Detecting the Impact of Parasitism and Egg Bank Recruitment on Host Genetic Diversity in a *Daphnia*-Parasite System

by
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To my grandmother, Mohtaram Khanum, who has always inspired me not to give up on my dreams,

and my lovely husband, Saeed, who has always been there for me.

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Abstract

In host-parasite systems, parasites can impose a strong selection force for resistance in host populations over the course of an epidemic, leading to rapid evolution of host resistance. Despite this reality, resistance rarely persists in the long term. In this study, we hypothesized that genetic diversity could be maintained in the host-parasite system through recombination events and reintroduction of new clonal genotypes from the diapausing egg bank. Our model system was the zooplankton *Daphnia dentifera* and its fungal parasite *Metschnikowia bicuspidata*. *Daphnia dentifera* reproduce asexually from spring to fall, but in early winter switch to sexual reproduction, forming diapausing eggs deposited into lake sediments that can hatch in spring (or remain in the egg bank). We investigated how the end-of-year sexual reproduction and recruitment from the egg bank impact the maintenance of genetic variation after a fungal outbreak in lakes. We found that one population, Midland Lake, had high genetic diversity at the end of the year, even after the epidemic. It then retained high genetic diversity through sexual reproduction, but, surprisingly, not through egg bank recruitment. The second population, Hackberry, had significantly reduced genetic variation in the parent population, despite there not being a high disease prevalence that year. Sexual recombination and egg bank recruitment restored the genetic variation in that population. Therefore, we did not observe a large change in Midland Lake genetic diversity. However, we did see changes in Hackberry, where genetic diversity started low and then increased due to sexual reproduction and recruitment from the egg bank.

Introduction

Parasitism is an interaction in which the parasite benefits and the host is harmed. Many studies have shown that parasitism imposes evolutionary pressure for increased resistance in hosts, often in the form of directional selection [1-3]. This directional selection causes a reduction in genetic diversity within host populations since it can lead to loss of unfavored alleles [4]. However, this is not the only possible outcome from parasitism: in some host-parasite interactions genetic diversity can be maintained through disruptive selection for resistance and fecundity [5].

Duffy and Sivars-Becker [1] have shown that the virulent parasite *Metschnikowia bicuspidata* can drive directional selection in its host, the freshwater zooplankton *Daphnia dentifera* such that *Daphnia dentifera* rapidly evolves resistance over the course of an epidemic or season. However, Hall *et al.* [6] have shown that *Daphnia dentifera* experiences a resistance-fecundity trade-off, meaning that fungal epidemics can drive disruptive selection, resulting in a host population made up of two extremes (very susceptible but fecund animals and very resistant animals with low reproductive success), as was seen in the population studied by Duffy *et al.* [7]. While these findings may seem incongruous, Duffy *et al.* [8] found infection prevalence likely determines the type of selection that occurs in this system.

It is important to note that *Metschnikowia bicuspidata* is not able to coevolve with its infected hosts since previous studies have been unable to quantify heritable variation in traits [1, 9, 10]. Therefore, the detected rapid evolution of host traits was parasite-driven and not related to coevolution of parasite.

While a large amount of research has studied rapid evolutionary events in *Daphnia dentifera* within a season, how these microevolutionary events translate

between years is still unknown. This is important to consider in organisms such as *Daphnia dentifera*, which experience harsh seasonal conditions (namely freezing during winter months) that result in local extinction each year. Under these conditions, the long-term persistence of *Daphnia* depends on sexually produced resting eggs which survive the harsh environment and hatch when conditions improve. Therefore, the translation of evolutionary events from one year to the next will depend on how the prevailing selective forces interact with the production of resting eggs and their recruitment from the egg bank [11-13].

Reproductive Cycle

Daphnia dentifera have a cyclically parthenogenetic reproductive cycle. They reproduce asexually during summer and early fall (when epidemics occur), and then switch to reproducing sexually in late fall (when epidemics decline). Sexual reproduction produces resting eggs, which are diapausing embryos encapsulated in a protective structure. Females drop these resting eggs in the lake sediment [14]. Many, but not all, of these eggs hatch the following spring. These diapausing eggs can remain viable for up to 150 years [15, 16], therefore unhatched eggs accumulate to form a long-standing egg bank much like the seed bank of plants [17, 18].

