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Uncovering The Variable Life History Traits And Strategies Of The Gregarine Parasite, Monocystis Perplexa, In Its Invasive Earthworm Host, Amynthas Agrestis

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UNCOVERING THE VARIABLE LIFE HISTORY TRAITS AND STRATEGIES OF THE GREGARINE PARASITE, *MONOCYSTIS PERPLEXA*, IN ITS INVASIVE EARTHWORM HOST, *AMYNTHAS AGRESTIS*

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ABSTRACT

Parasite life histories influence many aspects of infection dynamics, from the parasite infrapopulation diversity to the fitness of the parasite (the number of successfully transmitted parasites). Studies of medically important parasites, such as the parasite responsible for malaria (*Plasmodium* spp.), demonstrate the usefulness of investigating the life histories of parasites to better understand infection characteristics such as parasite load and probability of transmission.

The gregarines are a diverse group of apicomplexan parasites that infect invertebrates, and are particularly common in insects and annelids. Given the great biodiversity and importance of their hosts, coupled with their close evolutionary relationship with important human pathogens such as *Cryptosporidium* spp., relatively little is known about gregarine life histories. The exemplar gregarine genus, *Monocystis*, is an excellent example of how a well-known gregarine parasite can have relatively little known about its life history. Specifically, the low reproductive output of *Monocystis* spp. and the absence of asexual replication makes the currently accepted life cycle untenable. More data are needed on the life history traits and strategies of *Monocystis* spp. that allow the parasite to be maintained at high prevalence and parasitemia.

Here, a newly discovered species of *Monocystis*, infecting the invasive earthworm *Amynthas agrestis*, is described and investigated to determine key life history traits and strategies. First, I propose improvements to the current standard of gregarine species descriptions by standardizing nomenclature and biometrics and including molecular data. I described the newly discovered *M. perplexa* using the proposed improvements to gregarine species descriptions and found evidence of host species-specificity and widespread prevalence of the parasite in local earthworm populations. Such important data would not otherwise be collected with use of the current standard of gregarine species description and demonstrates the potential of implementing the suggested improvements.

The life history of *M. perplexa* was investigated by measuring various life history traits throughout the course of infection. Key life history traits such as parasite phenology, resource allocation, and reproductive output were measured for infections at three local earthworm populations to determine if there is variation in parasite life histories within or among earthworm populations. Substantial variation in parasite life history traits and trade-offs between traits were identified. Notably, there was a trade-off between timing to parasite maturity, resource allocation, and reproductive output. Surprisingly, there was a near-complete lack of transmissible stages produced in one population, despite being maintained at high prevalence and parasitemia.

To determine whether life history strategies, such as mode of transmission and asexual replication, can explain the currently untenable life cycle of *M. perplexa*, the presence of vertical transmission and asexual replication was revealed. Quantitative real-time PCR was used to detect and quantify minute amounts of parasite in the worm eggs and embryos and revealed a high rate of vertical transmission at all local sites. Evidence of parasite replication within the developing host embryo was found; however, no asexual replication was identified early in the host's season. Last, all worm tissues examined had high concentrations of parasite DNA, including the clitellum, the organ that produces the worm egg capsule.

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CHAPTER 1: Introduction and Literature Review

Parasitism is the most common lifestyle on Earth, with estimates of up to four parasite species to every host species (Rohde, 1982; May, 1992; Windsor, 1998; Poulin and Morand, 2000; de Meeûs and Renaud, 2002). Parasites themselves are very diverse, with life histories of parasites varying among closely related taxa and even within a species. The variation in life history traits and strategies may contribute to the success of parasites; this is because the environment of the parasite, its host, is often variable and the ability to adjust their life histories to their environment is advantageous (Poulin, 2007; Reece et al., 2009; Kochin et al., 2010). For example, the reproductive effort of the rodent malaria parasite, *Plasmodium chabaudi*, changes in response to the density and age of red blood cells. *P. chabaudi* exhibits an increase in reproductive effort when immature red blood cells are abundant as the environment becomes more resource-rich allowing the parasite to allocate more resources towards reproduction (Birget et al., 2017).

The life history of a parasite is crucial to understanding the infection dynamics and evolution of the parasite. The life history of an organism is the collection of traits that influence its life table, and includes such traits as timing of reproduction and reproductive output (Roff, 1992; Stearns, 1992; Eisen and Schall, 2000). The complex pattern of co-evolved life history traits constitutes life history strategies (Stearns, 1992), and for parasites, mode of transmission and life cycle are strategies that greatly influence parasites' fecundity, distribution, maintenance of infections, and genetic diversity.

The gregarines are a large clade of apicomplexan parasites that infect

invertebrates and are ubiquitous among insects and annelids as they frequently reach 100% prevalence in a host population (Desportes and Schrével, 2013). For example, *Lumbricus terrestris* earthworms in the wild are almost never found to lack infection by its *Monocystis* sp. gregarine parasite (Meier, 1956; Miles, 1962). Given their widespread prevalence in hosts that make up much of the biodiversity seen on Earth, it is surprising that relatively little is known about the life histories of the gregarines. The reclassification of *Cryptosporidium*, the second leading cause of childhood diarrheal disease, as sister to the gregarines further highlights the need to study the life histories of gregarines (Carreno et al., 1999; Barta and Thompson, 2006; Leander, 2007). Studying the life histories of gregarine parasites can contribute to our understanding of the evolution of parasite life histories and provide insight into the life histories of parasites of medical interest.

Monocystis is a genus of gregarine parasites that infect earthworm hosts and is an exemplar for the gregarines because it is frequently used as a representative of the gregarines in parasitology courses (Sheridan, 1986). Despite being studied for over a century, the life history and life cycle of *Monocystis* parasites has never been fully worked out (Miles, 1962; Fields and Michiels, 2006). The currently accepted life cycle of *Monocystis* parasites is described and depicted in Fig 1. In short, infection begins when an earthworm ingests the environmentally-durable oocyst. Upon ingestion, individual parasites termed sporozoites are liberated from the oocyst and migrate to the seminal vesicles where they feed on the earthworm's sperm morulae as trophozoites. After feeding, the parasites sexually reproduce (gametogony) to produce zygotes which then divide to form the infective sporozoites within an oocyst (sporogony). The oocysts are then released into the environment through an unidentified route, although multiple

routes of horizontal transmission have been proposed; these include the oocysts exiting the host via feces (Hahn, 1928), through the dorsal or male pores (Hesse, 1909; Fantham, 1932; Loubatières, 1955; Olsen, 1974), through copulation (Schmidt, 1854; Bhatia, 1924; Troisi, 1933), or through death and decay (Minchin, 1903; Hesse, 1909; Bhatia, 1924; Keilin, 1925). There has been limited support for all routes of transmission of the oocyst into the environment or to another host, particularly copulation and through feces, and many authors support death and decay as the likely primary route of transmission (Minchin, 1903; Hesse, 1909; Bhatia, 1924; Keilin, 1925; Miles, 1962).

The life cycle of *Monocystis* spp. is particularly interesting because it appears untenable given our current knowledge of the life history of *Monocystis*. Notably, *Monocystis* spp. have a low reproductive output for an r-selected species (Pianka, 1970), with a reproductive output on the order of hundreds as opposed to thousands for other apicomplexans; this is surprising because *Monocystis* spp. and other eugregarine parasites putatively lack asexual replication or merogony (Ellis et al., 1998; Levine, 1971; Vivier and Desportes, 1990). Other apicomplexan parasites, and even the closely related polyphyletic neogregarines (Levine, 1971), undergo merogony which dramatically increases the parasitemia within a host and can explain how few parasites can be transmitted and yet produce heavy infections (Smith et al., 2000; McKenzie et al., 2008; Rossouw et al., 2015). Given the low reproductive output yet high parasite loads and putative lack of merogony, ingestion of oocysts as the sole mode of transmission would require an improbably high survival and transmission rate of the oocysts.

The overall aim of this thesis is to describe the life history traits, strategies, and variation therein that allows *Monocystis* spp. to persist in earthworm populations at high

prevalence and parasitemia. To do this, I discovered a new species of *Monocystis* that infects an important invasive earthworm species, *Amynthas agrestis* (Callaham et al., 2003; Snyder et al., 2011; Zhang et al., 2010; Görres and Melnichuk, 2012). One advantage of working with this newly discovered parasite-host system is that the host is an annual earthworm, meaning it completes its entire life cycle within one season (spring–autumn); because of this, the entire life cycle of *M. perplexa* can be observed without interference from previous years' infections. Following the infection of *M. perplexa*, it is possible to describe the life history of the parasite, identify trade-offs between life history traits, and determine potential life history strategies that contribute to the success of the parasite.

First, I aim to produce a species description of *M. perplexa* that elevates the current standard of gregarine species descriptions (Chapter 2). A lack of standardization in nomenclature and biometrics have made comparisons among described gregarine species difficult (Clopton, 2004; Simdyanov et al., 2017). Further, a lack of molecular data for most known gregarines has hindered identification of species and resolving the early evolutionary history of the apicomplexans (Leander et al., 2003; Rueckert et al., 2011). Here, I have prepared a species description that complies with current gregarine species description requirements, proposes improvements to the current standard, and demonstrates the benefits of implementing the suggestions. Chapter 2 is written for ease of publication and follows the journal requirements, including organization and citation style, of the *Journal of Parasitology*; this journal was selected as it is a relatively high-impact journal in the field of parasitology and publishes influential species descriptions that address broader syntheses or critical issues.

Second, I aimed to conduct a comprehensive life history study to identify key traits and variation that contribute to the success and evolution of *M. perplexa*. To better understand its life history, infections were followed over the course of the host's season to measure various life history traits including parasite-host phenology, parasitemia, resource allocation, and reproductive output. Life histories were examined for three local populations of *A. agrestis* to determine if there is variability in life histories among host populations, potentially in response to their local environment. Chapter 3 has been written for ease of publication in *Parasitology*.

Third, I aim to identify important life history strategies that help explain the currently untenable life cycle and life history of *M. perplexa* (Chapter 4). Specifically, the presence of vertical transmission, transmission from the parent to the offspring, was evaluated to determine if *M. perplexa* is transmitted through mixed modes of transmission. The presence of merogony was also investigated. While the eugregarines are thought to lack merogony, the closely related polyphyletic neogregarines can undergo merogony which warrants investigation into whether merogony is present in *Monocystis* spp. and has simply not been detected due to the difficulty in providing evidence of merogony (Ray, 1930; Levine, 1974; Vivier, 1975; Desportes and Théodoridès, 1979; Gunderson and Small, 1986; Leander et al., 2003). To address these aims, I used quantitative real-time PCR (qPCR), a technique that allowed me to detect and quantify minute amounts of pathogens (Clark et al., 2011; Postollec et al., 2011; Yang et al., 2013). I followed the now standard methods to ensure high quality qPCR results by complying with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Chapter 4 has been prepared

for ease of publication in *Parasitology*.

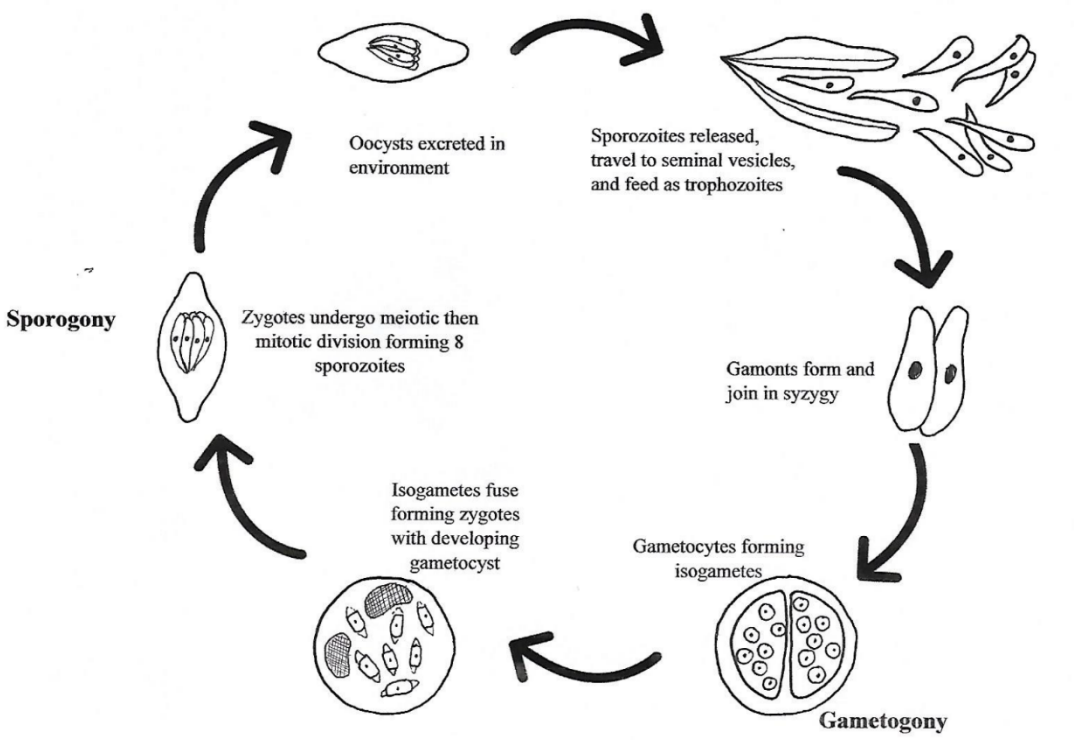

Fig 1. Currently accepted life cycle of *Monocystis* spp. Earthworm hosts become infected upon ingesting the environmentally-durable oocyst deposited in the soil. After ingestion, sporozoites are liberated from the oocyst and migrate to the seminal vesicles and begin feeding on host spermathecae as trophozoites. After feeding, the parasites develop into mature gamonts which then pair in syzygy. A membrane forms around the gametocytes which produce isogametes that fuse together to form diploid zygotes in a process known as gametogony. Within the developing gametocyst, the zygotes undergo meiotic then mitotic division to form eight haploid sporozoites inside an oocyst (sporogony). The gametocysts, or oocysts if they have been liberated, then exit the host into the environment by an unconfirmed route; in the case of *Monocystis perplexa*, likely when the worm dies in the late autumn. Figure drawn by Erin L. Keller.

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CHAPTER 2: *Monocystis perplexa* n. sp. (Apicomplexa: Monocystidae) from the Asian invasive earthworm *Amynthas agrestis* (Megascolecidae), with a proposal of an improved standard for *Monocystis* species descriptions

Summary

The gregarines are a highly diverse group of apicomplexan parasites that are ubiquitous among their invertebrate hosts; for example, species of *Monocystis* can often reach 100% prevalence in earthworm populations. While the gregarine importance in resolving the early evolution of apicomplexan parasites due to its early-branching position has been long recognized, the gregarines have been understudied compared to other parasites in the phylum. It was only recently that *Cryptosporidium*, the parasite that causes human diarrheal disease, was identified to be closely related to the gregarines because of the lack of genetic data for the gregarines. The current standard for gregarine species descriptions, particularly acephaline species, limits studies on the diversity and evolution of the gregarines. Here I describe *Monocystis perplexa* n. sp., a parasite of an important invasive earthworm, *Amynthas agrestis*, the first *Monocystis* described from a Japanese *Amynthas*. I propose an improved standard for gregarine species descriptions that i) standardizes the nomenclature to reduce synonymies, ii) utilizes a detailed and standardized biometrics set from Clopton (2004), iii) is informative about the host specificity of the parasite being described, and iv) provides molecular data that can be used to identify new species and improve phylogenies.

Monocystis perplexa is described using the proposed species description guidelines and key information about the life history of the parasite. First, comparing the biometrics and morphology of parasites in different local host populations showed that *M. perplexa* is ubiquitous among its earthworm host, *Amynthas agrestis*. Second, comparing parasite morphologies of *Monocystis* parasites in sympatric earthworm species indicates that *M. perplexa* is specific to *A. agrestis* and was likely introduced with its invasive host from Asia. Third, the use of the small subunit 18S rDNA gene was useful in constructing a gene tree that accurately clustered *M. perplexa* with the only other *Monocystis* species with molecular data available, *M. agilis*. The gene tree was concordant with other 18S phylogenies and, in combination with morphological data and other genes, has the potential to resolve lower-level taxonomic organization of the gregarines.

The use of standardized nomenclature, biometrics, and molecular data will improve our understanding of the diversity of the gregarines. As more data comes available for gregarine species, more robust phylogenies will be constructed, the diversity of the gregarines will become more apparent, and comparisons among gregarines can be more easily made. With the influx of biometric and molecular data, a uniform database housing information and genomic data of the gregarines should be implemented to facilitate retrieval of molecular data.

Introduction

The gregarines are a group of early-branching apicomplexan parasites that infect a broad diversity of invertebrate animals of marine, freshwater, and terrestrial environments (Desportes and Schrével, 2013). Like other apicomplexans, the gregarine life cycle includes sexual reproduction to produce the transmission stage (oocysts), but differs in lacking merogony (asexual replication) in most species. Also, unlike other apicomplexans, such as the haemoparasites and coccidians that are intracellular parasites, the gregarines are predominantly extracellular. The gregarines have historically been divided into three groups – the archgregarines, eugregarines, and neogregarines (Grassé, 1953) – based primarily on poorly-diagnostic morphological characteristics or the presence/absence of merogony (Levine, 1971; Leander et al., 2003b); however, polyphyly of the eugregarines and neogregarines suggests that these diagnostic characteristics are not sufficient in distinguishing these groups and the putative absence of merogony could result from lack of investigation into the life history strategy or simply the difficulty in obtaining evidence for merogony (Leander et al., 2003b; Levine, 1974; Vivier, 1975; Desportes and Théodoridès, 1979; Gunderson and Small, 1986).

While the gregarines are largely considered to impart little harm onto their hosts, some, particularly the neogregarines, which are diagnosed based on presence of merogony, have potential for use as biological control agents of insect vectors (Lantova and Volf, 2014). Despite their widespread presence in invertebrates and their potential importance as biological control agents of vector-borne diseases, less than 1% of invertebrates have been surveyed for gregarine infection and relatively few of likely gregarine species have been described (Clopton, 2000). Further, the lack of molecular

data, confusion of terms, and the use of poorly-distinguishing morphological characteristics has impeded our understanding of the evolutionary relationships among the gregarines (Rueckert et al., 2011; Simdyanov et al., 2017).

The evolution, life history, and diversity of the gregarines could be better understood by improving upon species descriptions of newly discovered gregarine taxa. First, the terminology used to describe diagnostic morphological features should be standardized. Even with Levine's widely used terminology for apicomplexan parasites (1971), certain terms have been misused and has led to confusion in understanding the evolution of important features (Simdyanov et al., 2017). For example, the attachment organelle should be referred to as "mucron" for aseptate eugregarines and as "epimerite" for septate eugregarines based on the current nomenclature (Levine, 1971); however, this terminology may require revising when evolutionary relationships become better resolved (Simdyanov et al., 2017). Further, lack of standardized biometrics used in species descriptions have made direct comparisons of diagnostic morphological features difficult. Using standardized nomenclature and biometrics, such as the detailed set presented by Clopton (2004), in species descriptions can help resolve our understanding of the evolution of important diagnostic traits. Comparison would also be further aided by improved quality of photographs used in species descriptions, particularly of the morphologically plastic Monocystids (Berlin, 1924, 1924; Rueckert et al., 2011). Fixed and stained preparations often result in distorted and shrunken parasite cells that make identifying features difficult (personal observations); therefore, live preparations of slides used with light microscopy is preferred.

Second, investigation into the gregarine component communities of invertebrate

species living in sympatry with the host species of interest can also provide important data in terms of gregarine host specificity. Although gregarines are assumed to be primarily host species specific (Clopton et al., 1992; Perkins et al., 2000), host specificity of gregarines has not been well studied (Clopton, 2009). Since many invertebrate host species of gregarines live in sympatry, such as earthworm communities, gregarine parasites likely encounter sympatric host species in the environment providing the opportunity to infect multiple host species. Investigating the component communities of sympatric host species can provide insight into the host specificity which is a key life history trait that influences the evolution and diversity of parasites.

Last, there is a dearth in molecular data for the gregarines that impedes identification of new species. DNA barcoding is a powerful method of identifying species based on molecular data and has greatly facilitated measuring biodiversity and accurately identifying species. Both nuclear and mitochondrial genes have been used as barcodes for eukaryotes, and the use of the co-oxidase I (COI) gene as a molecular barcode has been used successfully in some apicomplexa parasites, such as the coccidians (Ogedengbe et al., 2011); however, the COI gene in gregarines and the closely related *Cryptosporidium* spp. appears to be absent (Templeton et al., 2010; Putignani et al., 2004).

While more often used in resolving deep-branching phylogenies, the SSU rDNA 18S gene has also had success as a barcoding gene for apicomplexans. The 18S gene is useful as a barcoding gene because it harbors both conserved and variable regions, is present in multiple copies in the genome, and does not cross-react with host DNA making detection sensitive and accurate (Renoux et al., 2017). The 18S gene is thus appropriate as a barcoding gene for the gregarines and, because of its slower rate of evolution than

other barcoding genes like COI, can also be used to resolve higher taxonomic levels. For example, the use of SSU rDNA identified that *Cryptosporidum* spp. is more closely related to the gregarines than its previous classification in the suborder Eimerioirina Léger 1911 (Carreno et al.,1999; Relman et al., 1996; Barta et al., 1997; Morrison and Ellis, 1997; Tenter and Johnson, 1997; Carreno et al., 1998; Lopez et al., 1999). Use of the 18S gene as a barcode-like marker for the gregarines, in addition to accumulation of data for other genes, will aid in species identification. Primers and protocols used to obtain molecular data are also needed to promote the expansion of gregarine sequences available.

Here, I propose suggestions for gregarine species descriptions with the aim of increasing standardization among nomenclature and biometrics, better understanding host specificity, and increase the use of molecular data to identify species and elucidate unresolved evolutionary relationships. A new species description of *Monocystis* infecting the Asian invasive earthworm, *Amynthas agrestis*, is used to demonstrate the implementation of these suggestions. Standardized nomenclature and biometrics derived from Clopton's proposed set (2004) is used to describe *Monocystis* n. sp. Investigation of the component communities of local sympatric earthworm host species provides information on the specificity of *Monocystis* n. sp. Last, molecular data from multiple genes are provided for the species described and the 18S gene is used to construct a phylogeny of the gregarines to demonstrate the usefulness of submitting molecular data with species descriptions.

