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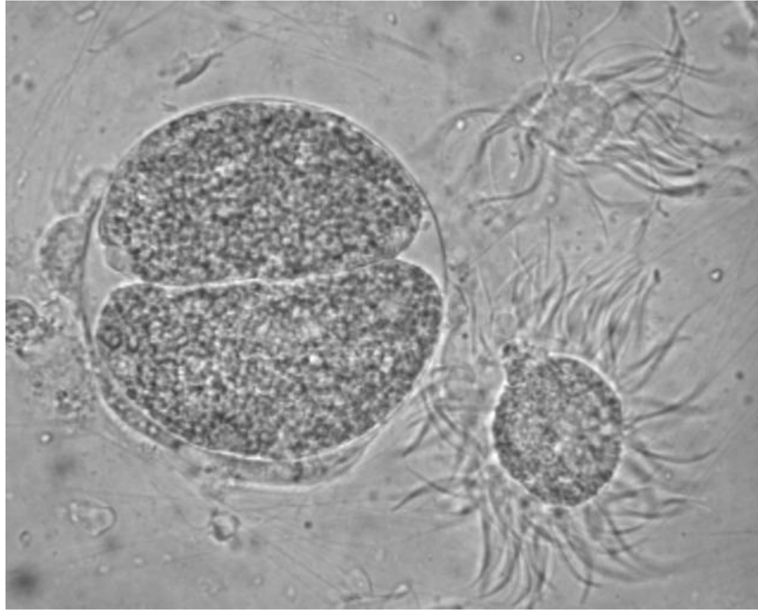
Life history of *Monocystis* parasites and genetic diversity of their hosts, the invasive *Amynthas* earthworms



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Honors Thesis

Defended April 27, 2016

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ABSTRACT

Monocystis is a genus of gregarine parasites in the phylum Apicomplexa; these parasites infect nearly 100% of earthworms sampled. Although the worms are heavily infected, the parasites have low survivorship, low production of transmissible gametocysts, and seemingly lack schizogony, the asexual stage typical for most apicomplexan parasites. The purpose of this study was to investigate the improbable lifecycle and life history traits of a Monocystis species of the invasive Asian earthworms, Amynthas agrestis and A. tokioensis at three sites in deciduous forests of northern Vermont: "Audubon", "Centennial Woods", and “Hort Farm".

Preliminary data suggested that Monocystis sp. vary in life history traits between three sites sampled; therefore, it was necessary to investigate its environment as a possible explanation for the variable life history traits; this meant investigating the parasite’s host, as the host is the parasite’s environment. The genetic variation of Amynthas spp. was studied as a possible influence as well as the mating system of Amynthas spp., because the mating system that an organism employs gives rise to the genetic variation of the species. Random Amplified Polymorphic DNA (RAPD) markers revealed genetic variation within and among sites of both Amynthas spp. which appear to employ a mixed-mating system of both sexual and asexual reproduction demonstrated by the presence of both clonal and unique genotypes.

The life history and life cycle of Monocystis sp. in A. agrestis was described by using classic microscopy techniques to measure phenology, parasite numbers, and relative proportions of parasite stages. The population in Audubon had a shorter season, measured in weeks from when earthworms first hatch to when the adults die in the fall, by two weeks; however, Audubon parasites were present earlier in the season and mean parasite numbers were higher in Audobon than in the other two population sites. The parasite success rate (proportion of individuals producing gametocysts) at Audubon was 113.8% compared to 73.6% at Hort Farm, and 0% at Centennial Woods. Regardless of survivorship, hosts sampled at Audubon had higher numbers of gametocyts overall but a lower mean number of sporocysts per gametocyst (127.0) than did hosts at the Hort Farm (145.8). These results suggest that Audubon has a higher rate of parasite development due to its host’s shorter season, and therefore, the parasite’s season, but overall less output of transmissible stages. Overall, it appears that Monocystis sp. at each site has different life history traits and schedules to compensate for the length of the season which can be a limiting factors in the number of transmissible stages of the parasite produced, and potentially for the genetic variation of its host.

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CHAPTER ONE

Introduction

At the center of ecological and evolutionary studies is the essential issue of life history which measures intrinsic and extrinsic factors that contribute to the variation of traits affecting the fitness of an organisms (Stearns 2000). Extrinsic factors include ecological impacts on survival and reproduction and intrinsic factors include the trade-offs among life history traits (Stearns 2000). Understanding the life history traits of a species provides insight into how the species has evolved over time and how it continues to be successful. For parasites, an integral part of their success is their transmission, the movement of a parasite from one host to the next (Burnet and White, 1972). Transmission depends on the sufficient output of an infective stage of the parasite into the environment or directly into the next host to establish a new infection.

The phylum Apicomplexa is a diverse phylum of parasites including well-known parasites such as the malaria parasites, *Plasmodium*. The life cycle begins with sporocysts which are transmitted into the new host via direct or indirect transmission. Inside the new host the parasite undergoes repeated asexual reproduction in a stage known as schizonts which allows for parasites to reach high densities very quickly because each schizont produces several to many daughter cells. The parasites then transition into their sexual phase of their lifecycle and sexually reproduce to produce transmissible sporocysts, which are then released into the environment or into a vector to continue the life cycle. One major clade of Apicomplexans, the gregarines (Fig. 1) generally do not include the asexual replication via schizonts in their life cycle.

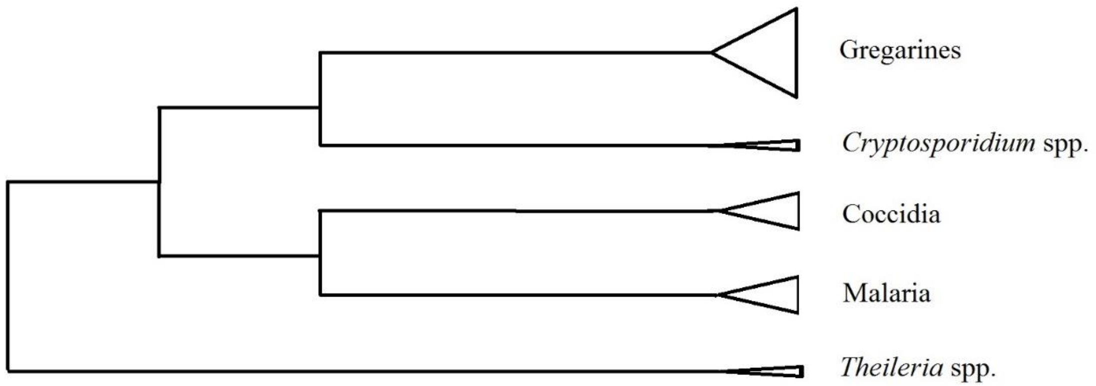


Figure 1. Phylogenetic hypothesis of the phylum Apicomplexa. Increased area at the end of a branch indicates higher diversity of species while smaller areas indicate lower diversity of species.

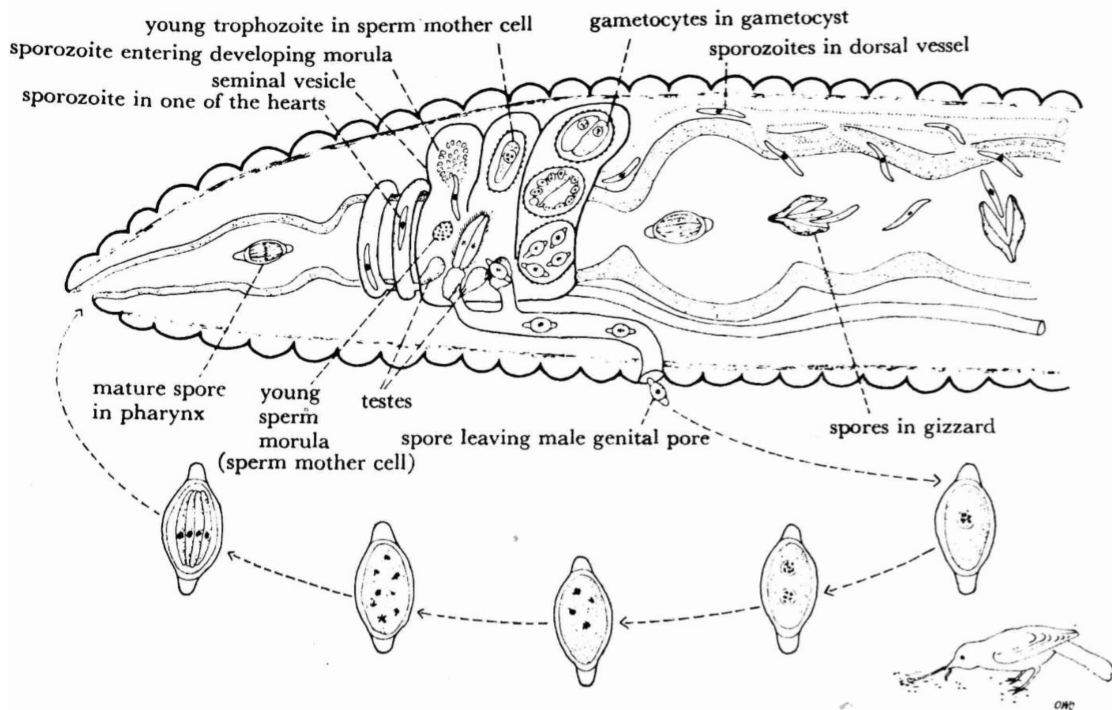


Figure 2. Graphical depiction of the life cycle of *Monocystis lumbrici* from Noble and Noble (1976) with an image of a bird consuming the *Lumbricus terrestris*, suggesting an indirect mode of transmission into a bird.

I studied the life history of a gregarine parasite within the Aplicomplexa phylum, *Monocystis* sp. *Monocystis* spp. are protist parasites that are extremely diverse, with over 6,000

of species described (Fig. 1) (Rueckert et al. 2010). I studied a *Monocystis* species of earthworms that infects nearly 100% of its hosts and is found wherever earthworms are present. The life cycle of *Monocystis* begins when parasites migrate from their unknown location of entry into the worm to the seminal vesicles of the earthworm where they feed as trophozoites and develop into their sexual stage. Mating parasites produce gametocysts containing sporocysts which are the infective stage of the parasite. Researchers in the past have suggested possible life cycles, such as a life cycle including an intermediate bird host (Fig. 2) (Noble and Noble, 1976); however, the actual life cycle of the parasite has yet to be confirmed. By an unknown mode of transmission, the sporocysts are able to infect new earthworms to maintain a nearly 100% prevalence and high density within each infected earthworm.

The parasite of interest in my thesis is an undescribed species of *Monocystis* that infects the annual earthworm, *Amynthas agrestis*. *Amynthas* spp. are invasive Asian earthworms that have been introduced into Vermont within the past few decades. The parasite has a perplexing life cycle and life history, as all sampled worms have high infections while the parasite has a low reproductive output of transmissible parasites. The combination of an unknown transmission mode, low reproductive output, and lack of schizogony makes the high prevalence of *Monocystis* infection perplexing. Furthermore, the parasite's host, *Amynthas*, is an annual species and, therefore, the parasite must also be on an annual life cycle in order to successfully infect new earthworms. The parasite must then be able to infect a new host, reproduce, and produce sufficient numbers of infective cysts in order to complete its life cycle.

Preliminary data collected at three different sites indicate that parasites within the different populations have different life history traits; therefore, in order to understand the variation in life history traits of the parasite, it was also necessary to study the genetic variation of its host at multiple sites. The original question concerning *Amynthas* spp. was how genetically

variable the earthworms are within a site and among sites. The presence of different genotypes at different sites may account for the substantial variability in life history traits of the parasites because they must adapt to their specific host. Looking at the genetic data of the earthworm sparked another question concerning the earthworm: what is the mating system of *Amynthas* spp? Morphological studies of *Amynthas* have suggested that they are parthenogenetic, but this is the first study to use molecular means to determine the mating system of *Amynthas* spp.

To determine the genetic variability and mating system of *Amynthas* spp., earthworms were collected over the course of Spring and Summer 2015. Random Amplified Polymorphic Data (RAPD) markers were used to establish a fingerprint of each earthworm sampled. These DNA fingerprints were then used to determine the genetic variability of earthworms within and among sites. These data were also used to determine whether or not the earthworms were parthenogenetic by looking at the number of clones and unique genotypes.

The life history and life cycle of *Monocystis* sp. was the other focus of this thesis. The main question that evolved into this thesis was how could a parasite with such a low reproductive output, an unknown mode of transmission, and lacks schizogony be so successful at infecting nearly all earthworms? To address this question, throughout the season of the earthworm, the infections of *Monocystis* sp. in *A. agrestis* were observed via microscopy. Absolute numbers, relative proportions, and descriptions of the parasite life stages were recorded to produce a timeline of infection and establish the survivorship and percent of parasites successful in reproduction. Using these data allowed for further insights into the life history and cycle of *Monocystis* and raise more questions into the mode of transmission and the possibility of the presence of unidentified schizogony in the parasite's life cycle.

In order to understand the life history of a parasite, it is necessary to study it alongside its host. In the case of the *Amynthas-Monocystis* system, both species contain mysteries in their life history traits and life cycle. This is the first study to examine the genetic variability and determine the mating system of *Amynthas* spp. Furthermore, this study adds to the body of research of the life history and cycle of *Monocystis* in an attempt to explain the impossible life cycle of the parasite. This research will also further our understanding of *Amynthas* and how it can be such a successful invasive across a broad range of habitats.

CHAPTER TWO

Genetic Diversity and Mating Systems of the Invasive Earthworms, *Amynthas agrestis* and *Amynthas tokioensis*

Introduction

The last major glaciation which ended approximately 12,000 years ago left central and northern North America without any native species of earthworms (Tiunov et al. 2006). For the 12,000 years without earthworms, the ecosystems of much of North America have missed the influence of earthworms until the early 17th century when European explorers began inadvertently introducing earthworms to North America by dumping their ship ballast weighed down with soil onto the shores of the east coast (Hendrix et al. 2002). Species such as the night crawler, *Lumbricus terrestris*, were introduced to northern North America centuries ago, whereas other species have only been introduced in the past few decades (Gates 1954).

