Interaction of Immune Status, Genetics and Environment in *Peromyscus maniculatus gracilis*

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ABSTRACT

Small, isolated populations often experience reduced genetic diversity; the reason why some do and some do not exhibit increased disease susceptibility has been much debated. Comprehension of these patterns is vital to informing management programs of wild and captive populations. In order to add to this understanding, I assessed the immune strength of inbred deer mice in Lake Michigan directly through antigen (PHA) challenge and indirectly through measurement of parasite loads and leukocyte counts.

Populations of deer mice on three islands, with areas of 2300, 3600 and 4600 acres respectively, were trapped during July 2009. Immune response, assessed as a 6 hour reaction to PHA injection, was measured on each mouse; blood and fecal samples were collected as well. Immunocompetence was assessed through total leukocyte counts, neutrophil: lymphocyte ratios, fecal oocyte counts and hematozoan prevalence. Genetic analysis was conducted with 5 microsatellite markers. Observed heterozygosity and heterozygosity by locus were used as measures of homozygosity and inbreeding, respectively.

I investigated the relationship between diversity, infection and immune status through a series of t-tests, ANOVAs and linear models. I found that parasite species richness and prevalence of endoparasitic but not hematozoan infection, generally decreased with island size. Smaller islands had higher levels of homozygosity and inbreeding. More homozygous individuals tended to be infected in all populations but there was no association with inbreeding. Relationships between stress level, inbreeding and intensity of infection were specific to each island population. Inbreeding was positively correlated with immune response but genotypes and environmental pressures drove how that relationship was exhibited. The results of my study emphasize the importance for management programs to understand the full ecological context of a population in order to forecast how introducing novel experiences will impact them.

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PREFACE

Climate shifts, landscape fragmentation and shrinking populations endanger communities by increasing the risk of disease emergence, transmission and introduction. Instances of extinctions and extirpations from bacteria, virus, fungi, and other parasites are mounting.[1-6] These patterns, of increased risks of extinction, have clear conservation and public health implications; they accentuate the urgency to understand and manage the risk and dynamics of disease in populations. Isolated, small populations are of particular concern because they are already at a high risk of extinction from stochastic events, and they experience reduced fitness from loss of genetic diversity.[7] Moreover, small populations with low genetic diversity also often exhibit increased rates of disease, though the underlying reasons are not completely clear.[6] One possibility is that the loss of genetic diversity reduces the immunocompetence of a population. Given that many endangered species tend to be low in abundance, and that habitats are becoming more and more fragmented, how will the inevitable loss of genetic diversity affect the ability of species to withstand disease?

The relative vulnerability of small populations to disease is likely due to the interaction of environment, genetics and the immune system.[8] Immunological investigations have identified specific genes involved in immune defenses.[9] Parasites are hypothesized to maintain diversity at these loci through mechanisms of balancing selection but, parasites also tend to be less diverse and abundant in small, isolated populations.[6,10] Isolated populations may thus be unable to resist exotic pathogens, due to both loss of functional gene diversity and reduced selection pressure on immune system related loci. In other words, there is no adaptive advantage to having a wide range of expensive immunological defenses in a pathogen-poor environment. However, general inbreeding depression may also play a role. Inbreeding reduces fitness and has been correlated with increased incidence of disease.[7,10] Physiological parameters that are involved in mustering immune defenses may be degraded under inbreeding and so lead to increased susceptibility to disease.

Further, species allocate their investments in immune response differently depending on life history characteristics and environmental factors, e.g., parasite species richness, predation pressures and resource availability.[11,12] The relative impact of these conditions change investments in the immune system and so alter the individual's relative ability to fight disease.

There is a connection between parasitism and genetic diversity as well as between parasitism and immune strength but results have been equivocal.[13-20] Most studies have looked at wild-caught captive or laboratory populations. Captive populations will likely differ from wild populations since they do not experience the multitude of environmental pressures faced by a natural population; these pressures create different trade-offs, making it difficult to determine causal factors. The majority of the natural populations investigated have been bird, reptile or invertebrate species. Nonetheless, there is support for a relationship between inbreeding and disease costs in vertebrates. Disease susceptibility varied with pathogen type in inbred sea lions, inbreeding increased the spread of morbillovirus in dolphins and parasite load and survival were correlated in a population of harbor seals.[20-22] However, comparing across the literature is difficult due to the use of different immunity measures and different environments. Therefore there is a continued need for studies examining the link between lowered genetic diversity, immunocompetence and disease resistance in order to firmly establish the impacts of environment and genetics on disease dynamics.

Here I add to the literature on this subject by assessing the immunological vigor of insular populations of the deer mouse, *Peromyscus maniculatus gracilis,* through direct challenge, and by linking the results with parasite loads and genetic diversity within an environmental context. In Chapter 1, I summarize literature that both provides background and speaks to the relevance of my question. I end the chapter with a brief description of my thesis and its predictions. Chapter 2 details the experimental design including habitat, species and statistical analysis. Results and discussion are presented in Chapters 4 and 5, respectively.

Chapter 1: Literature Review and Introduction

Import of Disease in Conservation

Although once largely overlooked, there is now no question that infectious disease contributes to the risk of extinction in populations. Two published cases have substantial evidence supporting a pathogen as the ultimate cause of extinction: a microsporidian parasite in a captive population of tree snails and chytridiomycosis (fungal) infection in the free-ranging sharp snouted day frog.[23,24] Historical extinctions of the thylacines, Hawaiian avifauna and the eel limpet have been hypothesized to be the result of infection by a protozoan, a virus and a slime mold respectively.[25-27] There is a strong correlation between a rodent species extinction and a trypanosome species in the early 20th century.[28] The proximate effects of a pathogen on a population range from reductions in fitness to reducing populations to unviable levels. For instance, disease has been implicated in increased sea lion pup mortality, decreased swallow fledgling output by mothers and massive extirpations of the American Chestnut tree and associated insect communities.[29-31] The question that then arises is, what are the mechanisms and dynamics governing how an agent of disease impacts a population?