Methods

Host collection and dissection

The invasive Asian earthworm, *Amynthas agrestis*, was introduced into the United States in the early 20th century and has since spread throughout the continental United States (Gates, 1935; Gates, 1937; Gates, 1954; Reynolds, 2018; Synder et al., 2011). Within the last decade, ongoing surveys began identifying the presence of *A. agrestis* in Vermont and these earthworms are now found throughout the state (Görres and Melnichuk, 2012; Reynolds et al., 2015; Reynolds, 2012). *A. agrestis* resides in the top 10cm of soils often sympatrically with the congeneric *A. tokioensis* and *Metaphire hilgendorfi* earthworms (Chang et al., 2016). Infected earthworms from the type locality were collected by manually sifting through the top 10cm of soil between July and October 2017. The new *Monocystis* species was studied in 19 *A. agrestis* earthworms from the type locality. Additional *A. agrestis* earthworms were collected from the type locality and two other local Vermont sites to determine prevalence and distribution of the parasite. There is no genetic structure between the Vermont populations of *A. agrestis* suggesting they may be from a single source population or connected through frequent migration (Keller et al., 2017). All adult earthworm samples were confirmed to be *A. agrestis* using a morphological key for pheretimoid earthworms (Chang et al., 2016).

Species Description Measurements

All earthworms were killed by submersion in 50% ethanol and the seminal vesicles of the earthworms were dissected and disrupted with 1x Ringer's solution and 2µL of the live preparations placed under a coverslip and examined with a light

microscope. Live preparations were preferred to fixed preparations because I observed dramatic distortion, shrinking, and loss of detail in cells caused by the fixing and staining. Further, Monocystids often distort their shape while moving so observing these morphological changes in live specimens can reduce false identification of new species based on morphological differences in fixed specimens. Measurements and photographs of the gamont, gametocyst, and sporocyst life stages were taken using Moticam 1000 1.3MP Live Resolution (Richmond, British Columbia) microscope camera and Motic Image Plus 2.0.11 computer program. Nineteen *Amynthas agrestis* used for the species description from the Green Mountain Audubon Center were dissected and gamonts, gametocysts, and oocysts, were measured according to the guidelines published by Clopton (2004) and photographed.

DNA Extractions and Sequencing

Oocysts from the seminal vesicles of earthworms were isolated by homogenizing the seminal vesicles with Ringer's solution and manually removing host tissue under a dissection microscope. The gametocyst samples were then strenuously vortexed with a small amount of 0.5mm zirconia beads for one minute to break open the gametocysts. The saline supernatant was then removed and the oocysts were treated with 10% sodium hypochlorite for 10 minutes to remove any remaining host DNA. The oocysts were then washed twice with saline and the oocysts opened by vortexing four times for three minutes at 4800 oscillations/minute in lysis buffer with a 1:1 ratio of beads to lysis buffer. Pure oocyst samples were then extracted using DNeasy ® Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and

stored at 4ºC until use.

The 18S SSU rDNA gene was amplified in a single fragment from the two oocyst samples using universal eukaryotic primers 5'- CGA ATT CAA CCT GGT TGA TCC TGC CAG T-3' and 5'- CCG GAT CCT GAT CCT TCT GCA GGT TCA CCT AC-3' previously described by Leander et al. (2003). The PCR conditions were as follows: Initial denaturation: 95ºC 2 min, followed by 35 cycles of 92ºC/45s, 60ºC/45s, and 72ºC/1.5 min, with a final extension at 72ºC for 5 min. The PCR yielded a single amplicon of approximately 1,700bp.

Generalized gregarine primers for amplifying a partial fragment of the actin gene were designed by aligning actin sequences from *Ascogregarina tawienensis* (GENBANK accession ABJQ00000000) and *Monocystis agilis* (GENBANK accession AY391264.1) and identifying consensus regions between the two; the actin primers designed are as follows and yield an amplicon length of 220bp: ActinF: 5' – GGG ATG ATA TGG AAA AAA TAT GGC ATC A – 3' and ActinR: 5' – ACG ATA CCA GTA GTA CGG CCA GA– 3'. The PCR conditions for the actin primers are as follows: Initial denaturation: 94ºC 4 min, followed by 32 cycles of 94ºC/30s, 49ºC/30s, 72ºC/1 min, with a final extension at 72ºC for 3 min.

Primers amplifying the partial end of internal transcribed spacer (ITS) ITS1, full 5.8S, full ITS2, partial 18S of *Monocystis* spp. were used to isolate the variable ITS region from *Monocystis* spp. (Velevan et al., 2010). The ITS primers are as followed and yield an amplicon length of approximately 430 base pairs: MITSF: 5' – GAG AAG TCT TGT AAA CCC AAT T – 3' and MITSR: 5' – GTT AGT TTC TTT TCC TCC – 3'. The PCR conditions were as follows: Initial denaturation: 94ºC 5 min, followed by 35 cycles

of 94ºC/1 min, 52ºC/1 min, 72ºC/2 min, with a final extension at 72ºC for 7 min. The amplicons for all genes were sequenced using Sanger sequencing.

Comparison to parasites of sympatric host species

Since *A. agrestis* earthworms are often found in sympatry with local and invasive earthworms of Vermont, the parasites infecting the seminal vesicles of six sympatric earthworm species were examined. All earthworm samples were collected within Chittenden County, Vermont and the dissection and live preparation of slides of the seminal vesicle infections was done as above. The following local earthworm species were sampled and examined for the presence of *Monocystis* n. sp.: *Aporrectodea turgida* (N=3), *Amynthas tokioensis* (N=8), *Lumbricus terrestris* (N= 5), *L. festivus* (N=1), *L. rubellus* (N=3). Apparent morphology of the observed parasite life stages for each host species were visually compared to that of *Monocystis* n. sp. to determine if *Monocystis* n. sp. was present in sympatric host species.

Phylogeny construction

To demonstrate the usefulness of the 18S rDNA gene in constructing gregarine phylogenies and the clustering of *Monocystis* n. sp. with another species in the genus, *M. agilis*, a gene tree was constructed using the 18S gene. The 18S gene sequences from 16 representative gregarine and outgroup species were obtained from GenBank (www.ncbi.nlm.nih.gov, Clark et al., 2016) and aligned through ClustalW (Thompson et al., 1994) in the bioinformatics software, Geneious 6.0.6 (http://www.geneious.com, Kearse et al., 2012). The codon positions for the resulting 1,738bp alignment were then

assigned using Mesquite (Maddison and Maddison 2018). The best partitioning scheme and models for substitutions for the data were determined using PartitionFinder 1.1.1 (Lanfear et al., 2012) using the *greedy* algorithm for all models. The Akaike Information Criterion corrected (AICc) was implemented to identify the best fit model.

The program Mr. Bayes 3.2.3 (Ronquist and Huelsenbeck, 2003) was used to perform the Bayesian inference for the data using the GTR+G+I model as this model was determined to be the most appropriate for the data. Three simultaneous and independent runs consisting of four Markov Chain Monte Carlo (MCMC, one cold and three heated chains) were run for 10 million generations each, with tree sampling every 1000 generations to calculate the posterior probabilities. The three runs were performed for the three partition blocks, by codon position, used in PartitionFinder. To determine if the MCMC has reached convergence, the program Tracer was used (Rambaut et al., 2014). Lastly, Mesquite was used to produce the Bayesian tree showing the posterior probability and branch lengths for each node.

GenBank accession numbers: XXX, *Monocystis* n. sp.; AF457127.1, *Monocystis agilis*; AY179988.1, saltmarsh sediment DNA alveolate clone CCA5; GU320208.1, *Gregarina ctenocephali*; FJ459755.1, *Paraschenideria metamorphosa*; AF372779.1, uncultured alveolate clone BOLA184; JX131300.1, *Ascogregarina taiwanensis*; AF129883.1, *Ophriocystis elektroscirrha*; AY334568.1, *Mattesia geminate*; KX965721.1, *Theileria buffeli*; KT184335.1, *Eimeria alabamensis*; KC8908803.1, *Selenidium terebellae*; AF151376.2, *Cryptosporidium serpentis*; LN901450.1, *Lecudina tuzetae*; FJ459754.1, *Leidyana migrator*; FJ459747.1, *Gregarina niphandrodes*.

Description

Phylum Apicomplexa Levine, 1988

Order Eugregarina Leger, 1900

Family Monocystidae Bütschli, 1882

Genus Monocystis Stein, 1848

***Monocystis perplexa* n. sp.** (Fig. 1)

Diagnosis: Infections are found primarily in seminal vesicles of *Amynthas agrestis* and occasionally seen in the body coelomic fluid. Mature gamonts are rhomboid in shape with length (L ± SD) 49.06 ± 12.43 μm, width (W ± SD) 27.20 ± 7.26 μm, and with a round sucker-like mucron with a retractile central tentacle located central laterally. Orbicular nuclei situated typically in the center of gamonts, with diameter (D ±SD) 10.33 ± 2.54 μm. Mature gametocysts are orbicular in shape with D 120.74 ± 16.43 μm. Oocysts are fusiform in shape with L 15.37 ± 0.74 μm and W 5.65 ± 0.53 μm (Fig.1).

Taxonomic Summary

Type host: Amynthas agrestis Kinberg 1867 (Megascolecidae).

Type locality: The Green Mountain Audubon Center, Huntington, Vermont, U.S.A (190 m elevation, 44.346774° N, -72.996216° W).

Additional localities: UVM Horticultural and Research Farm, South Burlington, Vermont, U.S.A (44.431489 ºN, -73.199211ºW); UVM Centennial Woods, Burlington, Vermont (44.475701ºN, -73.187088ºW). The three Vermont sites are all within 19km distance.

Type specimens: Holotype (XXX) deposited at the Smithsonian Museum of Natural

History, and paratype (XXX) deposited at University of Vermont Zadok Thompson Museum of Natural History.

Prevalence: Overall prevalence in *Amynthas agrestis* was 603 of 603 (100%) by mid-summer to time of seasonal host death in mid-autumn.

DNA Sequences: 18S small subunit ribosomal RNA gene (1,665bp, GenBank accession no. XXX); the partial end of ITS1, full 5.8S, full ITS2, partial 18S (427bp, GenBank accession no. XXX); actin nuclear gene (175bp, GenBank accession no. XXX).

Etymology: Monocystis perplexa, from the Latin word *perplexa* meaning intricate and complicated. *M. perplexa* is an appropriate name that refers to the protean morphology of this parasite's trophozoites as they appear to drastically change shape while moving. Further, many aspects of the life history and life cycle of *Monocystis* spp. remain unresolved and perplexing.

Remarks

This is the first species of *Monocystis* described from *Amynthas agrestis* and of any earthworm species from Japan. In concord with all parasites of the family Monocystidae (Bütschli, 1882), *M. perplexa* is characterized by solitary gamonts with little anterior differentiation and fusiform oocysts. An examination of earthworms of five species broadly sympatric in the region where *A. agrestis* has been surveyed, found all were infected with an apparent *Monocystis* species, but based on morphology and biometric measurements, *M. perplexa* is found only in *A. agrestis* earthworm hosts due to the distinct morphological differences among parasites in the other earthworms (e.g. S1). Further, *M. perplexa* appears to be widespread among local *A. agrestis* populations based on the presence of *M. perplexa* at two sites in additional to the type location. *M. perplexa*

can also be easily distinguished from other described *Monocystis* species found in *Amynthas* spp. (*Metaphire* spp.) earthworms based on gamont, gametocyst, and oocyst morphology. The two most similar *Monocystis* species to *M. perplexa* are *M. amynthae* from *A. hawayanus* and *M. metaphirae* from *Metaphire houlleti* and are compared below.

The gamonts of *M. amynthae* (Bandyopadhyay et al., 2006a) and *M. metaphirae* (Bandyopadhyay et al., 2006b) are longer, wider, and of different morphology than *M. perplexa* gamonts. *M. perplexa* gamonts are rhomboid, with tapered anterior and posterior ends, smooth edges, and no marked mucron, whereas *M. amynthae* and *M. metaphirae* gamonts have broad anterior ends, narrow posterior ends, and anterior mucrons. All three *Monocystis* species contain an irregularly shaped paraglycogen granules uniformly located throughout the gamont cytoplasm; however, *M. amynthae* gamonts are uniquely constricted between the anterior and posterior ends. At the center of *M. perplexa* gamonts and at the anterior end of *M. amynthae* gamonts, both species contain a round, cup-like depression in the center to anterior end with unknown function. Further distinctions between *M. perplexa, M. amynthae*, and *M. metaphirae* come from morphometric differences in gametocysts; *M. perplexa* gametocysts are nearly twice as large as *M. amynthae* and *M. metaphirae*, however, this may be due to differences in slide preparations. When preparing slides without a coverslip and with similar methodology to Bandyopadhyay (2006a, 2006b), *M. perplexa* gametocysts remain larger in diameter (75.02μm) than *M. amynthae* (58.0μm) but smaller than *M. metaphirae* (93μm). Additionally, *M. perplexa* oocysts are longer (15.4μm) and have a larger ratio of oocyst length to width (2.7) than *M. amynthae* (oocyst length 10.5μm; ratio 1.9) and *M. metaphirae* (oocyst length 9.0μm; ratio 1.6).

Perhaps the most useful distinction between *Monocystis* species is the molecular data of taxonomically informative genes. For example, *M. perplexa* can be easily distinguished using the 18S rRNA gene sequences from the only other *Monocystis* species available on GenBank (AF457127.1); the 18S sequence of *M. agilis* shares 83.0% identity with *M. perplexa* which highlights the usefulness of the 18S sequences as a barcoding gene for the gregarines. Further, the best supported gene tree from the Bayesian inference of the 18S gene supports the placement of *M. perplexa* in the genus *Monocystis*, as the two species were clustered together with an unidentified soil isolate which, based on its clustering with other *Monocystis* spp., suggests that the organism may be a *Monocystis* sp. (Fig 2). The topology of the gene tree is concordant with other studies using ribosomal genes to delineate gregarine relationships (Leander et al., 2003a; Cavalier-Smith, 2014).

Discussion

The gregarines are a diverse group of apicomplexan parasites that have the potential to further our understanding of the evolutionary relationships among parasites in the phylum. The gregarine's position as early-branching apicomplexans can provide resolution of the early divergence of parasites in the phylum (Leander et al., 2003a). Additionally, the relatively recent reclassification of *Cryptosporidium* as sister to the gregarines underscores the importance of better understanding these ubiquitous parasites. Further, some gregarine species may be useful as biological control agents of certain insects that function as vectors in some disease systems (Lantova and Volf, 2014). By studying the gregarines, important insights into apicomplexan evolution and gregarine

diversity can be made; however, in order for these insights to come to fruition, improved species descriptions of the gregarines are necessary.

One goal of this species description was to redefine and provide an example of what should be included in future gregarine species descriptions. First, standardized nomenclature and biometrics based on Clopton's set (2004) should be used to reduce synonymy in terminology. Second, in cases where the host species of interest inhabits a community with other potential hosts of gregarines (e.g. earthworm communities), the infection status of the sympatric species should be investigated to determine if gregarine species are shared between host species; this can provide important data regarding the host specificity of the gregarines. Last, molecular data should be provided with gregarine species descriptions to better identify gregarine species, contribute to the sequences available, and help produce more robust phylogenies.

Implementing the above suggestions in the species description of *Monocystis perplexa* provided important insight into the diversity, specificity, and distribution of *Monocystis* species. Notably, the host specificity of *M. perplexa* and other *Monocystis* spp. was found to be highly host species specific, with each species of earthworm appearing to have their own, morphologically distinct species of gregarine. *M. perplexa* infections were found in all *A. agrestis* earthworms sampled but, based on morphology, *M. perplexa* was not found in any other lumbricid or exotic earthworm species.

Beyond providing evidence for host specificity of *Monocystis*, the failure to identify *M. perplexa* in sympatric host species suggests that *A. agrestis* earthworms retain

their native infections when invading new communities. The role of parasites in the invasion success of their hosts has been of interest in studies of invasive species and it has been suggested that the success of an invasive species can be attributed to the loss of an invasive species' native parasite fauna ("Enemy Release Hypothesis") (Keane and Crawley, 2002; Torchin et al., 2002, 2003; Mitchell and Power, 2003). Alternatively, it has also been suggested that invaders are successful because they out-compete local species by infecting the naïve host species with their own pathogen ("Novel Weapons Hypothesis") (Callaway and Ridenour, 2004). In the case of the invasive *A. agrestis* earthworm, the observation that *M. perplexa* is found only in its native host suggests that neither the loss of their native parasites nor the gain of new parasites from local hosts plays a significant role in the success of *A. agrestis* as an invasive species. To further explore this, the infracommunity of parasites in *A. agrestis* earthworms should be compared between earthworms in their native location and the invaded location to see if the parasite species and parasite genetic diversity has been maintained through the invasion process.

Comparison of parasite component communities in *A. agrestis* earthworms between local populations and use of molecular data provided evidence that *M. perplexa* is widespread in earthworm populations in Vermont. The morphology, biometrics, and molecular data of parasite infections among local sites suggest a single parasite species; this is likely due to migration of hosts, and therefore parasites, between sites, and a possible single source population of *A. agrestis* (Keller et al., 2017). Identifying differences in life history traits of *M. perplexa* among host populations could provide

critical information on the plasticity of life history traits of gregarines in different host populations.

Lastly, the use of the 18S SSU rDNA as a barcoding gene is promising. The 18S sequences obtained from *M. perplexa* demonstrates that it is a new species and is found at all three local sites. The 18S gene sequenced from *M. perplexa* accurately clustered with *M. agilis*, as was expected based on the diagnostic characteristics of the genus (Stein). Beyond its usefulness as a barcoding gene, the 18S gene is also useful in resolving early evolutionary relationship. The 18S gene tree constructed was concordant with previous phylogenies using ribosomal genes. Phylogenies based on the 18S gene, however, are discordant with phylogenies based on morphological characters which result in monophyly of gregarine groups (Simdyanov et al., 2017). More genes are thus needed to elucidate the phylogeny of the gregarines. Genes such as the variable internally transcribed spacer (ITS) rDNA gene may provide great resolution at the genus and species levels. The ITS gene is particularly useful as it is a multi-copy gene that allows for amplification of small quantities of genomic DNA and is variable at the species-level making it another gene appropriate for species delimitations and phylogeny construction (Schoch et al., 2012; Man et al., 2010).

While reporting certain gene sequences such as 18S can contribute to the molecular data available for the gregarines, a comprehensive database for depositing and referencing gregarine genome sequences would certainly benefit studies of the gregarines. A unified database like this exists for avian blood parasites (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) and has significantly benefited the field (Bell et al.,

2015). The database, MalAvi (http://mbio- serv2.mbioekol.lu.se/Malavi/), was created in 2009 with the aims of providing an easily accessible comprehensive database to house information ranging from sequences, genetic markers, and lineage information and to standardize the nomenclature used in the field (Bensch et al., 2009). Before the implementation of this database, researchers studying avian blood parasites encountered similar challenges as researchers studying the gregarines, such as synonymy in terms and difficulty obtaining comparable genomic sequences. The success of MalAvi clearly demonstrates the potential of such database for the gregarines and should be strongly considered to remove confusion from the field and improve our understanding of the diversity and evolution of the gregarines.

Table 1. Measurements and samples sizes of key life stages of *Monocystis perplexa* with plane shape and corresponding measurements in accordance with Clopton's gregarine standardized nomenclature and biometric set (2004). Means and standard deviations for each life stage are given.

Characters	**Measurement**	***M. perplexa* n. sp.**	**Sample Size**
Gamonts (Rhomboid)	Length (distance along vertical axis of symmetry)	49.06 ± 12.43	591
	Width (distance along horizontal axis of symmetry)	27.20 ± 7.26	581
Nucleus	Diameter	10.33 ± 2.54	497
Gametocyst (Orbicular)	Diameter	120.74 ± 16.43	328
Oocyst (Fusiform)	Length	15.37 ± 0.74	227
	Width	5.65 ± 0.53	154

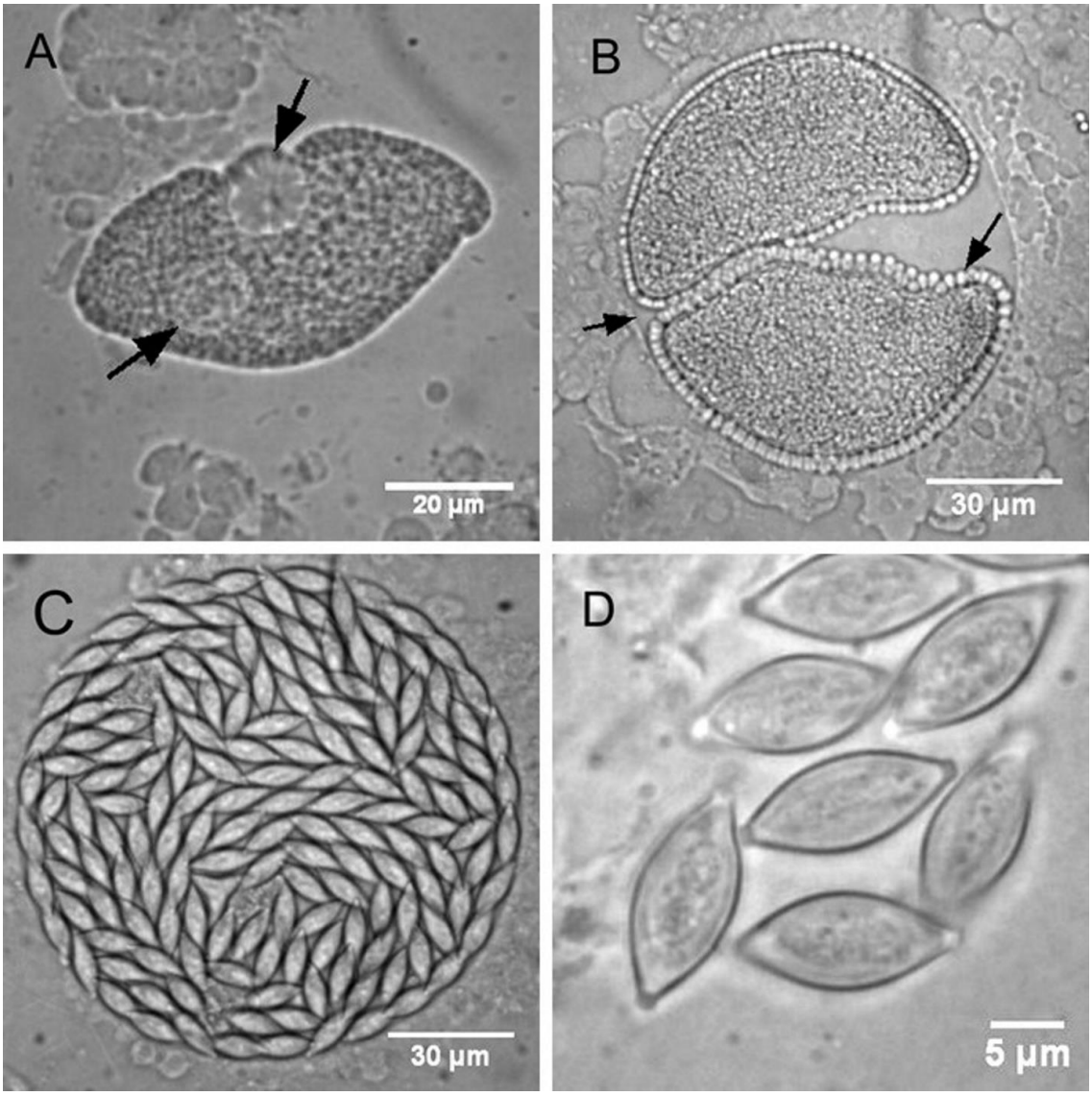

Fig 1. Photographs of key life stages of *Monocystis perplexa* taken from infections from *Amynthas agrestis* earthworms collected in Chittenden County, Vermont. A) Mature gamont with arrows pointing to the nucleus and cup-like depression of unknown function. B) Gametocytes enclosed by a gametocyst wall with gametes forming around the exterior of the gametocytes. C) Mature gametocyst containing infective oocysts. D) Fusiform oocysts that have been released from a gametocyst.

