counter-adaptation to infection (Poulin 1995). Numerous examples of this can be found in nature from insects to mammals (Hart 1990), but all involve changes in behavior or physiology that attempt to remove parasites, prevent infection, or minimize the effects of infection. One example is behavioral fever, which is a common strategy used by animals that involves simple behaviors to increase body temperature and effectively remove internal parasites (Kluger 1979). Other common examples include self-medication, grooming, and changes in typical mating patterns (Minchella 1985; Hart 1990).

One way to discriminate among the three hypotheses (parasitic adaptation, pathology, and host counter adaptation) is to examine the relationship between parasite development and the timing of modification in its host. The timing of modification is important because predation by definitive hosts will only result in successful transmission if the parasite has reached the developmental stage that is infective to the definitive host. Predation prior to this time would result in an early death because immature parasites possess underdeveloped attachment
structures (McCurdy et al. 1999; Sparkes et al. 2006; Poulin 2010; Perrot-Minnot et al. 2012; Bailly et al. 2018). Thus, if parasites are manipulating their host to increase fitness, modification would be most beneficial if it occurs when the parasite has reached the infective stage (Bethel and Holmes 1974; Sparkes et al. 2006).

Numerous studies have demonstrated this relationship between parasite development and timing of modification (e.g., Seidenberg 1973; Bethel and Holmes 1974; Brattey 1983; Pilecka-Rapacz 1986; McCurdy et al. 1999; Bailly et al. 2018). However, most have been run in laboratory-based settings, which can be problematic because they often do not present the complete range of scenarios that parasites and their hosts may encounter in natural settings (Mouritsen and Poulin 2003). For example, in laboratory predation experiments, infected hosts are often exposed to predators in controlled numbers (Thomas et al. 2005). In nature, parasites in intermediate hosts may be subjected to numerous predators, and not all of them may be suitable hosts. Thus, examining patterns of host-parasite interactions within their natural ecological contexts is crucial to understanding host modification (Thomas et al. 2005; Poulin 2010).

It has been widely demonstrated that host modification by parasites may result in behavioral differences between infected and uninfected individuals (Moore 2002). However, it may also be the case that pre-existing differences in behavior could explain the behavioral patterns seen after infection (Poulin 1995; Benesh et al. 2008; Barber and Dingemanse 2010). In other words, the behavior of one individual may make it more susceptible to infection relative to other conspecifics, and these differences could be maintained throughout the life of the host (Poulin 1995, 2010). As a consequence, differences in behavior that occur during late-stage infections may have existed prior to infection and could have even contributed to infection. However, little is known about how these differences relate to patterns of host behavior observed.
during late-stage infection by manipulative parasites. This can be studied in a relatively synchronous system because it becomes possible to observe behavioral patterns before infection and after the point when changes in behavior are expected to take place.

Conspecific parasites may successfully reach the target intermediate host, but host characteristics may still play a role in shaping patterns of parasite growth. For example, the isopod *Caecidotea intermedia* is an intermediate host to a manipulative parasite, *Acanthocephalus dirus*. The isopods are sexually dimorphic, where female isopods are smaller than male isopods. Because female isopods are smaller, the parasite may need to allocate more resources toward increasing the amount of available space as it grows. The reallocation of a finite amount of energy may be costly for the parasite in terms of its own growth, which is expected to increase parasite fitness (e.g., Lawlor et al. 1990; Poulin and Morand 2000; Dezfuli et al. 2001; Steinauer and Nickol 2002; Franceschi et al. 2010). Thus, parasites are expected to adopt different strategies in response to the different selection pressures they experience between female and male hosts (Park and Sparkes 2017). Sex-specific modification has been documented in cases of behavior (e.g., mating behavior, Oetinger 1987; Bierbower and Sparkes 2007; refuge use and activity, Park and Sparkes 2017), morphology (e.g., body size, Park and Sparkes 2017), and physiology (e.g., castration, Baudoin 1975).

Intermediate hosts provide more than transportation for trophically transmitted parasites, including resources in the form of nutrients and space (Dezfuli et al. 2001). After successful establishment in intermediate hosts, parasites need strategies to gain energy, grow within a confined space, avoid or mitigate immune responses from hosts, and ensure that the host lives to transmission (Poulin 2010; Caddigan et al. 2017). Additional demands may include paying costs associated with manipulation or in competition with other parasites present. Host sharing is
common in host-parasite systems and conflict may arise when immature and mature stages of parasites are present, because modification by mature parasites to increase conspicuousness may result in death for the other (Dianne et al. 2010). It has been shown that host sharing between conspecifics can have effects on growth and development (Dezfuli et al. 2001; Franceschi et al. 2008; Dianne et al. 2012). In other cases, behavioral modification may also be affected (Sparkes et al. 2004; Franceschi et al. 2008; Dianne et al. 2010, 2011, 2012; Caddigan et al. 2014). This conflict could cause parasites to adopt a strategy to mitigate effects of other competitors such as “sabotaging” the modification efforts of other manipulators (Thomas et al. 2002a). Consistent with a model proposed by Parker et al. (2009), a study showed that non-infective parasites prevented or delayed modification of anti-predator behavior of its host (“protection”) in the presence of infective conspecifics (Dianne et al. 2010). A subsequent study showed that the effects of protection by immature parasites reversed when the parasite reached the infective stage (Dianne et al. 2011). Some studies have demonstrated the relationship between host sharing and behavioral modification in the laboratory (e.g., Franceschi et al. 2008; Dianne et al. 2010), but few have used field-based approaches to quantify potential effects.

In order for selection to favor host modification over inducing simple pathological effects as a strategy, the benefits of the adaptation must outweigh potential costs (Poulin 2010). Benefits of modification should relate to measures of parasite fitness, such as increasing transmission to the target definitive host through predation. The relationship between host modification and predation success has been documented in the laboratory on numerous occasions (e.g., Camp and Huizinga 1979; Brattey 1983; but see also Perrot-Minnot et al. 2012). For example, the acanthocephalan *Polymorphus paradoxus* has been shown to modify the evasive behavior of its amphipod host, *Gammarus lacustris* (Bethel and Holmes 1973). This modification did not appear
until immediately after the parasite reached the infective stage (Bethel and Holmes 1974) and greatly increased the risk of predation to definitive hosts used in the study (mallards and muskrats, Bethel and Holmes 1977). In nature, parasites and their hosts are likely subjected to a wide variety of predators, and thus increased predation may not always mean increased transmission to the target definitive host (Mouritsen and Poulin 2003). There are very few empirical studies on the effectiveness of host modification to transmission, and even fewer of those studies have been conducted in natural settings (Mouritsen and Poulin 2003; Poulin 2010). Ultimately, parasites are expected to adopt strategies that increase the chances of completing their life cycle.

Acanthocephalans (also known as “thorny-headed worms” or “spiny-headed worms”) are endoparasites that require an arthropod intermediate host and vertebrate definitive host to complete their life cycles (Kennedy 2006). Modification of intermediate hosts occurs in almost all members of this taxonomic group and it is has been proposed that modification is an ancestral trait (Kennedy 2006). This group consists of four classes that contain 1293 species (Amin 2013): Archiacanthocephala (189 species, 15%), Eoacanthocephala (255 species, 20%), Palaeacanthocephala (845 species, 65%), and Polyacanthocephala (4 species, <1%).

In acanthocephalans, infection occurs when eggs are consumed along with food by the intermediate hosts. Inside the host, immature parasites (acanthors) burrow through the intestinal wall into the haemocoel of the arthropod. The parasite then matures through several non-infective (acanthellae) stages into the infective (cystacanth) stage, which involves development of reproductive organs and spiny proboscis. The proboscis contains several rows of hooks and serves as an efficient attachment structure in intestines of the definitive host when fully developed (Kennedy 2006). Successful transmission of the parasite occurs when the intermediate
host and parasite are consumed through a predation event and the parasite attaches to the gut of the definitive host. Inside the definitive host, male and female parasites reproduce sexually, and eggs are released into the environment. The distinct life cycle of acanthocephalans makes them attractive for studying questions related to development and host modification (Kennedy 2006).