Earthworms that are native in Europe are important because they act as ecosystem engineers that control resource availability by the ability to transform their physical environment, such as by churning soil, creating tunnel systems, and digesting plant litter (Jones et al. 1994). However, invasive non-native earthworms can substantially alter their environment in ways that contribute to loss of biodiversity and upset of ecosystems (Crooks 2002). Asian peregrine earthworms are of particular concern as they thrive on six of the seven continents and are spreading into areas previously devoid of recent invasive earthworms. The rapid anthropogenic introduction of these non-native earthworms into new habitats has caused the destruction of soil and forest communities (Hendrix et al. 2008).

The invasive Asian earthworms, *Amynthas* spp., commonly known as "jumping worms" were introduced into Vermont only a few decades ago and their impact on forest ecosystems has been substantial. These epi-endogeic earthworms, earthworms that reside in the surface layers of soil (Hendrix and Bohlen 2002), are known for devouring leaf litter and microflora and fauna

(Zhang et al 2010). The rapid depletion of leaf litter and accelerated decomposition can alter nutrient dynamics and soil structure, both of which have adverse effects on the ecosystem (Blouin et al. 2013). *Amynthas* also sequester high concentrations of mercury which leads to deposits high in mercury in the soil when the earthworms die and may harm birds who consume the earthworms (Richardson et al. 2015). In addition to the ability to consume large quantities of organic matter and sequester heavy metals, devastation of the forest floor is amplified due to the highly successful invasion of these earthworms. In just a matter of several years, a population of *Amynthas* can reach densities as high as 200 worms per square meter (J. Görres, personal communication) compared to densities of other earthworm such as the nightcrawler which only reach densities of 30 worms per square meter (personal observation). This high density is impressive because the worms are annuals in Vermont, and thus do not have overlapping generations; therefore, these worms must have a high reproductive rate. These worms also rapidly disperse to non-infested areas because of their ability to move quickly and tumble down slopes (Görres and Melnichuk 2012). The combination of their high reproductive rate and fast dispersal makes these earthworms highly successful invasive species.

What makes them even more successful as an invasive species is that some species of *Amynthas* may be parthenogenetic (below) and thus have the ability to start new populations with only one individual. At least 15 species of earthworms are known to be parthenogenetic and, therefore, have this dispersal advantage as it only takes one earthworm to begin a new population, or for a population to rebound after catastrophic decline (Terhivuo and Saura, 2006). Invasive species that are parthenogenetic also benefit from other aspects of parthenogenesis; for instance, parthenogenetic earthworms do not use valuable energetic resources finding a mate and copulating, are efficient at transferring resources to egg production, and two parthenogenetic

worms can produce one offspring each in the same time as it takes a mating pair to produce one offspring, effectively doubling their reproductive rate (Terhivuo and Saura, 2006).

Studies of the morphology of earthworms have hinted at parthenogenesis in one of the species of focus in this paper, *Amynthas agrestis*. While sex organs including the clitellum and female pores are always present, male pores are conspicuously absent in many, but not all, worms sampled (Reynolds, 1978). Furthermore, it has been recorded that spermatogenesis, the production of sperm, is terminated early on in the lifecycle of *A. agrestis* causing the male sex organs of the hermaphroditic earthworm to become sterile.

These morphological studies coupled with the rapid colonization and population growth of *A. agrestis* suggests that this species is parthenogenetic; however, there have been no previous studies looking at the genetic diversity and clonal structure of *Amynthas* spp. to substantiate these claims. This is the first study to evaluate the genetic diversity of *A. agrestis*, in order to determine its mating system. The genetic diversity of *Amynthas tokioensis*, a close relative of *A. agrestis*, was also evaluated because these worms cohabitate with populations of *A. agrestis* in Vermont forests and also reach high population density. Thus, *A. tokioensis* also may be parthenogenetic although there has been no morphological studies of this species to indicate parthenogenetic reproduction.

DNA fingerprints from Random Amplified Polymorphic DNA (RAPD) PCR were completed to describe and compare genotypes of *A. agrestis* and *A. tokioensis* at three different sites in Vermont. RAPD-PCR is a technique that has been used in the past with success to determine the genetic variability within and among invertebrate populations (Margonari et al., 2004). I will use these data to infer the likely reproductive system of *A. agrestis* and *A. tokioensis*. If these species are parthenogenetic as suggested by morphological studies, it is expected that there will be at least one clonal genotype, characterized by complete band sharing

at all loci for individuals in the population. If the species is newly parthenogenetic or there has been many introductions, I expect that there will be more genetic variation in the form of multiple clones (Jaenike and Solander 2015); also, if this is the case, I expect that there would be genetic variation between sites because the sites would conceivable have independent introductions by different clones. On the other hand, if the earthworms present in Vermont comes from a long-line of parthenogenetic species, then little genetic variation would be expected to be seen even over all the sites. A final possibility is that these worms are capable of both sexual and parthenogenetic reproduction which is supported by the presence of male pores and spermatogenesis in some individuals (Reynolds 1978). If either species employs a mixed-mating system, multiple clones would be present as well as many individuals with unique genotypes.

To determine the genetic diversity of *Amynthas* spp., RAPD-PCR will be used to determine the number of different genotypes, and possible clones, present and thus the number of introductions to the population. Genetic diversity between three sites in Vermont will also be addressed to determine whether they are independent populations or subpopulations. Finally, the genetic diversity provided by RAPD-PCR will be used to assign the most likely mating system that *Amynthas agrestis* and *A. tokioensis* employs.

Materials and Methods

Sampling and Sites

Three sites in Vermont were sampled during spring 2015. These sites are the University of Vermont Horticultural Research and Education Center, Centennial Woods Natural Area, and the Green Mountain Audubon Center (Figure 1). Permission to sample earthworms was granted at each of the three sites by the responsible authorities.

The University of Vermont Horticultural Research and Education Center (44.431489 ºN, -73.199211ºW) is a 97-acre active farm growing apples, rhododendrons, and other crops. Located at the far east end of the farm, hereby referred to as the "Hort Farm" or abbreviated as "HF", is a relatively flat deciduous forest. Collection of *Amynthas* spp. took place at the eastern-most part of the forest which backed up against residential fences. This area was at an elevation of 76 meters.

Centennial Woods Natural Area (44.475701ºN, -73.187088ºW) is a 70-acre forest containing hardwoods, confiders, wetlands, and streams and will be referred to as "Centennial Woods" and abbreviated as "CW". This natural area is open to the public and hosts frequent University of Vermont affiliated laboratory excursions along defined paths. Collection of *Amynthas* spp. took place alongside the main walking path in a mixed part of the forest containing both hardwood and coniferous trees. The elevation of the site is 80 meters.

The Green Mountain Audubon Center (44.346774ºN, -72.996216ºW) is a 250-acre outdoors educational and conservation center open to the public and contains forests, fields, and wetlands located in Huntington, Vermont and will hereby by referred to as "Audubon Center" or abbreviated as "AU". This site is the highest in elevation at 190 meters and the site itself is in a more mountainous area. The collection of *Amynthas* spp. took place alongside the main dirt road, Sherman Hollow Road, in a gully full of gravel and leaf debris.

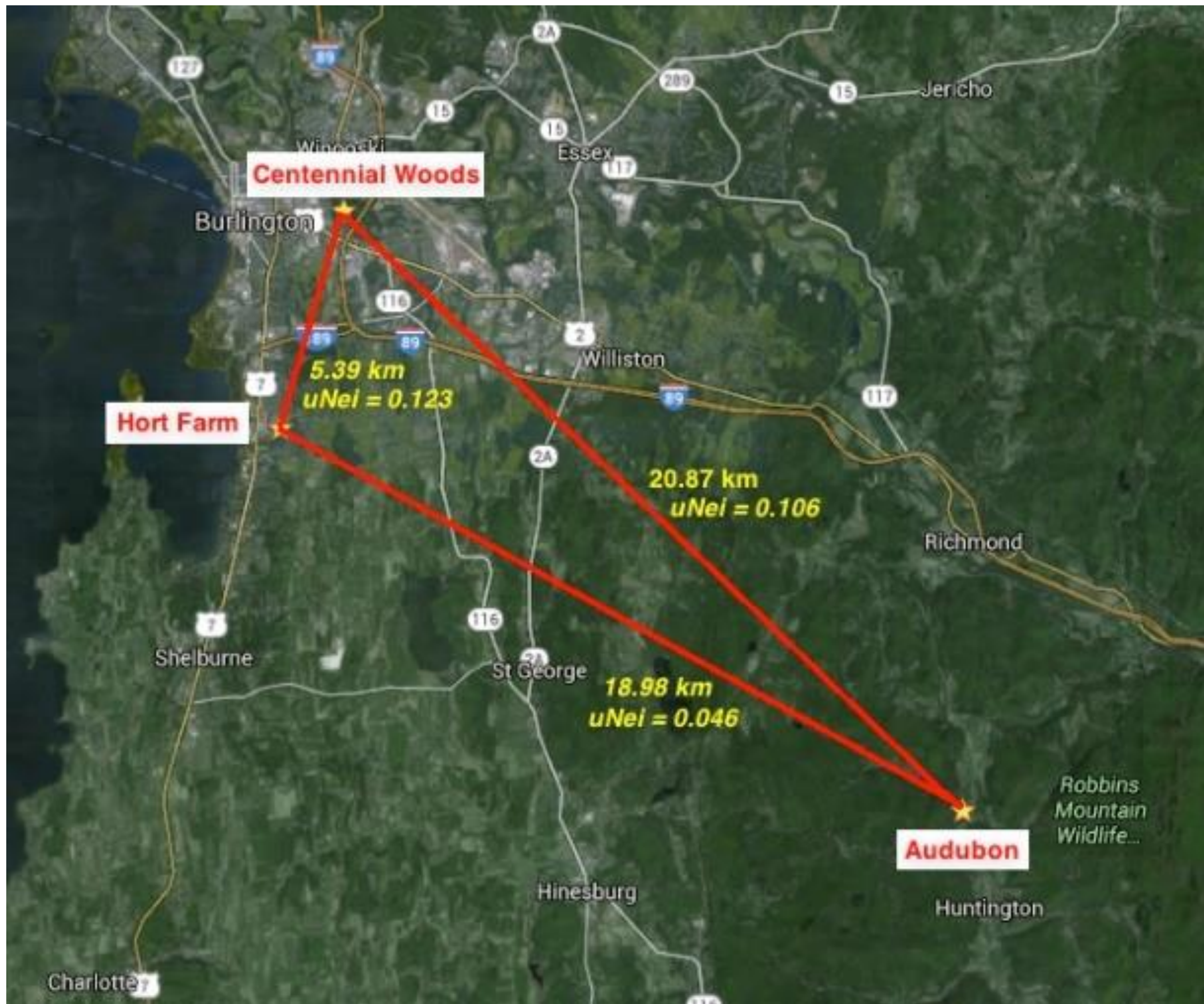


Figure 1. Satellite image showing the locations of the three study sites, Audubon Center, Hort Farm, and Centennial Woods. Distance (km) between each site is labeled as well as Nei's unbiased genetic distance for *Amynthas agretis* between sites.

Sampling of *Amynthas* spp. began on the week of May 7, 2015 when hatchling worms were first observed, and 30 worms were collected from each site on a weekly basis until June 24, 2015. *Amynthas* spp. were collected by manually sifting through the top five centimeters of soil and leaf debris, the A and O horizons. Sampling primarily took place over roughly five square meters at each of the sites. While sampling during the worms' early growth period, I could not readily identify the worms to species via morphology; therefore, there was no control on sample size and I needed to identify each worm using genetic methods (below).

DNA extraction

A total of 188 earthworms collected from all three sites between May 7, 2015 and June 24, 2015 were euthanized by submerging them in 50% ethanol for several minutes. Post-mortem length was recorded as well as the presence or absence of a clitellum. The first several anterior segments of each worm were cut off and used in the DNA extraction. The DNA extractions of the 188 samples were done using DNeasy ® Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

Species Identification

The species of each earthworm was identified by PCR using species-specific primers that resulted in different amplicon length products. First, the mitochondrially encoded cytochrome c oxidase I (Co1) gene for both *A. agrestis* and *A. tokioensis* was sequenced using primers conserved for animals and developed by Folmer et al. (1994). Species-specific primers were then developed by selecting an appropriate primer in common and two species-specific reverse primers from the sequences CoI gene that produced different amplicon lengths (primer in common: 5'-ataattggagcaggaataaga-3', *A. agrestis* primer: 5'-gccgaggacactaataaaat-3', *A. tokioensis* primer: 5'-cctgctaagtggagtgag-3'). PCR for each of the 188 worms was done using the three CoI primers in a single PCR reaction (1m @ 94, 32x, 30s @ 94, 30s @ 49, 1m @ 72, and a final extension of 2m @ 72). The PCR product was then run out on a 1% agarose gel and species were identified based on amplicon length, with bands at 387 base pairs indicating *A. tokioensis* and 268 base pairs indicating *A. agrestis*.

RAPD PCR and Band Scoring

Four RAPD primers originally developed by Operon Technologies Inc. (Alameda, CA, USA) were synthesized for use here by Invitrogen. PCR conditions were optimized by adjusting annealing temperature using a select panel of worms to produce the most reproducible and easily read bands. Three RAPD primers consistently produced easily read and reproducible bands and were used for this study (OPG 12: 5'-cagctcacga-3', OPP 04: 5'-gtgtctcagg-3', OPP 06: 5'-gtgggctgac-3'). PCRs using each RAPD primer was done for 179 earthworm samples using the thermal cycler program optimized for each primer; reactions were amplified through a PCR program comprised of 34 cycles with the following parameters: two minutes at 94ºC, one minute at 94ºC, seven minutes at the RAPD primer-specific annealing temperature, a final extension at 72ºC for two minutes, and final hold at 72ºC for ten minutes. The optimized annealing temperature used for OPG 12, OPP 04, and OPP 06 are 34.3ºC, 34.3ºC, and 41.5ºC, respectively.