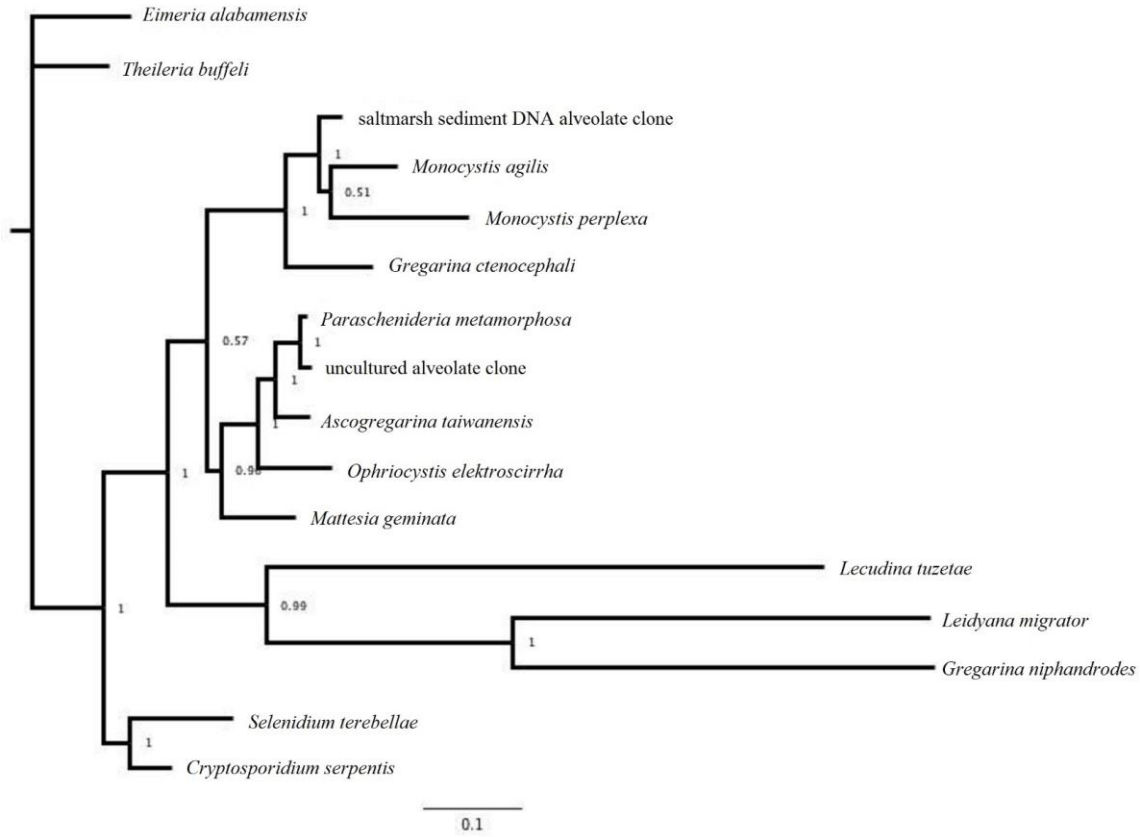

Fig 2. Consensus gene tree based on Bayesian analysis showing the relationship of *Monocystis perplexa* n. sp. to other gregarines, and that of gregarines to other apicomplexans inferred from 18S SSU rDNA sequences. Maximum parsimony trees were similar to the Bayesian trees for both genes (data not shown). The branch lengths are proportional to hypothesized evolutionary change as indicated (scale bar). For each branch, the posterior probability branch support is shown. The recovered phylogeny shows that the new species is sister to *Monocystis agilis*, a parasite of the European earthworm *Lumbricus terrestris*.

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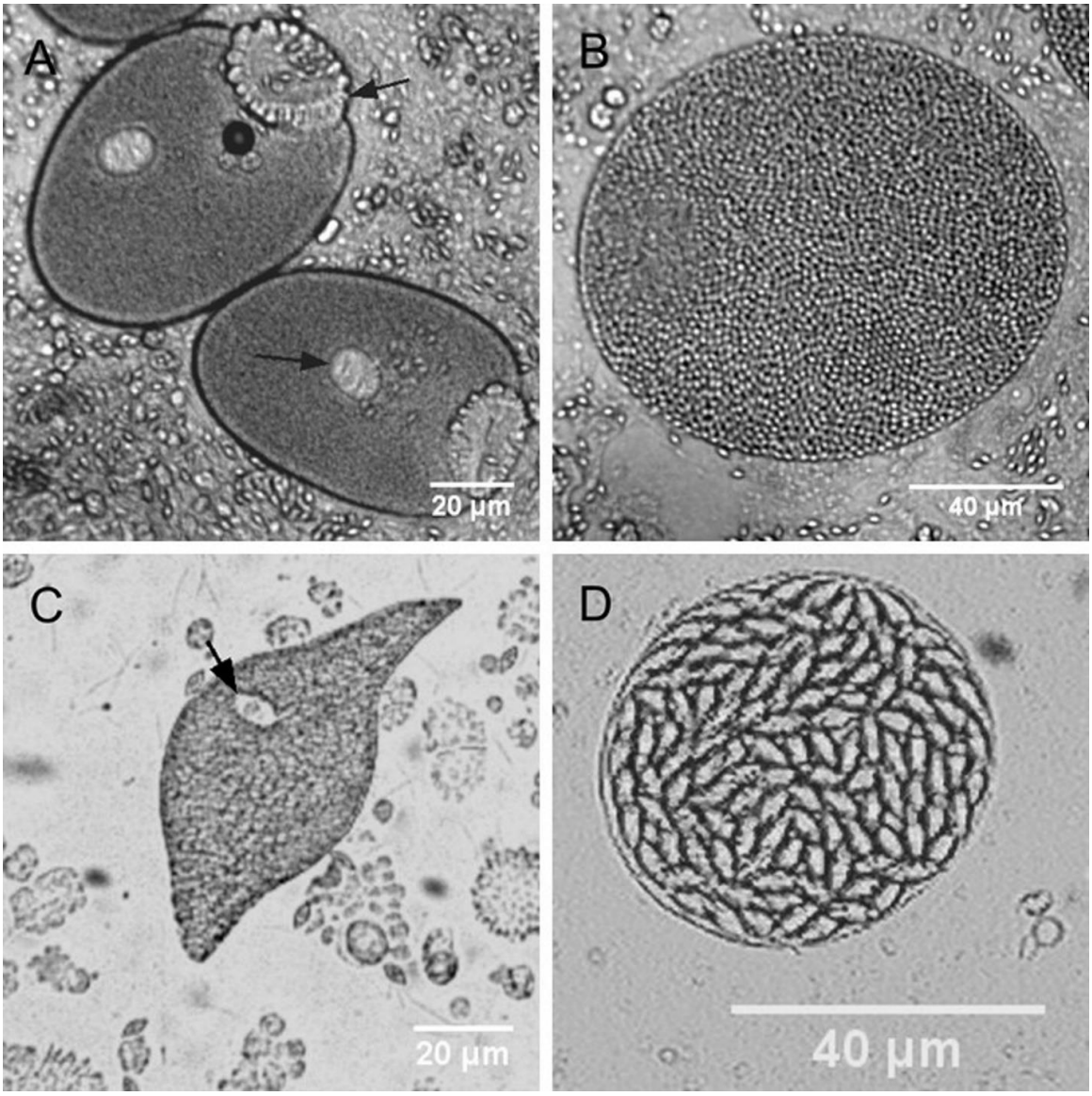
Supplemental Information



Suppl. Fig 1. Photographs of *Monocystis* spp. in two other local earthworm species, *Amynthas tokioensis* and *Lumbricus terrestris*. A) *Monocystis* sp. gamont in *A. tokioensis* with arrows pointing to the mucron and nucleus B) mature *Monocystis* sp. gametocyst in *A. tokioensis* C) *Monocystis* sp. gamont in *L. terrestris* with an arrow pointing to the nucleus D) mature *Monocystis* sp. gametocyst in *L. terrestris*.

CHAPTER 3: Substantial variation in the life history of the eugregarine parasite, *Monocystis perplexa*, among populations of its host, the earthworm *Amynthas agrestis* (Megascolecidae)

Summary

The life histories of parasites provide much insight into infection dynamics, including prevalence, distribution, parasitemia, and transmission success. Life history traits, and the variation therein, also contribute to the evolution of the parasite by influencing such attributes as fecundity, survivorship, and genetic variation. Life history traits are also used to determine the evolutionary relationships among parasites and have been the basis for the current organization of the gregarine clade (Apicomplexa: Gregarinomorpha); despite this, comprehensive life history studies have not been done for representative parasites, such as the eugregarine genus *Monocystis*. Here, the life history traits and variation are examined in the parasite *Monocystis perplexa* that infects the earthworm, *Amynthas agrestis*. This worm is an annual in the study region, and thus allows the entire life history of the parasite to be followed over the course of one warm season. Life history traits including parasite and host phenology, size at parasite maturation, and parasite reproductive output are examined and compared among host populations to understand how life history traits contribute to the seemingly untenable life cycle of the parasite.

Substantial variation was found in the life history traits of *M. perplexa*, within and among host populations. The prevalence of infection reached 100% for all populations by mid-summer, but the timing of infection and parasite stages varied by weeks among populations, a significant amount of time given the short lifespan of the host. Further variation was found in the size of mature parasite stages, representing resource acquisition, among two host populations; this variation likely influenced the variation in the reproductive output, the number of oocysts per gametocyst, among these sites. A trade-off was observed between timing to parasite maturity, resource acquisition, and reproductive output, with parasites in one population maturing faster at the expense of less resource acquisition and lower reproductive output compared to the infections at another host population. Perhaps the most striking difference in life history traits was the near-absence of gametocysts, the transmissible stage of the parasite, in one host population. Despite only ~5% of infections producing gametocysts, the parasite in this population is maintained at high prevalence and parasite load. The mechanism by which the parasite is maintained with extremely limited production of gametocysts remains to be determined; however, certain life history strategies, such as mode of transmission and asexual replication, may explain this phenomenon and is a promising avenue for future research.

Studying the life history of *M. perplexa* has highlighted the variability that exists within and among local host populations. Key life history traits such as time to maturity and reproductive output provide the variation that is needed for evolution and for the success of the parasite in variable environments.

Introduction

Parasitism is the most common organismal lifestyle (Windsor, 1998; de Roode, et al. 2008; Logan et al., 2012) and for every free-living host species it is estimated that there are four parasite species (May, 1992). One reason parasites are so diverse and successful is because they employ variable life history traits and strategies which allow them to adapt to unpredictable conditions (Poulin, 2007). The many challenges that parasites face in unfavorable host and external environmental conditions have led to the evolution of life history traits, including r-selection, which is responsible for producing enormous number of transmissible stages (Poulin, 1995), life cycle flexibility, and environmental durability of transmissible stages (Clopton et al., 2016). The life history of parasites has received a great deal of attention, particularly parasites associated with humans, domesticated animals, and livestock, and these studies have benefited our understanding of parasite evolution, transmission biology, and virulence (Taylor and Read, 1998; Bull, 1994; Ewald, 1994). For instance, Buckling et al. (1997, 1999) found that rodent malaria parasites (*Plasmodium chabaudi*) increase the production of gametocysts, the transmissible stage of the parasite, when the host environment deteriorates such as during malaria treatment with antimalarial chloroquine. This study revealed that certain intervention strategies may be less effective at clearing infections and reducing transmission than previously thought.

While life history studies of parasites have demonstrated great success in improving our understanding of infection dynamics and characteristics, certain parasite groups have been largely excluded. The gregarines are a large clade of apicomplexan

parasites that are ubiquitous in their invertebrate hosts (Desportes and Schrével, 2013). Despite their close association with biologically important invertebrate hosts, including vectors of human diseases (e.g. *Leishmania*), and their relationship with parasites causing human disease (e.g. *Cryptosporidium*), few studies have investigated the role of life history traits on the infection dynamics in gregarines. The gregarines are an ideal group to study life history variation due to their immense diversity and ubiquity among invertebrates.

Monocystis is a genus of gregarine parasite that is often used as a representative of the gregarines in literature and in parasitology courses (Sheridan, 1986). Despite being studied for over a hundred years, there have been few life history studies on *Monocystis* spp. and our knowledge of their life cycles is incomplete (Fields and Michiels, 2006; Miles, 1962). I discovered a new species of *Monocystis* in an invasive earthworm, *Amynthas agrestis* (Chapter 2), and this parasite provides a unique opportunity to study the entire life cycle of the parasite in one season due to its hosts' annual life cycle (Görres et al., 2016). *M. perplexa* frequently infects all hosts in a population at high parasitemia (personal observation) but has a surprisingly low reproductive output compared to other apicomplexan parasites (below). Additionally, the eugregarines lack asexual replication which is common in other apicomplexan parasites and allows them to rapidly increase parasitemia and, in turn, the number of transmissible stages. Horizontal transmission mode, the putative mode of transmission of the gregarines, is reliant on the number and distribution of infective oocysts in the soil; low production of these stages thus decreases the probability of encountering a host (Clopton et al., 2016). Furthermore, studies into the mode of transmission of *Monocystis* spp. are limited and few have been able to

demonstrate transmission via the oral-fecal route (Miles, 1962).

Investigating the variability in life history traits and strategies that *M. perplexa* employs may provide insight into how *Monocystis* parasites remain at high prevalence and parasitemia in host populations despite their low reproductive output. Comparing the variation of *M. perplexa* parasite's life histories among host populations will highlight their ability to adapt to local conditions, which may contribute to their overall success. Additionally, identifying key trade-offs that define life history traits of *M. perplexa* can point to important constraints imposed by the external environment, host, and the parasites themselves. Understanding how *M. perplexa* responds to these constraints will provide insight into the forces that drive gregarine evolution.

Methods

Site locations

Three study sites in Chittenden County, Vermont, USA were chosen that varied in elevation and soil types (Fig. 1), and permission to sample earthworms was granted for each site by the responsible authorities. The University of Vermont Horticultural Research and Education Center (hence HF or Horticultural Center) is a 39 ha experimental agricultural site (76 m elevation, 44.431489 ºN, -73.199211ºW), with a deciduous forest canopy of primarily sugar maple (*Acer saccharum*) and a soil of Windsor sandy loam. The University of Vermont Centennial Woods Natural Area (hence CW or Centennial Woods) is a 28 ha forest containing hardwoods, conifers, wetlands, and streams (80 m elevation, 44.475701ºN, -73.187088ºW). Earthworms were collected in Windsor sandy loam soil along a frequently used recreational path in a

mixed part of the forest containing both hardwood and coniferous trees. The Green Mountain Audubon Center (hence AU or Audubon Center) is a 100 ha outdoors educational and conservation center in Huntington, Vermont (190 m elevation, 44.346774ºN, -72.996216ºW). The area is a hilly habitat with mixed forests, fields, and wetlands with a gravel road bisecting the area. Earthworms were collected along the road's gully containing leaf debris, gravel, and road runoff.

Host collection and species identification

Collection of *Amynthas* spp. earthworms was done weekly from April 5, 2015 to November 15, 2015 at each site. Two species of *Amynthas* earthworms were collected, *A. agrestis* (the only host of *M. perplexa*) and *A. tokioensis* were common at each site, and *A. hilgendorfi* rare at HF. Overall, *Amynthas* earthworm density often reaches > 200/m^2 (Görres et al., 2016). Earthworms were collected by manually sifting through the top 10 cm of the leaf debris and soil (O and A horizons). Sampling of earthworms each week took place over approximately 5m^2 at each site. After collection, earthworms were stored at 16ºC in containers with soil and leaf litter from their site of origin. Adult earthworms were identified to species and separated using diagnostic morphological characteristics (Chang et al., 2016).

Earthworm host phenology and seasonal soil temperature

As noted above, elevation of HF and CW is similar, but higher by ~100 m at AU. Differences in lifespan of the host in this annual earthworm would also reflect on the time available for the parasite's development and production of transmission stages.

Therefore, earthworm phenology was determined at each site as date of first appearance of hatchlings, first mature earthworms (presence of the clitellum – reproductive tissue that forms a viscid sac in which the embryo develops), and last observation of live earthworms. Soil temperature was monitored at AU and HF with an iButton thermal logger (Thermochron iButtons, MaximIntegrated, San Jose, Ca.) six times a day starting in September 2014 with loggers placed 7 cm below the casting layer. Several analyses were done to determine if there was a difference in host season length between AU and HF. First, the weekly average temperature at both sites was graphed over two years. Second, earthworm season length was estimated by determining the longest period of consecutive days above 0ºC. Hard frosts occur at temperatures below 0ºC and results in high earthworm mortality (Görres et al., 2014); thus, consecutive days above 0ºC represents survivable conditions for *A. agrestis*. Last, cumulative growing degree days (GDD) was also calculated for 2015 and 2016 to determine differences in growth and development of *A. agrestis* at AU and HF. A base temperature of 5ºC was chosen because earthworm growth begins at this temperature (Görres et al., 2016). Cumulative GDD_5 was calculated as:

$$GDD_5 = \sum_{i=1}^{N} \left(\frac{(Tmax_i + Tmin_i)}{2} - 5 \right)$$

where $Tmax_i$ and $Tmin_i$ are the maximum and minimum temperatures on Julian data i. The accumulation rate of GDD_5, representing the rate at which energy accumulates in the soil, was calculated by finding the slope of GDD_5 between the first $GDD_5 > 0$ and the first $GDD_5 \geq 1000$. An endpoint of 1000 GDD_5 was used because development of *A. agrestis* adults occurs at 1000 GDD_5 after hatchlings appear (Görres et al., 2016).

Dissection of A. agrestis *and microscopy of* M. perplexa *infections*

Ten *A. agrestis* earthworms from each site were dissected within one to three days for 33 weeks for a total of 968 earthworms. Earthworms were killed by submersion in 50% ethanol, blot dried, and examined under a dissection microscope. Earthworm segments 1 - 20 were bisected on the ventral side and, if present, the seminal vesicles removed with forceps and placed in a finger bowl. Seminal vesicles were then manually thoroughly homogenized with forceps in a known volume of 1x earthworm Ringer's solution. The volume of the seminal vesicles was found by subtracting the volume of Ringer's solution from the total volume. One μl of the homogenized sample was used to make a live microscope slide preparation and observed under a coverslip.

Presence of *M. perplexa* infection was determined by scanning the preparation for parasites at 400x. If no parasites were observed in 1μl, up to 5 μl of seminal vesicle sample was scanned for parasites, and earthworms with no parasite present in 5 μl were considered not to be infected in the seminal vesicles. For infected samples, *M. perplexa* parasite stages were identified using the morphological and biometric description of *M. perplexa* (Chapter 2). Parasite stages are shown in Fig. 2 and scored as trophozoites, gametocytes forming gametes, immature gametocysts with developing oocysts, and mature gametocysts. Gametocytes were scored as mature when they contained completely developed oocysts. Total parasitemia of the earthworm's seminal vesicles was determined by multiplying the total number of parasites observed per unit volume by the total volume of the seminal vesicle.

Comparison of reproductive output at AU and HF

For some infected earthworms in 2015, when the presence of mature gametocysts was detected, gentle pressure was applied to the coverslip of the microscope slide to compress the oocysts into a single layer. Photographs of the compressed gametocyst were taken at 400x using a Moticam 1000 1.3MP Live Resolution (Richmond, British Columbia) microscope camera and Motic Image Plus 2.0.11 computer program (Suppl. Fig. 1). Thirty gametocysts were photographed for ten infections each at AU and HF for a total of 600 gametocysts, and counts of the number of oocysts per gametocyst were recorded. Gametocysts were rarely observed in CW infections over the course of the 33 weeks; thus, CW was excluded from this comparison. Additionally, to investigate the relationship between oocyst size and reproductive output, length and width measurements of five oocysts per gametocyst were taken from 30 gametocysts used to determine the reproductive output at HF.

To ensure reproducibility of the variation in reproductive output of *M. perplexa* parasites, measurements of resource allocation by parasites before reproduction was calculated by measuring gamonts (mature parasites capable of producing gametes) and gametocytes (sexually reproductive stage producing gametes). The size of mature parasites is representative of resource accumulation, which is associated with reproductive output (Jervis et al. 2008). During the week of July 23, 2017, gamonts and gametocytes were measured at AU (gamonts N=151 from five infections, gametocytes N=60 from four infections) and HF (N=101 from four infections, N=44 from four infections). The length and width of gamonts and gametocytes (Fig. 2) were measured at 400x as described above.

Statistical analyses

All statistical analyses were performed in the statistical computing software, R (R Core Team, 2013). The total parasitemia observed for each site and week was calculated by adding the total number of immature parasites (trophozoites, gamonts, and gametocytes) and two times the total number of gametocysts (to account for two gametocytes present in one gametocyst). Summary statistics for total parasitemia, total number of mature gametocysts, and total number of immature parasites (other than mature gametocysts) were calculated for each week and for each site.

The reproductive output of *M. perplexa*, or the number of oocysts per gametocyst, was compared using a nested analysis of variance (ANOVA) with counts for gametocysts nested within earthworm and earthworms nested within sites to determine if there is a statistical difference in the reproductive output among individual earthworms and among sites. In addition, the area of the rhombus-shaped gamonts and gametocytes was calculated and an ANOVA was run to determine if there was a significant difference in parasite size, and thus fecundity, by site. The area of the fusiform oocysts was also calculated and a linear regression was then performed to determine the strength and direction of the relationship between oocyst size and reproductive output.

Last, the transmission rate (number of transmission stages) of *M. perplexa* required to produce the observed parasitemia in the populations was calculated by first determining the total number of parasites over the season. This was calculated by adding the total number of immature parasites (trophozoites, gamonts, and gametocytes) and two times the number of gametocysts to account for two parasites forming one gametocyst.

The number of oocysts needed to be transmitted into the population to produce the observed parasitemia was calculated by dividing total parasitemia by eight, because each oocyst contains eight sporozoites giving rise to individual parasites (Noble and Noble, 1976). No information was available on the number of oocysts produced the prior year but, assuming the system is in equilibrium, I asked if the number of oocysts produced in the study year would be sufficient to yield a stable number of parasites the following year. The total number of oocysts produced by the population over the duration of infection was calculated by multiplying the total number of gametocysts by the average number of oocysts per gametocyst at that site. The minimum transmission rate, or the proportion of oocysts that are successfully transmitted into a new host, was calculated by dividing the number of required oocysts to produce the observed infection by the total number of oocysts produced. This is summarized as:

$$T_R = \frac{T_I + (2 \times T_M)}{8}$$

$$T_P = T_M \times T_S$$

where T_R is the total number of oocysts required to produce the observed parasitemia, T_I is the total number of immature parasites, T_M is the total number of gametocysts, T_P is the total number of oocysts produced from all infections, and T_S is the mean number of oocysts per gametocyst at a site. The ratio produced by dividing T_R by T_P represents the proportion of oocysts that must successfully be transmitted to a host to produce the observed parasitemia.