The parasite *A. dirus* is a member of Order Echinorhynchia and has been associated with numerous behavioral (e.g., refuge use and mating) and morphological (e.g., body size and color) modifications of its isopod intermediate host (reviewed in Park and Sparkes 2017). These parasites have the largest range of fish hosts and widest distribution in North America relative to two other species in the region (*A. alabamensis* and *A. tahlequahensis*; Amin 1985). Major populations within the United States likely stemmed from a population near the Mississippi River and were geographically separated 15,000 years ago. This separation is thought to have formed the current distributions in the Wisconsin-Lake Michigan and New England areas (Amin 1985). Its wide geographical distribution and ability to establish in numerous fish hosts suggest that *A. dirus* may be either an ancestor to more recently-derived taxa, or a new species that is relatively adaptable and successful at infecting hosts (Amin 2013). However, because of the dependence on its intermediate host for survival, it is likely that host range limits the distribution of *A. dirus* (Amin 1985; Kennedy 2006).

*Acanthocephalus dirus* parasites infect stream-dwelling isopods as intermediate hosts and several freshwater fish species as definitive hosts (Seidenberg 1973; Camp and Huizinga 1980). Development in the intermediate host from the non-infective to the infective stages takes two to three months (Oetinger and Nickol 1982; Sparkes et al. 2004, 2006). Changes in host phenotype have typically been examined after the parasite has reached the cystacanth stage (Seidenberg 1973; Camp and Huizinga 1979, 1980; Hechtel et al. 1993; Sparkes et al. 2004, 2006; Park and
Sparkes 2017). These phenotypic changes have been proposed to increase trophic transmission of the parasite through predation and have been seen in this system in a laboratory-based study (Camp and Huizinga 1979). This host-parasite system has a clear seasonality that begins with the initial infection of juvenile isopods in the summer, transmission to fish hosts in the fall, winter, and spring and ends when parasite eggs are expelled into the stream in the spring and summer (Sparkes et al. 2004). This pattern makes it possible to predict when parasites interact with their hosts and allows for investigation of development-related questions in nature (Seidenberg 1973; Camp and Huizinga 1980; Oetinger and Nickol 1982; Sparkes et al. 2006).

Previously, it has been shown that acanthocephalans, specifically in the A. dirus-Caecidotea intermedius system, provide opportunities to study development- and modification-related questions (Oetinger and Nickol 1982; Sparkes et al. 2006; Dianne et al. 2012; Franceschi et al. 2008). Life-history patterns for this system are predictable and relevant to other acanthocephalan systems. Additionally, refuge use is a commonly modified and easily measured antipredatory behavior that can give insight to the effectiveness of host modification (Camp and Huizinga 1979; Hechtel et al. 1993; Park and Sparkes 2017). Using this system, I aim to provide insight into the strategy of host modification through the lens of parasite development.

To evaluate parasite development, refuge use modification, and transmission patterns of A. dirus in relation to its hosts, isopod intermediate host (C. intermedius) and fish definitive hosts [creek chub (Semotilus atromaculatus), green sunfish (Lepomis cyanellus)], I used a comprehensive field survey to address the following four questions:

1. Do pre-existing differences in behavior explain behavioral patterns in late-stage infections? If behavioral patterns before infection are the same as patterns in late-stage infection, behavior is most likely shaped by factors other than parasite modification.
However, if behavioral patterns before infection are different from patterns in late-stage infection, behavior is most likely shaped by parasite modification.

2. Is sex-specific modification dependent on parasite development? If parasite development is an important factor in determining the timing of isopod behavior modification (refuge use), then modification of this trait is likely to increase transmission in ways that may be adaptive for the parasite. If parasite development is not an important factor, then other hypotheses may explain isopod behavior. Additionally, sex-specific infection may be associated with costs to parasite growth. If parasites in female and male hosts differ in size, then there are likely costs associated with sex-specific infection. However, if parasite size does not differ between females and males, then costs may not be associated with sex-specific infection.

3. Does sabotage play a role in sex-specific behavior modification? If there are no differences in behavioral modification between immature and mature parasites, then there is likely no conflict between developmental stages over the timing of host modification for that trait. However, if differences in behavior modification exist between immature and mature parasites, there may be a potential for conflict. Following this, the amount of modification induced in mixed-stage infections can give insights into how the conflict is resolved (e.g., in favor of immature or mature parasites).

4. Is behavior modification associated with transmission to definitive hosts? If the occurrence of parasites in definitive hosts coincides with the onset of behavioral modification, then it is plausible to interpret that behavior modification favors transmission.
Study system

Buffalo Creek is a low-order stream located in Lake County, Illinois approximately 50 km from the DePaul University, Lincoln Park Campus (Figure 1). Previous studies have shown that *C. intermedius* dominates the macroinvertebrate community in this site and that the prevalence of *A. dirus* is high in both the intermediate and definitive hosts (Sparkes et al. 2004, 2006). The life cycle of *A. dirus* in Buffalo Creek is shown in Figure 2. In the summer, isopods consume eggs of *A. dirus* along with their typical food (e.g., leaves) and the acanthors contained inside the eggs hatch. The parasites then undergo development from the non-infective (acanthella) to the infective (cystacanth) stage, and induce phenotypic changes in the host (Figure 3; Sparkes et al. 2004, 2006; Bierbower and Sparkes 2007; Kopp et al. 2016; Park and Sparkes 2017). Isopods are then consumed by a creek chub (*Semotilus atromaculatus*) or a green sunfish (*Lepomis cyanellus*), which serve as definitive hosts at this site. Inside the fish, male and female *A. dirus* mate and eggs are released into the stream either with the fecal matter of the fish or inside the intact bodies of *A. dirus* females that are expelled from the fish (Kopp et al. 2011; Wahl and Sparkes 2012). Any remaining adult isopods in the stream undergo senescence at the start of the summer (Wahl and Sparkes 2012).

METHODS

Field surveys of intermediate hosts and definitive hosts

To determine the relationships between parasite development, modification of intermediate host behavior (isopods) and transmission to definitive hosts (creek chub and green sunfish), I used year-long surveys in Buffalo Creek (i.e., the entire life cycle). For isopods, the survey included samples collected during three separate time-periods (2005: February-April;
2006-2007: June-September, November-February; 2016-2018: June-May), which were combined into one survey (Table 1). Samples were combined because logistical constraints prevented the collection of one continuous survey with sample sizes that would allow for the planned analysis. For definitive hosts, I collected creek chub and green sunfish each month from June 2016 to May 2017. The results obtained from the surveys of intermediate and definitive hosts were used to examine the four questions outlined in the Introduction.

a) Survey of intermediate hosts: *Caecidotea intermedius*

Isopods that were located out of refuge were picked by hand from the substrate, on top of rocks, or algal mats. Then, the isopods in refuge were collected by lifting rocks from the substrate and by picking individuals from the underside of rocks, or allowing contents to be washed into a hand net placed downstream (Figure 4a). Isopods were immediately preserved in 70% ethanol at the field site. In the laboratory, isopod body length, sex, infection status, and parasite intensity were determined using a stereoscopic dissecting microscope (Leica MZ12). The sex of each isopod was identified using morphological features (female = presence of oöstegites or brood pouch; males = presence of hemi-peni and enlarged gnathopods). Parasite intensity was determined by counting the number of *A. dirus* individuals present in each infected isopod following Bush et al. (1997).