Genetic Slippage and the Egg Bank Effect

Strong selection can act rapidly on asexual lineages, resulting in the rapid evolution of resistance; however, this evolution can be broken down by sexual recombination due to genetic slippage, which increases the expression of genetic diversity in sexually recombinant offspring and acts in opposition of selection [19]. Additionally, recruitment from the egg bank the following spring will determine the

genetic diversity of the population for that year, therefore how strongly evolutionary events carry over into the next year. The egg bank acts as a genetic archive, which can reintroduce genetic variation and provide temporal gene flow through recruitment from resting eggs deposited over many years in lake sediment [13]. This egg bank effect can have a large impact on the rate of evolution for the population, particularly in a variable environment. This becomes intuitive when we consider two potential outcomes: first, if the majority of eggs that hatch during the spring were deposited in the sediment months earlier, any selection that occurs during one year will carry across to the next, i.e. the genetic structure of the "new" springtime population will reflect that of the previous year. In this case, evolution of resistance should proceed with little egg bank effect. A second possibility is that most eggs that hatch will have been deposited over many years. This can result in increased genetic variation and a loss of any adaptive evolution that has been maintained despite genetic slippage. This will be especially true in a variable environment that selects for resistance one year and susceptibility the next, or sometimes results in disruptive selection.

In this study, we used *Daphnia dentifera* and *Metschnikowia bicuspidata* as our study system to understand how rapid resistance evolution in host population translates over years when sexual recombination and propagule production determine the long-term persistence of hosts. In other words, we aim to investigate 1) how sexual reproduction changes the genetic diversity in a parthenogenetic population, and 2) how recruitment from the egg bank determines how much genetic diversity changes, or stays the same, across local extinction events.

Methodology

Field Sampling

Using collection methods from Duffy *et al.* [1], uninfected female *Daphnia dentifera* bearing ephippia (i.e., sexually produced resting eggs) were collected from two lakes in Indiana, United States, during December of 2015. In Hackberry Lake, maximum disease prevalence was 0.05%. In Midland Lake, maximum disease prevalence was 17%. Based on the unpublished data obtained by Spencer Hall (Indiana University), Hackberry Lake was considered a "low-disease" lake while Midland Lake was deemed a "high-disease" lake.

The uninfected *Daphnia dentifera* bearing ephippia were brought to the laboratory, where they dropped their ephippia. We produced clonal lines of mothers and offspring by hatching the ephippia, then keeping both mothers and their offspring in optimal conditions (6 clones/30 mL water, 20°C, 14:10 light/dark cycle, fed 10^6 cells/mL *Ankistrodesmus* sp. 4 times weekly), causing both to revert to asexual reproduction. We sampled populations hatched from the egg bank the following spring and maintained clonal lines of these hatchlings using the same methods. These clonal lines of mothers, offspring, and egg bank hatchlings were maintained in the lab under optimal conditions and used for phenotypic assays and genotyping.

Genotyping

We genotyped one animal from every clonal line perpetuated in the laboratory. We extracted the DNA using a DNeasy Tissue Kit (Qiagen) [20]. In this study, we used six designed primer pairs for microsatellite genotyping [20]. These six primers can be found in Table 1.

Locus	Primer Sequence (5′– 3′)	Repeat	T_a (°C)	Size Range (bp)	No. Alleles	n	H_O	H_E	GenBank Accession	D. galeata galeata Amplification
Dgm 105	F: ATGTGAGCGCGCGAGCATTT R: GTCCAGCCGGCCCATTTCAGTT	$(CAG)_8AG$	58	188–197	3	103	0.58	0.56	AY542269	+
Dgm 106	F: ACCACCACCTCCTCCGCCACAT R: TTCGTCGATTTCCTCACCCATTTC	$(CAA)_8CCAA$	58	130–145	5	103	0.66	0.67	AY542270	+
Dgm 107	F: CCTTTGGCATCGTTTCTTATTCTT R: CCTGCCAACCTCCCAGTCCT	$(TGC)_7$	58	120–128	4	38	0.42	0.47	AY542271	+
Dgm 109	F: CCAGCTGTTGACCACCTG R: TGCGCGAGGATTTCCAACAC	$(ACC)_7AC$	58	258–266	6	102	0.57	0.66	AY542272	+
Dgm 112	F: GGAAATAGGCCTAGATGCTGTGT R: TTATTGATCTTCCGGCTGACTTTA	$(TGC)_6TGG$	58	121–130	3	39	0.49	0.54	AY542274	+
Dgm 113	F: TGCCACGAATCGTCTATAATGGTG R: AGCCCACATGTAGGCACAAGTCA	$(GCT)_7$	58	135–155	5	94	0.61	0.74	AY542279	+

Table 1. Characteristics of microsatellite loci for *Daphnia galeata mendotae*. $\mathbf{T_a}$, optimized annealing temperature; **n**, number of individuals genotyped; $\mathbf{H_O}$, heterozygosity observed; $\mathbf{H_E}$, heterozygosity expected [20].