Results

Parasite phenology

The earthworm and parasite phenology and infection characteristics are summarized in Table 1. Earthworms were considered not infected if they did not possess developed seminal vesicles, as seminal vesicles are the putative primary location of *Monocystis* spp. (Clopton, 2000; Noble and Noble, 1976; Wenyon, 1965). Infections were not observed in any earthworm sampled via microscopy from weeks 1 – 16, corresponding to weeks of April 5, 2015 – July 12, 2015. First infections, comprised of immature parasites, were observed at CW during the week of July 12, 2015, then observed at AU and HF infections within the following two weeks (Fig. 3). Trophozoites were the first parasite stage to appear in infections, followed by gamonts, gametocytes, and then gametocysts. Prevalence eventually reached 100% at all sites by July 19 – August 16, 2015, with earlier full prevalence at CW by five weeks compared to HF. At all sites, earthworm populations died after week 33 after hard frosts were experienced at the sites. Parasitemia generally increased after infections were first detected until peak parasitemia was reached, at which point parasitemia generally began decreasing. CW reached peak parasitemia earliest during the week of August 9, 2015, followed by HF five weeks later, and AU another three weeks later.

The decrease in parasitemia at AU and HF corresponded to the peak presence of gametocysts at AU at week 28 and at the first peak presence of gametocysts at HF at week 24 (Fig. 4). Gametocysts were first observed at weeks 17, 18 and 21 for AU, CW, and HF, respectively. Similar to the pattern seen in overall parasitemia, the number of gametocysts at AU and HF increased until peak number of gametocysts was reached,

followed by a decrease in the number of gametocysts. Consistent with the parasitemia observed, total AU earthworms produced many more gametocysts (4,695,963) than HF earthworms (2,466,684) over the course of the season. At CW, however, gametocysts were rarely observed as only nine out of the 179 (5.7%) earthworms sampled over the infection duration were observed to have gametocysts; this differs dramatically from the infection dynamics at AU and HF where, after the first gametocysts were observed, 77.8% and 90% of all sampled infections had gametocysts, respectively.

Reproductive output

There was a significant difference in the number of oocysts per gametocyst among individual earthworms and among sites ($p < 0.0001$ and $p < 0.0027$, respectively). The reproductive output of *M. perplexa* was higher at HF than AU, with a mean number of oocysts of 145.2 and 140.4, respectively (Fig. 5); however, the range of oocysts produced per gametocyst were similar for AU (73 – 207) and HF (78 – 223), and 97% of the variance could be attributed to differences among individual infections (Suppl. Fig. 2). AU infections appeared to be more variable than HF infections, with interquartile ranges of 120-157 and 132-158, respectively. Additionally, the sample variance for AU (830.15) was nearly double that of HF (428.78). The distribution of the number of oocysts per gametocyst was normal for HF (Shapiro-Wilk Normality Test $p > 0.05$) but not for AU (Shapiro-Wilk Normality Test $p < 0.01$) (Fig. 6). Thus, the parasites produced about 3% more oocysts per gametocyst, or 40 more parasite offspring (sporozoites) at HF than AU which had a broader range of oocysts. A moderate negative relationship was also observed between the number of oocysts per gametocyst and a measure of oocyst

size, the area of oocysts ($R^2 = 0.3048$, $p < 0.001$) (Fig. 7), such that oocysts are larger when fewer oocysts are produced.

A measurement of resource acquisition is the area of gamonts and gametocytes, and assuming that larger mature parasites could yield a larger output of oocysts, data from 2017 were analyzed and were concordant with the first estimate of reproductive output. HF gamonts were significantly larger in length, width, and total area with an average difference in gamont area between AU and HF of 160.1μm^2 ($p = 0.0037$) (Fig. 8). The area of gametocytes at HF were similarly larger, with a mean size of 1016.0μm^2 at HF and 674.9μm^2 at AU ($p < 0.001$) (Fig. 9). Consistent with the results from the number of oocysts per gametocyst, there was also a significant difference in gamont and gametocyte area among host individuals ($p < 0.001$); however, site accounted for much more of the variance (53.50%) for gametocytes than in any other comparison.

The transmission rate of *M. perplexa* was calculated for each site to determine what proportion of oocysts produced must be successfully transmitted into a host to produce the parasitemia observed. AU produced a total of 659,281,742 oocysts over the duration of the 2015 season, requiring ≥ 0.33% of all oocysts produced to be successfully transmitted to produce the observed parasitemia. HF produced overall fewer oocysts than AU, despite having a higher mean number of oocysts per gametocyst than AU, with 358,187,184 oocysts produced and a required transmission rate of ≥0.44%. As CW produced dramatically fewer gametocysts than the two other sites, only 2,154,878 oocysts were produced which would require an exceptionally high rate for a microbial parasite transmission rate ≥34.3%.

Site Season Length Analysis

Patterns of seasonal soil temperature for AU and HF were similar, with similar mean weekly temperatures throughout 2014 – 2016 (Fig. 10). In 2015, AU accumulated more growing degree days over the time periods measured than HF (Fig. 11). From January 1 - December 31, 2015, HF accumulated a total of 2048.25 GDD_{5S} while AU accumulated 2130.25 GDD_5. However, HF began accumulating growing degree days on April 14, 2015, 19 days before AU's GDD_5 exceeded 0ºC (May 3, 2015). Despite accumulating GDD_5 earlier than AU, HF did not reach 1000 GDD_5 until two days after AU on July 30. The slope between 0 and 1000 GDD_5 indicated that AU soil accumulated 11.94 GDD_5 per day whereas HF accumulated GDD_5 at a slower rate of 9.66 GDD_5 per day. The higher total GDD_5 and rate of the accumulation of GDD_5 at AU corresponds with the number of consecutive freeze-free days for *Amynthas agrestis* being longer than HF, with a total of 262 consecutive days above 0ºC compared to 252 days at HF.

The 2016 season lengths were similar for AU and HF. While the temperature data for 2016 is incomplete, between April 16 and November 11 AU accumulated 1954.75 GDD_5 and HF accumulated 1955.50 GDD_5 (Fig. 12). Since GDD_5 likely began accumulating before April 16, 2016, the slope from the first day with $GDD_5 > 0$ to the first day with $GDD_5 > 1000$ was not calculated; however, the curves of the accumulation of GDD_5 appear nearly identical indicating similar GDD_5 accumulation rate. In the same period, the season length measured in the number of consecutive days above 0ºC was 221 days and 207 days for AU and HF, respectively.

Discussion

Life history traits are crucial to the understanding of disease dynamics in a parasite-host system. Parasite phenology, life cycle, parasitemia, and reproductive output are all important traits that influence the severity and persistence of disease; these traits, however, cannot be studied in isolation and understanding the host life histories that allows parasite-host systems to persist is also necessary.

The genus *Monocystis* and its life history has been used as a prime example of acephaline gregarines in the literature as well as in parasitology courses (Sheridan 1986). Despite this, comprehensive studies of life history traits and the variation within these traits have been neglected for *Monocystis* spp. (but see Fields and Michiels, 2005). By taking advantage of the annual life cycle of its host, *Amynthas agrestis*, a comprehensive study of the life history of *M. perplexa* revealed substantial variation of life history traits both among infected earthworms and among host populations, as well as trade-offs between traits.

The phenology of *M. perplexa* was variable among sites, with the first parasites being observed at week 17 (mid-July) at CW and AU and one week later at HF. At AU only, gametocysts were observed the same week that infections were first detected; this could possibly indicate a faster maturation time for *M. perplexa* at this site compared to CW and HF which had gametocysts first observed at 18 and 21 weeks, respectively. The difference in maturation time from immature parasites to gametocysts could be in part responsible for the difference in reproductive output at AU and HF. HF produced on average 4.8 more oocysts per gametocyst than AU, resulting in a difference of 38.4 sporozoites. This difference is exemplified when considering how many potential

parasites are lost at AU given AU's total number of gametocysts; a loss of 38.4 sporozoites per gametocyst results in 180,324,979 potential parasites not produced at AU; however, the moderate negative relationship observed between oocyst size and reproductive output could indicate a trade-off in oocyst durability and the number of oocysts produced (Clopton et al., 2016). Investing more resources into fewer, more durable offspring could be a favorable life history strategy, particularly in poor environmental conditions that reduce parasite survival outside of the host. The difference in reproductive output is likely not due to genetic differences in *M. perplexa* among host populations, as previous studies found that the host species, *A. agrestis*, populations at HF, AU, and CW were not genetically distinct (Keller et al., 2017), which possibly suggests a recent origin of the earthworm populations from a common source or frequent migration among populations; however, the study found no overlap of clonal genotypes among populations which warrants future investigation into reproductive output in different clonal genotypes.

The reduction in reproductive output at AU compared to HF may be the result of a life history trade-off between reproductive output and time to parasite maturity. Parasites are thought to generally evolve to be more fecund than their free-living relatives (Poulin, 2007); however, reproductive output is often not maximized in parasites due to trade-offs that exist between traits imposed by the environment (Partridge and Harvey, 1988; Stearns, 1992). The environment offers a finite amount of resources in which the parasite must allocate between different traits, thus, investment in one life history trait can be detrimental to another (Roff, 1992; Stearns, 1992). One prominent trade-off that exists with respect to reproductive output is timing of reproduction or maturation time.

Reproducing later is favorable in stable conditions as the parasite can acquire more resources and thus produce more offspring, but the probability of survival to maturation typically decreases with increasing maturation time (Gemmill et al., 1999; Poulin, 2007). Both gamonts and gametocytes were larger at HF than AU, possibly indicating that AU parasites spend less time accumulating resources before reproducing; this supports the trade-off between resource accumulation and parasite fecundity. Alternatively, nutrient availability may be greater at HF where there is sufficient organic debris and less heavy metals in the soil than AU (Richardson et al., 2015). At AU, climactic conditions are likely not limiting to the parasite's maturation time because the season length of its host is longer by approximately two weeks in both 2015 and 2016. The rate of the accumulation of GDD_5, however, was greater at AU than HF, likely due to more direct sun exposure at AU than HF due to canopy cover; this corresponds to an increased rate of host development which may cause an increased rate of parasite development (Nealis et al., 1984).

The more rapid maturation rate of *M. perplexa* at AU is supported by both the faster rate of development of its host and by the number of weeks it takes for the parasite to mature and produce gametocysts. The trade-off between faster maturation time corresponding to faster generation time and lower reproductive output could be ameliorated if the oocysts produced during the season are excreted by the host and successfully transmitted into a host during the same season. If AU parasites are capable of multiple generations in a season and the oocysts produced can continually infect hosts throughout the season, then it is possible that more transmissible stages are produced through an increased number of generations than through an increased reproductive

output in less generations. For instance, if the oocysts produced in the first parasite generation infect hosts in the same generation, then over a billion more oocysts may be produced given the calculated transmission rate of the parasite and the average reproductive output stays the same across generations. In this scenario, optimization of fecundity may be due to a trade-off between parasite generation time and reproductive output; this is supported by the presence of both immature and mature parasites throughout the duration of infection indicating continual infection of hosts and the overall higher parasitemia at AU than HF.

While reproductive output determines how many possible transmissible stages there are in the environment, the transmission rate of the parasite determines how many of those transmissible stages successfully infect hosts. The required transmission rates at AU and HF to produce the observed parasitemia is high for r-selected species such as parasites (Pianka, 1970). The transmission rate of 34.3% at CW is exceptionally high due to only 5.7% of sampled earthworms containing gametocysts; this estimate is likely very high because of the heterogeneous distribution of oocysts in the soil due to the small proportion of infections producing gametocysts.

One possible explanation for the high transmission rate is that eugregarine parasites use environmentally persistent oocysts that allow the accumulation of oocysts in the soil over time increasing the chances of transmission (Clopton et al., 2016); however, because the host is an invasive species that has only been confirmed to be present in Vermont 10 years ago (Görres and Melnichuk, 2012), even ten years of accumulating oocysts would still require a high transmission rate of 3.4%. Oocysts remain viable for a finite amount of time depending on quiescent metabolic rate and amylopectin reserves,

and remaining viable for ten years is highly unlikely given other eugregarine oocysts persist from between 39 and 787 days (Clopton et al., 2016). Thus, the ability of *M. perplexa* to persist at high prevalence over multiple host generations may be best explained by the presence of a combination of previously undescribed merogony (asexual reproduction) and vertical transmission. Due to the parasite's proximity to its host's reproductive organs, vertical transmission may be a viable mode of transmission that would eliminate or reduce the need to produce gametocysts. While the primary mode of transmission in gregarines is horizontally via the oral-fecal route (Desportes and Schrevel, 2013), vertical transmission has been identified in several closely related genera and should be further investigated (Lantova and Volf, 2014; Warburg and Ostrovska, 1989).

The life history traits and their variation is critical to our understanding of disease dynamics of a system. By considering life history traits of both the parasite and its host, key trade-offs and indications of vertical transmission were identified; these findings shed new light on the life history strategies of *Monocystis* spp. and warrant further investigation into the life history of other gregarine parasites.

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Table 1. Summary of parasite phenology and important infection characteristics for each site. Week 1 is the first observation of hatchling earthworms in the soil (early April), and week 17 is mid-July.

	Audubon Center	**Horticultural Farm**	**Centennial Woods**
First infection observed (week)	17	18	16
100% prevalence observed (week)	20	21	17
First gametocyst observed (week)	17	21	18
Peak parasitemia observed (week)	28	25	20
Average peak parasitemia	219,855	217,945	91,970
Total earthworms sampled	161	151	179
Percent of infections with gametocysts after first gametocyst appears	50.3	45.4	5.8

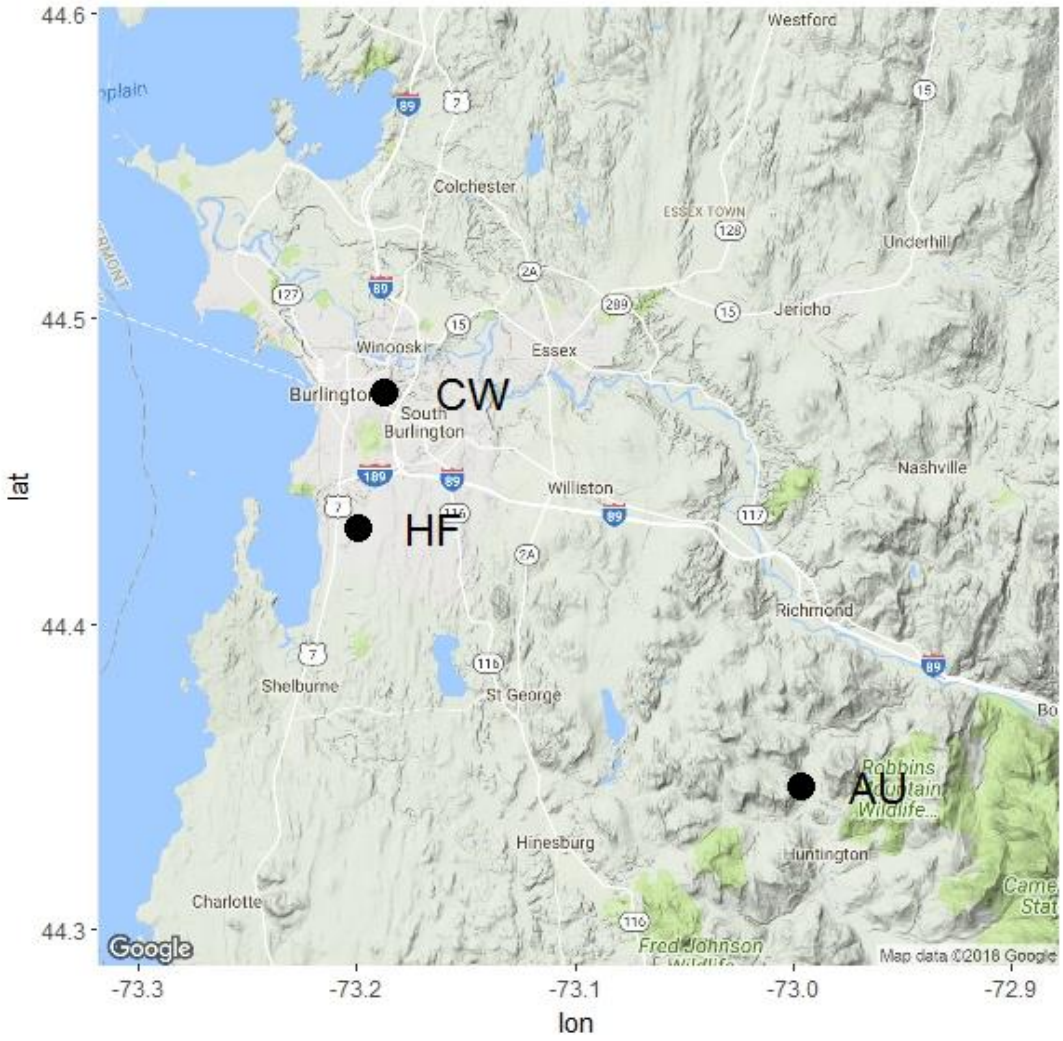

Fig 1. Site map of Centennial Woods (CW), Audubon Center (AU), and Horticultural Farm (HF) located in Chittenden County, Vermont, USA.

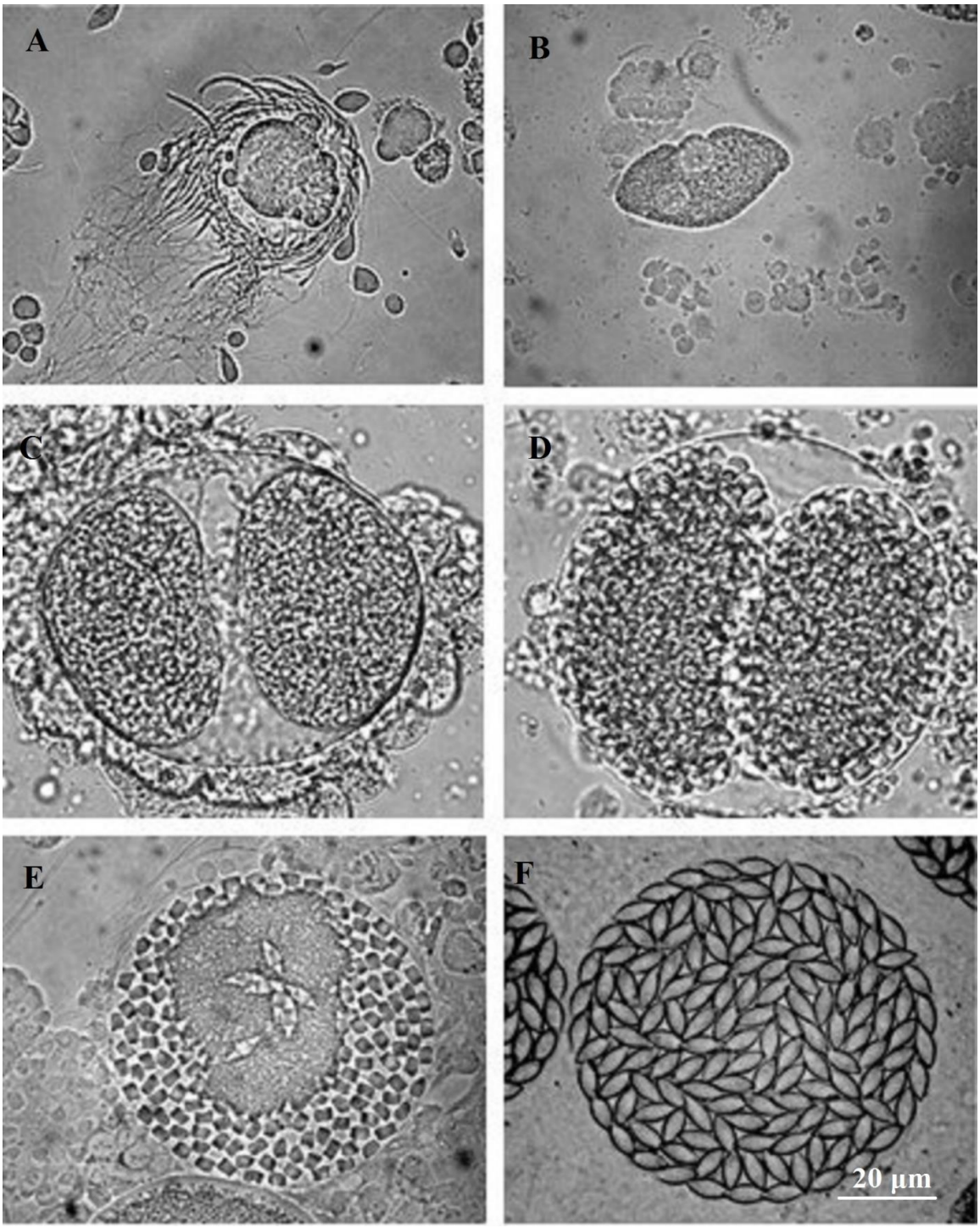

Fig 2. Key life stages of *Monocystis perplexa.* All photographs share the same scale as shown in Fig 2F. A) Immature trophozoite with sperm flagella surrounding the parasite B) Mature gamont C) Two gametocytes with a forming membrane D) two gametocytes forming gametes E) Immature gametocyst with developing oocysts F) Mature gametocyst with fully formed oocysts.