Parasites were recovered from the isopods and stored in ethanol until dissection. Developmental stage of each parasite (acanthella or cystacanth) was determined using morphological characteristics. Parasites were assigned to the cystacanth stage based on the presence of reproductive organs (female = ovarian balls; male = testes), invagination of the proboscis, and the presence of well-developed spines on the proboscis (Hasu et al. 2007).
Individual parasites that did not contain these features were assigned to the acanthella stage. Length and width were used to calculate parasite volume for samples collected between 2016 and 2018 \[\frac{\pi \times \text{length} \times \text{width}^3}{6};\text{ Dezfuli et al. 2001}\].

b) Survey of definitive hosts: *Semotilus atromaculatus* (creek chub) and *Lepomis cyanellus* (green sunfish)

Each month for 12 consecutive months between June 2016 and May 2017, a team of 3-5 people traveled to Buffalo Creek to collect fish samples. On each trip, a portion of the stream was blocked with a seine net so that water was able to pass through, but fish could not escape upstream (Figure 4b). Then, 1-2 people would use smaller hand nets to chase fish into another seine net placed downstream. This process was repeated in different sections of the stream until a total of 20 fish of either species were collected (creek chub, green sunfish). Only fish with total length >45 mm were collected. Each collection occurred over the course of 30-180 minutes between the hours of 9am-1pm. Fish were euthanized with an overdose of buffered MS-222 solution and immediately fixed with formalin (10%) at the field site. Samples were then transported to the laboratory and transferred to ethanol (70%) after 24 hours. For each fish (n = 240), total length was recorded in millimeters, and the intestines were examined for *A. dirus* and other macroparasites (e.g., trematodes). Additionally, gut contents were collected and stored in ethanol for a future project.
Testing hypotheses concerning 1) pre-existing differences in behavior, 2) parasite development and host sex, 3) intraspecific conflict, and 4) transmission.

1. Pre-existing differences in refuge use behavior

To determine if pre-existing differences in behavior occurred during early months of infection, I compared the number of infected and uninfected individuals that were in refuge to those out of refuge in June and July. This time period represents behavior that is expressed prior to or immediately after infection by early-stage parasites (Kopp et al. 2011). Values were analyzed using $\chi^2$ contingency tests and run separately for each sex.

2. Parasite development, refuge use, body size, and costs

To determine the relationship between parasite development, infection status and host body size, I compared sizes of infected and uninfected females and males during each time-period for the full survey. Body sizes were analyzed using individual Mann-Whitney $U$-tests for each time period. Effects of sample period on body size were analyzed separately for each group (uninfected female, infected female, uninfected male, or infected male) using Kruskal-Wallis tests.

To determine whether infection status and developmental stage of the parasites were predictors of refuge use behavior, I conducted analysis using the Akaike Information Criterion (AIC). AIC considers all possible combinations of predictors and interactions to create a candidate set of models. This type of analysis has growing support in the field of behavioral ecology because behavior is often influenced by several factors, and this approach allows for multiple hypotheses to be evaluated at one time (Garamszegi 2011; Richards et al. 2011).
Additionally, it does not use aspects of traditional null hypothesis testing (e.g., \( p \)- and \( \alpha \)-values) or model selection (e.g., backwards or stepwise). Instead, AIC measures the strength of evidence for several models using maximum likelihood and penalizes for a high number of parameters. Each model is associated with a log-likelihood value (LL) and a number of parameters (\( K \), including variance and intercept) that are used to calculate an AIC value or AICc values for small sample sizes. Both AIC and AICc tend to yield the same results. This value is then subtracted from the smallest AIC value to determine delta AIC (\( \Delta_i \)), thus, it is possible for a model to have a \( \Delta_i \) value of zero. Models with \( \Delta_i \leq 2 \) have substantial support, models with \( \Delta_i \geq 3 \) through 9 have considerably less support, and there is essentially no support for models with \( \Delta_i \geq 10 \). The AIC approach also provides a measure of uncertainty (\( w_i \)) that can be used to compare the weight of evidence for a given model. Assuming that all candidate models are biologically relevant, the set of models that can be used for interpretation (i.e., the 90% confidence set) typically contain \( w_i \) that individually add to \( \sim 0.90 \). Within the confidence set, the highest \( w_i \) is considered the best model.

Using this approach, I first examined the importance of infection status (infected vs. uninfected) as a predictor. In addition, I used other factors that may be related to observed behavioral patterns. Sample date and host size were important variables because these may be associated with the timing of behavioral changes. Interactions between infection status and sample date, and infection status and host length, were also considered because these variables have been shown to be related (Camp and Huizinga 1980; Park and Sparkes 2017). This was performed separately by host sex because sexual dimorphism in isopods may require different strategies for host modification and thus constrain parasite growth and development (Park and Sparkes 2017). Refuge use behavior was coded as a binary value (in refuge = 1; out of refuge =
0). Following this analysis, I used information about parasite characteristics to determine if developmental stage (acanthella or cystacanth) was an important predictor of refuge use behavior in isopods. In this analysis, I excluded uninfected isopods and individuals infected with both stages because mixed-stage interactions may play a role in determining behavior. This potential relationship is addressed in later analyses. Intensity was included as a predictor because it may also relate to intraspecific interactions, as well as an interaction between developmental stage and intensity. Sample date was listed as a factor because it has shown to be associated with development (Camp and Huizinga 1980; Sparkes et al. 2004, 2006). In addition, host length was included for similar reasons stated above. Since refuge use behavior was binary (in refuge vs. out of refuge), I compared generalized linear models (logistic regression) using AIC.

To determine if host sex impacted the timing of behavior modification, I calculated difference values for refuge use between infected and uninfected hosts for each time-period. This value was used as a measure of behavioral modification because it measured the number of individuals exhibiting atypical antipredatory behaviors for this system (e.g., out of refuge vs. in refuge). To determine if parasite body size (volume) was affected by host sex, I compared body sizes of mature stages using the 2016-2018 survey. Effects of host sex and sample date were analyzed using two-way ANOVAs.

3. Intraspecific conflict and modification of refuge use

To determine if intraspecific conflict was related to modification of refuge use, I conducted analysis for samples between August and January. These months represented an important development period for parasites and mixed-infections occurred during this time period. I examined cases where individuals had no parasites, acanthellae-only (non-infective),
cystacanth-only (infective), and infections where both stages of development were present. This was important because intraspecific interactions have been shown to affect patterns of modification in intermediate hosts (e.g., body color; Sparkes et al. 2004). To determine if sabotage played a role in behavior modification, I conducted $\chi^2$ contingency tests to compare refuge use behavior among the four groups (uninfected, acanthellae, cystacanth, and mixed-stage). In cases where there were significant differences among groups (i.e., a conflict may be occurring), individual $\chi^2$ contingency tests were performed for between-group analysis to determine if sabotage was occurring.

4. Refuge use and transmission to definitive hosts

To determine the relationship between the pattern of host modification and infection patterns in definitive hosts, I used measures of prevalence, abundance, and intensity in the common fish hosts. Prevalence is the percent of infected individuals within the population. Intensity is measured as the number of parasites within infected individuals and abundance is the number of parasites across all individuals (Bush et al. 1997). To determine if behavior modification was related to transmission to definitive hosts, I compared these values to the timing and magnitude of behavior modification across sample periods.

RESULTS

In total, analysis was conducted on 3616 isopods collected from Buffalo Creek (infected females: n = 591; uninfected female: n = 1046; infected male: n = 801; uninfected male: n = 1178). After combining samples, 26% of isopods were obtained from the survey conducted in 2016-2018 and 74% were obtained from previous surveys conducted from 2005-2007.
Additionally, there were 3046 parasites recovered from infected isopods in the sample (acanthellae: n = 1257; cystacanth: n = 1789).

1. Pre-existing differences in refuge use behavior

Isopods over 8 mm for females and 9 mm for males were not included in the sample for June-July because they were adults, and hence from the previous cohort. Additionally, some isopods were not included in sex-specific analysis because the sex was not distinguishable (n = 19). Refuge use behavior during initial infection is shown in Figure 5. During this time period, infected and uninfected isopods differed in refuge use for both females ($\chi^2 = 16.2, p < 0.001$) and males ($\chi^2 = 33.8, p < 0.001$). Infected females and infected males were found in refuge more often than uninfected members of the same sex.