All PCR products were run on a 1% agarose gel at 100V for 30 minutes; a 100 base pair ladder was included on each gel, in the first and last lanes, and pictures were taken of each gel (see Figure 2 for example of gel). A total of 564 gel lanes were examined (188 worms x 3 primers) to determine the total number of bands ever observed (15). The predetermined bands were selected by observing positions of bands from each primer and marking every position where a band was observed. Band scoring of the gels was then done by marking whether those 15 predetermined bands were present or absent for each sample and each primer. Thus, a total of 45 RAPD markers, regarded as 45 loci with two alleles that were characterized by the presence or absence of a band, were scored. RAPD markers are considered to be dominant; therefore, the presence of a band is a due to the worm being homozygous or heterozygous for the two alleles (Williams et al. 1990). The results of the band scoring were then converted into binary; a "1" indicated that a band was present at a specific locus while a "0" indicated that a band was absent.

Statistical Analyses

The binary data for the RAPD study were analyzed using the GenAlEx package (Peakall and Smouse, 2006). Genetic distance based on number of band differences was calculated for each pairwise comparison of individuals for each species at each site. The number of haplotypes for each site was determined by excluding individuals with zero band differences with another individual and summing the remaining unique haplotypes. Unique haplotypes from each site were then used to calculate Nei's unbiased genetic distance and identity (Nei, 1987) for each pairwise comparison of sites.

An analysis of molecular variance in GenAlEx was calculated for each species across all populations to determine the percentage of the total genetic variation was accounted for by within population and among population genetic variation. In addition, band frequencies, number of effective alleles, and Shannon's Information Index (I) were calculated for each population and species.

Results

Due to having to randomly sampled earthworms in regard to species, sample sizes varied for sites and species (Table 1). There were no individuals of *A. tokioensis* collected at Centennial Woods because the species was not found until later in the season after the sampling period had concluded.

Pairwise comparisons of individuals of the same species in a population yielded histograms of frequency of band differences (Figure 3). If two individuals shared the same banding pattern for all 45 loci, and thus were genetically identical for the markers, they would be scored as zero differences. Genetically identical individuals, also referred to as clones, were

common at each site. The pairwise comparison of band sharing between individuals calculated that 21/33 (63.6%) *A. tokioensis* at Hort Farm had at least one clone, and none of the eight *A. tokioensis* at Audubon had one clone. For *A. agrestis*, 4/14 (28.6%) had at least one clone at Hort Farm, 6/43 (13.9%) worms had at least one clone at Audubon, and 24/48 (50%) worms had at least one clone at Centennial Woods. I expect that scoring errors for one or two loci would lead to apparent differences for some genetically identical worms. Thus, inspection of the figures suggests that the number of genetically identical worms is substantially higher than these values, however, using the criterion of no band differences reveals identical groups of 2-9 worms (Table 2). No identical worms were seen across sites.

The high number of apparent clones, especially at Hort Farm where 63.64% of *A. tokioensis* were clones, and at Centennial Woods where 50% of *A. agrestis* were clones, is significantly greater than the number of individuals sharing an identical genotype with a population with a sexual mating system. The likelihood of finding two full sibs with identical genotypes can be estimated using the equation: $p = (0.25)^n$, where n is the number of loci being compared and 0.25 coming from the probability of sharing the same genotype at one locus given two heterozygous parents. Across 45 loci as used in this study, the likelihood of finding two full sibs with the same genotype is $8x10^{-28}$). At Hort Farm, there was not one pair of clones, but in fact 63.64% of individuals had at least one clone.

Table 1 presents various measures of genetic diversity. Percent of polymorphic bands, mean number of alleles, and mean effective number of alleles all reveal that genetic variation was present for all markers, and thus the RAPD primers used provided useful data for the study. The Shannon diversity measure showed similar values for all sites and species except for *A. agrestis* at Audubon which had a significantly higher measure of diversity. This result coincides with the lower proportion of clonal pairs of *A. agrestis* at that site.

Nei's unbiased genetic distance (Tajima and Nei, 1984) was calculated for each pairwise comparison of populations to determine the overall population structure of both worm species; specifically, to determine if the worms represent one population with a single source introduction or multiple introductions. Fig. 1 presents a map showing the results as well as Tables 3 and 4.

Table 1. Shannon's Information Index (I) and its standard error, the percentage of polymorphic bands, the mean number of alleles and number of effective alleles, and the unbiased heterozygosity for each site and each species calculated using GenAlEx.

Species	Site	Sample Size (N)	Shannon's Information Index (I)	-/+ 1 S.E. of I	Polymorphic Bands (%)	Mean Number of Alleles	Mean Effective Number of Alleles	Unbiased Heterozygosity
Amynthas agrestis	**Centennial Woods**	48	0.320	0.28-0.36	68.89%	1.467	1.335	0.212
	Hort Farm	14	0.379	0.341 - 0.417	77.78%	1.556	1.411	0.258
	Audubon	43	0.411	0.376 - 0.446	86.67%	1.733	1.445	0.272
Amynthas tokioensis	**Hort Farm**	33	0.304	0.260 - 0.348	62.22%	1.244	1.362	0.207
	Audubon	8	0.307	0.270 - 0.344	71.11%	1.444	1.309	0.207

Table 2. The number of groups of clones at each site for each species, where doublet refers to two clonal individuals and triplet refers to three clonal individuals and so on.

Species	Site	Doublet	Triplet	Quadruplet	Quintuplet	Sextuplet	Heptuplet	Octuplet	Nontuplet
A. agrestis	Centennial Woods	3	1	1	1	1	0	0	0
	Audubon	3	0	0	0	0	0	0	0
	Hort Farm	2	0	0	0	0	0	0	0
A. tokioensis	Audubon	0	0	0	0	0	0	0	0
	Hort Farm	3	2	0	0	0	0	0	1

Table 3. Nei's unbiased genetic distance between for *Amynthas agrestis* at each site is located beneath the diagonal while Nei's unbiased genetic identity for *Amynthas agrestis* between each site is located above the diagonal.

Site	Audubon	Centennial Woods	Hort Farm
Audubon	**---**	0.900	0.955
Centennial Woods	0.106	**---**	0.885
Hort Farm	0.046	0.123	**---**

Table 4. Nei's unbiased genetic distance between for *Amynthas tokioensis* at each site is located beneath the diagonal while Nei's unbiased genetic identity for *Amynthas tokioensis* between each site is located above the diagonal.

Site	Audubon	Hort Farm
Audubon	**---**	0.912
Hort Farm	0.092	**---**

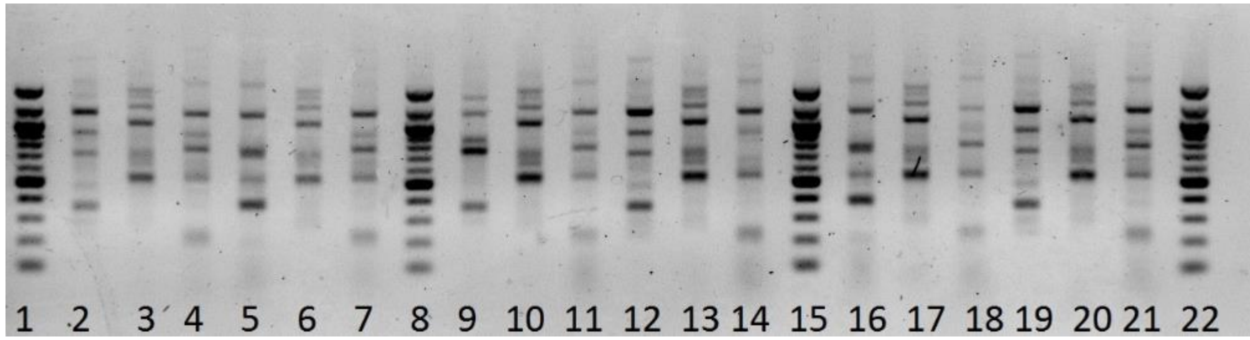
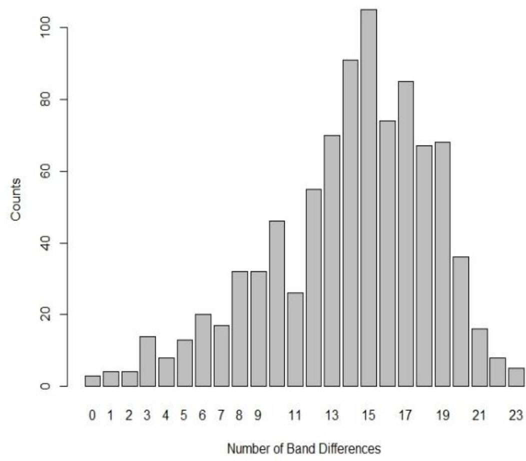


Figure 2. Example of RAPD primer OPG-12 ran out on 1% agarose gel using 3μL of DNA and running at 100V for 30 minutes. Lanes 1, 8, 15, and 22 are 100 base pair ladder, lanes 2-7 are Audubon worms, followed by Centennial Woods worms in lanes 9-14, and Hort Farm worms in lanes 16-21.



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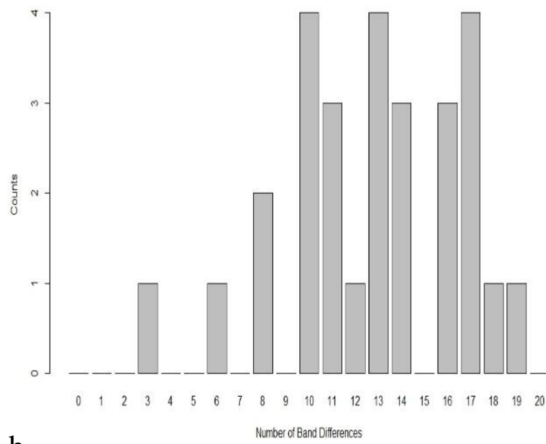

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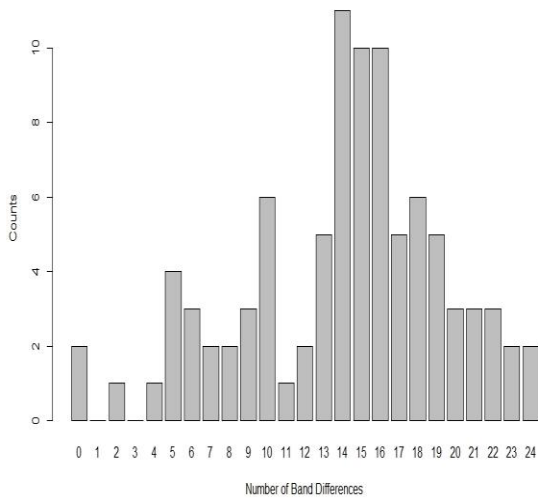

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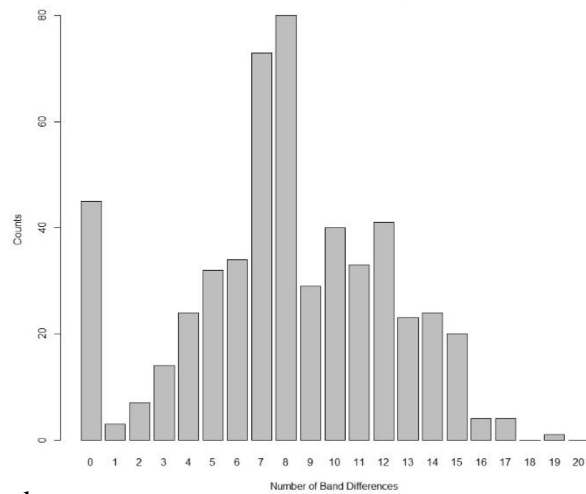

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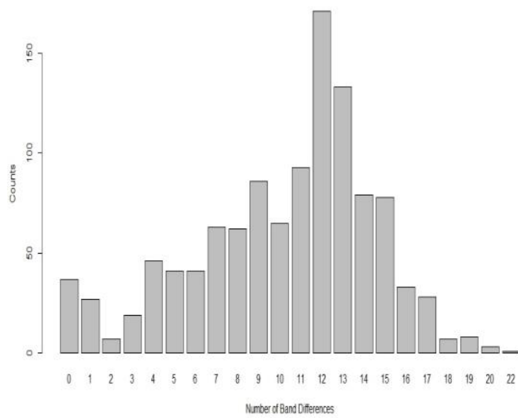


e.

Figure 3. Number of pairwise band differences between (a) *A. agrestis* at Audubon; (b) *A. tokioensis* at Audubon; (c) *A. agrestis* at Hort Farm; (d) *A. tokioensis* at Hort Farm; (e) A. *agrestis* at Centennial Woods.

Discussion

Morphological studies of *Amynthas* spp. suggest that *Amynthas* may reproduce parthenogenetically yet not all worms lack a complete male reproductive system. The purpose of this study was to determine whether clones were present for *A. agrestis* and *A. tokioensis* at three different sites and to use these data to infer a reproductive system. Finally, using the data from this study, indications as to what life history traits of these earthworms make them such successful invasive species was addressed.

The pairwise comparisons of individuals of the same species within each population yielded multiple pairs or groups of individuals (up to nine) sharing the same genotype across the 45 loci used in this RAPD study. The far higher prevalence of clones at each site than predicted for a sexual mating system suggests that parthenogenesis is part of the reproductive system of *A. tokioensis* and *A. agrestis*. The likelihood of two individuals having the same 45 locus genotype is very low in a sexually reproducing population ($8x10^{-28}$), so even if there were a sexually reproducing population of *Amynthas* with a high degree of inbreeding, the occurrence of so many identical individuals seems highly unlikely.

Despite the clones found at each site and in each species, there is still high genetic variation between clones and among individuals with unique genotypes. This high genetic variation, with upwards of 20 band differences between clones indicates that multiple genetically-distinct individuals originally underwent the switch to parthenogenesis to produce multiple clones. What is more perplexing is the high genetic variation between individuals with unique genotypes at each site; these individuals have many band differences between other individuals in the sample which suggests that they were the product of sexual recombination. If there are individuals with unique genotypes as well as a significant portion of the sample with two or more individuals sharing a common genotype, are *Amynthas tokioensis* and *Amynthas agrestis* truly parthenogenetic or sexual?