Polymerase chain reaction (PCR) amplifications were performed using QIAGEN® Multiplex PCR kit. We submitted the plates of PCR products to the University of Michigan DNA Sequencing Core. We scored the results of sequencing core using *GeneMarker* software.

To quantify how sexual recombination may increase genetic variation, we compared the clonal richness and diversity of populations of sexually recombinant offspring to their mothers. We quantified how temporal gene flow may increase genetic variation by comparing the clonal richness and diversity of each population of sexually recombinant offspring to the corresponding spring hatchling population within the same lake. We performed statistical analysis using the package *poppr* in R to measure clonal richness MLG/N (the proportion of different multilocus genotypes (MLG) in the sample

(N)), clonal diversity (the Shannon-Wiener index (H), Stoddart and Taylor's index (G), and Simpson's index (λ)) and clonal evenness [21, 22].

To analyze the collected data and obtain genotypic richness, diversity, and evenness in R language, we used the method explained by Kamvar *et al.* [22]. Based on this method, we used *poppr* library in R and created two data sheets in Excel using the standard *GenAlEx* format [22]. The first data sheet was the original collected data from *Genemarker*. The second data sheet was the modified version of collected data after rounding the peaks to their nearest integer and performing refinement based on Table 2.

Loci	Original Peak(s)	Modified Peak
Dgm 106	132	133
	135	136
	137	136
Dgm 109	248, 249, 251	250
	252	253
Dgm 112	110	109
	111	112
Dgm 113	154	153
	146	147

Table 2. Modification criteria for peaks by rounding the peaks to their nearest integer

It is important to mention that the primer indicated by Dgm 107 did not provide us with any detectable peaks. Therefore, we decreased the number of investigated loci from 6 to 5.

Results and Discussion

Analyzing the Impact of Recombination

One component of diversity is richness. Colwell [23] defined richness as, "the number of species in a community, in a landscape or marinescape, or in a region." In this study, the numbers of detected different multilocus genotypes (MLG) in the sample represents genotypic richness. In both lakes, we expected the fall offspring population to have a higher genotypic richness than the fall parent population since genetic recombination through sexual reproduction will increase diversity, which can be reflected as an increase of richness. There were 23 MLGs for the Fall Midland Lake Parent population and 25 MLGs for the Fall Midland Lake Offspring population (Table 3). The Fall Hackberry Lake Parent population had 10 MLGs and the Offspring population had 18. These results can support our expectation that sexual recombination will increase the genetic diversity (in this case, richness) of the population.

Lake	**Pop**	**N**	**MLG**	**eMLG**	**SE5**	**H**	**G**	**λ**	**E.5**	**H_{exp}**	**I_a**	**$\bar{r}_D$**
Midland	Parent	26	23	23.0	0.000	3.08	19.88	0.950	0.912	0.416	-0.0213	-0.00714
Midland	Offspring	30	25	22.2	0.826	3.15	21.43	0.953	0.912	0.420	0.0609	0.02049
Midland	Hatchling	44	21	14.8	1.494	2.62	8.27	0.879	0.573	0.340	0.3794	0.13216
Hackberry	Parent	26	10	10.0	0.000	1.50	2.52	0.604	0.438	0.241	1.5635	0.42219
Hackberry	Offspring	34	18	15.4	1.131	2.69	12.04	0.917	0.804	0.272	0.1308	0.04444
Hackberry	Hatchling	57	29	17.9	1.644	3.14	17.95	0.944	0.768	0.320	0.1476	0.04982
	Total	217	79	19.5	1.992	3.86	25.83	0.961	0.532	0.358	0.2189	0.06739

Table 3. The results table for obtained genotypic data in R. The listed parameters are explained in the Appendix I.