2. Parasite development, refuge use, body size, and costs

The relationship between body size and infection status for the entire cycle, including months of initial infection, is located in Table 2. Analysis revealed that there were pre-existing differences in body size prior to or during infection by immature parasites. Infected males were larger in volume than uninfected males, but infection status had no effect on body size for females during the early infection periods (June-July).

For females, significant size differences began to emerge the following time period (August-September). This size difference continued throughout the remainder of the year so that infected females were larger than uninfected females. For males, body size differences that were present during early infection persisted throughout the remainder of the year. In general, infected
individuals were larger than uninfected members of the same sex. Infected males were larger than infected females, followed by uninfected males and uninfected females.

I used Kruskal-Wallis tests to determine the relationship between sample date and body size. There was a significant effect of sample date on body size for all groups (infected females: $\chi^2_5 = 368.0$, $p<0.001$; uninfected females: $\chi^2_5 = 550.9$, $p<0.001$; infected males: $\chi^2_5 = 513.4$, $p<0.001$; uninfected males: $\chi^2_5 = 715.0$, $p<0.001$). Generally, isopods increased in body size throughout the year.

Isopod refuge use is shown in Figure 6. During initial infection periods, infected isopods were in refuge more often than uninfected isopods in both females and males. For females, this pattern continued until it reversed in December and January, so that infected isopods were out of refuge more than uninfected isopods. For males, the same reversal of refuge use behavior for infected and uninfected individuals occurred in October and November. By the spring months, infected isopods were found out of refuge more often than uninfected isopods.

I used AIC modeling to determine if infection status (infected or uninfected) was a main predictor of refuge use. Other potential predictors included in the generalized linear model were sample date combined into month groups (June-July = JJ, August-September = AS, October-November = ON, December-January = DJ, February-March = FM, April-May = AM), isopod length, and interactions between infection and length. Table 3 shows the models (90% confidence set) that best predict refuge use behavior. Female refuge use was best described by a model that included infection status isopod length, sample date, and an interaction between infection and length. The best model had the highest $AIC_C$ weight ($w_i$) of 0.588 indicating a lower level of uncertainty relative to other models and $\Delta_i$ of zero indicating a good fit. The next
best model included all predictors and had a smaller \( w_i \) of 0.284 and \( \Delta_i \) of 1.45, which also indicates a good fit.

For males, refuge use was best described using a model with infection status, length, and an interaction of infection and sample as predictors \( (w_i = 0.415, \Delta_i = 0) \). The next best model only included infection, length, and an interaction between infection and length but still had a relatively high \( w_i \), which also indicated a good fit \( (w_i = 0.335, \Delta_i = 0.43) \). For females and males, only the first models with \( w_i \) that summed to \( \sim 0.90 \) were used for interpretation \( (90\% \text{ confidence set}) \).

Parasite development inside isopod hosts is shown in Figure 7. Acanthellae parasites infected isopods during June-July. During the fall, acanthellae and cystacanths occurred at similar frequencies in the population until cystacanths dominated from December through May.

To determine if parasite development is a predictor of isopod refuge use behavior, I conducted AIC modeling. Other potential predictors included in the confidence model set were developmental stage (acanthella, cystacanth), sample date (JJ, AS, ON, DJ, FM, AM), host length, parasite intensity, and an interaction between developmental stage and intensity.

Individuals infected with both acanthellae and cystacanths were not included here because these values could confound analysis. This relationship was examined separately (see section next section addressing intraspecific conflict).

For female isopods (Table 4), the best model included length and sample as predictors \( (w_i = 0.317, \Delta_i = 0) \). The next best model included intensity as a predictor and had \( w_i \) of 0.287 and \( \Delta_i \) of 0.20, which also indicates a good fit. For male isopods (Table 4), the best model included developmental stage and sample date as predictors. This model had an AIC\(_C\) weight of 0.267. The next best models included intensity \( (w_i = 0.235, \Delta_i = 0.26) \) and an interaction between
developmental stage and intensity \((w_i = 0.223, \Delta_i = 0.35)\), respectively. The last model with \(\Delta_i < 2\) had an AIC\(_C\) weight of 0.104 and included only developmental stage, sample, and host length as predictors. For females and males, only the first models with \(w_i\) that summed to \(~0.90\) were used for interpretation (90% confidence set). Since developmental stage appeared in the best model for males only, developmental stage of the parasite appears to be important for male refuge use but not in females.

Figure 8 shows the relationship between parasite development and behavior modification. In males, infected individuals were in refuge during June-July while there were no cystacanths present in the male isopod population. From August through November, behavior modification increased so that the infected isopods appeared out of refuge in similar numbers to uninfected isopods. During this time period, cystacanths began to appear in the male isopod population. In December-January and throughout the remainder of the year, behavior modification increased so that there were more infected isopods out of refuge than uninfected isopods, and cystacanth presence in male isopods was highest. In females, infected individuals were in refuge from June through November. During this period, cystacanths began to appear in the female isopod population. Behavior modification that would cause females to leave refuge did not occur until December, and this continued throughout the year. In the spring, cystacanth presence in female isopods and behavior modification was highest.

Body size of female and male parasites within female and male hosts is shown in Figure 9. Female parasites within female and male hosts did not differ in size. Additionally, male parasites within female and male hosts did not differ in size. For parasite growth-related costs in female and male hosts, I ran a two-way ANOVA. In female parasites, there was an effect of sample \((F_{3,93} = 38.0, p < 0.001)\), but no effect of host sex \((F_{1,93} = 0.3, p = 0.6)\) or interaction
between sample and host sex ($F_{3,93} = 0.7$, $p = 0.6$) on parasite size (volume). In male parasites, there was an effect of sample ($F_{3,75} = 48.7$, $p < 0.001$), but no statistically significant effect of host sex ($F_{1,75} = 3.65$, $p = 0.06$) or interaction between host sex and sample ($F_{3,75} = 1.41$, $p = 0.2$) on parasite size.

3. Intraspecific conflict and modification of refuge use

To determine if conflict was occurring for the timing of behavior modification in mixed stage infections, I first conducted a $\chi^2$ contingency test for each sex. The relationship between refuge use and infection status are shown in Figure 10. In females, there was no significant difference in refuge use among the four groups ($\chi^2 = 4.8$, $p > 0.1$), and hence there could not be a conflict between acanthellae and cystacanths over the timing of host modification. In contrast, for males, there was a significant difference among groups ($\chi^2 = 36.9$, $p < 0.001$). Comparisons between male groups revealed that refuge use behavior did not differ between uninfected hosts, acanthella-infected hosts, and infected hosts that contained mixed-stage infections. Individuals with cystacanth-only infections showed significantly less refuge use behavior compared to other groups (Un vs. C: $\chi^2 = 24.4$, $p < 0.001$; A vs. C: $\chi^2 = 24.8$, $p < 0.001$; AC vs. C: $\chi^2 = 14.7$, $p < 0.001$).

These results also show that parasite development and timing of behavior modification is important in males but not in females during the time period examined (August through January). In females, refuge use behavior of cystacanth-infected isopods did not differ from other infection groups. However, in males, refuge use behavior of cystacanth-infected isopods was significantly different from other infection groups.
4. Refuge use and transmission to definitive hosts

A total of 240 fish were collected from Buffalo Creek in the 2016-2017 survey (Figure 11). In general, creek chub were more common in the stream than green sunfish, which was reflected in the samples (creek chub = 74%; green sunfish = 26%). Additionally, over half of fish collected were infected (infected = 61%; uninfected = 39%). From infected hosts, a total of 943 A. dirus were recovered. Two additional parasites were present (Allocreadium sp. and Posthodiplostomum sp.). A detailed analysis of these parasites will be included in a future project.