It is possible that both *Amynthas tokioensis* and *Amynthas agrestis* are capable of both sexual and parthenogenetic reproduction, as suggested by the presence of both clones and individuals with unique genotypes in the sample. Mating systems with both sexual and parthenogenetic reproductions is not uncommon in nature, with many species of aphids (Lushai et al. 1997) and *Drosophila* spp. (Chia-Chen et al. 2014) exhibiting both mating systems. In fact, invasive species biologists have suggested that species employing both sexual and parthenogenetic reproduction are the most successful invasive species (Shirk and Hamrick, 2014). When conditions are stable and mates are limited, species of a mixed mating system can have reproductive assurance by being parthenogenetic, as well as use facultative outcrossing to produce genetic variability in variable environments or when mates are common (Shirk and Hamrick, 2014).

The ability of these *Amynthas* spp. to generate variable genotypes as the product of sexual reproduction may have facilitated their establishment in temperate northern North America, which is considerably different from their native land of subtropical Asia (Hendrix et

al. 2006). Once populations of *Amynthas* spp. have been established, the earthworms may be able to increase their fitness by changing mating systems from inefficient and costly sexual reproduction to the efficient and prolific parthenogenetic mating system (Uyenoyama 1985). Not only does parthenogenesis produce more offspring, but it also has the advantage of increasing parent-offspring relatedness more so than in sexual mating systems (Unenoyama 1985). Thus, a mixed mating system potentially demonstrated in *Amynthas tokioensis* and *Amynthas agrestis* enables the earthworms to be successful invasive species in disturbed habitats.

An alternate hypothesis that would explain the presence of clones and individuals with seemingly unique genotypes has been proposed by Jaenike et al. (2014). In a similar study on the mating system of *Octolasion tyrtaeum,* Jaenike et al. found two common clones and several more uncommon clones seemingly coexisting. According to competitive exclusion, two species occupying the same niche likely arrived through drift and will be competing and one species will remain. Jaenike et al. suggest that the presence of multiple clones, including rare clones, is a transient event and a "general purpose" clone will eventually exist by itself.

I conclude that the high incidence of clones in each population of *A. tokioensis* and *A. agrestis,* including many individuals with unique genotypes suggest that *Amynthas* spp. found in Vermont are capable of both sexual and asexual reproduction. This mixed mating system may confer an advantage to these species as invasive species as they have the genetic variation to survive in variable and disturbed habitats while being able to proliferate quickly. Alternate hypothesis, such as that proposed by Jaenike et al. (2014) suggest that the individuals with unique genotypes may be rare clones; however, the high number of unique genotypes in less than 200 worms suggest that they are the product of sexual reproduction. In order to determine the exact mating system of *Amynthas* spp., which can give insight into the success of these species as invasive species, more research needs to be done. I suggest using molecular techniques, such as

microsatellite analysis, to reveal more information on the mating system and population makeup of the two species of *Amynthas*.

Literature Cited

Blouin, M., Hodson, M.E., Delgado, E.A., Baker, G., Brussaard, L., Butt, K.R., Dai, J., Dendooven, L., Peres, G., Tondoh, J.E., Cluzeau, D., and Brun, J.J. (2013). A review of earthworm impact on soil function and ecosystem services. *Eur. J. Soil Sci,* 64, 161–182. doi:10.1111/ejss.12025.

Chang-Chen, Ting C-T, Chang C-H, Fang S, Chang H-y (2014). The Persistence of Facultative Parthenogenesis in *Drosophila albomicans*. *PLoS ONE,* 9(11). doi: 10.1371/journal.pone.0113275.

Crooks JA. (2002). Characterizing ecosystem-level consequences of biological invasions: the role of ecosystem engineers. *Oikos*, 297(2),153-166. doi:10.1034/j.1600-0706.2002.970201.x.

Gates, G.E. (1954). Exotic earthworms in the United States. *Bull. Mus. Comp. Zool.*, Harvard 111, 219-258.

Görres, J.H., and Melnichuk, R.D.S. (2012). Asian invasive earthworms of the genus Amynthas Kinberg in Vermont. *Northeast. Nat*. 19, 313–322. doi:10.1656/045. 019.0212.

Hendrix PF, Baker GH, Callaham M a., et al. (2006). Invasion of exotic earthworms into ecosystems inhabited by native earthworms. *Biol Invasions*, 8, 87-100. doi:10.1007/978-1-4020-5429-7_9.

Hendrix, P.F., and Bohlen, P.J. (2002). Exotic earthworm invasions in North America: ecological and policy implications. *Bioscience*, 52, 801–811. doi:10.1641/ 0006-3568(2002)052[0801:EEIINA]2.0.CO;2.

Hendrix, P.F., Callaham, M.A., Jr., Drake, J.M., Huang, C.-Y., James, S.W., Snyder, B.A., and Zhang, W. (2008). Pandora's box contained bait: the global problem of introduced earthworms. *Annual Rev. Ecol. Evol. Syst*. 39, 593–613. doi:10.1146/annurev.ecolsys.39.110707.173426.

Jaenike J, Selander RK, Jaenike J. (2015). Evolution and Ecology of Parthenogenesis in Earthworms. *JSTOR*, 19(3),729-737.

Jones, C. G., Lawton, J. H., & Shachak, M. (1994) Organisms as Ecosystem Engineers. *Oikos*, *69*(3), 373–386. http://doi.org/10.2307/3545850

Lushai G, Loxdale HD, Brookes CP, von Mende N, Harrington R, Hardie J. (1997).Genotypic variation among different phenotypes within aphid clones. *Proc Biol Sci*., 264(1382), 725-730. doi:10.1098/rspb.1997.0103.

Margonari CS, Fortes-Dias CL, Dias ES. (2004). Genetic variability in geographical populations of Lutzomyia whitmani elucidated by RAPD-PCR. *J Med Entomol*., 41(2),187-192. doi:10.1603/0022-2585-41.2.187.

PEAKALL, R., AND P. E. SMOUSE. (2006). GENALEX6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes*, 6, 288-295

Reynolds, JW. (1978).The earthworms of Tennessee (Oligochaeta). IV. Megascolecidae, with notes on distribution, biology, and a key to the species in the state. *Megadrilogica*, 3(7), 117-129.

Richardson JB, Görres JH, Jackson BP, Friedland A. J. (2015). Trace metals and metalloids in forest soils and exotic earthworms in northern New England, USA. *Soil Biol Biochem*., 85, 190-198. doi:10.1016/j.soilbio.2015.03.001.

Shirk RY, Hamrick JL. (2014). High but variable outcrossing rates in the invasive Geranium carolinianum (Geraniaceae). *Am J Bot*. 101(7),1200-1206. doi:10.3732/ajb.1400224.

Tajima F, Nei M. (1084). Estimation of evolutionary distance between nucleotide sequences. *Mol Biol Evol*., 1(3), 269-285.

Terhivuo J, Saura A. (2006).Dispersal and clonal diversity of north-european parthenogenetic earthworms. *Biol Invasions,*8, 5-18. doi:10.1007/978-1-4020-5429-7_2.

Tiunov, A.V., Hale, C.M., Holdsworth, A.R., and Vsevolodova-Perel, T.S. (2006). Invasion patterns of Lumbricidae into previously earthworm-free areas of northeastern Europe and the western Great Lakes region of North America. *Biol. Invasions*, 8(6), 1223–1234. doi:10.1007/s10530-006-9018-4.

Uyenoyama M. On the evolution of parthenogenesis. (1985). II. Inbreeding and the cost of meiosis. *Evolution,* 39(6),1194-1206.

Williams JG, Kubelik a R, Livak KJ, Rafalski J a, Tingey S V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*.,18(22), 6531-6535. doi:10.1093/nar/18.22.6531.

Zhang, W., Hendrix, P.F., Snyder, B.A., Molina, M., Li, J., Rao, X., Siemann, E., and Fu, S. (2010). Dietary flexibility aids Asian earthworm invasion in North American forests. *Ecology*, 91, 2070–2079. doi:10.1890/09-0979.1. PMID:20715629.

Tiunov, A.V., Hale, C.M., Holdsworth, A.R., and Vsevolodova-Perel, T.S. (2006). Invasion patterns of Lumbricidae into previously earthworm-free areas of northeastern Europe and the western Great Lakes region of North America. *Biol. Invasions*, 8: 1223–1234. doi:10.1007/s10530-006-9018-4.

Uyenoyama M. (1985). On the evolution of parthenogenesis. II. Inbreeding and the cost of meiosis. *Evolution,* 39(6), 1194-1206.

Zhang, W., Hendrix, P.F., Snyder, B.A., Molina, M., Li, J., Rao, X., Siemann, E., and Fu, S. (2010). Dietary flexibility aids Asian earthworm invasion in North American forests. *Ecology*, 91, 2070–2079. doi:10.1890/09-0979.1. PMID:20715629.

CHAPTER THREE

The Life History of *Monocystis sp.* in the Invasive Asian Earthworm, *Amynthas agrestis*.

Introduction

At the center of the study of evolutionary ecology is life history theory (Stearns 2000). Life history theory attempts to explain the variation of traits that contribute to the fitness of an organism and states that life history traits are the product of intrinsic and extrinsic factors, the extrinsic factors are ecological impacts on survival and reproduction, and intrinsic factors include the trade-offs among life history traits (Stearns 2000). Life history traits include the assimilation of resources towards growth and reproduction, as well as the schedule of developmental and reproductive events (Eisen and Schall 2000). Understanding life history traits of a species provides insight into how evolution has shaped traits of a species which result in the highest fitness possible for individuals and as well as the complex strategies employed by species. Life history studies have primarily focused on free-living multicellular organisms; however, life history theory can be applied as well to single-celled parasites to further understand their infection biology and overall ecology (Eisen and Schall 2000). Some important traits to consider when studying the life history of single-celled parasites include rate of growth during asexual reproduction, peak density, and first production of mature cells (Eisen and Schall 2000).

The phylum Apicomplexa is a large and diverse group including malaria parasites and coccidians. For the purposes of life history studies, the phylum's great number of species and their complex life cycles provides excellent models to apply life history thinking to protists. One interesting group is the Eugregarinida subclass which is one of the most diverse groups in the phylum Apicomplexa with about 17,000 described species within about 244 genera (Clopton 2000). The gregarine family Monocystidae itself includes about 27 genera with more than 200 species.

Two genera of Monocystid gregarines, *Monocystis* and *Apolocystis*, are found worldwide wherever earthworms have been sampled. The primary literature on these two genera is an unpublished dissertation by Segun (1971) which focused on species found in England. Rees (1962) also reviews old literature concerning *Monocystis* but notes that it is difficult to diagnose these parasites because, “The morphology of monocystid gregarines is comparatively simple and there are very few features of the trophozoites which can be used as taxonomic characters.” In addition, the typical characters used for classifying these parasites are common throughout all gregarines. While others have researched and described *Monocystis* spp. (See Levine 1977, von Stein 1848, and Berlin 1924), there is no molecular data to work out the true relationships of the monocystid genera.

Beyond the limited and vague descriptions of species within the genus *Monocystis*, what is even more perplexing is the life cycle that has been worked out for species in this genus. All Monocystids occupy a single host via direct transmission (Levine 1977). Like all other Apicomplexa parasites, all stages are haploid except for the ephemeral zygote. The parasite enters its earthworm host by being ingested as a sporocyst which contains eight sporozoites. The sporozoites emerge and travel to the seminal vesicles of the earthworm and then develop into the feeding stage of the parasite called trophozoites. The trophozoite feeds on the sperm mother cells in the seminal vesicles until it can develop into a mature gamont. The gamont can then fuse with another gamont during syzgy and form a gametocyst. Each gamont produces gametes which mate with gametes from the other gamont and form a zygote. Finally, the zygote develops into a sporocyst containing eight haploid sporozoites which are thought to exit the worm into the environment as the transmissible stage. The sporocysts are unusually tough for protist cells which allows them to survive harsh environments outside of the worm. It is thought that the

sporocysts pass through the feces but there has been no conclusive study to identify the actual means of transmission (Field and Michiels 2006).

While the aforementioned aspects of the *Monocystis* life cycle matches other Apicomplexan parasites, the perplexing feature of this parasite is its seeming lack of schizogony. Schizogony is the asexual reproduction phase present in Apicomplexan parasites except for the gregarines. Asexual schizonts allow for infections to reach high densities in a short period of time because each schizont produces several to many daughter cells. The apparent lack of schizogony in these parasites make their naturally high prevalence and density within hosts mysterious. Fields and Michiels (2006) conducted a controlled study of the transmission of parasites by feeding *Lumbricus terrestris* with 20,000 sporocysts. With the lack of an asexual stage, the 20,000 sporocysts containing eight sporozoites could produce 160,000 parasites yielding 80,000 mating pairs as two gamonts must come together for sexual reproduction. If each mating pair produced around 200 sporocysts, the total output of sporocysts would be 16,000,000 sporocysts in a single worm; however, Fields and Michiels found only between 38,000 and 589,000 sporocysts per worm giving a survival rate of the parasite of only 0.2-3.7%; wild worms had infections with upwards of 29,100,000 sporocysts. This would mean that wild worms would have to eat 91,000,000 sporocysts to reach that high infection rate with a survival rate of 3.7%!

The enormous numbers of sporocysts that would have to be consumed if the parasite is transmitted via the fecal-oral route makes this scenario problematic. Thus, the apparent lack of schizogony and the high mortality rate of the parasite lead to important questions on the actual life cycle and life history of *Monocystis* spp. This study seeks to investigate a species of *Monocystis* found in the invasive Asian earthworm, *Amynthas agrestis*, in Vermont. This worm is an annual in Vermont, with its life cycle completed from late April to early November (Görres et al., 2016), and thus the parasite must also have an annual cycle. Using classical parasitology

techniques with a modern ecological lens, the life stages of this species will be examined and described. Furthermore, the relative proportions and counts of each stage will be recorded including the dates that different stages appear in the life of the annual earthworm host. The description of life stages and the analysis of the abundances of parasites over the season will be used to construct the life history of this parasite which can be used to understand how the parasite is almost universally prevalent in earthworms and how it is different from other Apicomplexan species.

The purpose of this study is to describe the life history of the perplexing parasite, *Monocystis* sp. in *A. agrestis.* The timeline of the infection over the course of the parasite and earthworms' season was established by sampling infections on a weekly basis. Total numbers and relative proportions of the life stages of the parasite were recorded each week. This information will be used to construct a life table which will then be used to infer survivorship and total number of infective sporocysts, which can infect new earthworms, at the end of the season. Since *A. agrestis* is an annual earthworm, I expect that the parasite must also be an annual species and produce the highest amount of infective gametocysts at the end of the season in November. For the parasite to reach as high numbers as has been recorded (Fields and Michiels, 2006), I expect that the gametocysts will contain hundreds of infective sporocysts. Finally, as two gamonts must mate to create a gametocyst, I expect there to be at least twice the amount of asexuals as there are gametocysts.