It is important to note that the sample size (N) is different in each population (N=26 for the Fall Midland Lake Parent population, N=30 for the Fall Midland Lake Offspring population, N=26 for the Fall Hackberry Lake Parent population, and N=34 for the Fall Hackberry Lake Offspring population). A more appropriate comparison of richness between populations is the eMLG value, which is an approximation of the number of genotypes that would be expected at the largest shared sample size (N= 26 for the Fall Midland Lake populations and N=26 for the Fall Hackberry Lake populations) based on rarefaction [24]. Rarefaction is a technique used in ecology to compare species richness among different samples by taking into account the difference in sample size. In order to obtain the rarefaction curves, the pool of N samples are randomly re-sampled multiple times and the average number of species found in each sample is plotted. Therefore, using this method, we can generate the expected number of species in a small number of samples, n, drawn randomly from the large pool of N samples [24]. Thus, after taking the sample size difference into account, as shown in Figure 1, the eMLG for the Parent and Offspring populations of Fall Midland Lake were 23 and 22.2, respectively. This result shows a slight decrease in the richness of offspring population in the Midland Lake, which fails to support our expectation. However, in the Fall Hackberry Lake populations, eMLG is 10 for the Parent population and 15.4 for the Offspring population (Figure 1). The result in the Hackberry Lake supports our expectation that recombination will increase genetic diversity.

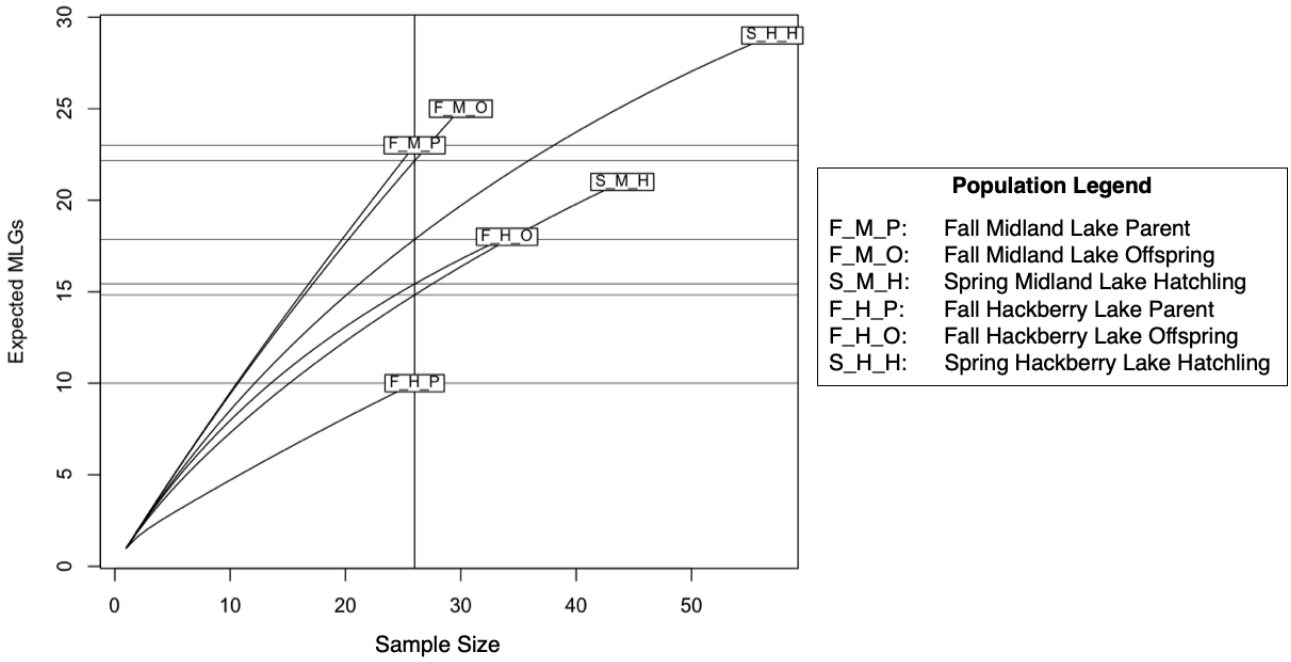

Figure 1. The rarefaction curves representing the richness in each population. The vertical line shows the largest shared sample size (N=26), and the horizontal lines show the expected MLGs for each population at the largest shared sample size.