Prevalence, abundance, and intensity of infection in the fish hosts are shown in Table 5. The relatively high values in all three measures for the June-July sample likely indicate that these samples reflect members of the previous cohort. Fish generally expel the parasites throughout the summer, and are infected with the new cohort of parasites beginning in the autumn. Prevalence of parasites in fish showed that cystacanths began appearing in August-September and increased throughout the year so that it was highest in the spring. Intensity and abundance also increased throughout the same time period.

The pattern of behavior modification in isopods and prevalence of parasites fish hosts are shown in Figure 12. Prevalence was used here, rather than intensity or abundance, for ease of visualization. This increase of parasite prevalence in fish coincided with an overall increase in behavior modification, so that infected isopods were out of refuge in August-September. Behavior modification also continued to increase throughout the winter and spring months.

The relationship between behavior modification and parasite prevalence in female and male isopods is shown in Figure 13. Parasite prevalence in fish hosts was again shown here to illustrate this relationship, and was used instead of abundance or intensity for ease of
visualization. From August to January, parasites began to appear in the fish population. In males, behavior modification increased so that infected males appeared out of refuge in August-September. This increased throughout the remainder of the year and was highest during the spring months. In females, behavior modification occurred later in the year so that females did not appear out of refuge until December. Behavior modification in females increased throughout the remainder of the year and was highest during the spring months.

**DISCUSSION**

I examined the relationship between modification of refuge use behavior associated with *A. dirus* infection and transmission to definitive hosts in nature. I also examined how parasite development, intraspecific conflict, and host sex can play a role in the process and outcome of host modification. This was one of the first studies to examine these relationships using a long-term, field-based approach. The findings shown here can also be applied to several of the other host-parasite systems that involve modification. For all parasites, development is a critical process related to parasite fitness, intraspecific interactions are common, and the host environment may provide additional selective pressures that require specific adaptations for parasite survival. The relative synchrony of the *A. dirus* system allows for an understanding of these interactions at the host-parasite and parasite-parasite levels, and can provide insight into the roles of adaptive manipulation, pathology, and host counter-adaptations in transmission. The relationships between *A. dirus* infection, behavior modification, intraspecific conflict, and transmission were addressed using four approaches, which are discussed in detail below.
Pre-existing differences in refuge use behavior

The first part of this study examined if there were pre-existing differences in behavior between infected and uninfected isopods that could explain differences in refuge use that had been observed previously during late-stage infections (Kopp et al. 2016; Park and Sparkes 2017). This is important because it gives insight into how existing patterns of behavior may affect the observed modification of those traits. The prediction was that if infection-related refuge use behavior during initial infections was consistent between early- and late-stage infections, then differences in behavior that have been attributed to parasite infection can be explained instead by these pre-existing differences. Alternatively, if refuge use behavior during early-stage infections was different from patterns seen during late-stage infections, then behavior modification is more likely to be associated with effects of infection.

The results obtained showed that infection-related variation in behavior during early-stage infections was not consistent with the patterns in late-stage infections. During the early stages of infection, infected isopods were more likely to be in refuge than uninfected isopods. In contrast, during late stages of infection, infected isopods were more likely to be in the open than uninfected isopods. Thus, the commonly observed decrease in refuge use in late-stage infections could not be explained by pre-existing differences in refuge use behavior. Instead, the decreased refuge use observed in late-stage infections is more likely to be associated with effects of infection.

The differences in behavior that were identified during early-stage infections were also of interest because these differences may give insight to the early interactions that occur between A. dirus and its isopod host. I found that infected isopods were more likely to be in refuge than uninfected isopods during early infection. Two potential hypotheses can be used to explain the
patterns above: 1) parasites may be actively reducing risky behaviors of the host during early-stage infection, or 2) natural variation in behavior within the isopod population makes some individuals more susceptible to infection than others.

During the non-infective stage, parasites are often underdeveloped and lack attachment or reproductive structures (Kennedy 2006). For trophically transmitted parasites, predation of the host during this time could be detrimental to their fitness. Thus, parasites may “protect” the host by increasing antipredatory behaviors, such as refuge use, to reduce the likelihood of predation (Dianne et al. 2011). Thus, the differences in refuge use between infected and uninfected isopods during early-stage infection may be the result of protective effects by early-stage *A. dirus*. This hypothesis relies on the assumption that changes in behavior occur shortly after infection during which time the parasites are in very early stages of development.

An alternative explanation for differences in refuge use behavior during early stages of infection is that the isopods vary in personality and this variation influences the likelihood that infection occurs. Animal personality is typically described as behavioral differences among individuals that are consistent over time and between contexts (Sih et al. 2004, Réale et al. 2010). Previous studies in this system have shown that adult isopods exhibit personalities for refuge use (Park and Sparkes 2017). If this type of personality is also present in juveniles, and if being in refuge increases encounter rates with *A. dirus* eggs, then individuals with this personality may be more likely to become infected. Consistent with this hypothesis, eggs of *A. dirus* possess fibrils that attach to leaves (Pfenning and Sparkes 2019), which often accumulate under rocks as leaf packs. The potential importance of animal personality to host-parasite dynamics has been emphasized in recent years (Barber and Dingemanse 2010; Poulin 2013).
**Parasite development, refuge use, body size, and costs**

Host modification is the process in which host phenotypes are changed following parasite infection (Moore 2002). Modification of body color (Oetinger and Nickol 1982), phototaxis (Bethel and Holmes 1973), body size (Oetinger 1987), and activity (Park and Sparkes 2017) are examples of traits that can potentially increase conspicuousness to visually-hunting predatory hosts. Historically, host modification has often been interpreted as an adaptive parasite strategy that favors transmission. However, changes in host traits can also be due to a pathological effect of infection (i.e., sickness behavior), or be a counter-adaptation by the host to mitigate negative effects of infection (Poulin 1995, 2010; Thomas et al. 2005).

One way to discriminate between the hypotheses is to assess the relationship between parasite development and the timing of host modification (Bethel and Holmes 1973; Sparkes et al. 2006). To maximize parasite fitness, changes should occur when the parasite reaches the developmental stage that is infective to the definitive host. Predation during this period would increase the likelihood of successful transmission and completion of its life cycle. Additionally, modification during the non-infective stage may result in an early death for the parasite within the definitive host. Thus, I predicted that if manipulation was adaptive, parasite development should then be directly related to the timing of modification of refuge use, such that modification does not occur until the parasites have reached the cystacanth stage.

Phenotypic differences have been observed for several traits in this system and several of these effects were sex-specific (Park and Sparkes 2017). Consistent with this pattern, I found that the pattern of modification of refuge use behavior was sex-specific. In both sexes, infection status was an important predictor of refuge use behavior (specifically decreased refuge use), which indicates that parasite infection appears to play a role in determining variation in this
behavior in both females and males. However, analysis within the infected isopods revealed that the factors that best explained variation in refuge use behavior differed between the sexes.

In males, parasite developmental stage was a predictor, along with sample date. This indicates that development from the acanthella into the cystacanth stage, which is infective to the definitive host, is associated with timing of behavior modification. Thus, the timing of modification in males appears to be adaptive, and hence modification of refuge use behavior in general is likely due to adaptive manipulation by the parasites. These results support the interpretation that modification of refuge use behavior is likely due to adaptive manipulation by the parasites in males. In contrast, parasite developmental stage was not a predictor of female isopod refuge use behavior. Instead, female isopod body size and sample date were predictors, indicating that the transition into the cystacanth stage did not have a direct effect on the timing of behavior modification. Thus, these results do not support the interpretation that the timing of modification of refuge use behavior is due to adaptive manipulation by the parasites in females. However, modification of refuge use behavior in females may still be adaptive if it is not due to a direct relationship between the timing of modification and parasite development (see further discussion below). Alternatively, changes in refuge use behavior may also be explained by pathological effects of infection, or the result of a host counter-adaption.