Materials and Methods

Sampling of Amynthas agrestis

Three sites in Vermont were sampled over Spring and Summer 2015, the University of Vermont Horticultural Research and Education Center (Hort Farm, or HF), Centennial Woods

Natural Area (CW), and the Green Mountain Audubon Center (AU). Details on the location and natural history of these sites has been given in Chapter 1, and Fig. 1 in that chapter. These three sites were sampled for *Amynthas* on a weekly basis starting for the week of April 5, 2015. Two species of *Amynthas* were present at each site*, A. agrestis* (the target species) and *A. tokioensis,* a related species of earthworm with its own *Monocystis* species, so care was taken to include only the target host and parasite in the study. Each week, at least 30 *A. agrestis* were sampled by scratching around the first 5cm of leaf litter and soil (the O and A horizons). The collected earthworms were stored in a 16° C incubator in containers with soil and leaf litter from their site of origin.

Dissection of Amynthas spp. *and extraction of seminal vesicles*

Ten earthworms from each sample were dissected within hours to a week of collection. To euthanize the earthworms, they were submerged in 50% ethanol for several minutes. Using dissection scissors, the underside of the anterior end of the earthworms were carefully bisected so that all of the seminal vesicles could be extracted using forceps.

The seminal vesicles were then placed in a clean finger bowl and homogenized using forceps. A known amount of saline was added to the seminal vesicles and mixed. The volume of the homogenized solution was then determined by sucking up the solution into a pipette. The amount of saline added was subtracted from the total volume of the homogenized solution to yield the volume of the seminal vesicles and this volume was recorded.

Counting and identifying parasite stages

One microliter of the homogenized seminal vesicle solution was pipetted onto a microscope slide with a cover slip. Using a light microscope (400 x), the parasites were located

and each stage in the parasite's life cycle was counted. Morphological features including size and shape of each parasite stage was observed and recorded using the Moticam 1000 1.3MP Live Resolution (Richmond, British Columbia) microscope camera and Motic Image Plus 2.0.11 computer program. Relative proportions of each stage was determined by dividing the abundance of the stage of interest by the total number of parasites counted. The total parasite load in the 1µL sample was converted into the total parasite load in the earthworm by multiplying the total number of parasites by the total volume of the seminal vesicle.

At least 30 pictures of each stage of parasite were taken using a microscope camera. For determining the number of sporocysts per gametocysts, 30 photographs were taken of whole gametocysts in ten worms from both Audubon and Hort Farm for a total of 600 gametocysts. For each of the 600 gametocysts, the total number of sporocysts present in the gametocyst was counted and recorded.

Statistical analysis

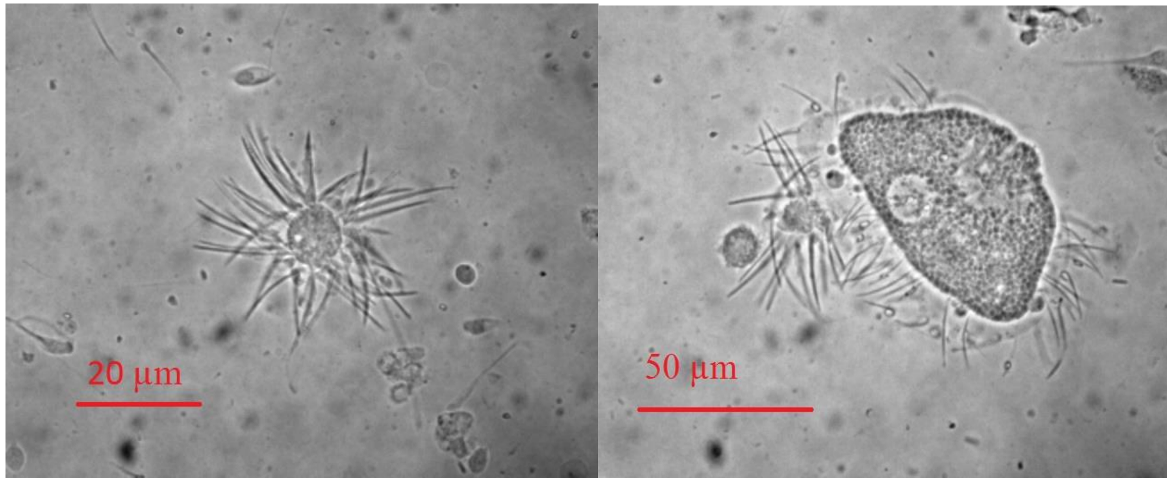
A nested AMOVA of the total sporocyst counts per gametocyst of both Audubon and Hort Farm was done using JMP Pro 12. The histogram of sporocyst counts for both Audubon and Hort Farm was also graphed using JMP. Finally, the mean abundance of each stage of parasite and the date on which it was first observed was graphed for each site to depict the life history and cycle of *Monocystis.*

A life (L_X) table for the parasite was determined by dividing the mean abundance of the parasite stage of interest by the mean abundance of the preceding stage to determine the probability of survival between parasite stages. The mean total number of transmissible parasite stages, sporocysts, was calculated by multiplying the mean number of gametocysts in an earthworm by the mean number of sporocysts per gametocysts determined above.

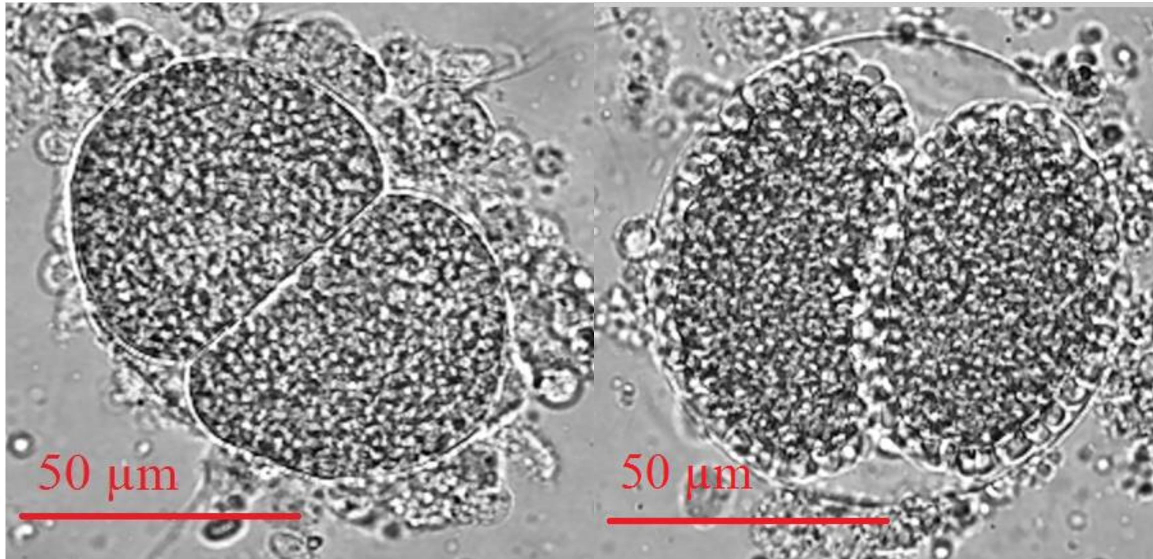
Results

Parasite morphology and life stages

Life cycle stages counted in the study are shown in Fig. 1. Immature trophozoites are circular with sperm flagella often attached to the cell membrane and have a diameter of approximately 10µm. (Fig. 1a). The trophozoites feed on the worm's sperm cells, and thus the sperm flagella cover the cell. Mature trophozoites then develop to be larger, with a length of approximately 50µm, and often circular or oblong (Fig. 1b). These trophozoites then develop into gamonts that mate with another gamont to form a mating pair (Fig. 1c). The mating pair develop a surrounding wall and produce gametes which then combine to form zygotes (Fig. 1d). Immature gametocysts are formed as the zygotes develop into sporocysts and feed off of material in the gametocyst (Fig.1e). Lastly, once all of the sporocysts are formed, the circular mature gametocyst contains upwards of 200 sporocysts and measures approximately 100µm in diameter (Fig. 1f). After observing the parasites in *Amynthas* spp. of Vermont and comparing their morphology to other described species of *Monocystis*, it is clear that this is a unique and undescribed species of parasite (Rees 1962, Segun 1971, Cognetti de Martiis 1923).



a. **b.**



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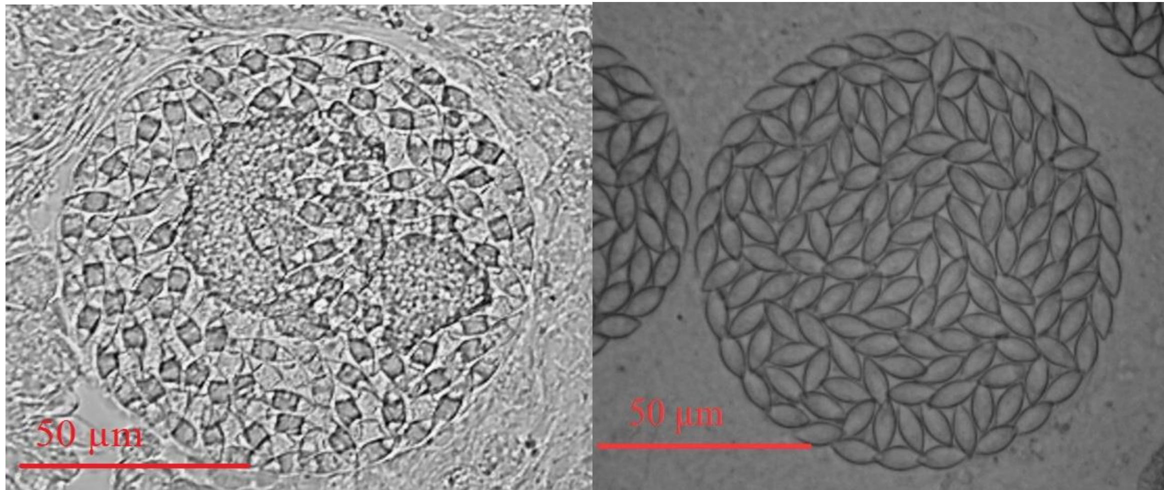


e. **f.**

Figure 1. a) Immature trophozoite with sperm flagella surrounding the parasite b) Mature trophozoite with surrounding sperm flagella and an immature trophozoite located next to the mature trophozoite c) Two gamonts forming a mating pair d) two gamonts forming gametes in a mating pair e) Immature gametocyst with developing sporocysts f) Mature gametocyst with fully formed sporocysts.

Worm and parasite phenology

Amynthas spp. hatch from their cocoons as early as April; the hatchlings were found even under the late melting snow. First appearance of hatchlings was recorded as Week 1. The worms grow rapidly and adult worms (defined as those with a clitellum) were seen starting on Week 17. At Audubon and Centennial Woods, asexual stages of *Monocystis* are present beginning 17 weeks after *Amynthas* hatchlings appear. Parasites were not observed in Hort Farm worms until one week later at 18 weeks after worms hatch. The broad schedule of parasite development is shown in Fig. 2. Presence of *Monocystis* parasites seen beginning at week 17 for Audubon and Centennial Woods worms. Parasites were not observed via microscopy for Hort Farm worms until week 18. Mature gametocysts were observed in Audubon worms beginning week 18 and were present each week until week 32, the final week of sampling. Mature gametocysts were observed for Hort Farm worms starting week 21. Only one worm from Centennial Woods had mature gametocysts detected by microscopy at week 19, otherwise, only 9 of 134 Centennial Woods worms had mature gametocysts detected by microscopy during the season.

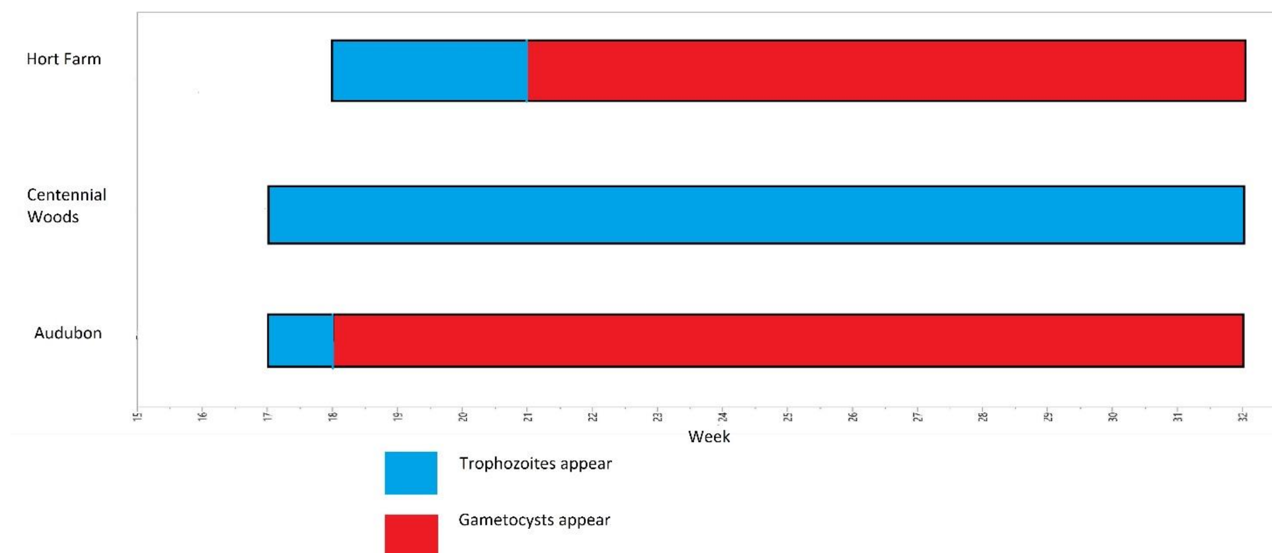


Figure 2. Graphical depiction of parasite schedule, with blue representing when trophozoites are first seen and red representing when gametocytes are first seen by week at Hort Farm, Centennial Woods, and Audubon. Only trophozoites are indicated in the figure for Centennial Woods because very few infections included any gametocysts.