Colwell [23] defines the diversity index as a diversity measurement that combines data regarding richness and evenness of the studied population. Based on the method we used in our analysis [22], the Shannon-Wiener index (H), Stoddart and Taylor's index (G), and Simpson's index (λ) are genotypic diversity indices that are employed by *poppr*. In this study, comparing the diversity of the parent population to that of the offspring population in both lakes (Midland Lake and Hackberry Lake) shows that both the Shannon-Wiener index and Stoddart and Taylor's index are greater for the offspring population. Thus, our expectation that the offspring population has a higher diversity than the parent population due to sexual reproduction is supported.

The other diversity index that we used is the Simpson index (λ), which is defined as one minus the sum of squared genotype frequencies [22]. This index scales from 0 (no genotypes are different) to 1 (all genotypes are different), and indicates an estimation of the probability of two randomly selected genotypes being different [22]. λ for the

Offspring population of the Fall Midland Lake was slightly higher than that of its Parent population (0.953 vs. 0.950; Figure 2). Moreover, λ for the Fall Hackberry Lake Offspring population was greater than that of the Fall Hackberry Lake Parent population (0.917 vs. 0.604). Thus, data obtained based on the Simpson index support our expectation as well. To account for the difference in sample size, the Simpson's index can be corrected by multiplying λ by N/(N−1) [22]. In Table 4, we listed the results of correction of Simpson's index for sample size for all populations. Based on the corrected Simpson's index, our expectation was supported once again since the genotypic diversity in the offspring population was higher than the parent population.

Analyzing the Impact of Recruitment from the Egg Bank

We expect hatchling populations to reflect recruitment from egg bank in spring in both lakes (Midland Lake and Hackberry Lake), i.e., spring hatchlings should have greater genetic diversity than the sexually produced offspring from the fall due to temporal gene flow from resting eggs deposited in lake sediment throughout years [13]. Thus, the richness is expected to be higher in the spring populations than in the fall offspring populations.

As shown in Figure 2, the Fall Midland Lake Offspring population (MLG = 25) had a higher richness than the Spring Midland Lake Hatchling population (MLG = 21). The same pattern can be observed in comparing the eMLGs in these two populations (Figure 1). These results fail to support our expectation that recruitment from the egg bank would increase diversity in Midland Lake. However, our expectation was supported in Hackberry Lake: we observed a higher richness in the Spring Hackberry Lake Hatchling population compared to the Fall Hackberry Lake Offspring population. Both MLG and eMGL are higher in the Spring Hackberry Lake Hatchling population than the Fall Hackberry Lake Offspring population (Figure 1). The Fall Hackberry Lake

Offspring population has MLG of 18 and eMLG of 15.4 (Table 3). The obtained MLG and eMLG values are 29 and 17.9 for the Spring Hackberry Lake Hatchling population, respectively (Table 3).