One possible explanation for the sex-specific differences in behavior patterns and their relationship to parasite development may be associated with the fact that female and male hosts can create different types of environments for growth and development. In C. intermedius, adult females are smaller than adult males (Keogh and Sparkes 2003). This dimorphism between sexes may pose a challenge for parasites because there may be less space available for growth in females compared to males. Additionally, females generally require more energy for
reproduction than males, and this energy requirement may constrain allocation to somatic growth (Baudoin 1975). Thus, parasites in female hosts would benefit by inducing a reallocation of energy assigned for reproduction towards somatic growth. Consistent with this interpretation, reproduction in female hosts is often affected more drastically than males (e.g., Dezfuli et al. 2007), and body size can increase following infection (e.g., Minchella 1985). This type of modification may be important if host size influences parasite growth because parasite body size has been associated with numerous fitness-related measures [e.g., establishment success, male-male competition, and female fecundity inside the definitive hosts (Lawlor et al. 1990; Dezfuli et al. 2002; Poulin and Morand 2002; Steinauer and Nickol 2002)].

My results were consistent with the predictions described above concerning host body size and its effects on parasite body size. I found that there was no relationship between infection status and body size in females during initial infection, which indicates that there was no pre-existing difference in size prior to infection. Following this period, infected and uninfected females differed in their pattern of growth throughout the year, which resulted in infected females having larger body size than uninfected females. Previous studies in this system have shown that this increase in growth of infected females is also associated with decreased reproduction and atrophy of the reproductive organs (Sparkes et al. 2006). This contrasted with males because infected males were larger than uninfected males during initial infection. This difference then continued throughout the year. Collectively, this pattern of energy reallocation in females resulted in more space, and possible energy, available for growth. Consistent with this interpretation, parasites in female hosts did not differ in body size from parasites in male hosts (Figure 9). Thus, it appears that the strategy of host modification differs between male and female hosts. In male hosts, parasites can grow and develop unconstrained and modify host
behavior as soon as they make the transition into the cystacanth stage. In contrast, parasites in female hosts must first modify the pattern of energy allocation to improve conditions for growth and development so that they can attain an optimal size for transmission. Modification of behavior would then occur later in female hosts than it does in male hosts, which would disrupt the tight association that occurs between development and modification in male hosts.

The explanations for modification of female and male host behavior outlined above rely on mechanisms that benefit the parasites (i.e., adaptive manipulation). Differences in refuge use behavior between infected and uninfected isopods could also potentially be explained by pathological effects of infection or as host counter-adaptations to infection. Infection by parasites has been associated with a disruption of neurochemical pathways that change the behavior of the infected individual, and this change in behavior may not benefit either the host or the parasite (reviewed in Helluy 2013; Moore 2013). It is possible that *A. dirus* parasites disrupt neurochemical pathways in isopods in ways that cause females to display sickness behaviors. Some general examples of these behaviors are commonly seen in vertebrates and include lethargy or drowsiness (Adelman and Martin 2009), but can also be applicable to invertebrate systems because these behaviors are often controlled by similar pathways (e.g., cytokines, Adelman and Martin 2009; Helluy 2013). Thus, the loss of anti-predator behavior (refuge use) of female isopods may be due to lethargy as immune functions respond to infection (Dantzer 2001). This explanation may contribute to changes in female behavior but seems unlikely to explain changes in male behavior since the onset of this change in males is coupled tightly to parasite development. However, this possibility cannot be excluded at this time.

Another potential mechanism that could explain differences in refuge use behavior is that individuals may move out of refuge to mitigate the effects of infection (i.e., as a counter-
adaptation, Minchella 1985; Poulin 1995). Two resources are located out of refuge that may be of importance in this context: high-quality food and mates. Firstly, the process of body size modification by parasites may reduce the amount of energy available for the female. Thus, infected isopods may leave refuge to forage for additional or alternative food sources (Médoc et al. 2011). For example, diatoms provide a high-energy food supply for isopods and they are typically located out of refuge (Torres-Ruiz et al. 2007). Secondly, parasitized hosts may adjust typical mating patterns to compensate for effects of infection (Minchella 1985). These explanations can also not be excluded at this time.

Intraspecific conflict and modification of refuge use

Previous research has shown that both intraspecific and interspecific conflicts can occur over host modification (Thomas et al. 2002a; Sparkes et al. 2004; Dianne et al. 2012). Natural selection is expected to favor parasites that use strategies to resolve the conflict, which may include sabotage of manipulation attempts by other parasites to prevent transmission (Thomas et al. 2002a). Intraspecific conflict over the timing of host modification can occur when immature (non-infected) and mature (infected) parasites share a host (Sparkes et al. 2004; Dianne et al. 2010). In contrast, conflict over modification is not expected to occur if all parasites are at the same developmental stage or if development is not important to modification (Sparkes et al. 2004; Dianne et al. 2010). In the field surveys from Buffalo Creek, there were numerous cases of mixed-stage infections, which occurred between August and January. This occurrence allowed for the potential importance of conflict to be analyzed.

The results showed that female and male hosts differed in refuge use behavior in ways that impacted the potential for conflict. In female hosts, there was no difference in the level of
behavior modification regardless of infection status (uninfected, acanthellae-only, cystacanth-only, or mixed-stage). Thus, there was no potential for conflict over the timing of behavior modification. In contrast, modification of refuge use behavior in males differed between the non-infective (acanthella) and infective (cystacanth) stages, which established the potential for conflict. In male hosts, cystacanth-infected individuals were more likely to be located out of refuge than both uninfected and acanthellae-infected individuals. If being out of refuge increases the risk of predation, then acanthellae-stage parasites that share a host with cystacanths may benefit from preventing (sabotaging) this behavior modification. Analysis of refuge use behavior for isopods that contained mixed-stage infections revealed that the conflict appeared to be resolved in favor of the non-infective parasites. That is, non-infective stages delayed modification of host behavior. This would benefit non-infective parasites because they could remain in refuge until they have completed development into the infective stage.

An additional example of conflict over trait modification occurs in the A. dirus–C. intermedius system for variation in body color (Sparkes et al. 2004). Cystacanth-infected isopods typically differ in color from uninfected isopods because they do not produce the pigment that gives rise to the brown body color (Oetinger and Nickol 1981). The lack of color increases conspicuousness of infected isopods because they are light-colored against the dark sediment in the stream (Seidenberg 1973). In acanthellae-infected isopods, this color change occurs but is not as severe (40% vs. 80% color loss). This conflict was resolved in favor of the cystacanths, since mixed-stage infections induced color modification that was similar to cystacanth-only infections. Thus, non-infective stages did not sabotage modification of color in mixed-stage infections. To contrast, an example of sabotage occurs in the amphipod Gammarius insensibilis infected with two different parasite species. The amphipod serves as a host for a trematode (Microphallus
papillorobustus) and a nematode (Gammarinema gammari), and both parasites use the host for different purposes (Thomas et al. 2002a). In trematode-infected amphipods, individuals swam to the surface of the water column in the presence of a mechanical disturbance, which is likely to increase predation in nature. In nematode-infected amphipods, however, individuals did not swim towards the surface. This behavior was considered the normal antipredatory response for amphipods. In mixed infections, amphipods exhibited normal behavior. This indicated that the conflict was resolved in favor of the nematode.

Refuge use and transmission to definitive hosts

I predicted that the occurrence of parasites in definitive hosts should coincide with the onset of behavior modification if leaving refuge increases risk of predation. At the population-level, I found that behavior modification of isopods and prevalence of the parasite in fish hosts were somewhat consistent in that the highest levels of modification and prevalence occurred between December and May. However, the relationship between behavior modification and transmission appeared to be more aligned in male isopods than female isopods. For males, infected individuals were typically in refuge during June-July, which began to change in August-September, resulting in infected males generally being out of refuge from December through May. In contrast, infected females were generally in refuge from June-July through November, after which time infected females decreased their use of refuge from December through May. During this period, prevalence in fish hosts was lowest in August-September, increased to February-March, and appeared to plateau through May. This initial increase coincided somewhat with the onset of behavior modification in male hosts, but not in females, because the onset of changes in refuge use behavior for females did not occur until December-January. Overall, the
results obtained provide some support for the interpretation that behavior modification is associated with transmission in nature. Additional support for this interpretation has been provided from studies that showed direct evidence of infection-related predation in *C. intermedius* (Camp and Huizinga 1979; Cronin T, unpublished data). Several other studies have examined this relationship between host modification and predation but results have often been inconsistent (e.g., Perrot-Minnot et al. 2012), indicating that this relationship is likely complex and system-specific.