The requirement for a threshold level of hosts prevents most pathogens from causing true extinction, i.e. if the pathogen kills enough hosts to substantially reduce its probability of transmission, it will die out before the host population.[32] Pathogens may escape or avoid this threshold if they are able to lie dormant in the environment, survive in a reservoir host or if their transmission is frequency dependent.[33] However, with or without a threshold, disease can promote extinction by preventing recovery from or causing greater susceptibility to stochastic, anthropogenic and genetic issues.[34] The exacerbation of these factors by pathogenic agents is more important in smaller populations.

This pattern of small populations being more vulnerable to the damaging effects of disease is particularly alarming in the face of increasing habitat fragmentation and the recent surge in emerging infectious diseases.[35] Emerging infectious diseases (EIDs) are pathologies that have suddenly become more frequent, invaded new geographical regions, expanded their host range or mutated into a new strain of contagion.[36] Although new agents may evolve, usually the emerging pathogen is endemic, and simply taking advantage of opportunities created by a shift in population dynamics and structure.[37]

Such opportunities are often afforded through human actions that provide exposure to novel experience or place stress on the population (e.g. pollution, introduced species, climatic shifts and deforestation).[6,38,39] Failure of wolf expansion in Wisconsin, Montana and Minnesota has been blamed in part on increased pup mortality from parvo virus, a disease likely contracted from domestic dogs.[40,41]Vehicle tracks and bush walkers distributed a fungus all over an Australian forest, exposing naïve plants and causing subsequent death in the canopy and insect communities changing the overall canopy structure.[42] The agent of whirling disease, affecting trout and salmon across North America, was carried by a stocked European fish, introduced in the 1950s.[43] In the southwest, a Hantavirus pulmonary syndrome outbreak in humans was traced to an increase in the virus' reservoir host, *Peromyscus maniculatus*; their increase was traced to El Nino induced changes in precipitation.[44] This example, illustrating how changes can drive outbreaks, raises concerns about the relationship between predicted weather patterns from climate change and disease dynamics. Deforestation in the Amazon led to increased mosquito bites and increased incidence of malaria.[44] When disease leads to decreased population viability, it is usually a generalist pathogen invading a small, isolated population.[6]

Vulnerability of Island Populations

Islands are often used as proxies for habitat fragmentation; island species illustrate the overall high vulnerability of small, isolated populations

due to loss of genetic diversity and other factors. Low numbers, absent to minimal migration, drift processes and bottleneck events all contribute to the loss of heterozygosity and inbreeding.[10] In some instances, inbreeding can reduce fitness by causing an overabundance of homozygous loci and increasing the probability of deleterious rare alleles leading to inbreeding depression.[10]

Evidence for inbreeding depression includes the following: increased mortality in topminnows, embryo developmental instability in frogs, reduced birth weight in sheep and impaired predator induced plasticity response in snails.[45-48] Inbreeding depression has a greater effect on traits that are related to fitness and its strongest impact is in harsh or stressful environments.[7,8] Indeed, a recent study showed that populations of fish eagles have persisted for centuries with low genetic diversity, and the author acknowledges that this may be due to the relative constancy of their environment.[49] Given climate change and expanding human development, constant environments will be rare; therefore, small, inbred populations experiencing reduced fitness may not be able to adapt, driving home the need to understand their relative risk in the face of disease.

Relationship Between Fitness and Immune Response

Immunocompetence, which encompasses the ability of the host to detect and respond as well as the parasite's avoidance strategies, is a trait that seems to mediate fitness and is affected by stress.[50] Immune defense is a complex mixture of physical barriers, hormonal and cellular players, that help protect a species from attacks against its health by something foreign (an antigen).[9] The immediate reaction against an invasion or injury is the innate response, triggered by the presentation of foreign peptides by certain cell types and composed of non specific inflammatory proteins and phagocytic cells.[9] A second time-delayed reaction, also reliant on foreign peptide presentation, is the adaptive response: a cascade of cellular and chemical production that results in a series of cloned cells targeted against a specific antigen and the ability to more promptly defend upon re-exposure.[9] There are likely two

significant costs to an immune response: mustering a response associated with protein availability, production and metabolism and the tissue damage inflicted by that response.[51] However, for there to be a link between the immune system and fitness, costs and benefits associated with immuncompetence must influence fitness traits like survival and reproduction, and the costs of the pathogen have to outweigh the costs of immunity.[51]

Basic evidence that energy is associated with immune defense can be seen in the reduced activity, sociality and foraging exhibited by individuals fighting off an illness.[52] The relative age and health of the animal reflects its resistance as well. For instance, older tropical pythons had lower humoral responses after challenge with an antigen.[53] Downgrade or upgrade of the immune system has been coupled with multiple fitness-related traits across taxa including sexual signaling in mallards, social rank in cichlid fish and mating in damsel flies.[54-56] The ability to modulate the immune response makes intuitive sense because there may be times when it is more or less beneficial to have a strong immune system.[57] Mice have stronger immune responses during winter, and pregnant Siberian hamsters have lower levels of circulating antibody partially due to gestational energy expenditure.[58,59]

If an immune response ultimately detracts from other physiological necessities, then it is not needed and should be down regulated.[60] Cotton rats given immune-enhancing supplements and low quality protein showed no difference in immune function as compared to a control.[61] However, northern bobwhite chicks when deprived of appropriate protein showed lower immune response than other nestlings.[62] In the first case, since neither group had a requirement for up-regulation of the immune system, low protein had no effect. In the second case, the energy required for growth by the nestlings is immense, and the group receiving the lowest protein channeled energy away from the immune system into growth. Interestingly, the group receiving mid levels of protein had some reduced growth and normal immune response, indicating that growth is not always prioritized over defense. Enhanced immune function reduces growth in both great tit and blue tit nestlings but only in the presence

of ectoparasites; otherwise, supplementation to enhance immune function decreases nestling growth.[16,62,63] Thus the literature supports the idea that immune function is energetically costly but necessary in the face of pathogens, and that its benefits are weighed against other needs to optimize investment.