Parasite density and relative proportion of parasite stages

Figures showing the raw data for parasite counts (trophozoites and gametocysts) for each site are placed in the Appendix. Figure 3 shows the total number of parasites for each site throughout the season. Within a week, the number of parasites seen in the seminal vesicles rose from none detected to tens of thousands.

Figures 4-6 show the relative proportions of trophozoites and gametocysts at each site throughout the season. The asexual parasites increased in numbers for the first few weeks of the season, with more than double the number of asexual parasites between weeks 17 and 18 for Audubon and Centennial Woods parasites and weeks 20 and 21 for Hort Farm Worms (Fig. 3). Immature trophozoites were the most abundant stage of the parasite for the first half of the season with gradually increasing numbers of mature trophozoites. As the season progresses, more mature trophozoites and mating pairs were observed. Gametocysts were present in Audubon worms at week 17, the first week the parasite was seen at that site (Fig. 5); whereas Hort Farm gametocytes did not begin to show up until week 21, three weeks after the first parasites are seen (Fig. 6). At Audubon, gametocysts continue to increase in numbers and made up a larger proportion of the total number of parasites every subsequent week. Beginning at week 27, the proportion of the sexual stage of the parasite, the gametocysts, was larger than the proportion of asexual parasites; this trend continued for the remainder of the season with the majority of the parasites observed being gametocysts (Fig. 5). The parasites at Hort Farm followed the same pattern as the proportion of gametocysts increase each week, and at week 31, the majority of the observed parasites are gametocysts.

The increase in the proportion of gametocysts at both Audubon and Hort Farm coincide with an overall decrease in parasite abundance. The decrease in parasite numbers is due to the

mating between two gamonts to form a single gametocyst. In *Monocystis*, two gamonts join to form a mating pair and the two parasites produce gametes. These gametes join, forming zygotes which develop into sporocysts inside a large gametocyst. This mating of two parasites to form one gametocyst partially accounts for the large decline of parasites. The production of parasites and their transition to the transmission stage is summarized in Table 1.

Table 1. Mean number of asexual parasites (trophozoites) during the first half of the season and the mean number of sexual parasites (gametocysts) during the second half of the season for each site. Percent successful reproduction calculated from dividing twice the sexual parasites, as two parasites are required to make a gametocyst, by the mean asexual parasites.

Site	Season Length (Week Numbers)	Mean Asexual Parasites During First Half of Season	Mean Sexual Parasites During Second Half of Season	Percent Successful in Reproduction
Hort Farm	18-33	56,241	20,683	73.55%
Centennial Woods	17-32	50,222	22.0	0.0088%
Audubon	17-32	67,763	38,544	113.76%

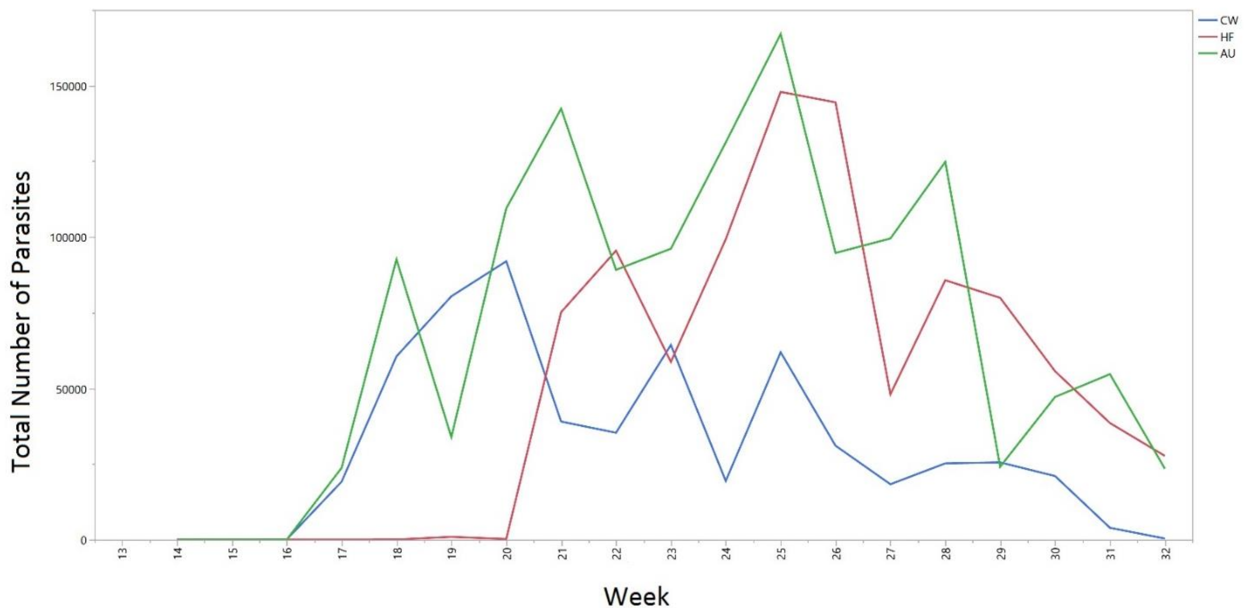


Figure 3. Mean total number of parasites from weeks 14 to 32 for CW, AU, and HF sites.

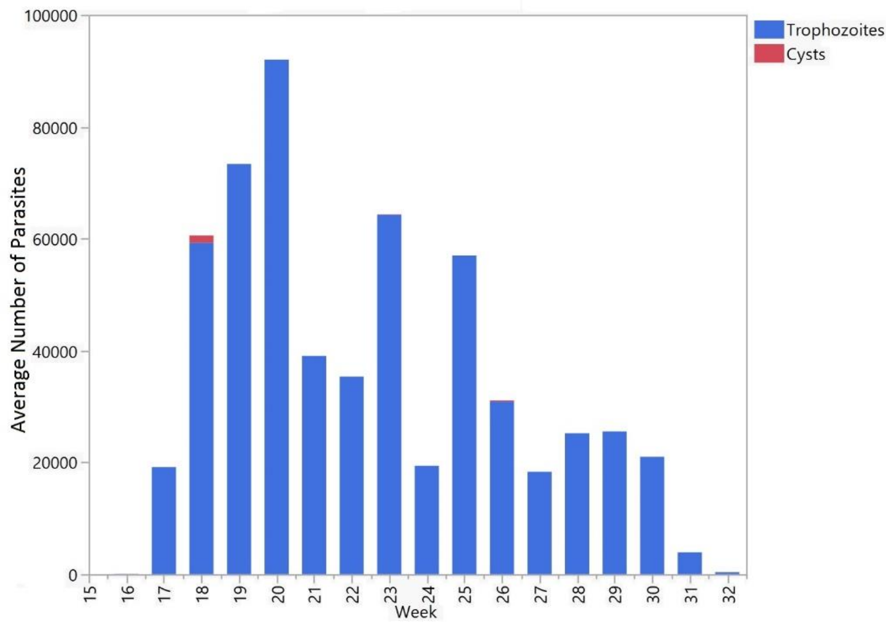


Figure 4. Relative proportions of the mean number of trophozoites and the mean number of gametocysts each week from Centennial Woods worms.

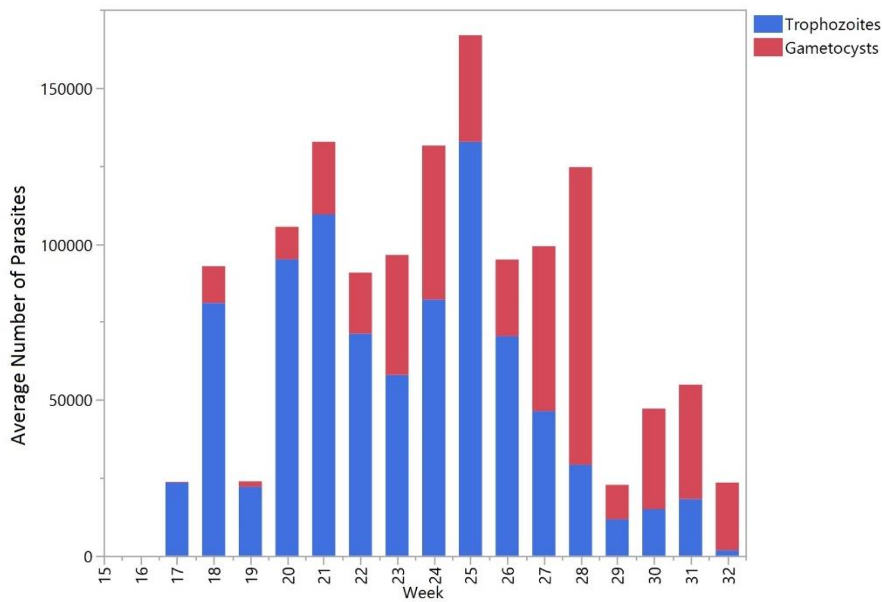


Figure 5. Relative proportions of the mean number of trophozoites and the mean number of gametocysts each week from Audubon worms.

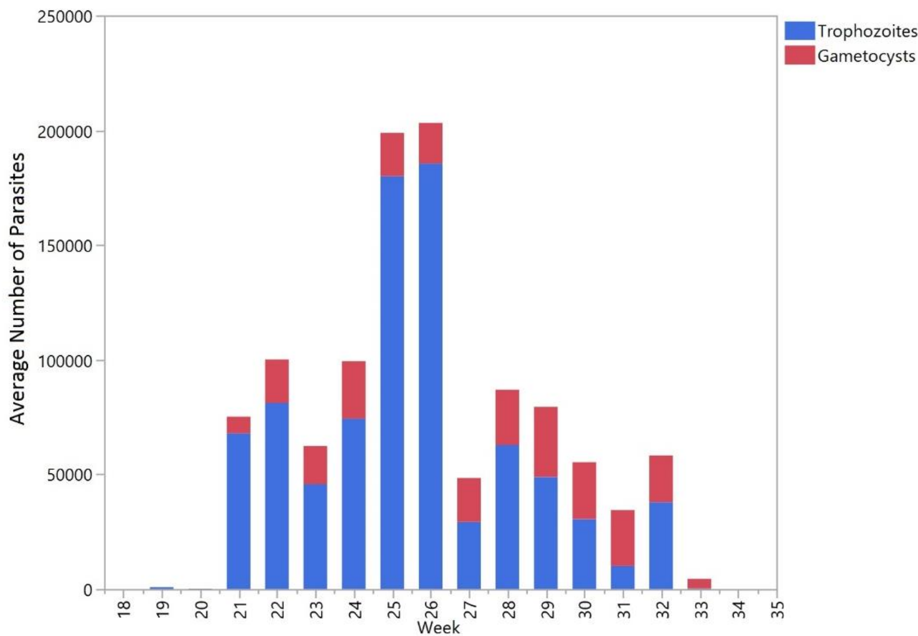


Figure 6. Relative proportions of the mean number of trophozoites and the mean number of gametocysts each week for Hort Farm worms.

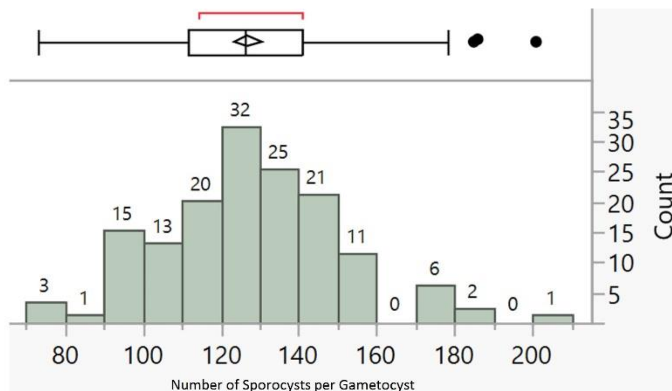
Sporocyst production by gametocyts (offspring numbers)

A total of 600 gametocysts were counted in order to determine the number of sporocysts per gametocyst at Audubon and Hort Farm. Very few infections produced gametocytes at Centennial Woods, so no counts were made for that site. Fig. 7 illustrates the distribution of sporocysts per gametocyst. Table 2 reports the mean number of sporocysts, the range of sporocysts per gametocyst, the standard deviation, and the 95% confidence interval of sporocysts at Audubon and Hort Farm. To determine if the distribution of sporocysts per gametocyst was normal, a Shapiro-Wilk W Test was conducted and yielded a W value of 0.9961 ($P = 0.157$). The distribution was not significantly different from normal, and thus a nested ANOVA was appropriate to examine the treatment effect of worm and site on number of sporocysts per gametocyst. A nested ANOVA yielded an analysis of variance F value of 21.64 ($P < 0.0001$). The effect tests of sites and individual worms produced F values of 9.08 and 22.34 ($P = 0.0027$

and <0.0001 respectively). Thus, both worm and site had an effect on number of cysts produced. The number of sporocysts produced per mother cyst was significantly smaller for Audubon, the site where the parasite produced gametocyts earlier. Because each gametocyst is a result of two mating parasite cells, the reproductive success of mating cells was ~60 for Audubon and ~70 for Hort Farm.

Table 2. The mean number and range of sporocysts per gametocysts, the standard deviation, and the 95% confidence interval of sporocysts at Audubon and Hort Farm.