In addition to providing insights to the overall pattern of modification and transmission, analysis of the sex-specific nature of host modification yielded new insights into the source of parasites that contribute to overall transmission patterns and the potential fitness payoffs to the parasites that occupy female versus male hosts. Based on sex-specific differences in the timing of modification of refuge use, in which infected males were out of refuge earlier in the year than infected females, it is likely that the increase in parasite prevalence in definitive hosts between August and November is due to predation on infected males. After this time, when infected females leave refuge, predation of infected females also contributes to overall parasite prevalence. This type of pattern of sex-specific modification of behavior and transmission may also have implications for parasite fitness. For example, if this is the case, parasites that develop in male hosts may be transmitted to fish hosts sooner in the year than parasites that develop in female hosts. This could mean that parasites from male hosts have more opportunities to mate because they occupy the host for longer periods of time. Future work is needed to determine the potential biological significance of this type of relationship.
Conclusion

In this study, I addressed questions related to host refuge use behavior, parasite development, intraspecific conflict, and transmission to definitive hosts using the system *A. dirus* and *C. intermedius*. I found pre-existing differences in refuge use behavior between females and males in that infected isopods were in refuge more than uninfected isopods during early infection. Behavior differences during early-stage infection likely do not affect behavior differences seen during late-stage infection, and can instead be explained by effects of infection.

I found that the timing of refuge use modification was sex-specific. In males, I found that the timing of refuge use modification was directly related to parasite development, which indicates that modification of this trait is likely adaptive. In females, I found that the timing of refuge use modification was not directly related to parasite development. However, modification of behavior may still be adaptive if an indirect relationship to parasite development increases female transmission to definitive hosts but other explanations cannot be excluded at this time.

Sex-specific modification of refuge use may be explained by size constraints present in female hosts that are not present in male hosts. Parasites within females may need to modify host body size before growth and development can occur, which could delay the onset of modification. Consistent with this interpretation, infected females were larger than uninfected females so that they were similar in size to males, and parasite body size was not affected by host sex. Infected males were also larger than uninfected males throughout the year, but the pattern is likely explained by pre-existing differences in body size.

In females, there was no conflict over host modification between mixed-stage infections. In males, the potential for conflict was present and resolved in favor of non-infective parasites.
This indicates that non-infective parasites may use strategies such as sabotage to prevent early transmission to definitive hosts, and the conditions that influence this may also be sex-specific.

The pattern of transmission coincided with the overall pattern of behavior modification, and is likely to be adaptive for parasites. Further analysis within females and males showed that the pattern of transmission was also sex specific. It is likely that male isopods are unconstrained in energy and space available for growth, and are able to grow, modify, and be transmitted to fish sooner in the year relative to females. Thus, parasites that appear in fish during the fall months likely originated from a male isopod and are more likely to have higher fitness payoffs (increased mating encounters in the definitive host) than parasites in female isopods.
REFERENCES


De Moraes CM, Stanczyk NM, Betz HS, Pulido H, Sim DG, Read AF, Mescher MC (2014) Malaria-induced changes in host odors enhance mosquito attraction. PNAS 111:11079-11084.


Table 1. Field survey sampling periods between 2006 and 2018.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Month</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>July</td>
<td>2006, 2016</td>
</tr>
<tr>
<td>August-September</td>
<td>August</td>
<td>2006, 2016</td>
</tr>
<tr>
<td></td>
<td>September</td>
<td>2006, 2016</td>
</tr>
<tr>
<td>October-November</td>
<td>October</td>
<td>2016, 2018</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>2006, 2016</td>
</tr>
<tr>
<td>December-January</td>
<td>December</td>
<td>2006, 2016</td>
</tr>
<tr>
<td></td>
<td>January</td>
<td>2007, 2017</td>
</tr>
<tr>
<td></td>
<td>March</td>
<td>2005, 2017</td>
</tr>
<tr>
<td>April-May</td>
<td>April</td>
<td>2005, 2017</td>
</tr>
<tr>
<td></td>
<td>May</td>
<td>2005, 2017</td>
</tr>
</tbody>
</table>
Table 2. Relationship between body size and infection status. Shown are median values (in mm) with lower and upper quartile ranges in parentheses. Sample period is shown in month groups (June-July = JJ, August-September = AS, October-November = ON, December-January = DJ, February-March = FM, April-May = AM). ** indicates p < 0.001, *** indicates p <0.0001. Degrees of freedom = 1 in all cases.

<table>
<thead>
<tr>
<th>Host sex</th>
<th>Sample</th>
<th>Infected</th>
<th>Uninfected</th>
<th>$\chi^2$</th>
<th>p-value</th>
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<td>Female</td>
<td>JJ</td>
<td>4.7 (4.2-5.6)</td>
<td>4.9 (4.1-5.9)</td>
<td>0.33</td>
<td>ns</td>
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<tr>
<td></td>
<td>AS</td>
<td>5.5 (5.0-6.1)</td>
<td>4.4 (3.8-5.1)</td>
<td>50.1</td>
<td>***</td>
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<tr>
<td></td>
<td>ON</td>
<td>8.1 (7.0-9.4)</td>
<td>6.3 (5.8-7.0)</td>
<td>47.0</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>DJ</td>
<td>9.4 (7.5-12.3)</td>
<td>7.7 (6.8-8.5)</td>
<td>24.8</td>
<td>***</td>
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<tr>
<td></td>
<td>FM</td>
<td>11.0 (8.6-12.8)</td>
<td>7.8 (7.0-8.5)</td>
<td>136.1</td>
<td>***</td>
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<td></td>
<td>AM</td>
<td>12.8 (10.9-14.7)</td>
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<td>***</td>
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<td>4.9 (4.1-6.0)</td>
<td>4.3 (3.2-6.1)</td>
<td>8.0</td>
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<tr>
<td></td>
<td>AS</td>
<td>5.3 (4.4-6.3)</td>
<td>4.3 (3.6-5.1)</td>
<td>37.2</td>
<td>***</td>
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<tr>
<td></td>
<td>ON</td>
<td>8.5 (7.2-9.9)</td>
<td>7.2 (6.3-8.3)</td>
<td>32.8</td>
<td>***</td>
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<tr>
<td></td>
<td>DJ</td>
<td>10.7 (7.9-13.7)</td>
<td>8.6 (7.3-9.5)</td>
<td>34.4</td>
<td>***</td>
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<tr>
<td></td>
<td>FM</td>
<td>11.7 (9.0-14.3)</td>
<td>9.3 (8.2-10.5)</td>
<td>34.1</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>AM</td>
<td>13.6 (11.5-15.8)</td>
<td>10.5 (8.9-11.6)</td>
<td>70.6</td>
<td>***</td>
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Table 3. AIC table for the 90% confidence set of generalized linear models of female and male isopod refuge use behavior to determine effects of infection status. The predictor variables for isopod refuge use (in refuge = 1, out of refuge = 0) were infection status (IS; infected, uninfected), sample date (SD; June-July, August-September, October-November, December-January, February-March, April-May), host length (HL), an interaction between infection and host length (IS:HL), and between infection status and sample date (IS:SD). Log-likelihood (LL), number of parameters (K), AICc value, delta AICc ($\Delta_i$), and AICc weight ($w_i$) are also shown below. Best model is listed in bold.