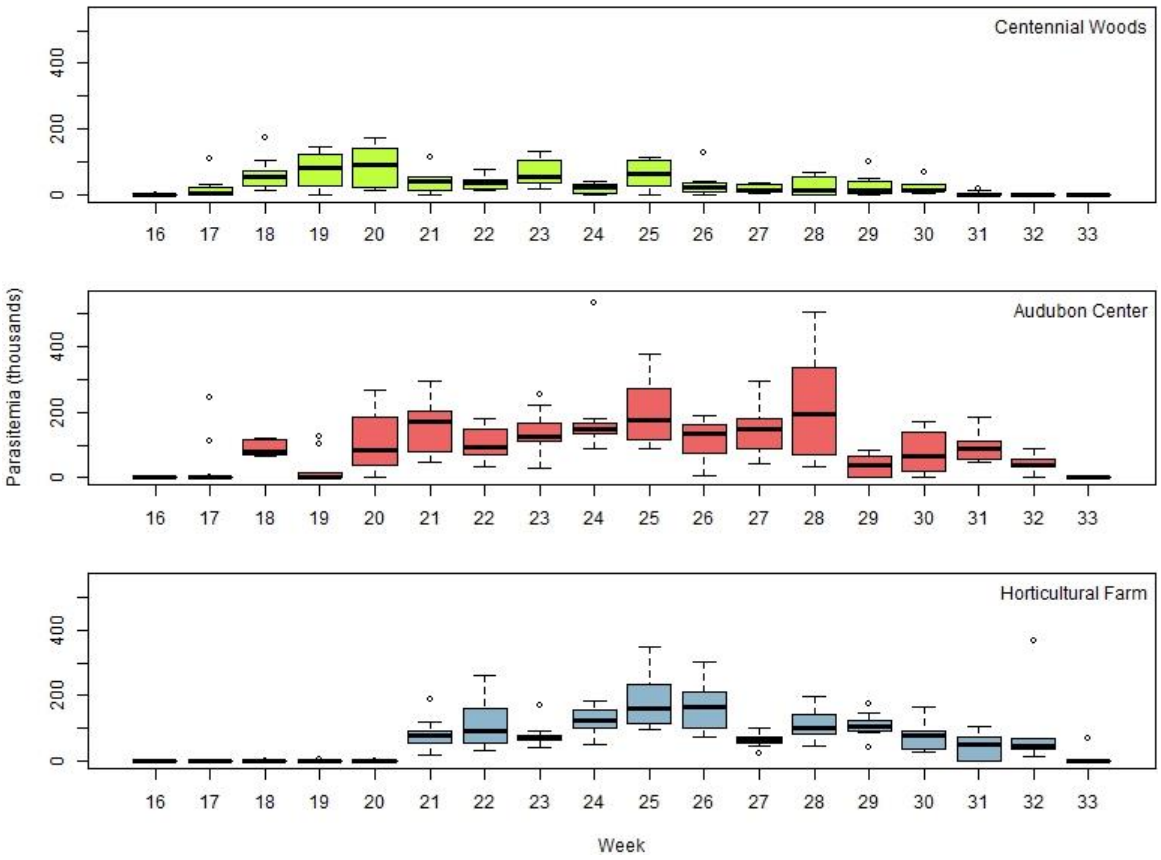

Fig 3. Box and whisker plot of the total number of immature parasites for Centennial Woods, Audubon Center, and Horticultural Farm for the duration of observed infection (week 16 – week 33 corresponds to weeks of July 19, 2015 – November 15, 2015). Black horizontal bars within each box represents the median of the data, the box represents the interquartile range, and the whiskers indicate range. Values that exceeded 1.5x the third quartile were considered outliers and are represented by dots.

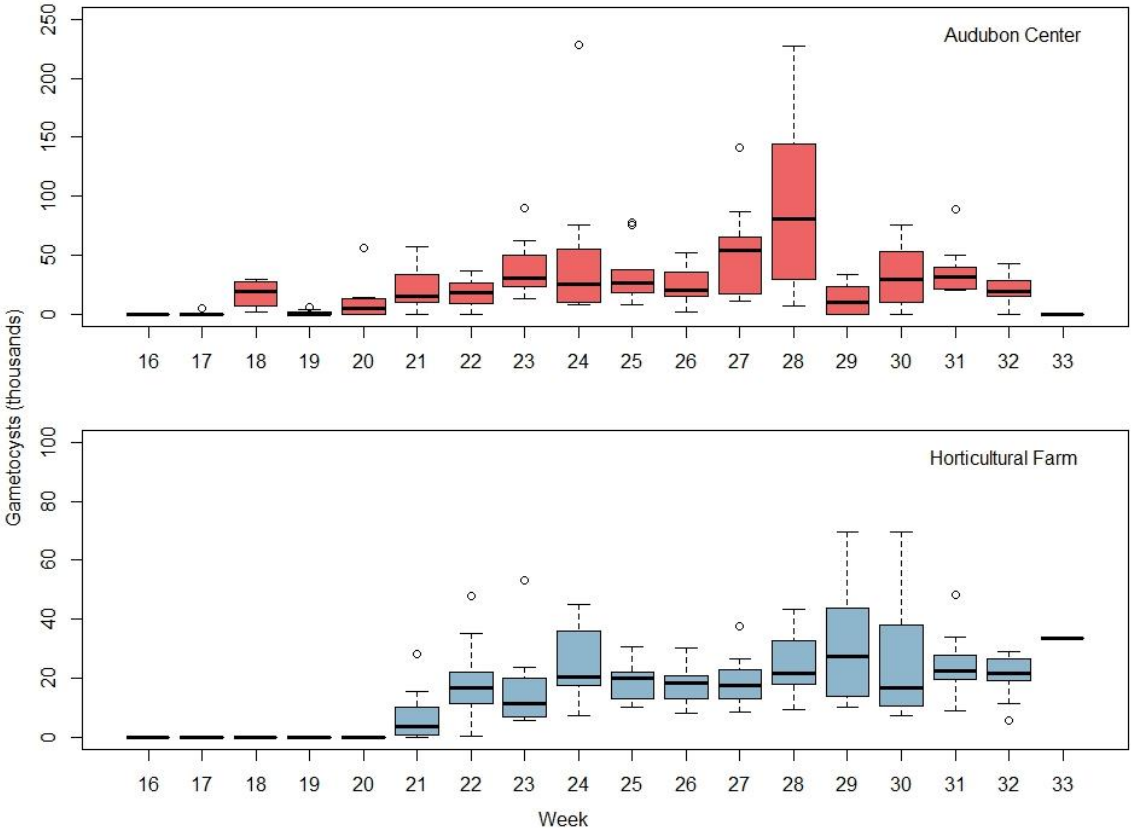

Fig 4. Box and whisker plots of the total number of gametocysts in thousands at Audubon Center and Horticultural Farm over the duration of observed infection (weeks 16-33 correspond to weeks of July 19, 2015 – November 15, 2015). Black horizontal bars within each box represents the median of the data, the box represents the interquartile range, and the whiskers indicate range. Values that exceeded 1.5x the third quartile were considered outliers and are represented by open circles.

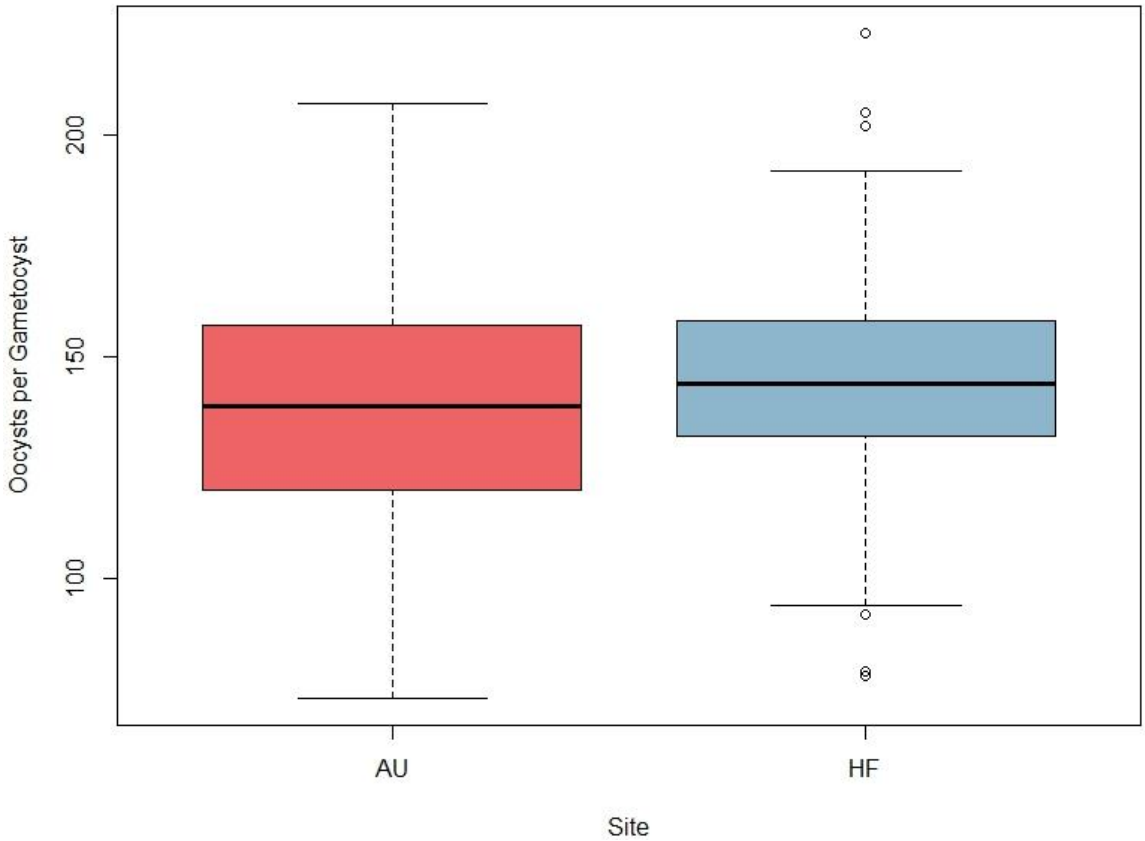

Fig 5. Box and whisker plot of the number of oocysts per gametocyst at Audubon Center (AU) and Horticultural Farm (HF). The horizonal black bar within the box represents the median (AU = 139.0, HF = 145.2), the box represents the interquartile range, and the whiskers indicate the range of the data. A total of five outliers were identified at HF, as the number of oocysts per gametocyst were less than or more than 1.5x the first quartile or third quartile.

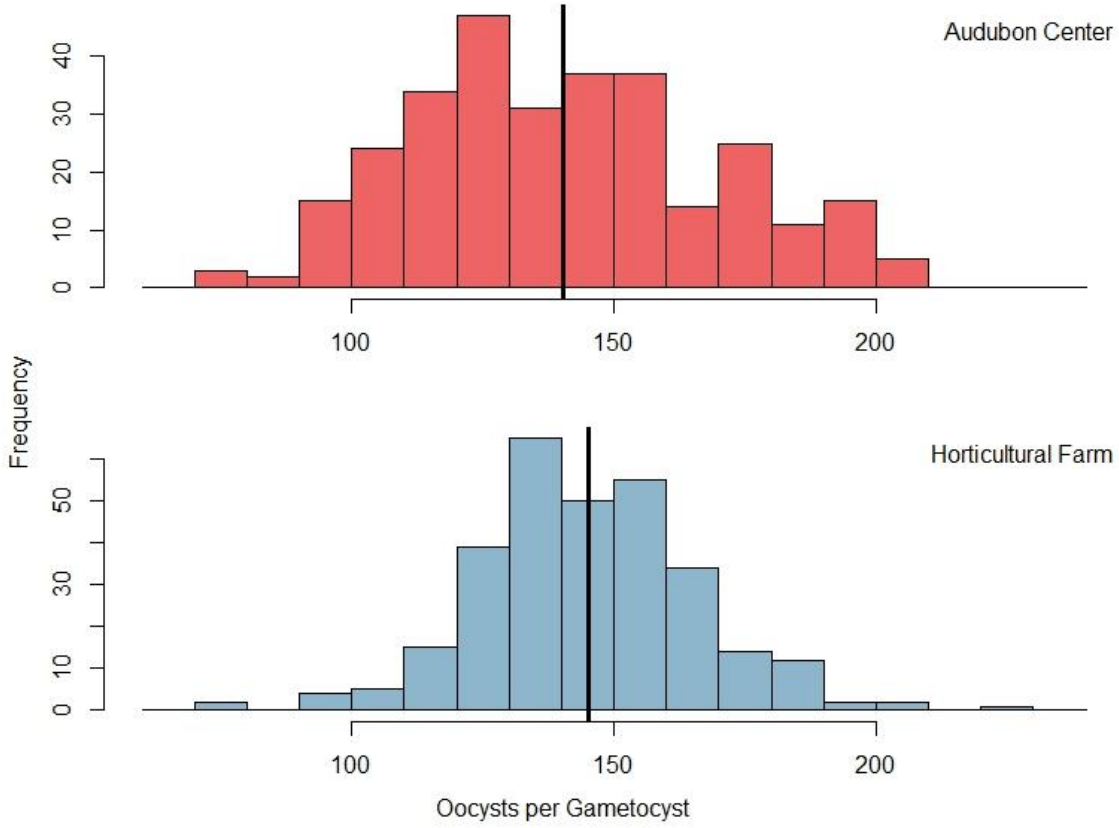

Fig 6. Histogram of the distribution of oocysts per gametocyst at Audubon Center (N = 300) and Horticultural Farm (N = 300). The vertical black bar indicates the mean of the data (AU = 140.4, HF = 144.0).

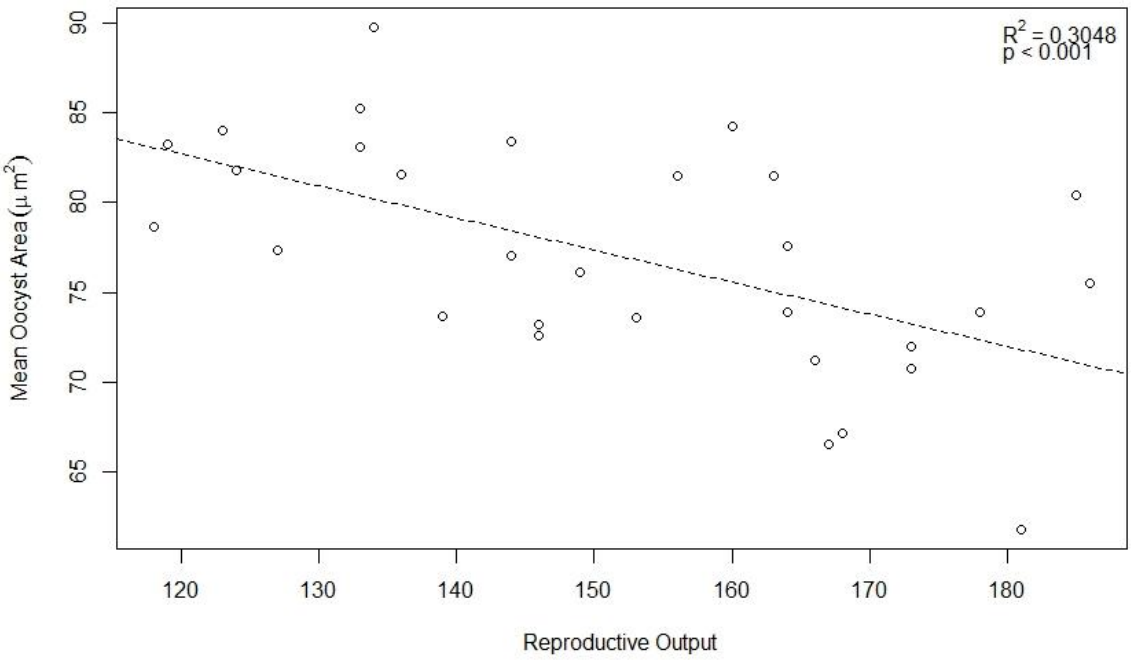

Fig 7. Scatterplot of the mean oocyst area (μm^2) by reproductive output (number of oocysts per gametocyst). Linear regression of the data yielded a negative relationship between mean oocyst area and reproductive output. A total of 30.48% of the variability in oocyst area can be attributed to reproductive output ($p < 0.001$).

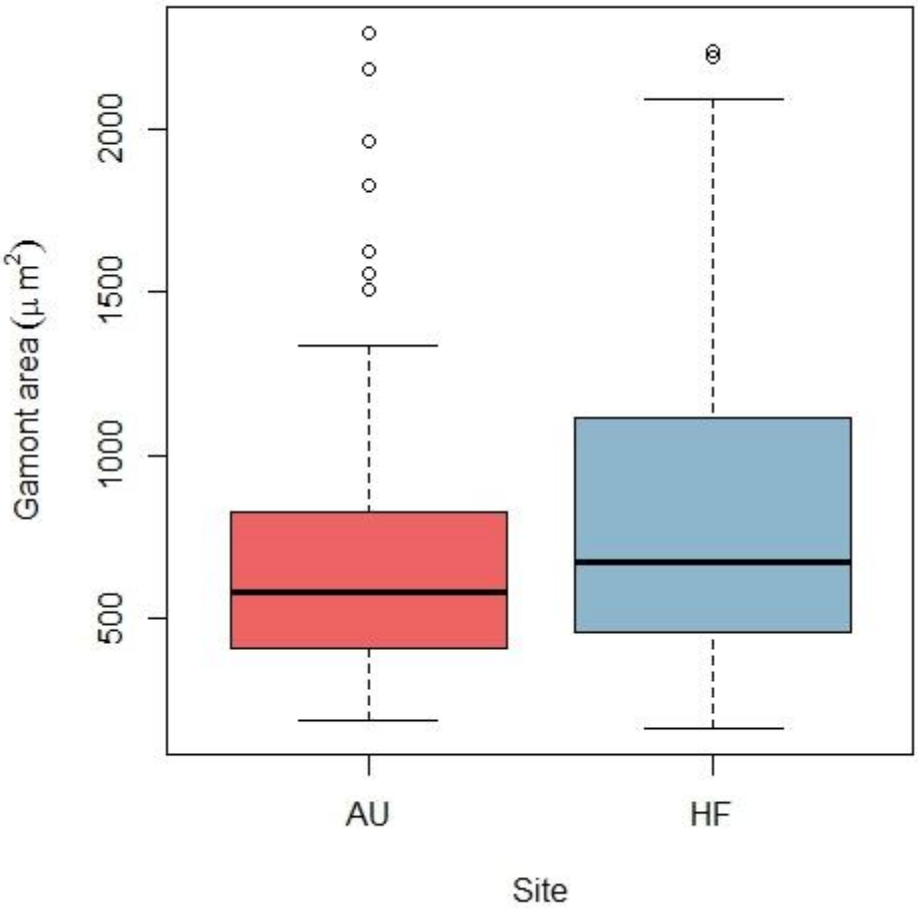

Fig 8. Boxplot of the distribution of gamont area (μm^2) by site. The length and width of 211 and 150 from AU and HF, respectively, were measured and the area of the rhombus-shaped gamonts was calculated.

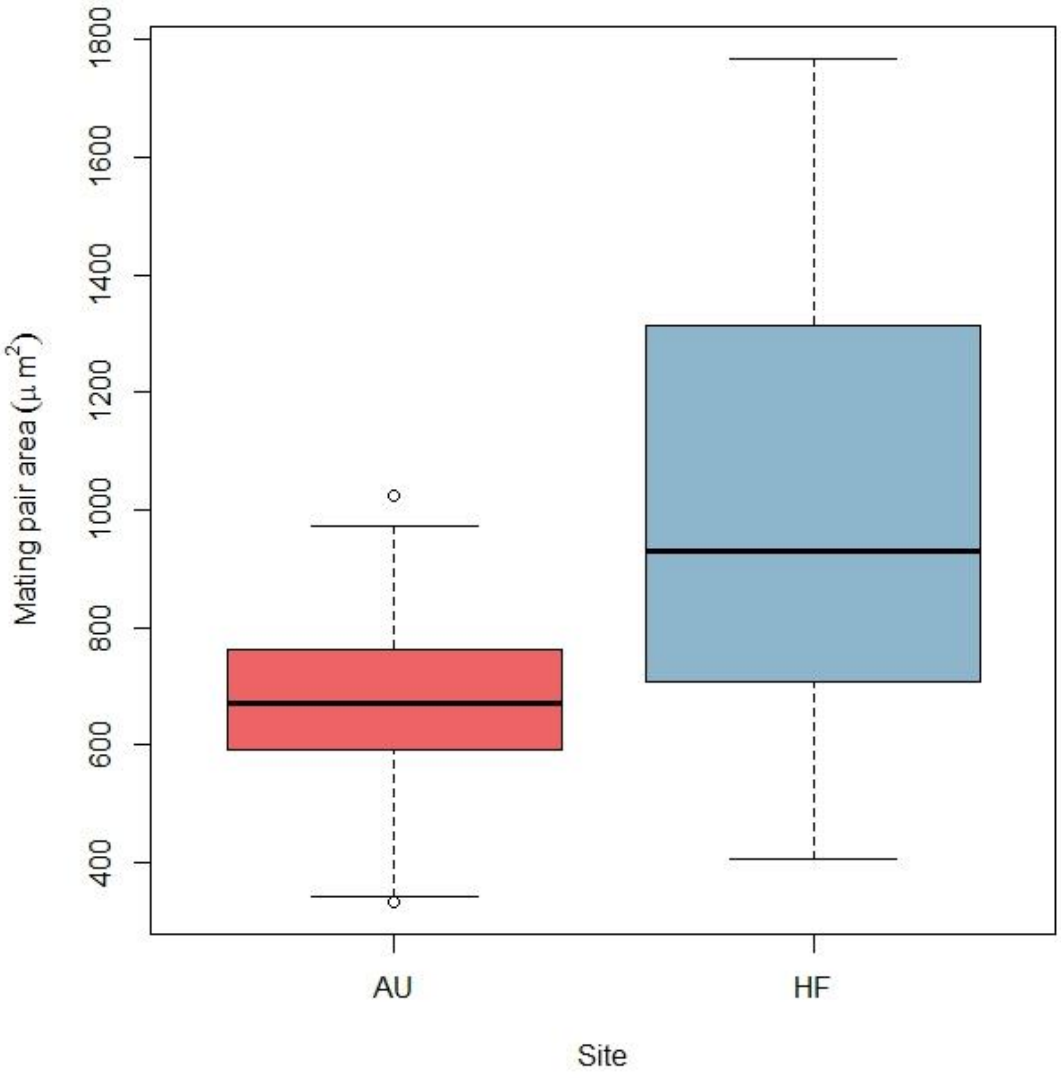

Fig 9. Boxplot of the distribution of gametocyte size, in area (μm^2), between AU (N= 60) and HF (N = 44) measured during the week of July 23, 2017.

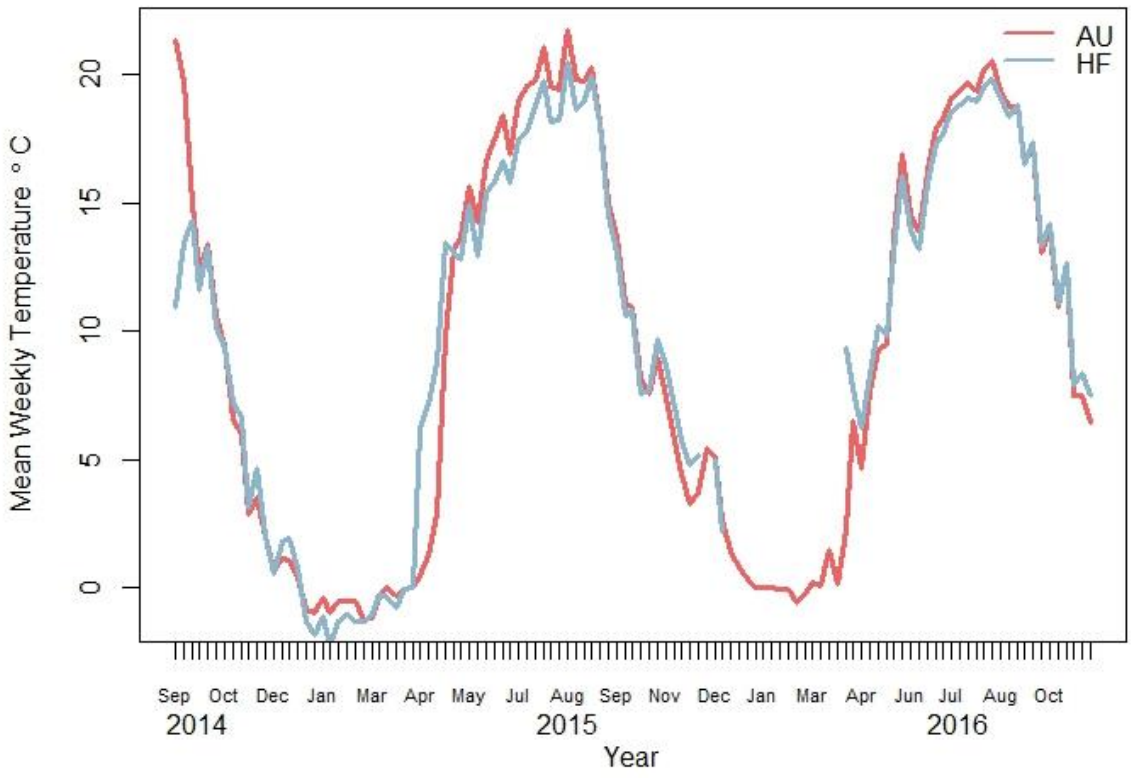

Fig 10. Mean weekly temperature (ºC) for Audubon Center and Horticultural Farm from September 18, 2014 to November 8, 2016.