Site	Mean Number of Sporocysts per Gametocyst	Range	Standard Deviation	95% Confidence Interval
Audubon	127.0	73-201	22.998	123.242-130.663
Hort Farm	145.8	78-223	20.643	142.786-148.858



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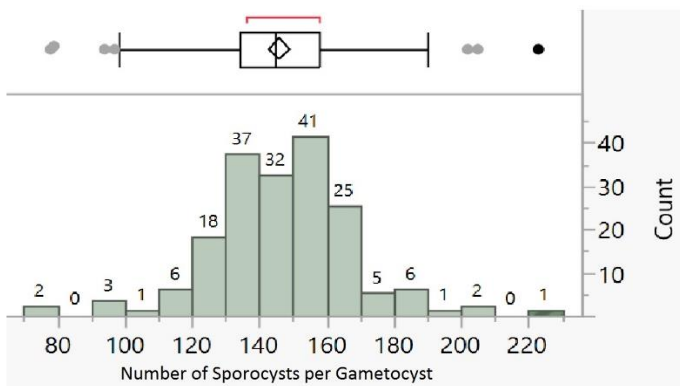

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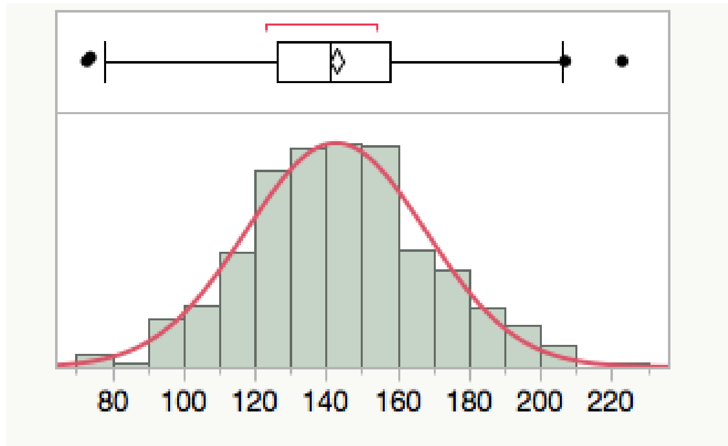


c.

Figure 7: a) Histogram of the number of sporocysts per gametocysts of 300 gametocysts from *Amynthas* spp. from Audubon b) Histogram of the number of sporocysts per gametocyst of 300 gametocysts from *Amynthas* spp. from Hort Farm c) Histogram of the number of sporocysts per gametocysts at both Audubon and Hort Farm for *Amynthas* spp. A normal distribution line is overlaid on the bar graph.

Discussion

This report is the first to study the entire life cycle of a gregarine parasite to determine any variation in life history traits. Studying a *Monocystis* sp. allowed for the entire life cycle to the parasite to be studied because its annual cycle matches that of its host, *A. agrestis*. The purpose of this study was to determine the life cycle and life history traits of *Monocystis* sp, in *A. agrestis* to gain insight into how almost all earthworms sampled contain high infections of this parasite even though the parasite seems to lack schizogony, an asexual reproduction stage found in all other apicomplexan parasites. Infections were observed by microscopy starting approximately 17 weeks after earthworms hatched, and Audubon and Hort Farm worms followed a relatively similar schedule in terms of proportions of life stages and timing; however, only 9 of 134 earthworms in Centennial Woods were found to have gametocysts, and those were in low densities compared to the other sites.

The pictures of the parasites in Figure 1 include most stages of the parasite's life cycle. After observing the parasites in *Amynthas* spp. of Vermont and comparing their morphology to

other described species of *Monocystis*, it is clear that this is a unique and undescribed species of parasite (Rees 1962, Segun 1971, Cognetti de Martiis 1923).

Amynthas agrestis hatch from their cocoons as early as April and begin growing and maturing rapidly. Asexuals are first observed via microscopy approximately four months later and quickly begin to increase in numbers. During the first half of the season, asexuals make up the majority of parasite cells. Over this period, the abundance of parasites more than double in numbers, even over the course of several weeks. The parasites then mature into mating gamonts and mate to produce the dispersal stage of the parasite, the gametocyst. The second half of the season is characterized by the growth of gametocyst numbers.

Audubon parasites appear to be on an early schedule than those of Centennial Woods and Hort Farm. Asexual parasites appear in Audubon worms at Week 17 and as well as a few gametocysts (Fig 2). Gametocysts in Audubon worms make up a large proportion of the total parasites even in the first half of the season and quickly outnumber asexuals (Fig. 5). In addition to the early occurrence of gametocysts, Audubon has the high production of gametocysts out of all of the sites even though it has the shortest season due to hard freezes. Hort Farm parasites appear later than Audubon parasites with asexuals showing up at Week 18 (Fig 2). At Week 18 gametocysts were observed and followed the trend of increasing in numbers until the end of the season (Fig 6). Centennial Woods is an exception to the aforementioned pattern, as asexual parasites show up at Week 17 and increase in numbers, however, only nine worms are found to have a few gametocysts over the remainder of the season (Fig 4). Overall, Audubon parasites show up earlier than Hort Farm worms and quickly begin producing transmissible gametocysts earlier in the season and make up a greater proportion of parasites than other sites.

The parasites at Centennial Woods seem to have an impossible life cycle because the parasites do not seem to produce gametocysts. Gametocysts are the transmissible stage of the

parasite that allows for new infections, therefore, the absence of gametocysts would mean that no new infections are being made. How can infections persist at high densities and in all worms at a site where no gametocysts are being produced? One possible explanation is that the parasite migrates into an earthworm egg and establishes a new infection within a cocoon. This hypothesis would explain how all earthworms are infected and how they reach high densities quickly; the earthworms do not need to ingest any sporocysts as the parasite is already present inside of the cocoon. This possible mode of transmission has not been studied but may provide an explanation into the nearly universal occurrence of parasites within *Amynthas* spp.; therefore, further research should investigate this possible mode of transmission.

The increase in the proportion of gametocysts at both Audubon and Hort Farm coincide with an overall decrease in parasite abundance. The decrease in parasite numbers is due to the mating between two gamonts to form a single gametocyst. In *Monocystis* spp., two gamonts join to form a mating pair and the two parasites produce gametes. These gametes join, forming zygotes which develop into sporocysts inside of a large gametocyst. This mating of two parasites to form one gametocyst partially accounts for the large decline of parasites.

Another possible explanation for the decline of parasite numbers is that mature gametocysts may exit the worm into the soil to be ingested by another worm. The gametocysts may exit the worm in feces and this loss would account for the net decrease in parasites in the seminal vesicles (Fields and Michiels 2006). Death or failure to successfully reproduce may also account for the decrease in parasites in the seminal vesicles during the second half of the season. Survival between the asexual and sexual life stages of the parasite was estimated by dividing twice the mean sexual parasites of the second half of the season by the mean asexual parasites of the first half of the season. Hort Farm parasites had a percent survivorship between their asexual and sexual stages with a survivorship of 73.55%. Audubon parasites had a percent of parasites

successful in reproducing of 113.76%. This value exceeds 100% likely because there were asexuals present in the first half of the season and the second half of the season that were successful in reproducing that were not accounted for in this calculation. Centennial Woods parasites had the lowest survival at just 0.022% (Table 1).

The large difference between survivorship rates for Hort Farm and Audubon parasites may be due to life history trade-offs. Audubon parasites develop gametocysts earlier in the season at week 17 while Hort Farm parasites do not develop until week 21 (Figure 2). The season at Audubon is slightly shorter than the season at Hort Farm, as Audubon is at a higher elevation and the ground remains frozen for about two weeks longer at the beginning of the season and experiences a hard freeze before Hort Farm (Görres, pers. comm.). It may be that in order for the parasite to reach its transmission stage and enter the soil by the end of the season, gametocysts need to develop earlier, but at a cost of lower survivorship between asexual and sexual stages. While there appears to be a trade-off between survivorship and time to develop gametocysts, Audubon still produces the highest mean number of gametocysts at every week observed (Figure 5 and Table 1).

The fitness trade-off of Audubon parasites is also demonstrated in the decreased number of sporocysts per gametocyst. At Audubon, the mean number of sporocysts per gametocyst is 126.9 while at Hort Farm, the mean is 145.8. The nested AMOVA conducted for number of sporocysts per gametocyst among both individuals and among sites indicated that the number of sporocysts per gametocyst were significantly different among both individuals and sites (Table 2). The decreased number of sporocysts could possibly be another fitness consequence of early development of gametocysts and, therefore, reduces Audubon parasites' fitness to closer to that of Hort Farm parasites.

The life history trade-offs at Audubon decrease the parasites' fitness closer to Hort Farm parasites' fitness, with mean sporocysts produced from Audubon being, 4,895,138 and the mean number of sporocysts produced from Hort Farm being 3,011,442. Worms at both Audubon and Hort Farm then are potentially releasing three to four million sporocysts into the soil at the end of each season. The question that remains is, are the worms of the next generation ingesting thousands of sporocysts to yield the high numbers of parasites observed over the course of an infection? A possible explanation to this perplexing situation is that there may be an asexual stage of the parasite that has not been previously reported for any *Monocystis*. All parasites in the phylum Apicomplexa, except for the gregarines, have an asexual stage known as schizogony. This asexual stage at the beginning of an infection would greatly increase the number of parasites and would explain the high density of parasites in wild worms. Asexual reproduction of the schizonts early in the season would lead to high numbers of trophozoites being produced in a short period of time; this pattern is seen in our data, as there appear to be no parasites, or what have been conclusively identified as parasites, until trophozoites appear at great numbers (Fig. 3). As of yet, no *Monocystis* spp. schizonts have been identified as being present in an infection; however, this does not mean that they are absent. It is possible that a cell that has been referred to as a worm cell may actually be a schizont. These cells are round and filled with granules with a similar appearance to trophozoites (pers. obs.). These cells have been suggested to be immune cells of the worm, but no molecular work has been done to determine whether these cells are parasite or host (Stein et al. 2005), Positively identifying these cells as schizonts would solve the conundrum of the high abundance of parasites in wild worms, because this asexual stage could drastically increase parasite numbers quickly.

Monocystis is a commonly known parasite that are present in many different species of earthworms. The parasite is clearly successful, as almost all earthworms in an infected area have

high numbers of parasites; however, it remains a mystery how the parasites enter and exit the earthworm, and how they reach such high numbers. This commonly known parasite is actually not so well understood, and further research on the transmission mode of the parasite and its life stages needs to be conducted to further understand the life history of this parasite. I suggest that future research focus on identifying schizonts in an earthworm by molecular means to explain this phenomena of nearly universal infection and high parasite abundance.

Literature Cited

Berlin, H. (1924). Untersuchungen über Monocystideen in den vesiculae seminales der schwedischen Oligochaeten. *Archiv fur Protistenkunde,* 48,1-124.

Clopton, R. E. (2000). Order Gregarina leger, 1900. pp 205-288. In J. L. Lee, G. F. Leedale, P. Bradbury (Eds.) An Illustrated Guide to the Protzoa, 2nd Edition. Society of Protozoologists, Lawrence Kansas, 2 Vols. Vol 1 689 pp.

Cognetti de Martiis, L. (1923). Sul genere Monocystis. *Monitore zoologico italiano*, 34, 250-

Eisen RJ, Schall JJ.(2000). Life history of a malaria parasite (*Plasmodium mexicanum*): independent traits and basis for variation. *Proc Biol Sci*., 267(1445),793-799. doi:10.1098/rspb.2000.1073.

Field, S. G. and N. K. Michiels. (2006). Acepheline gregarine parasites (*Monocystis* sp.) are not transmitted sexually among their lumbricid earthworm hosts. *Journal of Parasitology* 92, 292-297.

Görres, J. and Melnichuk, R. (2016). Asian Invasive Earthworms of the Genus *Amynthas* Kinberg in Vermont. *Northeastern Naturalist*, 19(2), 313-322.

JMP®, Version Jmp Pro 12. SAS Institute Inc., Cary, NC, 1989-2007

Levine, N. D. (1977). Revision and checklist of the species of the aseptate gregarine family Monocystidae. *Folia Parasitologia*, 24, 1-24.

Rees, B. (1962). Studies on monocystid gregarines. Two new monocystid genera *Cephalocystis* and *Denrocystis*. *Parasitology*, 52, 1-15.

Segun, A. O. (1968). Studies on acephaline gregarines in British earthworms – their systematics, occurrences, and possible mode of transmission. Ph.D. Dissertation, University of London. 319 pp.

Segun, A. O. (1971). Acephaline gregarines of British earthworms – their possible host specificity. *Parasitology*, 62, 389-396.

Segun, A. O. (1971a). Further additions to the British records of acephaline gregarines of earthworms. *Parasitology*, 62, 397-407.

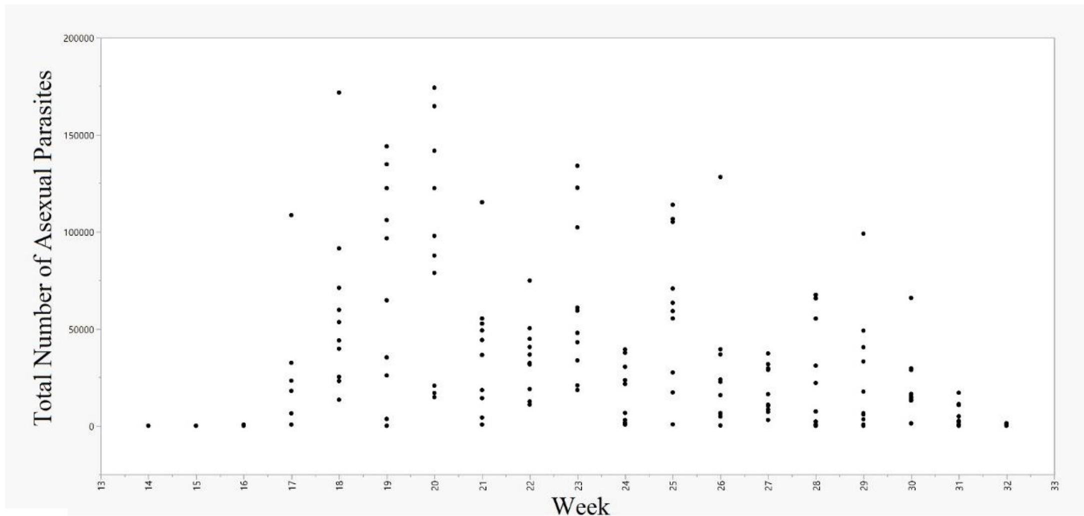
Segun, A. O. (1972). Distribution of acephaline gregarines of British earthworms and their comparison with those of other neighboring European countries. *Parasitology*, 65, 47-53.

Stearns, SC. (2000) Life history evolution: Successes, limitations, and prospects. *Naturwissenschaften*, 87(11), 476-486. doi:10.1007/s001140050763.