Relationship Between Genes and Immune Defense

Aside from health status, environmental factors and fitness needs, another major determiner of immunocompetence appears to be in the variation of genes configuring the structure and function of the immune system. Genes encode antigen-presenting cell receptors and production of leukocytes, and they enable the diversity of antibody production.[9] Additionally, genes control many life history characteristics, hormone production and organ function that indirectly support the immune system. Genetic diversity gives a population the elasticity to adapt to new situations.[7]

There are three proposed mechanisms why the loss of genetic diversity would affect fitness: direct, local and general. The *direct effect* describes a situation where an individual heterozygous at a gene coding for the trait of interest functions better than a homozygous individual. [60] The investigator needs to look directly at that locus to test this mechanism. Neutral markers are microsatellites, non-coding, repeating segments of the genome that are often used to assess levels of genetic diversity in a population. Local and general effects explain why neutral markers might show a correlation between loss of heterozygosity and fitness. The *local mechanism* presumes that the microsatellite and fitness loci are in linkage disequilibrium, which is something that often happens in bottlenecked populations.[60] The *general effect* presumes that the loss of microsatellite diversity is correlated with homozygosity at multiple loci throughout the genome, which is something that is more likely in inbreeding situations.[60]

Investigations into the connections among genetics, disease and the immune system have focused of late on individual genes or gene families. Individual genes may directly confer resistance to specific pathogens such as with the tapeworm *Hymenolepis citelliin* in deer mice; mice with the dominant

form of the gene are resistant to re-infection.[64] The Major Histocompatibility Complex is a family of genes responsible for recognition of material as self or foreign. An individual's MHC composition confers resistance or susceptibility to specific disease agents; high polymorphism at these alleles is at least partially pathogen-mediated.[65,66] For instance, in a natural population of sticklebacks, high diversity at MHC class IIB loci was seen within habitats that had greater macroparasite species richness.[67] Mainland kestrels had higher MHC diversity and more diverse parasitic loads than insular kestrals.[68] However, other studies have found no, or negative, correlations between disease resistance and general MHC diversity.[69,70] This is likely due to the studies not looking at a diverse enough range of parasites to capture the trend. Since MHC alleles confer resistance to specific pathogens, it would be unlikely to find a link between general heterozygosity and a single pathogen. For instance, a study looking at mouse lemurs found correlation between nematodes and specific MHC alleles but no association with general MHC heterozygosity.[71]

Although the MHC is unquestionably important, it would be a mistake to ignore overall genetic diversity's effects on immunity. Multiple physiological parameters and mechanisms feed into the healthy functioning of an immune response, not just immune-specific genes. Further, there is evidence that MHC tends to retain heterozygosity for a longer time than other parts of the genome. For instance, the island foxes of the California Channel Islands are extremely monomorphic at most neutral loci but retain high MHC diversity; nevertheless, they are plagued by higher prevalence of heartworm than their mainland counterparts. [71,72] If the MHC is still relatively diverse, what is causing the increased disease susceptibility? There is relatively little strong evidence for a mechanistic link between MHC variations with overall increased disease susceptibility; moreover these investigations often do not account for general inbreeding effects.[73]

Direct evidence for correlations between inbreeding with reduced immune response is harder to find. Comparing studies is problematic because

they tend to use different measures of immune strength including intensity of infection cellular immune responses and antibody levels.[14,17-19,74-76] Most studies on neutral measures of inbreeding and disease are in wild-caught captive or laboratory populations; few are on mammalian vertebrates. Additionally, results from the studies are contradictory. For instance, bacterial infections caused lower mortality in wild, inbred topminnows compared to normal populations.[77] Nevertheless, the literature supports that there is a connection between immune response and loss of general genetic diversity, though the underlying mechanism remains unclear.[78]

Role Of the Parasite

The third major factor that needs to be considered is the parasite itself. The interaction of pathogen and host is thought to drive both the evolution of complex immunological mechanisms in a species and innovative escape strategies in the parasite: a co-evolutionary relationship.[79] Infectious pathogens may be grouped into two general types: the microparasites (including bacteria and viruses), and the macroparasites such as helminths.[35] While varying widely in their makeup and dynamics, most of these agents require other species as hosts, and all are capable of short generation time (allowing for rapid responses to environmental pressures). They affect the fitness and survival of their hosts in many ways; in turn, their hosts have evolved processes to combat and defend against them: the immune system. Parasites then develop ways to evade the immune system, such as antigen masking or the production of immunosuppressive hormones. Evidence exists that parasites not only drive selection at immune-related genes, but also help to maintain diversity across the genome.[79]

Parasites affect the fitness parameters of their host in both direct and indirect ways. They divert nutritional resources and cause tissue damage.[51] Laboratory mice carrying helminthes, protozoa and nematodes emitted an odor that was offensive to other mice thereby altering social dynamics.[52] The presence or absence of malaria appeared to determine the presence of certain lizard species in the Caribbean.[80] Laboratory populations of bank voles showed

decreased survival and reproduction when infected with parasites. Further, they tended to have higher immune responses in environments with parasites then in environments without parasites, indicating that there is a benefit to fighting off infection.[58]

Brief Description of Thesis

In summary, inbreeding depression and lowered genetic diversity may lead to a reduction in immune strength either through loss of functional genes or a general loss of ability to support the immune system. Parasites may help maintain diversity, but they also lower organisms' fitness by changing their allocation of resources.[81] Due to ever-shrinking habitat and quickly changing environments, there is a pressing need to understand the drivers behind environment, gene and parasite interactions in disease ecology.

To disentangle the possible relationships, I looked at the immunocompetence of four insular populations of deer mice, *Peromyscus maniculatus gracilis*. The expectation was that both genetic diversity and parasite load would increase with island size.[82-,86] Preliminary work by Meagher[87] in the 1980s investigating allozymes supported the first and second expectation. A more recent publication by Taylor[88] et al describing lower diversity in some islands in the system supported the first. I investigated relationships among genetic diversity, immune system status and parasite loads to elucidate the effects each factor has on the other. For instance, finding a negative relationship between parasite load and genetic diversity independent of island would support the general belief that inbred populations are more susceptible to disease.

Thesis Predictions:

Hypothesis: Inbreeding reduces immune response due to genome-wide effects that decrease fitness and ability of the animal to muster an immune response.

1. Immune response will be negatively correlated with inbreeding. If populations have similar levels of inbreeding, the level of immune response will depend on exposure to parasites or other environmental factors.