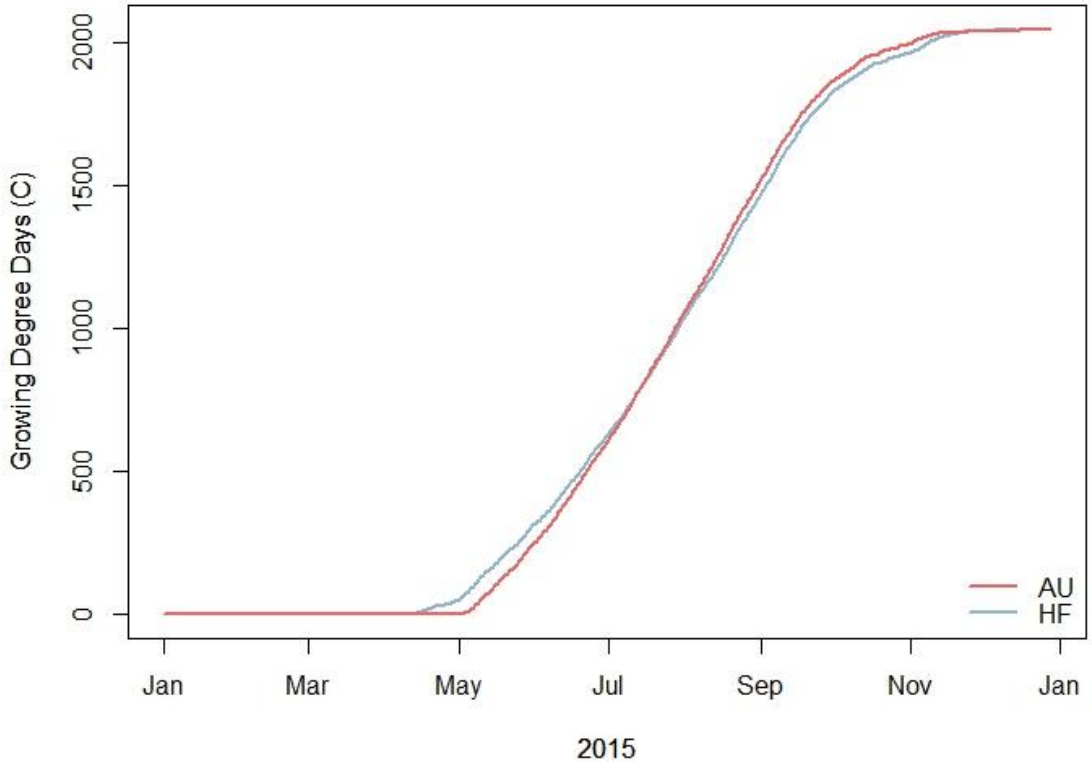

Fig 11. Graph of the total growing degree days (ºC) for Audubon Center and Horticultural Farm over the course of 2015.

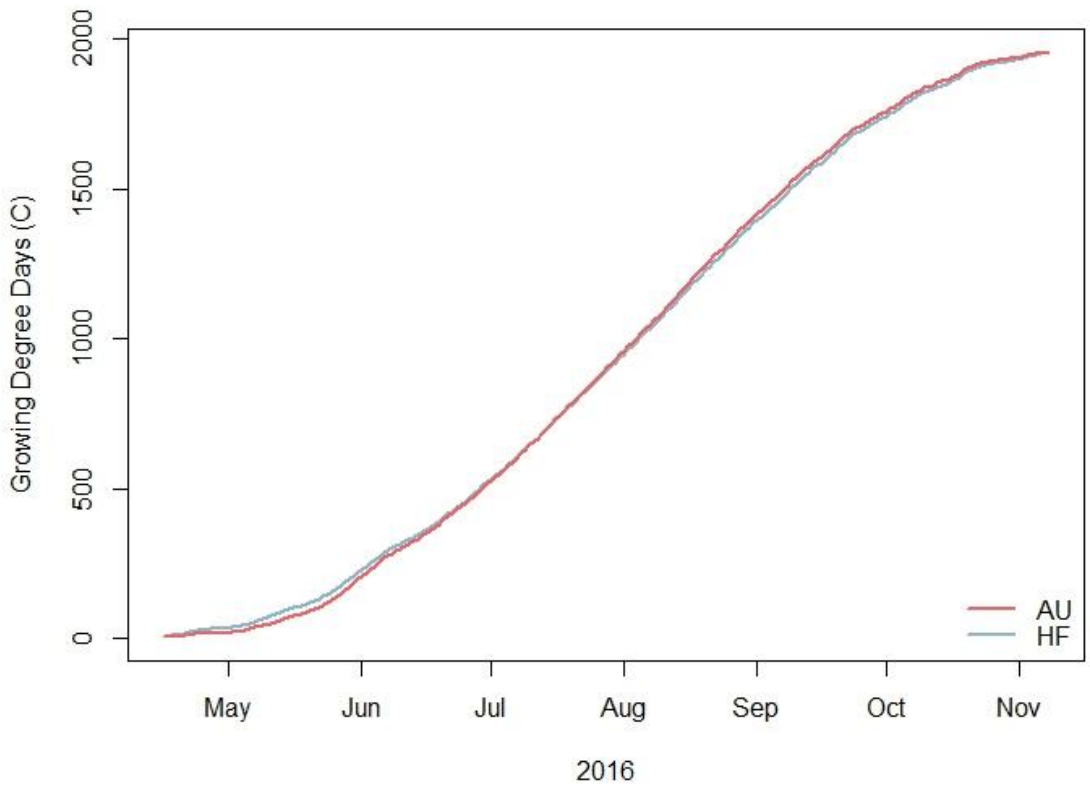

Fig 12. Graph of the total growing degree days (ºC) for Audubon Center and Horticultural Farm over the course of 2016.

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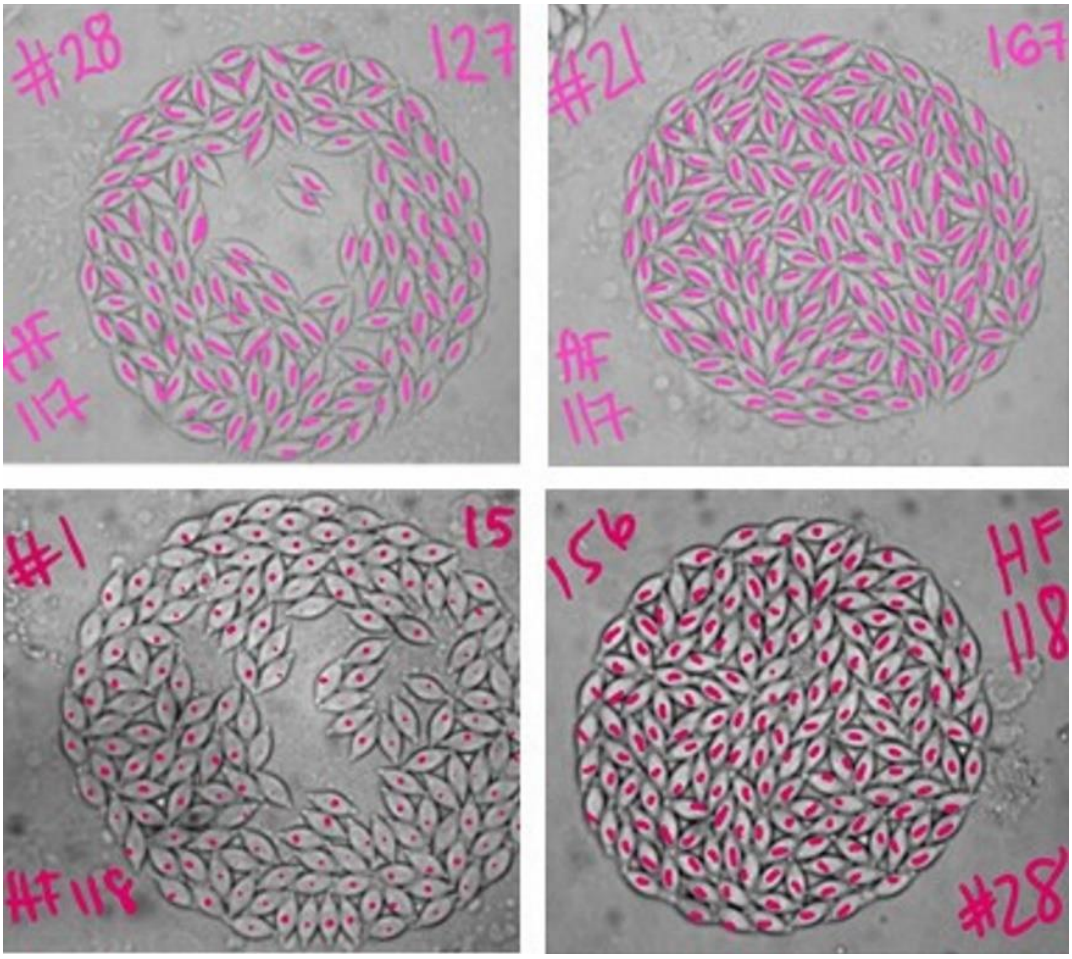
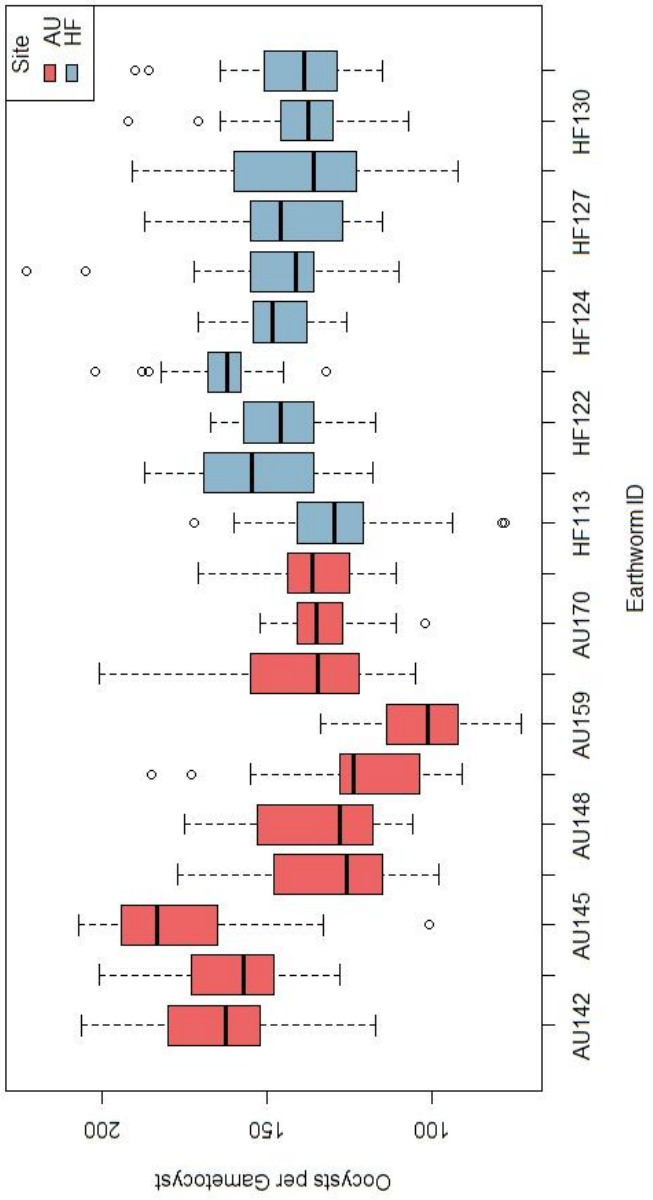
Supplemental Materials



Suppl. Fig 1. Examples of gametocysts from two infections at HF (top: HF 117; bottom: HF 118) that were measured for their reproductive output (number of oocysts per gametocyst). Photographs were taken at 400x.



Suppl. Fig 2. Box and whisker plots of the number of sporocysts per gametocysts for each earthworm from Audubon Center (N = 10) and Horticultural Farm (N = 10). The horizontal black bar within the box represents the median number of sporocysts per gametocyst, the boundaries of the box represent the interquartile range, and the whiskers indicate the range of the data.

CHAPTER 4: Vertical and horizontal transmission by a gregarine parasite, *Monocystis perplexa* (Apicomplexa: Eugregarinoida)

Summary

Transmission biology is central to the life history of parasites because it influences the prevalence, distribution, genetic diversity, and maintenance of parasitic infections. Additionally, asexual replication is a life history strategy that is responsible for increasing the parasite load in a host and increasing the probability of transmission. In this study, the presence of vertical transmission, a mode of transmission that is often underreported and not well-known in gregarines, was investigated using the gregarine parasite, *Monocystis perplexa*. The presence of asexual replication was also evaluated during embryonic development of the host, *Amynthas agrestis*, during the first month after earthworms hatch. The density of parasites in various host tissues was also evaluated. Quantitative real-time PCR was used to detect minute amounts of *M. perplexa* DNA and to quantify the difference in parasite loads. Evidence of vertical transmission was found in the three host populations studied, with 44.3% of all embryos tested found to be infected with *M. perplexa.* There was also a moderate increase in the parasite load over the course of embryonic development which suggests that parasites may be replicating within the embryo. High density of parasite DNA was also found in all host tissues examined.

While large proportions of recent earthworm hatchlings were infected early in their season, there was no overall increase in parasite load over the four-week study; this suggests that asexual replication is not present early in the earthworm season although it may be present during embryonic development. Further investigation into the presence of merogony in different host tissues and during different time periods in the host's season may reveal that eugregarine parasites, like the polyphyletic neogregarines, are capable of merogony.

This study is the first to find clear evidence of vertical transmission in the long-studied genus *Monocystis*. Vertical transmission appears to be a widespread phenomenon among local host populations and likely contributes to the overall prevalence and maintenance of *M. perplexa* in its annual earthworm host. Future studies could benefit from investigating the genetic diversity of *M. perplexa* infections associated with horizontal *vs*. vertical transmission to better understand how transmission mode influences the evolutionary history and potential of this parasite. Due to the identification of vertical transmission in multiple gregarine species, the currently accepted life cycle and life history strategies that the gregarines employ needs to be reconsidered with vertical transmission and possible merogony in mind.

Introduction

Parasitism is the most common life strategy on Earth with up to 70% of higher taxa having a parasitic representative (Windsor, 1998; de Roode et al., 2008; Roberts and Janovy, 2009). Perhaps the reason parasitism is so widespread is because of the great diversity of life history strategies parasites employ, including transmission modes and life-cycle complexity (Poulin, 2007). These strategies govern the frequencies of interactions between parasites and hosts and provide insight into the evolutionary history and potential of the parasite system (Barrett et al., 2008). Transmission mode is one of the most crucial life history strategies of parasites as the fitness of parasites is measured in transmission success – i.e. the total number of new infections. The mode of transmission also influences many other life history traits, the genetic diversity of the infrapopulation, and parasite evolution by shaping the timing and probability of transmission and the genetic diversity of parasites that are transmitted (Barrett et al., 2008).

The two modes of transmission – vertical and horizontal transmission – constitute an evolutionary trade-off between the production of transmissible stages and virulence (Antonovics et al., 2017). Horizontal transmission, or the transmission of a pathogen through direct or indirect routes among individuals in the same generation or production of a durable transmission stage in the environment, typically favors high parasite burdens, increased pathogenicity, and increased prevalence; this is because transmission is dependent on the high production of parasites to overcome various environmental and host conditions that can greatly reduce transmission success

(Dunn and Smith, 2001; Chen et al., 2006). One advantage to horizontal transmission is the dispersal between unrelated hosts resulting in an increased chance of encountering a new lineage of hosts. Vertical transmission, on the other hand, is constrained to the direct transmission of a pathogen from the parent to its offspring; because of this, vertical transmission favors low virulence and long-term persistence because the pathogen's success is dependent on the survival and reproduction of its host (Herre et al., 1999; Ewald, 1987; Bull et al., 1991; Chen et al., 2006). Due to reduced pathogenicity and numbers of parasites produced, vertical transmission is often underreported, and many studies instead focus solely on horizontal transmission (Dunn and Smith, 2001; Chen et al., 2006). For this reason, the presence of vertical transmission may be overlooked in a system that utilizes both horizontal and vertical modes of transmission, as horizontal transmission may be more obvious to researchers given its higher pathogenicity and production of more-easily identifiable transmissible stages.

Despite the trade-offs between the two modes of transmission, some parasite systems employ a combination of vertical and horizontal transmission. In fact, intermediate transmission strategies can be found across parasitic taxa (e.g. malaria, microsporidia, myxozoans, and bacteriophages) and increase the overall prevalence in populations (Antonovics et al., 2017). The increase in pathogen prevalence in a population can be attributed to the complementary transmission into hosts that would otherwise escape infection by one mode of transmission alone; this 'Law of Diminishing Returns' posits that there is a decrease in the number of susceptible individuals with increasing horizontal transmission until the point where contact with the remaining susceptible individuals is unlikely. Vertical transmission, when present with horizontal

transmission, can infect what would be the remaining noninfected individuals (Lipsitch et al., 1995). This intermediate form of transmission consisting of the presence of vertical and horizontal transmission allows for persistence and high prevalence of a pathogen; however, mixed transmission modes are often understudied and underreported (Russell et al., 2017).

The gregarines are a diverse clade of parasites in the phylum Apicomplexa that parasitize a broad range of invertebrates (Desportes and Schrével, 2013). The life cycle involves replication in the host during sexual reproduction (sporogony), and horizontal transmission via when a host ingests the transmissible oocyst stage. A few species (the neogregarines which form a polyphyletic group) are capable of asexual replication (merogony) in the host. Most species, the eugregarines, are not thought to undergo merogony (Leander et al., 2003; Clopton, 2002). Gregarines are ubiquitous among their hosts, frequently reaching 100% prevalence in host populations, and exhibit high parasite density (parasitemia) in individual hosts (Desportes and Schrével, 2013). This is perplexing given the low reproductive output compared to other apicomplexan parasites (only hundreds of oocysts produced per parasite in an infection *vs*. millions) and the presence of putatively only horizontal transmission. The durability of oocysts in the environment is known for only a few species, but certainly cannot explain these patterns (Clopton et al., 2016).

The eugregarine parasite, *Monocystis perplexa*, is an excellent example of how a eugregarine parasite can have high parasitemia and prevalence while having low reproductive output (Chapter 3), horizontal transmission, and putatively no merogony. *M. perplexa* parasites infect the invasive annual earthworm, *Amynthas agrestis*, and reach

100% prevalence and high parasitemia in the seminal vesicles, the primary site of infection of *Monocystis* parasites, within several months after earthworms hatch in spring (Chapter 3); this means that the earthworm host must consume potentially many thousands of oocysts within three months in order to produce the parasitemia observed in the wild in the absence of merogony. Further adding to the mystery of how *M. perplexa* accomplishes high parasitemia and prevalence is the observed near-complete absence of gametocysts, the transmissible stage of the parasite containing the infective oocysts, in a population of *A. agrestis* earthworms in Vermont (Chapter 3). The lack of the horizontally transmissible stage of the parasite coupled with the maintenance of infection at the site over host generations suggests that important alternative life history strategies could be present in *M. perplexa*.

Vertical transmission is a strategy that could explain the maintenance of high prevalence *M. perplexa* infections through host generations. While gregarines are largely transmitted through the oral-fecal route, resolving the route of transmission has been challenging and a matter of debate in some gregarines, notably *Monocystis* (Miles, 1962, Fields and Michiels, 2006; Hesse, 1909; Bhatia, 1924, Keilin, 1925; Hahn, 1928). Further, some gregarine parasites are capable of vertical transmission. *Ascogregarina* spp. and *Psychodiella chagasi,* for example, are parasites of insects that are vertically transmitted when host larvae consume oocysts attached to the exochorion of eggs (Lantova and Volf, 2014; Warburg and Ostrovska, 1989). In *M. perplexa*, vertical transmission could be accomplished by infecting earthworm cocoons – environmentally durable cases containing the embryo – by infecting the embryo itself or the contents of the cocoons that are ingested upon development and hatching. Vertical transmission

could help explain how the parasite is maintained in a host population with an extremely low production of horizontally transmitted parasite stages.

Another life history strategy that could allow *M. perplexa* parasites to reach the high parasitemia observed in the wild is the presence of merogony. Infections in *A. agrestis* seminal vesicles can be undetectable via microscopy and within days reach parasitemia in the millions (Chapter 3). While eugregarines are thought to not undergo merogony, the neogregarines are capable of merogony and are polyphyletic to the eugregarines (Cavalier-Smith, 2014). In fact, the only distinguishing characteristic between the eugregarines and neogregarines is the presence of merogony. Thus, it is possible that merogony has simply not been observed in the gregarines rather than the neogregarines only secondarily evolved merogony; this warrants an investigation into whether *M. perplexa* can undergo merogony which allows this parasite to reach the high parasitemia observed.

Here, the life history strategies of vertical transmission and merogony are investigated to determine if they are present in the eugregarine parasite, *M. perplexa*, and whether they have a significant role in influencing infection dynamics. First, the presence of vertical transmission is investigated using quantitative PCR (qPCR) to detect and quantify minute amounts of parasite that may be missed through microscopy methods alone. I hypothesize that vertical transmission will be common in all host populations sampled because of the high infection prevalence observed early in the earthworm's life. Next, the presence and density of parasite in various host tissues is investigated to determine important sites of infection outside of the seminal vesicles. I hypothesize that major sites of infection are the coelomic fluid, gizzard, and clitellum. The coelomic fluid

is the site of infection of other gregarines, the gizzard is thought to be involved with release of sporozoites from the oocyst, and the clitellum produces the protective shell around the embryo. Last, the presence of merogony is investigated by quantifying the amount of parasite in recent earthworm hatchlings over time. I hypothesize that if the parasites undergo merogony early in their host's life, then an exponential increase in the density of parasite over time will be observed consistent with multiple rounds of asexual replication. By identifying locations of infection and the transmission mode, the infection dynamics of *M. perplexa* will be elucidated and will provide insight into the life history strategies of other gregarines.