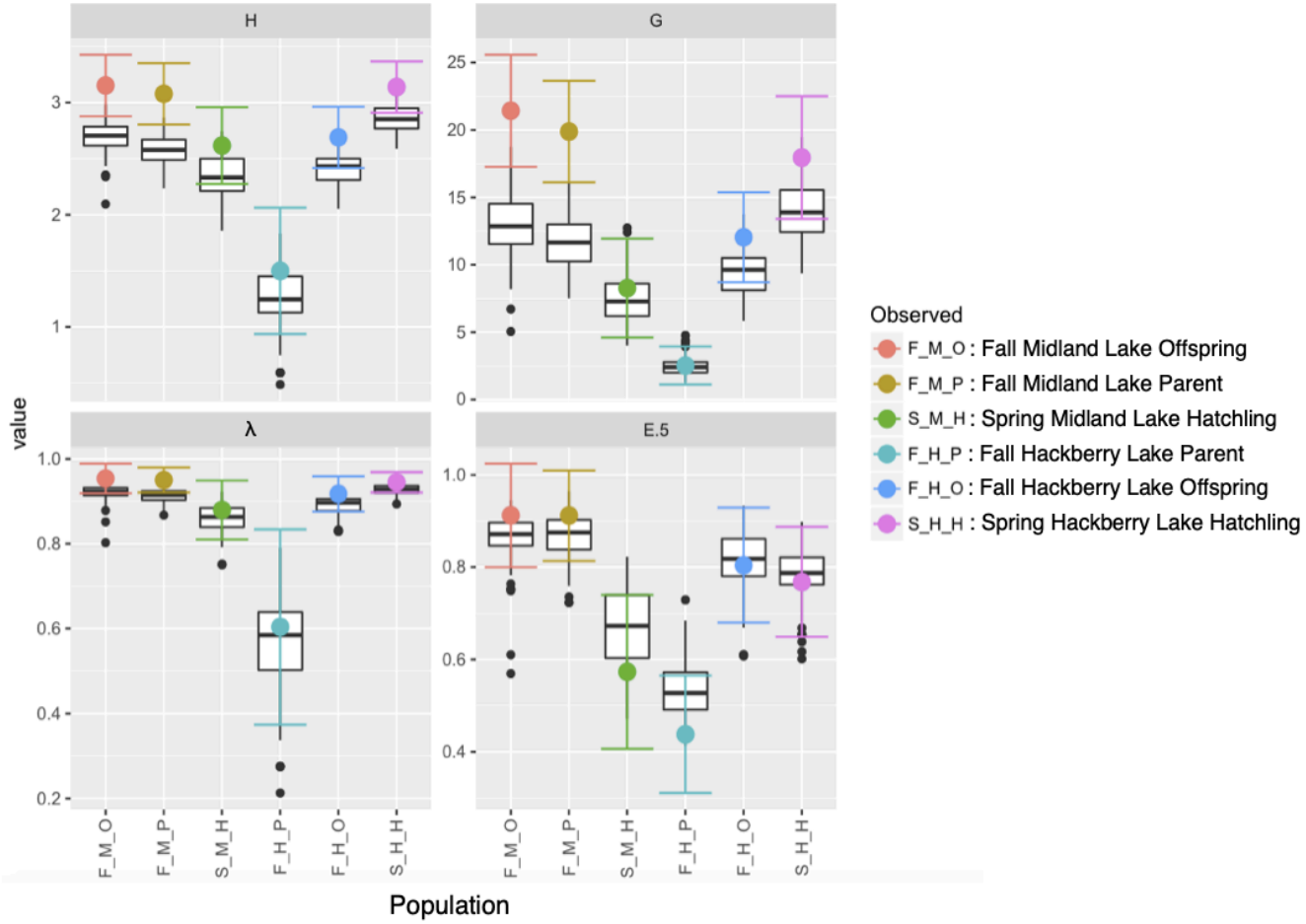

Figure 2. The observed value for the diversity indices (H index, G index, and λ) and evenness (E.5) for each population (colored dots) along with the corresponding 95% confidence intervals (colored lines). The boxplots represent the actual values from the bootstrapping, which will often appear below the estimates and confidence intervals [22].

The Shannon-Wiener index (H), Stoddart and Taylor's index (G), and Simpson's index (λ) were all greater for the Fall Midland Lake Offspring population than for the Spring Midland Lake Hatchling population (Figure 2). Thus, our expectation for higher diversity in the spring hatchling population was not supported. This may be explained partially by the sensitivity of these indices to genotypic richness in the uneven sample

sizes. Since the sample size used to calculate these diversity measures was different from population to population, the comparison between populations based on Shannon-Wiener index (H), Stoddart and Taylor's index (G), and Simpson's index (λ) may not be best reflective of real difference in genotypic diversity. Thus, the corrected Simpson's index can resolve this issue of difference in sample size and may provide more accurate results. However, in this case, the results from the corrected Simpson's index shows the same results, again rejecting our expectation. Genetic diversity was not higher for the Spring Midland Lake Hatchling population compared to the Fall Midland Lake Offspring population (0.900 for the spring population vs. 0.988 for the fall offspring population).

On the other hand, in Hackberry Lake, the Shannon-Wiener index (H), Stoddart and Taylor's index (G), and Simpson's index (λ) were higher in the Spring Hackberry Lake Hatchling population compared to the Fall Hackberry Lake Offspring population. If the differences in sample size are taken into account, the results of corrected Simpson's index provide the same pattern, with the Spring Hackberry Lake Hatchling population having a greater genetic diversity than the Fall Hackberry Lake Offspring population. These results support our expectation regarding increase of genetic variation in the spring population as a result of recruitment from the egg bank.

Lake	Population	Corrected Simpson's index
Midland	Parent	0.986
Midland	Offspring	0.988
Midland	Hatchling	0.900
Hackberry	Parent	0.628
Hackberry	Offspring	0.945
Hackberry	Hatchling	0.961
	Total	0.966

Table 4. The Corrected Simpson's index for all populations.