Populations with high exposure to parasites will have a higher immune response than populations with lower exposure to parasites if their levels of genetic diversity are similar.

2. Parasite abundance, prevalence and species richness will increase with island size. Inbred populations will have a higher number of infected individuals and inbred individuals will have more intense infection, independent of island effect.

Chapter Two: Materials and Methods

Experimental Design

Species

Populations of the deer mouse, *Peromyscus maniculatus gracilis*, living on islands within the Beaver Island Archipelago (Figure 1) in Lake Michigan were investigated. This species is arboreal, preferring to nest in tree recesses and forage for seeds, nuts and insects at night.[89] Habitats may be coniferous, mixed or hardwood forests but these mice tend to be found near white pine, beech and maple trees.[89] A deer mouse's life span in the wild is around 6 months.[90] Females give birth to an average of five young per litter and are seasonally polyestrous. Their home range is between 242 to 3000 square meters with males roaming farther than females.[90]

The Beaver Island Arichipelago emerged from glacial ice approximately 10,000 years ago; Garden Island, High Island and Beaver Island were fully isolated from the coast 7000 years ago (Myers, 2010, unpublished). However, during the next several thousand years, water level fluctuations periodically submerged parts or all of the archipelago's islands. Hog Island emerged approximately 4000 years ago. (Myers, 2010, unpublished). Therefore, *Peromyscus maniculatus* populations on Garden Island and High Island have been isolated for at least 7000 years and the populations on Hog Island have been isolated for approximately 4000 years. Thus they are likely to exhibit high levels of inbreeding. I also chose these populations because there is extensive knowledge about the species that aids in interpretation of the results.

Habitat

Garden Island is 20 km^2 (4600 acres) and is approximately 11.4 miles from the mainland. Garden Island is more frequently visited by hikers and boaters so there is some chance of migration of mice from Beaver Island or the mainland. Its forests mainly consist of maple, beech and spruce trees.

The coast of High Island, approximately 14 km^2 (3600 acres), is lined with sandy beaches that serve to draw a fair number of visitors. Forests are

made up of red and white pine, some maple, oak and spruce trees. The closest mainland point is at a distance of 14.5 miles.

Hog Island, approximately 8 km^2 (2300 acres) is composed of lake marshes surrounding forest stands of spruces, maples, beech and old growth northern hardwood. The shortest distance to the mainland is 13 miles.

I selected sites on each island based on the relative abundance of oak, maple, white pine and beech trees. Each site was no more than 1.5 miles from the coast of the island. Two sites were trapped on Garden Island, separated by a distance of 0.25 miles. Birds of prey such as osprey and owls have been reported on all the islands; mammalian predators capable of swimming are potentially on all the islands as well.

Trapping

Ten by ten meter grids, composed of 50 to 100 Sherman traps baited with oat seed, were placed in late afternoon. Traps were checked at around 6 the following morning. Distance was measured with GPS and pacing. Two localities on Garden Island were trapped for two nights each. The localities were no more than a mile apart and were evaluated as one population. One locality on High Island was trapped for two nights, and one locality on Hog Island was trapped for one night. Trap success was calculated by dividing number caught per 100 trap nights. Density was calculated as mice per square meter. Sample size and densities of each island are summarized in Table 1.

Sample Collection

Due to differences in immune status, juveniles were not used in this study; any mouse weighing less than 14 gm was assumed to be juvenile. Sex, weight, tail length, body length and ear length were recorded. Data are summarized in Table 2. Biopsies were taken from the right ear with a 2 mm ear punch. Tissue was stored in 95% ethanol. Drops of blood were obtained via a 25 gauge needle inserted into the ventral tail vein. The blood was smeared on a glass microscope slide and the slides were fixed in methanol. Fresh feces were collected during restraint or from the trap. The feces were stored in polyvinyl alcohol.[91]

Antigen Challenge

Phytohemagglutin is a mitogen; post injection swelling is correlated with immune response strength in deer mice.[92] One mg of phytohemagglutinin was dissolved in 25 ml of phosphate buffered saline; the resulting solution was divided into 5 ml aliquots. In the field, the solution was stored on ice in a cooler. All injections were given to the mice after sample collection. Prior to the injection, the diameter of the right thigh was measured with a digital microcaliper. Injections of 0.1 ml of the solution were administered subcutaneously in the right lateral thigh. The measurement was then repeated three times and the average was recorded. Mice were put back into the trap with oats and apple.

The traps were placed in a shaded area and checked periodically over six hours. After six hours, the mice were taken out of the trap and their hind limbs were measured in the manner described above. The mice were then released. The PHA response was calculated as the pre PHA measurement minus the post PHA measurement. Percent change was then determined by first dividing the response and the pre PHA measurement and then multiplying by 100.

Parasite Evaluation

Preserved feces were evaluated through direct and indirect methods. The direct method involved smearing part of the fecal sample on a slide and examining it under a microscope. For the indirect method, the remaining feces were placed in a tube, suspended in zinc sulfate and centrifuged at 12,000 rpm for five minutes.[93] The tubes were filled with zinc sulfate until a meniscus formed. A coverslip was placed on top of the meniscus. After ten minutes, the cover slip was examined for parasites under a microscope.

Average parasite abundance was calculated from the counts. Parasites were identified to the level of genus or class. Prevalence, calculated as number of individuals infected divided by total number of individuals checked, was determined for each population. Intensity, which is the average number of parasites per mouse within the infected individuals, was also determined.

Average abundance, prevalence and intensity of infection of total parasites and of each type of parasite were determined.

Leukocyte Counts and Blood Parasites

Blood smears were stained using a Giemsa-Wright protocol.[94] Two slides from each mouse were examined for both parasites and leukocytes. The number of cells per ten high-power fields was recorded for each slide. The final leukocyte count was calculated by taking the average of these two slides and multiplying by 1000. Each slide was examined for three minutes for the presence of blood parasites. Due to experimental design constraints, only prevalence was considered for blood parasites. When considering total prevalence for each islands, both endoparasite and blood parasite infection were taken into account.