Methods

Cocoon collection, dissection, and DNA extraction

Soil samples composed of the top 10cm of soil (O and A horizons) were collected at three sites in Vermont: Audubon Center, Hort Farm, and Centennial Woods (see Chapter 3 for full description of sites). Only two earthworm species are found in the soils, *A. agrestis* and the congener *A. tokioensis*. Cocoons were extracted from the soil by manually sifting through soil samples. Upon extraction from the soil, cocoons were sorted by size to identify earthworm species; only cocoons measuring >3mm, corresponding to cocoons of *Amynthas agrestis*, were used in this study (Görres et al., 2018). Cocoons were then thoroughly rinsed in distilled water to remove any external parasite contamination and stored in moist Petri dishes at 10ºC until dissection.

Cocoons were rinsed in 10% sodium hypochlorite and then thoroughly rinsed in

distilled water to remove any external parasite contamination on the cocoon. Using sterile forceps, gentle pressure was applied to the cocoon to release the cocoon contents into a 1.5mL vial. After removing the cocoon shell, the cocoon contents were observed under a light dissection microscope to determine the stage of embryonic development. Embryonic development was categorized and recorded as follows: Undeveloped – absence of any tissue development; Stage I – absence of segmented body parts; Stage II –presence of unpigmented partially segmented body parts; Stage III – fully developed with unpigmented or pigmented segmented body (size of a hatchling worm).

Dissected cocoons were then extracted using Qiagen's DNA Tissue & Blood Extraction Kit (Valencia, CA) according to manufacturer's protocol in a separate laboratory where *Monocystis* spp. has not been studied to eliminate contamination of DNA samples. Further, each small batch of extractions were done with a negative water control to identify cross-contamination during the extraction process. All extracted DNA samples were stored at 4ºC until use.

Earthworm hatchling collection and dissection

Young *Amynthas agrestis* earthworms were collected from each of the three sites on June 5, 2017 by manually sifting through the top 10cm of soil. These worms were small (~2-4cm), and likely hatched in early April (hatchlings ~ 1 cm). Upon collection, each earthworm was thoroughly rinsed in distilled water and stored in ventilated plastic containers with parasite-free medium. The parasite-free medium is constituted of the O and A horizons of soil collected from Camel's Hump mountain in Huntington, Vermont (elevation: 1,244m) known to be devoid of all earthworm species

and thus lacking transmissible *Monocystis* spp. oocysts. Hatchlings were raised in these artificial habitats for four weeks, with ten earthworms per site sampled each week from June 5, 2017 – June 26, 2017 for a total of 113 earthworms (CW experienced a die-off before week 4, resulting in only three earthworms sampled during week 4). The earthworms sampled weekly from each site were then rinsed in distilled water, killed via freezing in unique 1.5mL vials, and stored at -20ºC until dissection.

Dissections were performed under a light dissection microscope using sterile Petri dishes, disposable forceps, and razor blades. Earthworm segments nine through 17, containing the reproductive organs of *A. agrestis* – and the site of infection of *M. perplexa* - were removed and masticated with the razor. Tissue samples were stored at -20ºC until DNA extraction.

Lastly, tissues from various parts of the earthworm were obtained to determine the presence of parasite in host tissue other than the seminal vesicles, the primary site of infection. Earthworms were dissected in sterile finger bowls after cleaning the earthworm in 10% sodium hypochlorite, rinsing in distilled water, and blot drying. Samples of various tissues were collected for DNA extraction; these included the clitellum – a reproductive tissue that produces a viscid sac in which earthworm eggs are deposited – as well as the skin, coelomic fluid, and gizzard – a muscular organ that grinds food and is thought to help liberate sporozoites from oocysts.

All DNA extractions were done with Qiagen's Blood & Tissue Extraction Kit (Valencia, CA) according to the manufacturer's protocol following the same precautions as above. Additionally, the DNA concentration and purity was determined for each sample using a Nanodrop ® ND-1000 Spectrophotometer (TheromFisher Scientific,

Waltham, MA). Any samples with DNA concentrations >100 ng/μL were diluted with elution buffer to 100 ng/μL, per the qPCR manufacturer's recommendation. Poor quality samples (260/268 DNA/protein < 1.8) and low concentration samples (< 10 ng/μL) were excluded from testing to reduce false negatives. All DNA samples were stored at 4ºC until further use.

Molecular determination of infection

An optimized quantitative real-time polymerase chain reaction (qPCR) protocol was designed and implemented to quantify the parasitemia of *M. perplexa* in the embryo and recent hatchling samples. Primers specific to the 18S SSU rDNA gene of *M. perplexa* were designed by aligning 18S sequences of *M. perplexa* (GenBank accession number), *M. agilis* (AY391264.1), *Ascogregarina taiwanensis* (ABJQ00000000), and *Amynthas agrestis* (GenBank accession number) using the bioinformatics platform Geneious version R6 (Kearse et al. 2012). Variable regions in the alignment were located and the following primers were designed to produce a 99bp amplicon: MP250F 5' GGT GAT CCA TAA TAA TGT CGC AGA 3' and MP277R 5' CGG TAG GAC AAT ACC CGA CTG 3'; this primer pair was verified to be *M. perplexa*-specific by testing the primers using conventional PCR against *M. agilis* and *M. perplexa* samples. Only *M. perplexa* samples amplified indicating species-specificity of the primers.

The qPCR protocol was optimized in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009). Three primer concentration ratios, 100nM:100nM, 100nM:200nM, and 200nM:200nM, and three annealing temperatures, 53ºC, 55ºC, and 57ºC, were tested to

determine the most efficient and specific qPCR conditions. A 100nM:200nM primer ratio and an annealing temperature of 57ºC was used as these conditions eliminated the formation of primer-dimers and amplification in the non-template control (NTC). Optimized reactions were performed in 10µL reactions with 5µL Luna® Universal qPCR Master Mix (New England Biolabs ®, Inc.), 3.7µL PCR-grade H_2O, 0.1uL MP250F primer, 0.2µL MP277R primer, and 1µL of sample DNA. The reactions were carried out in an Applied Biosystems® 7500 Real-Time PCR machine with the following thermocycling protocol: initial denaturization 95ºC 1m, 40 cycles of denaturization at 95ºC for 15s and extension at 57ºC for 30s, followed by a melt curve analysis starting at 60ºC. The qPCR protocol amplifies a single 99bp amplicon of the *M. perplexa* 18S gene with an experimentally determined melt temperature of 80.9 ± 0.08 ºC.

A 10-fold serial dilution was produced by purifying the target amplicon resulting from conventional PCR using the qPCR conditions and primers. Serial dilutions ranging in DNA concentrations from 8.23 to $8.23x10^{-8}$ ng/µL, determined using a Qubit® 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA), were run in triplicate to generate a standard curve for absolute quantification and to determine the efficiency of the reaction (below). Additionally, a second standard curve was made using a 10-fold serial dilution of parasite genomic DNA to determine if genomic DNA, which is used for testing samples, is amplified with the same efficiency as PCR product used for absolute quantification.

The qPCR reaction plate preparation and the addition of genomic DNA were done in two separate laboratories to eliminate contamination during the reaction set-up. Embryo and hatchling samples were tested in duplicate initially and in triplicate if the

initial replicates had a Cq (quantitation cycle when amplicon is detected) difference greater than one or discordant results (i.e. failure of one replicate to amplify). Samples were considered positive if at least one replicate in the rerun amplified below the limit of detection. All plates contained at least three NTC to detect contamination, non-specific amplification, or primer-dimerization; however, no NTC amplified throughout the study.

Calculations and Statistical analyses

The total number of 18S molecules amplified was determined by using absolute quantification from the standard curve. First, the number of 18S molecules was theoretically determined by using the following formula:

$$Number\ of\ copies\ (molecules) = \frac{Xng\ \times 6.022E23\ molecules/mole}{N \times 660\ g/mole \times 1.0E9\ ng/g}$$

Where X is the mass of amplicon and N is the length (bp) of the double stranded DNA amplicon. Next, the standard curve was generated by graphing the quantification cycle (Cq) value on the y-axis and the log base 10 number of 18S molecules amplified on the x-axis. A linear regression was performed to determine the relationship between the Cq value and the log number of 18S molecules. The equation of the line of best fit was then used to convert Cq values for unknown samples to the log number of 18S molecules:

$$y = mx + b$$

Where y is the Cq value, m is the slope of the line of best fit, x is the log number of 18S molecules, and b is an estimation of the Cq value obtained for a single molecule (Fig 1). The qPCR conditions yielded a highly efficient reaction with an efficiency of >99% and met all MIQE guidelines (Bustin et al., 2009). The reaction done with parasite genomic

DNA also had an efficiency >99%; thus, absolute quantification from the PCR-product standard curve can be reliably used to determine the number of parasite molecules in genomic samples.

For detection of parasite in embryos, first, a Chi-squared test was performed to determine if there is a difference in the proportion of embryos infected by site. Next, a Kruskal-Wallis test was used to determine if there is a significant difference in the number of *M. perplexa* 18S molecules (hereafter referred to as 'number of parasite molecules') in infected embryos among sites. Due to the low sample size and potential misidentification of embryonic development stages, non-developed and Stage I embryos were combined to form a "no-early development" group and Stages II and III embryos were combined to form a "late development" group. A Mann-Whitney U-test was then performed to determine if there is a significant difference in the number of parasite molecules among embryonic development stages.

For analysis of the earthworm hatchling samples and tissue samples, the analyses were done for both the absolute and adjusted number of parasite molecules. The adjusted quantity of parasite DNA is the number per ng of DNA extracted (log number of 18S molecules divided by the total amount of extracted genomic DNA) which corrects for the efficiency of the DNA extraction process itself. Such a correction was not done for the embryos because the goal was to determine presence and density of parasite DNA in the entire embryo. To determine if there is a difference in the absolute or adjusted number of parasite molecules among host tissue types, a Kruskal-Wallis test was performed using the infected clitella, gizzard, and skin tissue samples; the coelomic fluid samples were excluded from this analysis due to the insufficient sample size (N = 2). For investigation

of asexual replication, an ANOVA test was performed to determine if there is a significant difference in the absolute or adjusted number of parasite molecules among weeks of the asexual replication study. To determine if there was an overall difference in the proportion of hatchlings infected by week, a Chi-squared test was performed. All graphics and analyses performed were done in the statistical programming language, R (R Core Team, 2013).

Results

Presence of parasite in earthworm embryos

Monocystis perplexa DNA was detected in embryos from all sites with 44.3% of 70 embryos tested infected (Table 1). There was a significant difference in the proportion of embryos infected by site ($\chi 2 = 7.5$, $p = 0.023$); AU had the highest percentage of embryos infected, followed by CW and then HF (Table 1). There was no statistical difference in the log number of parasite molecules among sites (Fig 2.; $p = 0.75$); however, the quantity of parasite DNA varied greatly among infected embryos (Fig. 2; 0.744 – 4.04 log number molecules or 6 – 10,964 number of molecules). The number of parasite molecules was significantly different among no-early development embryos and late-development embryos (Fig. 3, $p = 0.027$). The mean quantity of parasite DNA increased with embryonic development stage, and Stage III embryos had more than six times more parasite molecules than undeveloped and Stage I embryos and nearly two times more parasite DNA than Stage II embryos (Table 2).

Presence of parasite in different host tissues

Parasite DNA was detected in all samples of all tissue types (Table 3) (Fig 4). As expected, the seminal vesicle sample used for a base-line estimate of the number of parasite molecules in the primary site of infection had the highest number of parasite molecules (6.935 log number of parasite molecules). While only two coelomic fluid samples had high quality and quantity of extracted DNA, both samples had the highest absolute number of parasite molecules after the seminal vesicles. In fact, the mean log number of parasite molecules in coelomic fluid is similar to the base-line estimate of the seminal vesicles. Skin, clitella, and gizzards all had a similar absolute and adjusted number of parasite molecules ($p > 0.05$). While the coelomic fluid samples could not be included in the analysis due to sample size, qualitatively, coelomic fluid samples had many more parasite molecules than clitella, gizzard, and skin samples. Further, coelomic fluid samples had the highest adjusted number of parasite molecules, indicating that the coelomic fluid has a higher ratio of parasite to host DNA than any other tissue, including the seminal vesicles.

Presence of asexual replication

Presence of *M. perplexa* in hatchlings kept in parasite-free media was detected during each week and in hatchlings collected from each site (Table 4; Fig. 5). Combining sites, there was no difference across the four sample periods in the proportion of worms infected (Table 4; $\chi 2 = 4.12$, $p = 0.248$). Breaking out the data by sample period shows CW had the lowest percentage of hatchlings infected, ranging from 20-68%, whereas both HF and AU had >90% of worms infected by week 2 (Table 5). There was

a significant difference in the total quantity of parasite DNA in infected earthworms among sites ($p = 0.001$), with AU having more parasite DNA than HF and CW ($p = 0.002$ and $p = 0.023$, respectively); however, there was no difference in the quantity of parasite DNA among weeks at all sites ($p = 0.435$) nor among weeks at each site independently ($p > 0.3$ for all sites) (Fig 5).

Discussion

Life history strategies are fundamental in shaping the infection dynamics of a host-parasite system. In particular, mode of transmission influences many aspects of the biology of parasites including virulence, prevalence, and parasitemia. For some parasites in the phylum apicomplexa, such as the coccidians and malaria parasites, the ability to undergo asexual replication, or merogony, is a life history strategy that greatly influences infection dynamics because it allows for parasites to rapidly increase in numbers within individual hosts, as well as produce huge numbers of transmission stages. In contrast, the gregarines, including *Monocystis*, are held to lack this asexual replication, and thus far fewer transmission stages are produced. To understand the life history of a gregarine parasite, *Monocystis perplexa*, two critical life history strategies, vertical transmission and possible merogony, were investigated to gain insight into how this parasite is ubiquitous among its earthworm hosts.

The presence of parasite DNA in a large portion of embryos tested suggests that *M. perplexa* is being transmitted vertically by its host, *Amynthas agrestis*. The route of vertical transmission may be through infecting the embryo directly (transovarial) or by

infecting the cocoon shell or other cocoon contents (transovum) (Ewald, 1987). Both transovarial and transovum transmission are likely possible because presence of parasite was detected both in the embryo and in the clitellum. With approximately 40% of all embryos and 100% of clitella infected with *M. perplexa*, large portions of the new generation of *A. agrestis* earthworms are likely infected upon hatching. The high rate of vertical transmission could explain how *M. perplexa* infections are maintained at 100% prevalence throughout multiple generations, even with extremely limited production of the horizontally transmitted stage of the parasite (Chapter 3). With predominately low-virulence pathogens like the gregarines, excluding the neogregarines capable of merogony (Levine, 1985; Lantova and Volf, 2014), 100% prevalence in host populations can be maintained through high rates of vertical transmission with little horizontal transmission (Lipsitch et al., 1995; Mims, 1981). The phenomenon at Centennial Woods, where 100% of hosts are infected but only ~5% of infections produce gametocysts, could thus be explained by high rates of vertical transmission.

Further, the presence of parasite in the embryo did not appear to harm the development of its host as vertical transmission can in other gregarine parasites, primarily neogregarines, such as *Ophryocystis elektroscirrha* (de Roode et al., 2008). Undeveloped and early-development embryos contained a lower number of parasite DNA than late-development embryos which suggests that the amount of parasite is not responsible for deterring host embryonic development; however, it is interesting to note that 100% of undeveloped embryos were infected while only ~40% of developing embryos were infected. The increase in the number of parasite molecules in embryos over their development indicates that there is parasite replication during embryonic development.

There is more than a 6x increase in the mean number of parasite molecules in Stage III embryos compared to non-developed and Stage I embryos. The difference in parasite load may be better understood in terms of the difference in the number of parasite cells. While data on the number of 18S gene copies present in the gregarine genome is limited to *Cryptosporidium* spp., using the calculated 18S gene copy number from *Cryptosporidium* spp. of five copies per genome can provide an estimate for the number of *M. perplexa* cells (Le Blancq et al., 1997). Using an 18S copy number of five and multiplying the number of cells in 1μL by 60 to account for the total volume of DNA extracted, the increase in parasitemia during embryonic development is an increase from ~600 cells in undeveloped embryos to ~4,260 cells in Stage III embryos; this estimate is reasonable given that the lowest number of parasite molecules that was detected was ~5 which would correspond to a single parasite cell. Several rounds of asexual replication could explain this increase in parasite molecules and, by increasing the number of parasites in the developing embryo, *M. perplexa* infections in the embryo may be more likely to survive to hatching.

Vertical transmission likely partially contributes to the number of infections early in the earthworm season. At both the Horticultural Farm and Audubon Center, the vast majority of earthworms were infected within several weeks of hatching likely due to a combination of vertical and horizontal transmission. Gametocysts remaining in the soil from the previous season could continue to infect new hatchlings so that, within a month of hatching, 100% of hatchlings can be infected. At Centennial Woods, however, the observed lack of gametocysts produced and deposited in the soil likely explains why the proportion of early earthworms infected at Centennial Woods is lower than the other

two sites and is more similar to the proportion of embryos infected. Without an abundance of gametocysts in the soil, the earthworms at Centennial Woods infected likely became infected through vertical transmission as embryos rather than by horizontal transmission.

While hatchlings were infected during each week of the asexual replication study, there was no increase in the quantity of parasite over the four weeks; this suggests that merogony is not taking place early in hatchling development. Comparing the quantity of parasite DNA in hatchlings to the quantity of parasite DNA in the seminal vesicles of mature earthworms (approximately four weeks after the end of the asexual replication study) indicates a substantial increase in parasitemia in the same tissues. The increase in parasitemia in the reproductive tissues could be explained by continual infection via horizontal transmission and/or asexual replication occurring during this time. Future studies should look at whether tissues or fluids other than the seminal vesicles may be the site of merogony. The coelomic fluid, in particular, is a candidate site of asexual replication as this fluid had the densest and highest amount of parasite after the seminal vesicles. The presence of parasite in all tissue types tested also suggests that estimates of parasitemia based solely on the parasitemia of seminal vesicles may be a substantial underestimate of the total parasitemia in a host. For example, in a small sample of coelomic fluid that is widespread throughout the host's body cavity, the parasitemia can be as high as the total parasitemia in the seminal vesicles. Previous estimates of the transmissible oocyst survival rate needed to produce the parasitemia observed based on the seminal vesicles alone are severe underestimates given the new evidence of heavy parasite loads throughout the host body (Chapter 3); this further suggests that the

currently accepted life cycle of *Monocystis* spp. is untenable and more research is needed on quantifying the rates of the different modes of transmission and investigating the possibility of merogony to better elucidate infection dynamics.

Future studies could also benefit from determining the mechanism by which the embryo becomes infected. *A. agrestis* can be both parthenogenetic and sexually reproducing (Keller et al., 2017), therefore, presence of parasites among the sperm in the seminal vesicle may not contribute to infection of the embryo in parthenogenetically reproducing earthworms. The female pore – the location where the earthworm egg is released – may then be another site of infection that allows transmission of the parasite into the embryo. The parasite itself may be partially responsible in influencing the reproductive strategy of its host, as a decreased production of sperm from parasite feeding may limit the efficiency of sexual reproduction. Another avenue for future research could investigate the routes of vertical transmission in sexual and parthenogenetically reproducing earthworms and whether infection of *M. perplexa* in the seminal vesicles influences the rate of sexual versus parthenogenetic reproduction.

The presence of vertical transmission is an important discovery in understanding the life history and infection dynamics of gregarine parasites and this is the first study to find clear evidence of vertical transmission in the long-studied genus *Monocystis*. Previously, only two genera of eugregarine parasites have been found to undergo vertical transmission (Lantova and Volf, 2014; Warburg. and Ostrovska, 1989); however, the identification of vertical transmission in *Monocystis* sp. suggests that vertical transmission may be a more widespread strategy than current gregarine literature suggests. Vertical transmission may be an important strategy for maintaining the ubiquity

of gregarines that is observed in natural populations of invertebrates, and future consideration of multiple modes of transmission in gregarine parasites may elucidate the evolution of virulence, host specificity, and diversity of gregarine parasites.

Table 1. Summary table reporting the total number of embryo samples tested per site, the total number of infected embryos, percent of embryos infected at each site, and the mean and standard deviation of the log number of parasite molecules.

Site	Total	Total Infected	Percent Infected (%)	Log 18S molecules
Centennial Woods	24	10	41.7	2.062 ± 0.914
Horticultural Farm	23	7	30.4	2.112 ± 0.708
Audubon Center	23	16	69.6	1.808 ± 0.556
Total	70	31	44.3	1.949 ± 0.703

Table 2. Summary table reporting the sample size, percent infected, and the mean and standard deviation of the log number of parasite molecules by embryonic development stage.

Embryo development	Total	Total Infected	Percent Infected (%)	Log 18S molecules
Undeveloped	10	10	100	1.717 ± 0.479
Small	26	12	46.2	1.756 ± 0.627
Medium	24	7	29.2	2.272 ± 0.937
Large	10	4	40	2.548 ± 0.573

Table 3. Summary table reporting the sample size for each tissue type tested for presence of *M. perplexa*. All tissue samples were positive for the presence of *M. perplexa*, and the mean and standard deviation of the absolute and adjusted log number of 18S molecules are given.

Tissue	Total	Log 18S molecules	Log 18S molecules per ng of DNA
Seminal vesicle	1	6.935	0.123
Clitellum	10	3.376 ± 1.178	0.155 ± 0.350
Skin	10	3.866 ± 1.081	0.075 ± 0.091
Gizzard	8	2.818 ± 1.034	0.032 ± 0.012
Coelomic fluid	2	5.901 ± 0.874	1.241 ± 0.509

Table 4. Summary table reporting the sample size of the recent earthworm hatchlings, percent of hatchlings infected, and the absolute and adjusted log number of parasite molecules by week.