Analyzing the Impact of Disease Prevalence on Genotypic Diversity

Hackberry Lake was a "low-disease" lake with maximum disease prevalence of 0.05% while Midland Lake was a "high-disease" lake with maximum disease prevalence of 17%. It's very important to note that these different populations have undergone different selective events in the past, and that we did not sample the genetic diversity of the populations at the beginning of the year. We expected less genetic diversity in the Fall Midland Lake Parent population than in the Fall Hackberry Lake Parent population since Midland Lake had high infection prevalence by the fungus, *Metschnikowia bicuspidata,* therefore there was a stronger selection pressure for resistance on the population which could result in greater clonal loss. When we compare Shannon-Wiener index (H), Stoddart and Taylor's index (G), and Simpson's index (λ) between the Fall Midland Lake Parent population and the Fall Hackberry Lake Parent population, we can see that the Hackberry Lake population had a lower genetic diversity than the Midland Lake population. This result is contradictory to our expectation. One possible explanation is that other selective events occurred in Hackberry Lake, such as different parasite epidemics, changes in resources, or an increase in predation. Such changes in lake ecology could result in clonal loss in *D. daphnia*, but would go undetected due to our sampling process.

It is also notable that we used a small number of loci to delimit MLGs. The number of MLGs can underestimate the true number of different clones, since clones may differ at non-investigated loci [25]. However, it is important to note that information on the number and diversity of MLGs is still useful for investigating the clonal structure in natural populations [25]. Overall, we suggest that in future research studies more loci be investigated to provide a better representation of the sampled population.

Conclusion

Hosts can rapidly evolve resistance in response to parasite outbreaks, yet host populations also remain susceptible to infection over the long term [5]. We studied the impact of sexual production of diapausing eggs on the maintenance of genetic variation using the ecologically important zooplankton *Daphnia dentifera* and its virulent fungal parasite *Metschnikowia bicuspidata*. To investigate changes to host diversity we compared the genotypic diversity of parents to their sexually produced offspring in two lake populations: one that had experienced a large epidemic, and one that had not. We used three diversity indices (Shannon-Wiener index (H), Stoddart and Taylor's index (G), and Simpson's index (λ)) to compare diversity across populations. However, since sample sizes were different for each population, we used the corrected Simpson's index for more robust comparisons. Based on the corrected Simpson's index, genotypic diversity increased in sexually produced offspring compared to their parent population in both lakes. Thus, our expectation that sexual recombination would increase genotypic diversity was supported.

To investigate the effect of the egg bank on genetic variation, we compared the fall offspring populations to the spring hatchling populations in both lakes. In the low-disease lake the spring hatchling population had a higher corrected Simpson's index compared to the sexually produced offspring in fall, indicating an increase in genetic variation due to recruitment from the egg bank. These results supported our hypothesis. However, in the high-disease lake the genotypic diversity index was lower for the spring hatchling population compared to the sexually reproduced offspring in fall. This particular result failed to support our hypothesis. These mixed results suggest further investigations on the egg bank effect will be necessary. We also note that using methods

which allow for more fine-scale genetic differentiation within a population may be useful in future studies.

In conclusion, this study explored how changes in the genetic diversity of host populations translate over years when sexual recombination and propagule production are linked. Our results showed high genetic diversity in the parent population of one lake even after high disease prevalence. This high genetic diversity was maintained through recombinant events, but not through egg bank recruitment. In the second lake, the parent population had significantly reduced genetic variation despite experiencing almost no disease. In this lake, the genetic variation was restored through sexual reproduction and egg bank recruitment. We believe the expected effects were detected in this lake because the parent population had low genetic diversity to begin with, which then increased due to sexual reproduction and recruitment from the egg bank. It is notable that in both lakes the genetic diversity of the springtime hatchling population was different from (either greater than or slightly less than) the genetic diversity of the sexually produced offspring that went into the egg bank in the fall. This suggests that any adaptive evolution that may take place during a single year will be impacted by the egg bank effect in ways that this study was not able to measure or predict. Further study is needed to determine the factors that most strongly influence which eggs will hatch in the spring, since this will determine the rate at which adaptive evolution will occur in egg banking populations.