Neutrophil:lymphocyte ratios were also calculated.[95] Neutrophils are primarily involved in inflammation and stress response while lymphocytes have a more diverse role, ranging from inflammation to immunoglobulin production.[9] The other cell types tend to be associated with specific immune response; for instance, eosinophils are linked to allergies and parasitic infections.[95] Leukocytes, particularly lymphocytes and neutrophils, are modulated by the glucocortocoid axis. Stress levels rise and the numbers of lymphocytes drop while neutrophils increase.[9] Therefore a high neutrophil ratio is a measure of stress, whether through disease or some other factor. Moreover, these ratios apparently remain elevated and so indicate chronic rather than acute stress.[95] High ratios have been associated with high stress levels and increased risk of mortality in birds and humans.[96] On the other hand, high ratios have been shown to be evidence of an efficient immune response in birds. Dominant birds with bright plumage had higher ratios than birds that were not dominant.[95]

Genetic Preparation

DNA was extracted from the ear tissue using Qiagen DNeasy kits and confirmed by running the solution on a 1% agarose gel. Successful extractions

were completed on 13 High Island samples, 22 Hog Island Samples and 17 Garden Island samples.

Six microsatellites were chosen to assess genetic diversity within each population. Primers are listed in Table 3. Primer sequences were obtained from three different papers.[97-99] Ten microliter PCR reactions were prepared with the following components: 1 microliter DNA template, 0.8 microliter $MgCl_2$, 1 microliter 10x PCR Buffer, 2 mM dNTPs, 1 microliter forward primer (Hex labeled), 1 microliter reverse unlabeled primer and0.045 microliters Platinum Taq polymerase. Ultrapure water was added to reach the final volume of 10 microliters.

After verification on 2% agarose gels, a portion of each sample reaction was diluted, depending on the strength of the band signal. Optical AB plates were prepared for sequencing. A microliter of one of the dilution, HiDi formamide and LIZ ladder were placed in each well of the plate. The plate was then submitted to the University of Michigan Sequencing Core for genotyping.

Genetic Analysis

PCR analysis runs the risk of overestimating heterozygosity due to null alleles, stuttering and large allele drop-out. Null alleles occur when one allele fails to amplify and the locus is falsely identified as homozygous.[100] The large allele drop out is due to the preference of primers for smaller runs of bases. [100] Stuttering describes slippage during the PCR process causing an incorrect product to amplify. [100] In order to check for these errors, the genetic data of each population was put through Microchecker software.[100] If stuttering, drop-out or null alleles were present, the microsatellite was not used.

I assessed the genetic diversity of each population in two ways: by determining the number of heterozygous loci per individual and by estimating levels of inbreeding. A general increase in homozygosity in small, isolated populations due to genetic drift events or bottlenecks could result in decreased immunocompetence. In that case, neutral microsatellites would detect this if the neutral marker was linked to a locus involved in immune function or the microsatellite diversity represented genome-wide diversity. An increase in the

level of inbreeding in a population could result in decreased immunocompetence because of high proportions of deleterious alleles or due to over-dominance.[7] Over dominance refers to idea that the phenotypic expression of a heterozygous genotype is distinctly different from and may confer an advantage over the homozygous phenotype. In that case, to detect immunological changes related to inbreeding depression via microsatellites, neutral microsatellite diversity would need to represent genome wide inbreeding or be linked to a functional locus.

First, I looked at the levels of homozygosity in the population. I used GeneAlex software to calculate observed heterozygosity, expected heterozygosity, allelic richness and inbreeding coefficient (F_{is}).[101] Observed heterozygosity is the number of heteorzygotes at a locus divided by the number of individuals who carry that locus.[7] On an individual level, it is proportion of heterozygous loci within an individual. Expected heterozygosity is the proportion of individuals that would be heterozygous at a randomly selected locus; this is based on known allele frequencies.[7] Allelic richness is the average number of alleles per locus. The inbreeding coefficient, also based on known allele frequency, is the probability that two alleles at a randomly selected locus are identical by descent.[7] Expected and observed heterozygosity are numbers between zero and one where one is fully heterozygous. The inbreeding coefficient is also between zero and one; a more inbred population will have values closer to one.

GenePop software was used to check for Hardy Weinberg equilibrium.[102] One issue with small populations in genetic analysis is that homozygosity may be overestimated; to avoid this bias, a rarefaction technique was applied using HPRARE software.[103,104] A linear model was also applied to determine if there was a relationship between sample size and observed heterozygosity. FStat software was used to compare variation within and among populations as well as measures of genetic diversity.[105] Any finding of Hardy-Weinberg equilibrium deviation was investigated further to determine if the deviation was due to artifact or some other reason.

Once the loci were found to be suitable for analysis, correlations between immune measures and observed heterozygosity were tested in linear models (described in the next section). In order to evaluate if a locus was potentially linked to a functional gene, the models were repeated after removing each locus one at a time.[106] For instance, a model testing the relationship between PHA response and the average observed heterozygosity was run. If a correlation was found, the observed heterozygosity was recalculated after the removal of Pm-101 and the model was re-run. Then, the observed heterozygosity was recalculated including Pm-101 but excluding Pm104. The model was then re-run and this procedure was repeated with the removal of each locus. Essentially, this procedure checks to see if any one locus is having a disproportionate effect on the whole; that is, if the correlations seen are due to all the loci combined or just one locus.[106]

Second, to estimate levels of inbreeding, I used the index Heterozygosity By Locus.[106] This statistic is the sum of the expected homozygosities of all of an individual's loci divided by the sum of the expected heterozygosities plus the sum of the expected homozygosities.[105] Since expected heterozygosity and expected homozygosity are based on allele frequency, a locus will have more weight if its alleles are highly frequent and diverse. Thus Heterozygosity By Locus reduces the chances of overestimating homozygosity by prioritizing the level of polymorphism at a locus in its calculations.[106] The resulting number is between zero and one; one is fully inbred and zero is fully heterozygous. Calculations for Heterozygosity By Locus were done in an excel spreadsheet developed in a paper by Amos.[107]

Data Management

Data distributions were graphed using SPSS software. Normality was assessed by examining histograms and by running both Kolmogorov-Smirnov and Shapiro-Wilk tests. Since normality tests have low power with small samples, more weight was given to visual comparison of the observed distribution to a normal curve. All non-normal distributions were transformed to normality as necessary.