Stein, E., Avtalion R., and Cooper, L. (1977). The coelomocytes of the earthworm *Lumbricus terrestris*: morphology and phagocytic properties. *Journal of Morphology*, 153(3), 467-477.

von Stein, F. (1848). Oeber die Natur der Gregarinen. *Archiv für Anatomie, Physiologie und Wissenschaftliche Medicin,* 182-223.

Appendix



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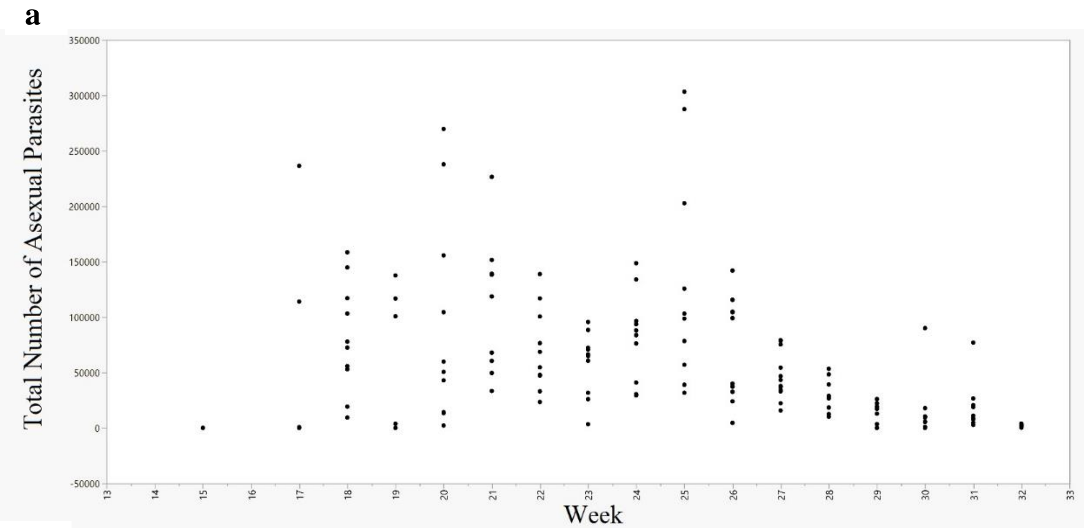

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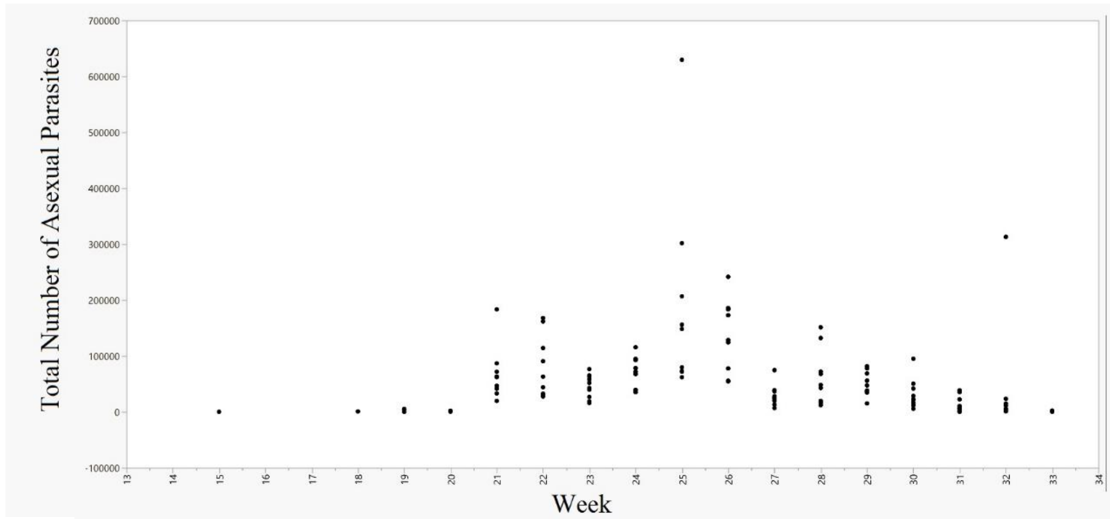

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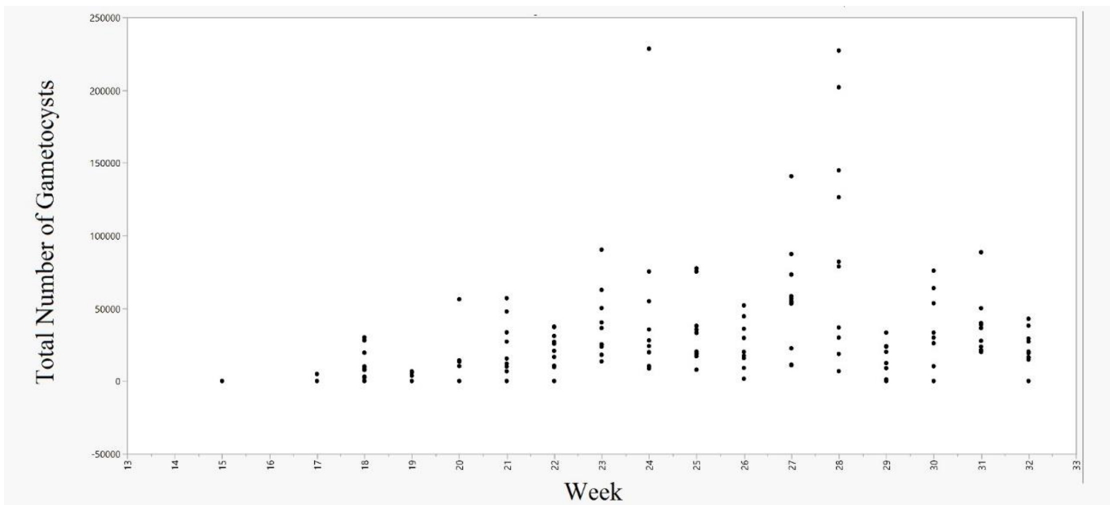

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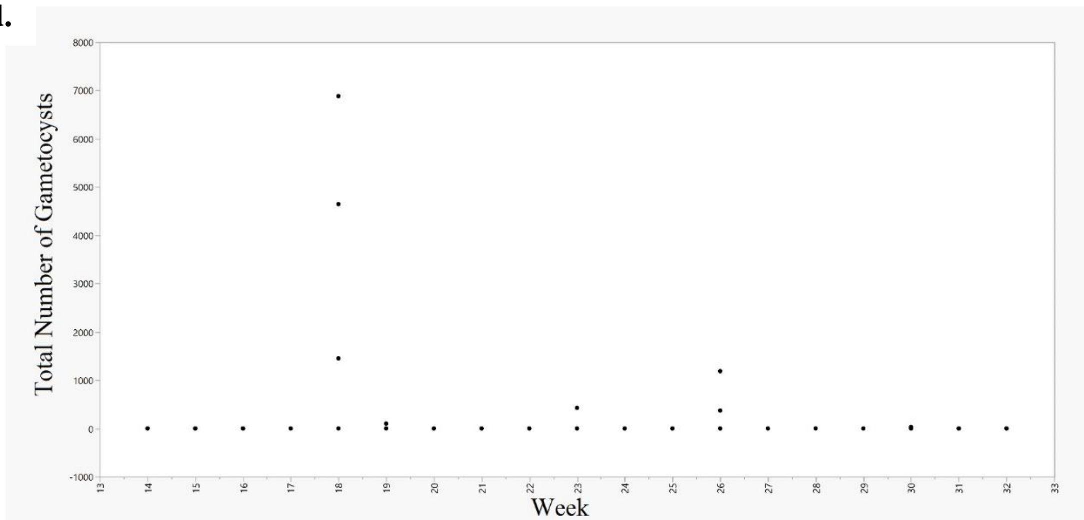

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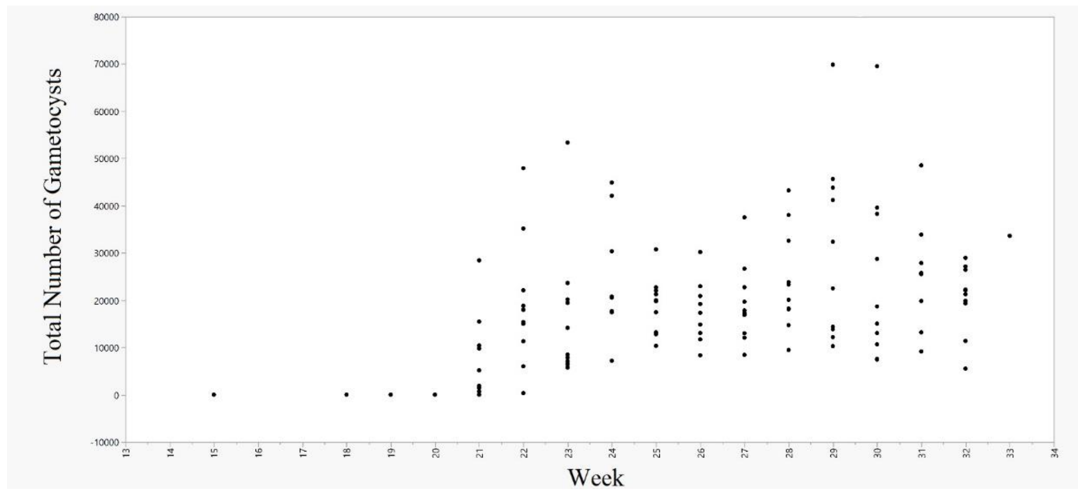

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Figure 1. a) Total number of asexual parasites at Audubon each week b) Total number of asexual parasites at Centennial Woods each week c) Total number of asexual parasites at Hort Farm each week d) Total number of gametocysts at Audubon each week e) Total number of gametocysts at Centennial Woods each week f) Total number of gametocysts at Hort Farm each week

CHAPTER FOUR

Conclusions

Parasite-host systems are often complex as they have coevolved with each other; therefore, it is important when studying a parasite to also study its host. Studying the host can provide useful indications into aspects of the parasite's life cycle and life history. The purpose of this paper was to study the host-parasite system of the invasive Asian earthworm, *Amynthas agrestis* and its parasite, *Monocystis* sp.

Amynthas agrestis is an annual earthworm that has been extremely successful in invading new areas and subsequently damaging its new environment. One hypothesis explaining why this earthworm has been successful as an invasive species is that it is parthenogenetic; parthenogenesis allows for a new population to be established with as little as one introduced individual and can rebound after a population decline (Terhivuo and Saura, 2006). Morphological studies have suggested that *Amynthas* spp. are parthenogenetic because this species often lack male pores and demonstrate early termination of spermatogenesis (Reynolds, 1978).

The present study sought to determine the mating system of *A. agrestis* and *A. tokioensis* using genetics Using RAPD markers, 536 *Amynthas* spp. were sampled from three sites in Vermont, Centennial Woods, Audubon, and Hort Farm. Analysis of the RAPD study indicated the presence of clones in populations of *A. agrestis* and *A. tokioensis* at all three sites, with up to 63.6% of sampled earthworms having at least one clone. While there is evidence of parthenogenesis from the presence of clones, there is high variability within these sites with many unique genotypes. The presence of both clones and unique genotypes suggests a mixed mating system consisting of both asexual and sexual reproduction. A mixed mating system offers

advantages such as being able to generate high genetic diversity in variable or disturbed environments while parthenogenesis allows for rapid population growth of a successful genotype (Shirk and Hamrick, 2014).

While there have been both morphological studies, and now a molecular study, on the mating system of *Amynthas* spp., I suggest that further research be done to determine when these earthworms are parthenogenetic versus when they are sexual, the genetic diversity of the earthworm, and how many introductions of *Amynthas* spp. have occurred in an area.

The purpose of the present study was also to determine the life history of *Monocystis* sp. This well-known parasite has a perplexing life cycle, as it seemingly lacks the asexual reproduction stage, schizogony. Weekly dissection of earthworms detected *Monocystis* sp, infections after approximately 17 weeks after earthworm hatchlings appeared in the soil. Within a matter of one to two weeks the parasite density reached high levels at each of the sites with predominantly asexual stages. At the end of the season in November, Audubon and Hort Farm infections consisted mainly of gametocysts, while only nine earthworms sampled at Centennial Woods were found to have gametocysts.

The proportion of asexuals that successfully mate and produce gametocyst is considerably lower at Audubon than at Hort Farm, with only 28.44% of asexuals producing gametocysts versus the 73.55% survivability at Hort Farm; however, the mean number of gametocysts was higher at Audubon than at Hort Farm. These gametocysts are the infective stage of the parasite responsible for infecting new earthworms, although the exact mode of transmission has yet to be determine and further research needs to be done to complete our understanding of the life cycle.

The most perplexing aspect of *Monocystis* spp. is its lack of schizogony. Infections increase rapidly in numbers during the first several weeks of observed infection. A possible explanation for this is the presence of the undiscovered schizont life stage. If schizonts are present in the *Monocystis* spp. lifecycle, it would explain the rapid increase in numbers of parasites in a limited amount of time. An earthworm consuming relatively few sporocysts could develop a high number of parasites as the schizonts rapidly undergo asexual reproduction. Further research needs to be done to determine whether or not shizogony is present in the lifecyle of *Monocystis* spp.

The parasite-host system of *Amynthas agrestis* and *Monocystis* sp. is incredibly interesting and complex. The invasive Asian earthworm is successful in establishing new populations and destroying forests while its parasite is successful in establishing its own infections in almost all earthworms sampled. Both *Amynthas* and *Monocystis* lifecycles and life history traits need to be further investigated to fully understand the complexities of this system and provide insight into both invasive earthworms and gregarine life history traits.

Acknowledgements:
I would like to thank Dr. Schall for years of his dedication to helping me with this project, unwavering encouragement, and priceless guidance and advice. I would also like to thank Dr. Görres for introducing me to *Amynthas* spp. and teaching me all about these species. In addition, I would like to express my appreciation for Dr. Gotelli for his support as a member on my thesis committee. Dr. Kilpatrick also donated his time and expertise to helping me with my analysis which I am extremely thankful for. Further thanks go to incredible undergraduate students, Naomi Cunningham and Jessa Mason, for helping me with collection and molecular work. Finally, I would like to express my thanks for the Honors College for their continued support and the Office of Undergraduate Research for both the mini-grant and summer grant I received which made my molecular work possible.