Week	Total	Total Infected	Percent Infected (%)	Log 18S molecules	Log 18S molecules per total DNA (ng)
1	30	17	56.7	2.074 ± 1.249	0.036 ± 0.056
2	30	22	73.3	2.507 ± 0.938	0.047 ± 0.038
3	30	24	80	2.124 ± 0.706	0.028 ± 0.014
4	23	16	69.6	2.542 ± 0.919	0.030 ± 0.011

Table 5. The percent of *A. agrestis* hatchlings raised in parasite-free medium tested that were infected with *M. perplexa* by week. All N = 10, except for CW week 4, N = 3

	Week			
Site	**1**	**2**	**3**	**4**
Centennial Woods	20%	30%	50%	67%
Audubon Center	50%	100%	100%	80%
Horticultural Farm	90%	90%	90%	60%

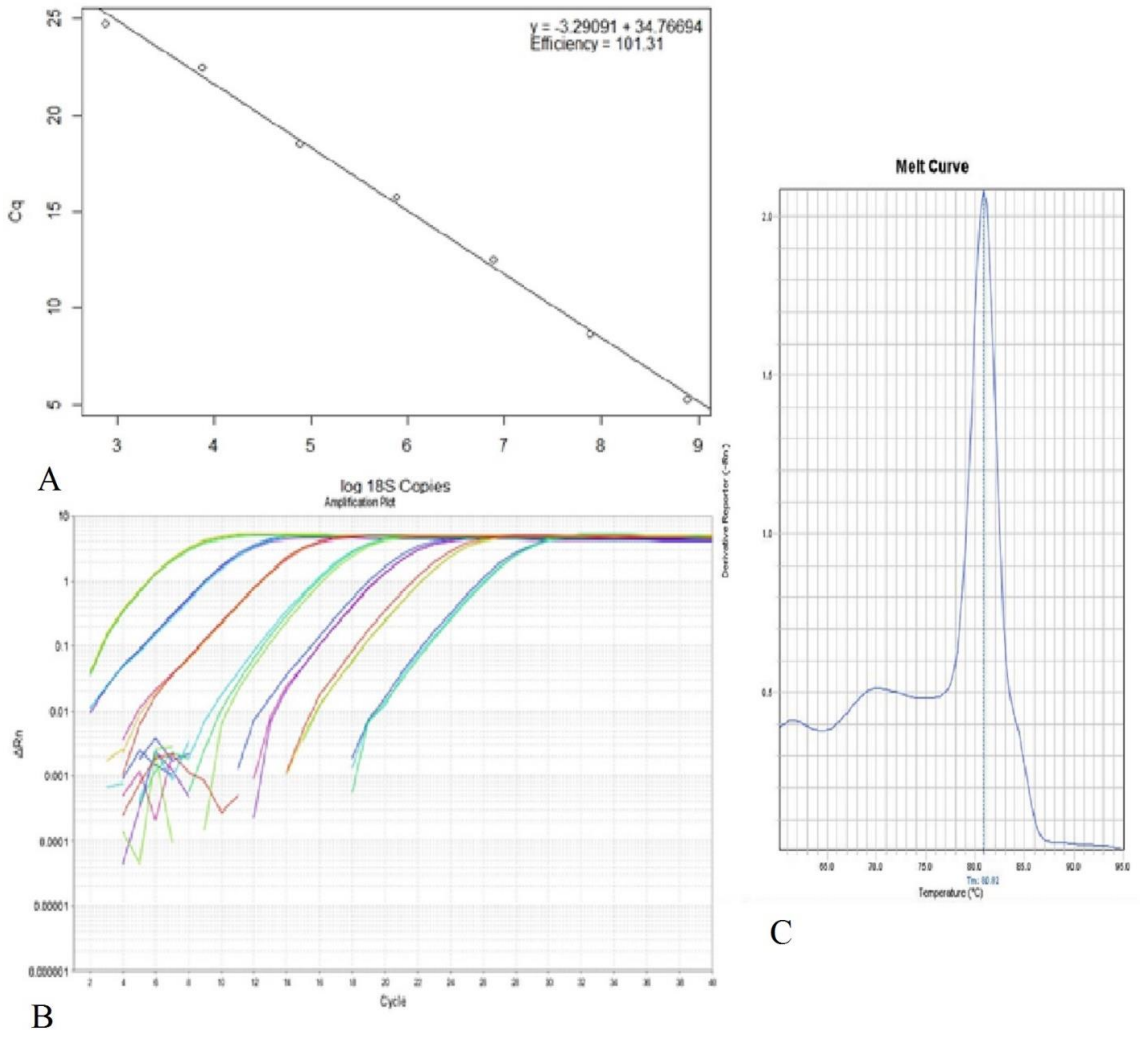


Fig 1. Relevant graphs and figures associated with generation of the standard curve using the 18S gene qPCR program for absolute quantification. A) Linear regression of the serial dilution used to generate the standard curve for the MP250 qPCR protocol. The relationship between the mean Cq values and the log number of 18S copies for each dilution sample yields a primer efficiency of 101.31 and an $R^2 = 0.9964$ B) example amplification plot of the standard curve C) example melt curve for the known-positive sample used for the standard curve.

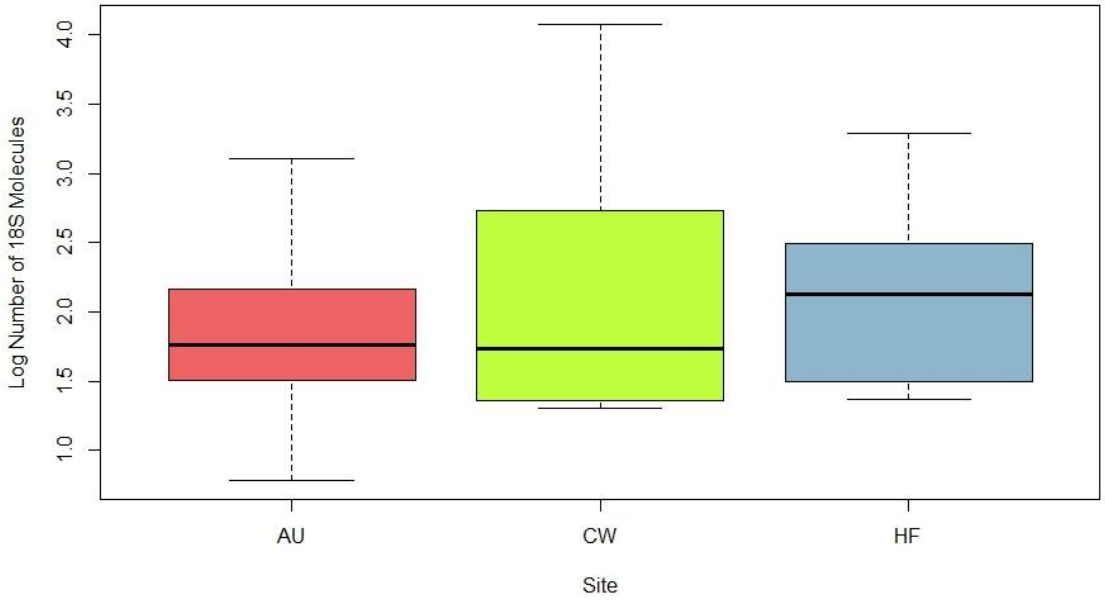


Fig 2. Boxplot depicting the distribution of the log number of *M. perplexa* 18S molecules in embryos by site: Audubon Center (AU, N = 23), Centennial Woods (CW, N = 24), and Horticultural Farm (HF, N = 23). Black horizontal lines depict the median log number of parasite molecules, the box represents the interquartile range, and the whiskers represent the range of the data. Sites were not significantly different in the log number of 18S molecules in embryos (p = 0.542).

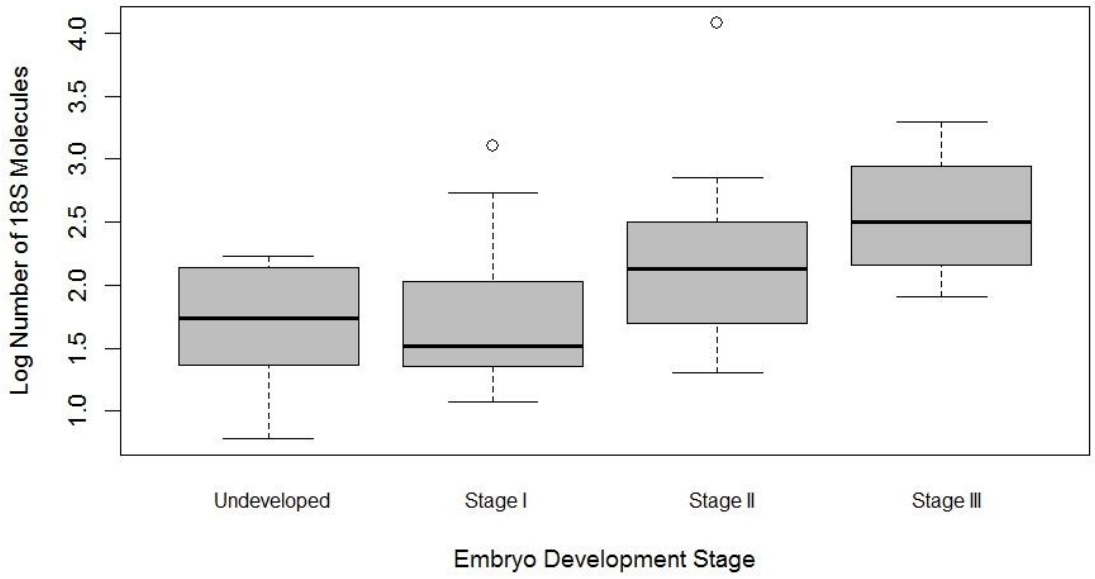


Fig 3. Boxplot of the distribution of the log number of 18S molecules in different embryo development stages: no development (N = 10), absence of segmented body parts (N = 26), presence of unpigmented partially segmented body parts (N =24), and fully developed with unpigmented or pigmented segmented body (N = 10). There was a significant difference in the log number of parasite molecules and early vs. late embryonic development (p = 0.027).

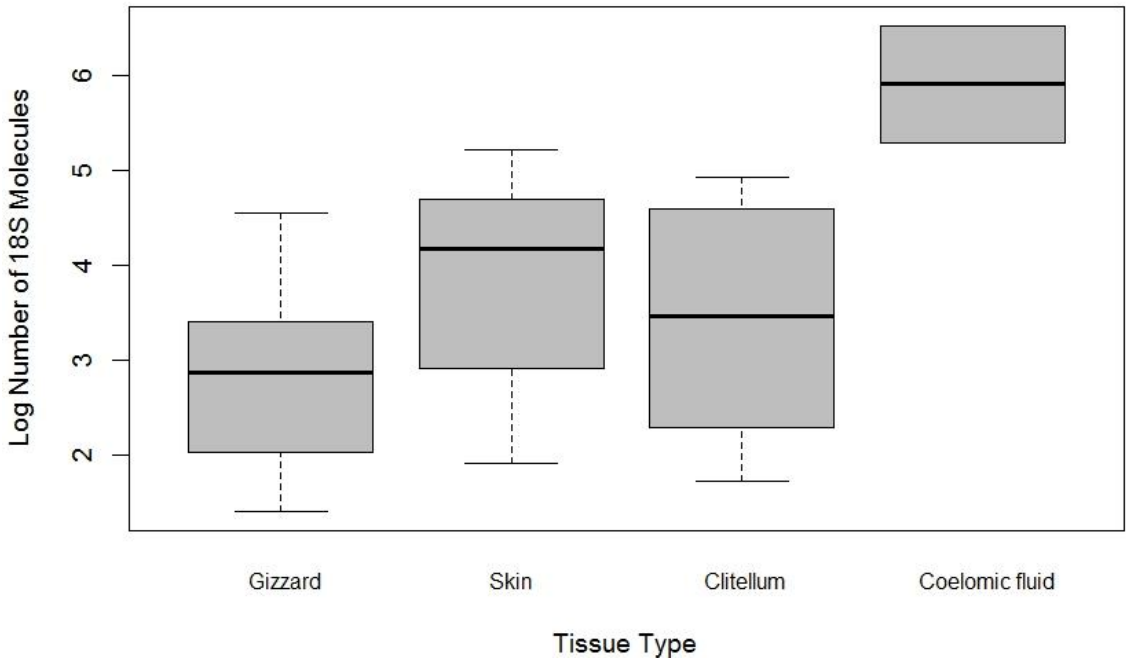


Fig 4. Boxplot depicting the distribution of the log number of *M. perplexa* 18S molecules by tissue type: gizzard (N = 8), skin (N = 10), clitellum (N = 10), and coelomic fluid (N = 2). There was no significant difference in the log number of 18S gene copies among gizzard, clitellum, and skin tissues ($p > 0.05$); however, coelomic fluid samples have qualitatively more log number of parasite molecules than the other tissues. Coelomic fluid samples had a similar mean number of log 18S copies to the putative primary site of infection (6.935), seminal vesicles (not shown).

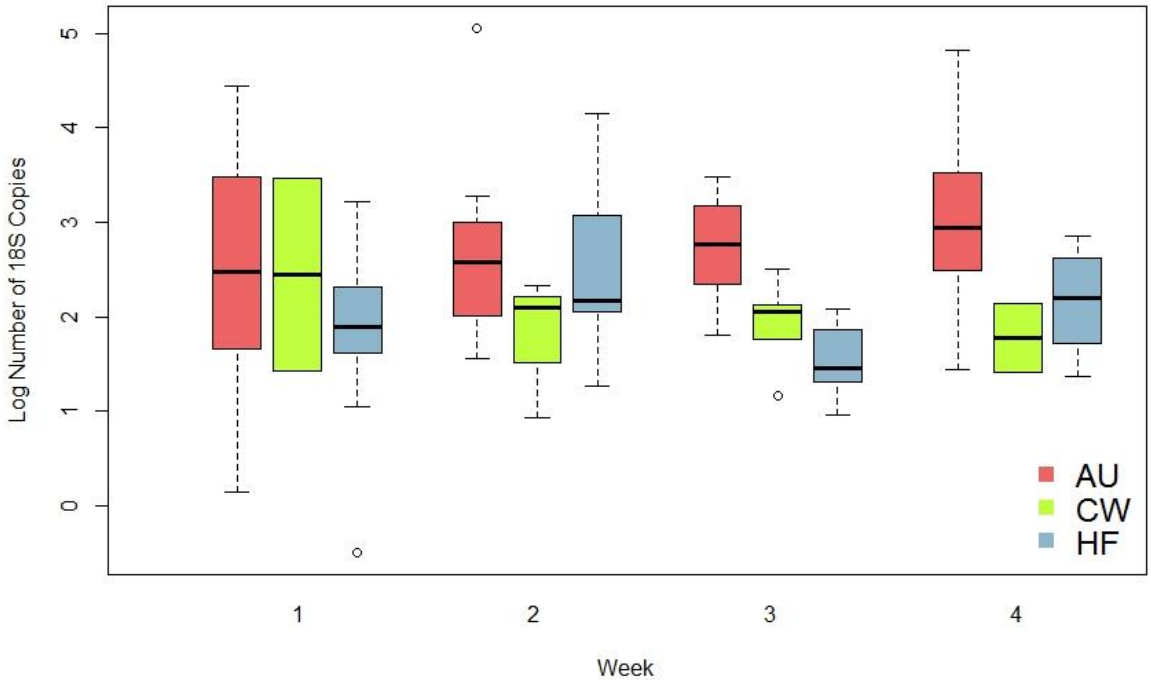


Fig 5. Boxplot of the log number of 18S molecules in hatchlings by week and divided by site.

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CHAPTER 5: Summary and Future Directions

Life history traits, strategies, and their variation govern parasite infection dynamics by influencing the fecundity, survival, and genetic diversity of the parasite (Poulin, 2007). Studies of the evolution of life histories of parasites has, therefore, provided much insight into disease dynamics, diversity of parasites, and the variability of strategies parasites employ that contribute to the success of parasitism as a form of life. Our understanding of human-parasitic disease has greatly benefited from studies of life histories on parasites such as *Plasmodium* spp. (e.g. Buckling et al., 1997), but studies on parasite life histories of invertebrate hosts has lagged behind. One of the clearest demonstrations of this is the relative dearth of life history studies of the apicomplexan group of parasites, the gregarines. The highly diverse gregarines that infect putatively all invertebrates have the potential to resolve the evolutionary history of the apicomplexans (Leander et al., 2003a; Templeton et al., 2010; Simdyanov et al., 2017), be used as biological control agents for controlling vector-borne diseases (Lantova and Volf, 2014), and provide insight into the infection dynamics of important human parasites, such as the closely related *Cryptosporidium* (Barta and Thompson, 2006; Leander, 2007). In this thesis, a newly discovered parasite species, *Monocystis perplexa*, is described and its life history investigated. Substantial insights into the life history and transmission of *M. perplexa* were revealed, which highlight our current lack of understanding of the gregarines and underscore the importance of comprehensive life history studies.

First, the species description of *M. perplexa* implementing a new proposed standard for gregarine species descriptions provided evidence of host species specificity

and widespread prevalence across earthworm populations. Current gregarine species descriptions often omit data on host specificity and distribution which causes parasitologists to rely on "common belief" rather than clear evidence. The species description of *M. perplexa* (Chapter 2) provides the first clear evidence of the host specificity of *Monocystis* spp., a representative genus of acephaline gregarines. Additionally, molecular data from multiple genes were included to assist in the identification of the parasite beyond morphology alone. From this molecular data, the accurate placement of *M. perplexa* into the genus *Monocystis* was demonstrated through the construction of a gregarine gene tree that clustered *M. perplexa* with *M. agilis*. The improvements made to the current standard of gregarine species descriptions yielded important data concerning the life history of *M. perplexa*; the successful implementation of these improvements in this species description demonstrates the benefits of including standardized nomenclature and biometrics, host breadth, and molecular data.

Second, a comprehensive life history study of *M. perplexa* uncovered substantial variation in life history traits among local host populations of *Amynthas agrestis* (Chapter 3). The genus *Monocystis* is often used as a representative parasite for the acephaline gregarines (Sheridan, 1986); however, many elements of the genus's life history have eluded researchers studying *Monocystis* over the past century. One obstacle to conducting a comprehensive life history study of *Monocystis* spp. may be the multi-year lifespan of many earthworm hosts (e.g. *Lumbricus terrestris*), making it difficult to follow the complete life cycle of *Monocystis* spp. By taking advantage of the annual life cycle of its earthworm host, the entire life cycle of *M. perplexa* was studied for three local host populations. Notably, *M. perplexa* infections were widespread throughout the host

populations, with all sites reaching high parasite loads and 100% prevalence by mid-summer; this was particularly surprising because of short lifespan of the host and the low reproductive output of *M. perplexa* (on the order of hundreds per pair of parasites). The reproductive output of *M. perplexa* was variable within and among host populations, and a trade-off was observed between time to parasite maturity and reproductive output. Perhaps the most perplexing finding of the life history study was the near-complete lack of gametocysts at Centennial Woods. The low production of the environmentally-durable transmission stage suggests that the currently accepted life cycle of *Monocystis* spp. needs to be reevaluated with special consideration paid to multiple modes of transmission and asexual replication.

Third, the life history strategies of *M. perplexa* were investigated to determine if certain strategies, such as transmission mode and asexual replication, could help explain the apparently untenable life cycle and life history of *Monocystis* spp. (Chapter 4). The mode of transmission of *Monocystis* spp. has been a debate for many years (Hesse, 1909; Bhatia, 1924, Keilin, 1925; Hahn, 1928; Miles, 1962, Fields and Michiels, 2006) and the putative lack of asexual replication in the eugregarines has been a point of contention for systematists (Leander et al., 2003b; Cavalier-Smith, 2014). The study conducted in this thesis (Chapter 4) is the first to use modern molecular techniques (i.e. quantitative real-time PCR) to evaluate the presence of these life history traits. The high rate and widespread presence of vertical transmission of *M. perplexa* is an important discovery for the genus *Monocystis* and gregarines as a whole; this is the first study to identify vertical transmission in a *Monocystis* parasite and the first to use molecular methods to identify this mode of transmission in a gregarine parasite.

Further, the detection of the parasite in all host tissues tested indicates that measurements of parasitemia based on the seminal vesicles alone is likely a gross underestimate of total parasitemia; this finding in particular emphasizes the currently acceptable but untenable life history of *Monocystis* because that would lead to a requirement of >100% success of the transmission stage. Lastly, while asexual replication was not identified in early earthworm hatchlings, there is evidence of parasite replication during embryonic development. The findings from this study highlights the need to investigate the presence of less conspicuous strategies, particularly vertical transmission, in gregarines, and indeed in other parasites.

The results from this thesis, from the variability of life history traits to the presence of vertical transmission, provide an excellent starting point for future avenues of research concerning *M. perplexa* and other gregarine parasites. For example, the *M. perplexa – A. agrestis* parasite-host system is an appropriate system to study the dynamics of parasite fauna in an invasive species. As discussed in Chapter 2, *A. agrestis* earthworms are a successful invasive species originating in Asia that have been introduced into the United States, with recent expansions of their range into New England within the last 50 years (Gates, 1954; Snyder et al., 2011). While gregarines are primarily thought to cause minimal harm to their hosts (Desportes and Schrével, 2013), future studies should determine the extent of the harm done on the hosts, if any, as a possible avenue for biological control for this earthworm. Further, investigating the parasite fauna in *A. agrestis* in the earthworm's native and introduced ranges could shed light onto how parasite infections change or are maintained as an invasive species invades new communities. As discussed in Chapter 2, *M. perplexa* appears to be restricted to *A.*

agrestis earthworms indicating the parasite is host species-specific; however, the parasites of *A. agrestis* in its native range must be investigated to clearly determine if its parasite fauna changes with invasion (e.g. "Novel Weapons Hypothesis" and "Enemy Release Hypothesis").

Another promising avenue for future research concerns the genetic diversity of *M. perplexa* in host populations. Studying the genetic diversity and structure of *M. perplexa* can reveal the evolutionary potential of the parasite, estimate the rates of horizontal and vertical transmission, and allow interpretation of the effects of certain life history strategies (Barret et al., 2008). For example, infections originating from vertical transmission are expected to be less genetically diverse than infections resulting from horizontal transmission due to a bottleneck in the transmission of parasites from the parent to the offspring host (Russell et al., 2017). The presence of asexual replication may also reduce the genetic diversity of parasite infrapopulations, because high parasite loads may reflect clonal replication of few original parasites. Studying both the life history strategies and the genetic variation of a parasite is needed to interpret the effects each has on one another (Barret et al., 2008).

One tool that could help elucidate signatures of modes of transmission, such as mixed-modes of transmission, is genomics. Unlike other methods for studying population genetic structures, genomic data can help identify unique patterns of genome evolution consistent with different modes of transmission (Russell et al., 2017). As discussed above, infections resulting from vertical transmission are expected to be less diverse than infections from horizontal transmission because of bottlenecks during vertical transmission and because horizontally transmitted parasites are exposed to the

environment and thus must maintain diversity (Gomes et al., 2014; Salem et al., 2015; Russell et al., 2017); however, other signatures of transmission mode may be missed without the use of genomics. For example, vertical transmission also results in the accumulation of deleterious mutations with a gradual genome size loss (McCutcheon and von Dohlen, 2011; Russell et al., 2017). Thus, genomics may be the best tool to uncover the population genetic structure of *M. perplexa*, identify mixed modes of transmission, and understand patterns of genome evolution in the context of transmission.

In summary, the thesis provided here benefits the field of ecological and evolutionary parasitology by preparing a detailed species description with molecular data, comprehensively investigating the diversity of life history traits, and providing the first clear evidence of vertical transmission in *Monocystis* spp. The results from these studies are an excellent foundation for future gregarine studies focused on life histories and transmission and highlight the need to continue researching these diverse and informative parasites.

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