I wanted to make sure that each population was responding to PHA. In other words, if there was not a difference on average between a measurement before injection of PHA and after the injection of PHA, I could not argue that PHA response was indicative of anything. The pre-injection PHA values and the post-injection PHA values were both normal distributions. Paired t tests were run to compare pre-injection and post-injection PHA values in each population to confirm there was a change.

I also wanted to ensure that there was no bias due to confounding factors such as sex, weight or size. Comparisons of immune measures (i.e. PHA response, total leukocyte counts, neutrophil: lymphocyte ratios) and disease load measures (i.e. prevalence, intensity of infection) were run between each of these variables before analyzing the entire dataset.

Once I had transformed the data if necessary and established that there were no apparent confounding variables, I grouped individuals based on specific conditions or statuses. To see if there were differences between a particular condition of an animal and its diversity or immune measure, I grouped the animals in fours ways: infection status, response to PHA, island and category of genetic diversity. These groupings are described below.

I grouped individuals according to infection status to determine if there was a difference in observed heterozygosity, Heterozygosity By Locus or immune measures between infected and non–infected individuals. An individual was classified as *one* if they were infected with any kind of parasite or as *zero* if they were free of all parasites. This grouping was done within each population as well. A difference in immune measures (e.g. PHA response, leukocyte count, neutrophil:lymphocyte ratio) between these groups would indicate a relationship between disease and immune strength. A difference in levels of diversity (e.g. observed heterozygosity, Heterozygosity By Locus) between these groups would indicate a relationship between disease status and genetics. I labeled this grouping: *INFECTION STATUS.*

I also grouped individuals according to how strongly they responded to PHA (e.g. the magnitude of the swelling). Magnitude of response to PHA

varies in mice but the percent change in size tends to be around 50% after 24 hours.[56,92] Based on this information, I calculated how much of a change would be present after six hours and estimated that a normal PHA response would show at least a 15% increase at the 6 hour measurement. Therefore, I considered any response under 15% to be a low immune response and anything over 15% to be a normal immune response. I placed individuals into this group across the sample size and I also did this within populations to see if the trend held true within each island. I labeled the low responders *zero* and the normal responders *one.* This grouping was to enable comparison of diversity and infection status between low and normal responders to PHA. I labeled this grouping: *RESPONSE.*

The third group was simply comparing variables between islands. The size of the island is expected to have an effect on the diversity of the population; levels of diversity should decrease with island size. Additionally, the parasite abundance and parasite species richness is expected to decrease with island size. I labeled this grouping: *ISLAND.*

Lastly, I grouped individuals into two categorical ranges: one based on their observed heterozygosity and one based on their Heterozygosity By Locus value. For the first, I divided observed heterozygosity into four parts: 0-.25, 0.26-0.5, 0.51-0.75 and 0.76-1. Individuals with observed heterozygosity within each range were then categorized together. For instance, a fairly heterozygous mouse with an observed heterozygosity of 0.25 would be in category #1 and an overall homozygous mouse with an observed heterozygosity of 1 would be in category #4. These categories labeled as *one, two, three* and *four*, respectively, group individuals according to their level of homozygosity. I labeled the observed heterozygosity grouping: *HO GROUPING.*

I also divided the range of Heterozygosity By Locus into three parts: 0 to 0.33, 0.331 to 0.66 and 0.661 to 1. These categories, labeled as *one, two* and *three*, respectively, group individuals according to their estimated level of inbreeding. For instance, highly inbred mice would fall into category 3 and a

highly outbred mouse would fall into category 1. I labeled the Heterozygosity By Locus grouping: *HL GROUPING.*

Statistical Analysis- Comparison of Groupings

Independent t-tests were run comparing measures of diversity and measures of immune response within the *INFECTED* grouping. The measures of diversity were Heterozygosity By Locus and observed heterozygosity. The measures of immune response were percent PHA response, total leukocyte count and neutrophil:lymphocyte ratio.

Independent t-tests were run comparing measures of diversity and measures of disease load within the *RESPONSE* grouping. The measures of diversity were Heterozygosity By Locus and observed heterozygosity. The measures of disease load were endoparasite abundance, parasite species richness and intensity of endoparasitic infection.

An ANOVA was run comparing measures of immune response and measures of disease load within the *HO GROUPINGS* and *HL GROUPINGS* groups. Measures of immune response were percent PHA response, total leukocyte count and neutrophil:lymphocyte ratios. Measures of disease load were endoparasite abundance, parasite species richness and intensity of endoparasite infection.

An ANOVA was run comparing variables measuring immune status, diversity and disease within the *ISLAND* grouping. Variables of immune status were percent PHA response, total leukocyte count and neutrophil:lymphocyte ratio. Variables of diversity were Heterozygosity By Locus and observed heterozygosity. Variables of disease were parasite abundance, species richness and intensity of endoparasite infection.

Statistical Analysis- Comparison of Prevalence and Intensity

In the section above, I described how I compared average parasite abundance and species richness between various groups. However, another important variable to consider is the proportion of individuals that are infected on an island, i.e. prevalence. Macroparasite load distribution is usually right skewed with a few individuals carrying the highest loads and the majority

having low loads or remaining uninfected; therefore tests with assumptions of normality are not appropriate for comparing prevalence. The software, Quantitative Parasitology 3.0, runs specific tests for these types of non-normal data.[108]

The software was used to calculate prevalence and mean intensity of total endoparasite infections as well as of individual endoparasite type for each island. Total parasite prevalence and intensity of infection were compared across islands. Prevalence and intensity of infection for each class of parasite was also compared across islands.

Due to the differences in disease transmission and host immune system interaction, blood borne parasites were considered apart from the endoparasites. Only prevalence of total blood parasites and each type of parasite was compared using Quantitative Parasitology 3.0. The animals I examined were more likely to be chronically infected, which reduces the likelihood of finding organisms in the blood. Because I did not test for antibodies or do necropsies, comparing intensity of infection or overall abundance was not possible.