<table>
<thead>
<tr>
<th>Host sex</th>
<th>Model Terms</th>
<th>LL</th>
<th>K</th>
<th>AICc</th>
<th>$\Delta_i$</th>
<th>$w_i$</th>
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<tr>
<td>Female</td>
<td>IS + HL + SD + IS:HL</td>
<td>-945.771</td>
<td>6</td>
<td>1901.6</td>
<td>0.00</td>
<td>0.588</td>
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<td></td>
<td>IS + HL + SD + IS:HL + IS:SD</td>
<td>-945.490</td>
<td>7</td>
<td>1903.0</td>
<td>1.45</td>
<td>0.284</td>
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<tr>
<td>Male</td>
<td>IS + HL + SD + IS:SD</td>
<td>-1207.177</td>
<td>6</td>
<td>2424.4</td>
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<td>0.415</td>
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<td>IS + HL + IS:HL</td>
<td>-1208.395</td>
<td>5</td>
<td>2424.8</td>
<td>0.43</td>
<td>0.335</td>
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Table 4. AIC Table for the 90% confidence set of generalized linear models of female and male isopod refuge use behavior to determine effects of parasite development. The predictor variables for isopod refuge use (in refuge = 1, out of refuge = 0) were developmental stage of the parasite (DS; acanthella, cystacanth), sample date (SD; June-July, August-September, October-November, December-January, February-March, April-May), host length (HL), parasite intensity (IN), and an interaction between developmental stage and intensity (DS:IN). Log-likelihood (LL), number of parameters (K), AICc value, delta AICc ($\Delta_i$), and AICc weight ($w_i$) are also shown below. Best model is listed in bold.

<table>
<thead>
<tr>
<th>Host Sex</th>
<th>Model Terms</th>
<th>LL</th>
<th>K</th>
<th>AICc</th>
<th>$\Delta_i$</th>
<th>$w_i$</th>
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<tr>
<td>Female</td>
<td><strong>HL + SD</strong></td>
<td><strong>-296.394</strong></td>
<td>4</td>
<td>598.8</td>
<td>0.00</td>
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<td></td>
<td>HL + SD + IN</td>
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<td>599.0</td>
<td>0.20</td>
<td>0.287</td>
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<td>HL + SD + DS</td>
<td>-296.385</td>
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<tr>
<td></td>
<td>HL + SD + IN + DS</td>
<td>-295.410</td>
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<td>600.9</td>
<td>2.10</td>
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<tr>
<td></td>
<td>SD + IN</td>
<td>-298.152</td>
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<td>602.4</td>
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<tr>
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<td>HL + SD + IN + DS + DS:IN</td>
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<td>4.14</td>
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<tr>
<td>Male</td>
<td><strong>DS + SD</strong></td>
<td><strong>-378.691</strong></td>
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<td>763.4</td>
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<td>0.267</td>
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<tr>
<td></td>
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<td>-377.808</td>
<td>3</td>
<td>763.7</td>
<td>0.26</td>
<td>0.235</td>
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<tr>
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<td>-376.843</td>
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<td>763.8</td>
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<tr>
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<td>DS + SD + IN + HL</td>
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<td>6</td>
<td>765.7</td>
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<td>0.086</td>
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Table 5. Prevalence, abundance, and intensity of infection in fish collected between June 2016 and May 2017. Sample date is shown in month groups (June-July = JJ, August-September = AS, October-November = ON, December-January = DJ, February-March = FM, April-May = AM).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Prevalence</th>
<th>Intensity</th>
<th>Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>JJ</td>
<td>82.5</td>
<td>10.8</td>
<td>8.9</td>
</tr>
<tr>
<td>AS</td>
<td>27.5</td>
<td>3.4</td>
<td>0.9</td>
</tr>
<tr>
<td>ON</td>
<td>40.0</td>
<td>3.3</td>
<td>1.3</td>
</tr>
<tr>
<td>DJ</td>
<td>67.5</td>
<td>4.2</td>
<td>2.8</td>
</tr>
<tr>
<td>FM</td>
<td>72.5</td>
<td>6.7</td>
<td>4.9</td>
</tr>
<tr>
<td>AM</td>
<td>75.0</td>
<td>6.3</td>
<td>4.7</td>
</tr>
</tbody>
</table>
**Figure 1.** Depiction of field site, Buffalo Creek, in Lake County, Illinois.
**Figure 2.** Life cycle of *Acanthocephalus dirus* in isopod intermediate hosts and fish definitive hosts in Buffalo Creek. Isopods consume parasite eggs from the stream, where parasites mature from the non-infective (acanthor, acanthella) to the infective stage (cystacanth). Fish (creek chub and greens sunfish) are infected after consuming infected isopods, and parasites mate inside the fish gut. Mature parasite eggs are then released back into the stream.
Figure 3. Infected and uninfected isopods (*Caecidotea intermedius*). a) Ventral view of isopod with *Acanthocephalus dirus* parasites. b) Infected isopod (left) and uninfected isopod (right).
Figure 4. Field sampling techniques. a) Collection of isopods using hand net. b) Fish blocking procedure with seine net.
Figure 5. Percent of female and male isopods in refuge during early-stage infections for June and July.
Figure 6. Refuge use and infection status (infected versus uninfected). Sample date is shown in month groups (June-July = JJ, August-September = AS, October-November = ON, December-January = DJ, February-March = FM, April-May = AM). a) Percent of female isopods in refuge by infection status. b) Percent of male isopods in refuge by infection status.
**Figure 7.** Parasite development from immature (acanthella) to mature (cystacanth) stage in isopod hosts. Sample date is shown in month groups (June-July = JJ, August-September = AS, October-November = ON, December-January = DJ, February-March = FM, April-May = AM).

a) Parasite development in female isopods. b) Parasite development in male isopods.
Figure 8. Relationship between presence of mature parasites (cystacanth) and behavioral modification (decrease in refuge use) in isopod hosts. Sample date is shown in month groups (June-July = JJ, August-September = AS, October-November = ON, December-January = DJ, February-March = FM, April-May = AM). Difference values were calculated using the percent of individuals out of refuge and in refuge as a measure of behavioral modification. a) Mature parasites and behavior modification of female hosts. b) Mature parasites and behavior modification of male hosts.
**Figure 9.** Body sizes (volume in mm$^3$) of female parasites in female hosts, female parasites in male hosts, male parasites in female hosts, and male parasites in male hosts.
Figure 10. Percent of individuals in refuge by possible infection states (Un = uninfected, A = acanthellae-only, AC = acanthellae and cystacanth, C = cystacanth-only) from August to January. a) Female isopods in refuge with no significant differences (ns) between groups. b) Male isopods in refuge.
Figure 11. Percent of each fish species (creek chub, *Semotilus atromaculatus*; green sunfish, *Lepomis cyanellus*) caught during 2016-2017. Sample date is shown in month groups (June-July = JJ, August-September = AS, October-November = ON, December-January = DJ, February-March = FM, April-May = AM).
**Figure 12.** Behavioral modification of intermediate hosts and transmission of *Acanthocephalus dirus* to definitive hosts. Shown is prevalence of infection in fish hosts. Behavior modification is shown as difference values (gray bars). Difference values were calculated using the percent of individuals out of refuge and in refuge as a measure of behavioral modification. Sample date is shown in month groups (June-July = JJ, August-September = AS, October-November = ON, December-January = DJ, February-March = FM, April-May = AM). June-July value represents previous cohort.
Figure 13. Behavioral modification of female and male isopods and transmission of *Acanthocephalus dirus* to definitive hosts. Shown is prevalence of infection in fish hosts and June-July prevalence value represents parasites from the previous cohort. Behavior modification is shown as difference values (gray bars) and was calculated using the percent of individuals out of refuge and in refuge as a measure of behavioral modification. Sample date is shown in month groups (June-July = JJ, August-September = AS, October-November = ON, December-January = DJ, February-March = FM, April-May = AM). a) Modification of female isopods and parasite transmission b) Modification of male isopods and parasite transmission.