Appendix I

List of Parameters

Parameter	Description
Pop	Population name
N	Number of individuals observed
MLG	Number of multilocus genotypes (MLG) observed.
eMLG	The number of expected MLG at the smallest sample size ≥ 10 based on rarefaction
SE5	Standard error based on eMLG
H	Shannon-Wiener Index of MLG diversity [26]
G	Stoddart and Taylor's Index of MLG diversity [27]
λ	Simpson's Index [28]
E.5	Evenness [29]
H_{exp}	Nei's unbiased gene diversity [30]
I_a	The index of association [31, 32]
$\bar{r}_D$	The standardized index of association [33]

Appendix II

Genotypic Evenness

Genotypic evenness measures how genotype abundances are distributed. Evenness equals to one for a population with equally abundant genotypes and is close to zero for a population dominated by a single genotype [22]. The evenness is indicated in our analysis by E.5 in Table 3. Below, in Figure A1., the abundance of each MLG in each population is shown.

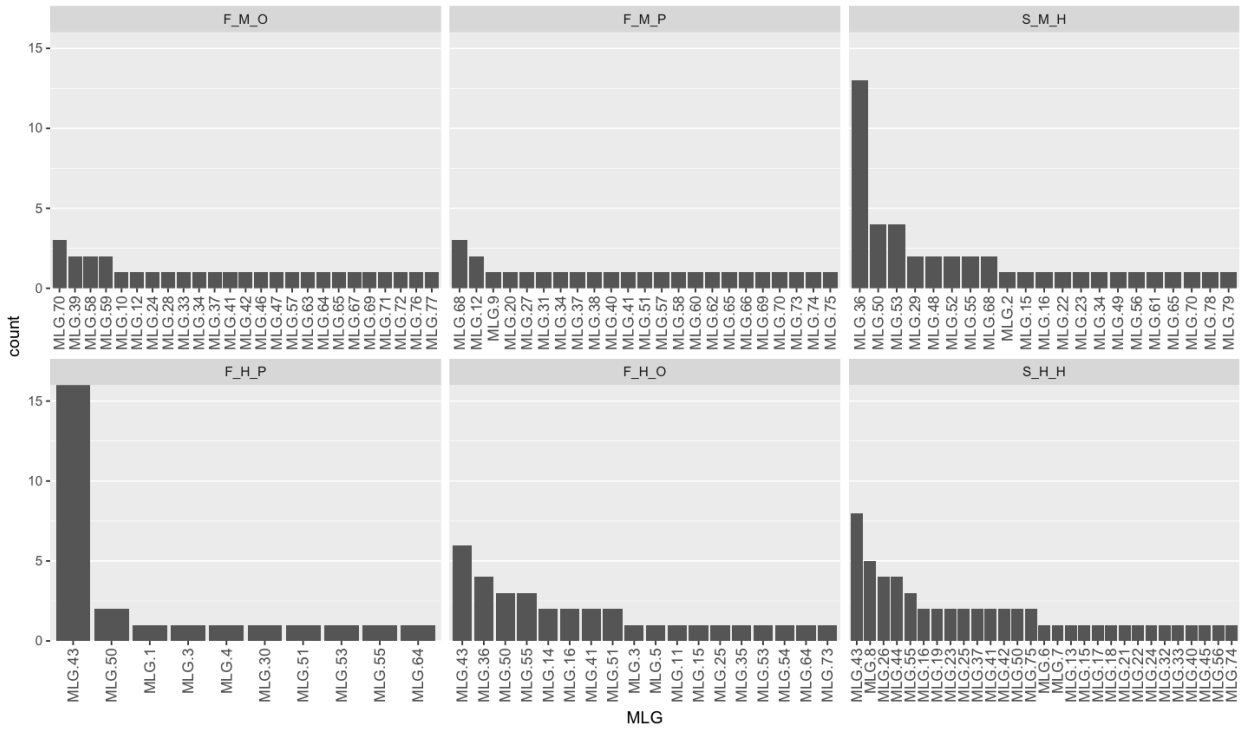

Figure A1. The abundance (Y-axis) of each MLG (X-axis) in each population. **Top row:** F_M_O: Midland Lake sexually produced offspring population, F_M_P: Midland Lake parent population, S_M_H: Midland Lake egg bank hatchlings population. **Bottom row:** F_H_P: Hackberry Lake parent population, F_H_O: Hackberry Lake sexually produced offspring population, S_H_H: Hackberry Lake egg bank hatchlings population.

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