Generalized Linear Models-Assessing Interactions

In my system, the islands differed in density and parasite abundance; both of these affect immune response and infection status. Linear models were prepared to assess the relationship between my immune measures and genetic diversity, independent of other factors. The models were designed to answer the following questions: Do island effects predict immune response or disease load? Is immune response a function of an individual's genetic diversity? Does individual genetic diversity drive probability and magnitude of infection? I ran a series of mixed models to see if there was a quantitative relationship between diversity (i.e. heterozygosity and inbreeding) and immune status. However, immune responses could be modulated by the presence of infection, stress and intensity of infection or even the presence of other immune system components. For instance, an animal's increased PHA response could be due to a rapid inflammatory response from high amounts of circulating leukocytes.

Endoparasites have been correlated with decreased immune response which may be due to immunosuppression.[108] Additionally, factors such as density, habitat quality or being trapped could trigger a stress response and subsequent immunosuppresion.[9]

Therefore I set up these basic models:

1.) PERCENT PHA RESPONSE = DIVERSITY + INTENSITY + ISLAND + LEUKOCYTE COUNT + NEUTROPHIL:LYMPHOCYTE RATIO

2.) LEUKOCYTE COUNT = DIVERSITY + NEUTROPHIL: LYMPHOCYTE RATIO + ISLAND + INTENSITY

2.) NEUTROPHIL:LYMPHOCYTE RATIO = DIVERSITY + INTENSITY OF INFECTION + ISLAND + LEUKOCYTE COUNT + PHA RESPONSE

In order to see if diversity measures had an effect on immune response independent of island area or population density, I put island as a random factor into the model. Since the presence of parasites could modulate the immune response, I weighted the covariates with abundance of total parasites. The total parasite abundance was the oocyte count per individual. If the model's resulted indicated that the random factor's effects (i.e. island) was significant, I re-ran with the model with island as a fixed factor. Variables were removed if they were not significant and the model was rerun. I also ran these models within populations if possible. To determine if there was any locus with a disproportionate effect (i.e. a neutral marker linked to a functional gene), I removed each locus as described prior in the paper and re-ran the model.[105]

I also wanted to know if diversity had a relationship with susceptibility to disease and the severity of the disease. Susceptibility has more to do with how easily an animal becomes infected and severity has more to do with how well the animal is controlling the disease once it has infected them.

Again, several factors may complicate this relationship. For instance, increased density of a population increases the transmission of many pathogens.[33]

Therefore, in order to evaluate if the presence of infection could be predicted by their level of heterozygosity or inbreeding, I set up the following generalized univariate models.

1.) TOTAL PARASITE ABUNDANCE = DIVERSITY + DENSITY + PHA RESPONSE + LEUKOCYTE COUNT + NEUTROPHIL:LYMPHOCYTE RATIO

In order to evaluate if the intensity of infection could be predicted by an individual's level of heterozygosity or inbreeding, I set up the following generalized univariate model:

1.) INTENSITY OF INFECTION = DIVERSITY + DENSITY + PHA RESPONSE + LEUKOCYTE COUNT + NEUTROPHIL:LYMPHOCYTE RATIO.

Variables were removed if they were not significant and the model was rerun. I also ran these models within populations if possible. If a positive relationship was found for either dependent variable and observed heterozygosity, the model was re-run several times with the removal of each one of the loci.

Chapter Three: Results

Genetic Analysis:

Microsatellite loci were all highly polymorphic. On average per population, Pm101 had 8.2 alleles, Pm104 had 10.4 alleles, PO26 had 5.8 alleles, PLGT15 had 7.4 alleles and PLGT62 had 6.5 alleles per loci. A total of five individuals had some loci that did not amplify. A member of the Garden Island population did not amplify at PO-26 or at PO35. PCR using PO-35 failed to produce a product for three individuals in the High Island population.

Genotype frequencies were tested to see if they were in Hardy-Weinberg equilibrium. Deviations from Hardy-Weinberg equilibrium could indicate non random mating, mutations, selection on that allele, linkage disequilibrium or inbreeding in small populations.[9] Additionally, they may indicate artifacts from stuttering, drop out or null alleles. Therefore, if there are genotypic frequencies that are not in Hardy-Weinberg equilibrium, it is important to rule out causes such as artifacts before proceeding with analysis.

There were some loci that contained genotypes that were not in Hardy Weinberg equilibrium: Pm101 in the High, Hog and Garden Island populations, Pm104 in the High island population, PO26 in Garden and Hog Island populations, PLGT15 in High and Hog Island populations, PLGT62 in High and Hog Island populations and PO35 in the High island population. Microchecker software ruled out the possibility of artifact in all of these loci. However, null alleles were detected in PO-35 in three populations and so that marker was not used in the analysis.

The next step was to determine why the other genotypes were not in Hardy Weinberg equilibrium. Based on the Markov chain method in GenePop, the following primers were in linkage disequilibrium: Pm101 and Pm104 in the High Island population ($p=0.015$), and PLGT62 and PO26 in the Hog Island population ($p=0.04$). These findings are likely an error from low number of markers and high inbreeding since the markers have been used in other

studies and the loci were linked in only one population. Therefore all loci were treated as independent.

Rarefaction and linear regression confirmed that sample size was not affecting diversity measures, particularly since the largest sample size had the lowest heterozygosity. As expected, the Garden Island population had the most genetically diverse population. However, the High Island population, on the second largest island, had slightly higher levels of inbreeding (as measured by Heterozygosity By Locus) than the Hog Island population (Table 4).

As shown in Figure 1, the frequency of individuals on Garden Island that exhibit higher numbers of homozygous loci is lower than for either of the populations of Hog or High Island. In fact, the populations of Hog and High Island have similar frequencies of observed heterozygosity (Figure 1). On the other hand, the distributions of inbreeding within each island population reveal different trends. As shown in Figure 2, the frequency of individuals that exhibit inbreeding (i.e. higher values of Heterozygosity By Locus) on Garden Islands is still much lower than for either of the Hog and High Islands. Indeed, the High Island population contains only individuals that are highly inbred (Figure 2). Although the inbreeding distribution of the Hog Island population also has more relatively inbred individuals, it does contain some individuals that are fairly outbred (Figure 2). Put another way, Garden Island's population tends to have heterozygous individuals while the High and Hog Island populations tend to have more homozygous individuals (Figure 3). In regards to inbreeding, the High Island population has on less variation around its average Heterozygosity By Locus than either the Garden or the Hog Island populations (Figure 4).

Data Management

The majority of the data were non-normal and needed to be transformed to be appropriate for statistical comparisons. Heterozygosity By Locus, total leukocyte count, neutrophil:lymphocyte ratio and percent PHA response were ln(x+1) transformed. Total parasite abundance (oocyte count)

and intensity of endoparasite infection (oocyte count) were both $\log_{10}$ transformed. Observed heterozygosity was arcsin transformed.

The pre and post PHA measurements both had normal distributions. Average pre and post PHA measurements were compared within each population with paired t tests. On average, there was a significant difference between thigh measurement prior to injection and after injection indicating that all islands contained individuals that responded to PHA (Garden, $p=0.005$; High and Hog, $p<<.001$).

Sex, weight and body size did not appear to have a relationship with measures of immune status, disease or diversity. Individuals were successfully placed into *INFECTION, RESPONSE, HO CATEGORY* and *HL CATEGORY* groupings. The sample sizes within each group across the entire sample and within each population are summarized in Table 5.

Statistical Analysis- Comparisons of Groupings

Differences Between Islands

As expected, observed heterozyogosity generally decreased with island size (Table 4, Figure 3) but contrary to expectations, inbreeding did not (Figure 4). An ANOVA comparing observed heterozygosity between islands indicated that the Garden Island population had more heterozygous individuals than the High Island population ($p=0.02$) and the Hog Island population ($p=0.009$). An ANOVA comparing Heterozygosity By Locus between islands indicated that the Garden Island population had fewer inbred individuals than the High Island population ($p=0.001$) and the Hog Island population ($p<.001$). Levels of observed heterozygosity and levels of inbreeding in the High Island population were not significantly different from each other.

There was not a large amount of significant difference found between average immune measures across islands. An ANOVA comparing the percent PHA response indicated that the average response was lower in the Garden Island population as compared to the Hog Island population ($p=0.036$). However, the PHA response does appear to be increasing with island size (Figure 5). An ANOVA found no difference between average leukocyte count

or neutrophil:lymphocyte ratio between island populations. However, as a general trend, the neutrophil:lymphocyte ratio, which is a measurement of stress, decreased with island size (Figure 6).

Parasite classes and genera identified on one or more islands were cestodes, nematodes, trematodes, *Giardia* and *Coccidia* species. Cestode and nematode eggs were identified in the feces of some individuals on all the islands. All the parasite groups were present on Garden Island. Trematode species were not found in the High Island population. *Giardia* and *Coccidia* were not identified on Hog Island. Only cestode, nematode and total parasite infection counts were used in comparisons and models because cestodes and nematodes were the only classes detected on all islands.

An ANOVA indicated that parasite species richness, which includes blood parasites and endoparasites, decreased with island size ($p=.002$). The parasite abundance, which accounts only for endoparasites, showed a slightly different pattern (Figure 7). An ANOVA demonstrated that the Garden Island and the High Island population had significantly more parasites than the Hog Island population ($p=0.013$, $p=0.033$). Overall, the Garden Island population and the High Island population had similar levels of endoparasite abundance and were much higher than the Hog Island population.

Differences Between Infected and Non-Infected Individuals

On average, inbreeding levels of mice infected with endoparasites and mice free of all parasites were the same. However, an independent t test comparing infected versus non-infected mice revealed that more homozygous individuals (i.e. observed heterozygosity) were more likely to be infected ($p=0.027$). Average diversity and infection status was also considered within populations. Within the Garden Island population, no association between inbreeding or heterozygosity with infection status was detected. Within the High Island and Hog Island populations, more homozygous individuals tended to be infected (High: $p<.001$; Hog, $p=.045$). In general, inbreeding had no relationship with infection status but heterozygous individuals were less likely to be infected (Figure 8).

Some interesting patterns emerge when looking at differences in measures of immune status between infected and non-infected individuals. Across the sample, the percent PHA response did not differ significantly between the groups. However, in the Garden and Hog Island populations the infected individuals had a lower PHA response ($p<0.001$) but this trend was not apparent in the High Island population (Figure 9). Across the sample, the infected individuals had lower neutrophil: lymphocyte ratios (Figure 10). While this trend held true within each population, only in Hog island, was the lower neutrophil:lymphocyte ratio in infected individuals significant ($p=0.003$). Generally, infected individuals had lower PHA responses except on High Island and all infected individuals had lower neutrophil:lymphocyte ratios.

Difference Between PHA Responders

Independent t-tests were run comparing individuals that had a PHA response less than 15% to individuals that had a response greater than 15%. Across the sample, more inbred individuals tended to have higher responses ($p=0.003$). This trend held true within the Garden Island population ($p=0.044$) and the Hog Island population ($p=0.009$) but not the High Island population (Figure 11). Individual observed heterozygosity was not different between the two groups. No differences between endoparasite loads per individual or intensity of infection was detected.

Differences Between Categories of Heterozygosity

Individuals were grouped based on their relative proportions of homozygous loci. An ANOVA comparing intensity of infection across these categories showed that the most homozygous mice in category #4 tended to have more intense infections than mostly heterozygous mice in category #1 ($p=0.034$). No relationship was found between these categories and any other disease or immune measure. Essentially, these findings indicate that extremely homozygote individuals had more intense infections than extremely heterozygote individuals.

Differences Between Categories of Inbreeding

Individuals were grouped based on their relative level of inbreeding as estimated by their Heterozygosity By Locus value. An ANOVA comparing percent PHA response indicated that the outbred mice in category #1 had lower PHA responses than moderately inbred mice in category #2 ($p=0.021$) or highly inbred mice in category #3 ($p=0.024$). This trend repeats itself in every island: the more inbred the individual, the higher their PHA response is except that the difference is less extreme in the High Island population (Figure 12).

Quantitative Parasitology

Macroparasite distributions are right skewed and non-normal therefore non-parametric tests were run using Quantitative Parasitology software. Median intensity of endoparasite infection and nematode infection is highest in the High Island population and lowest in the Hog Island population ($p=0.019$) (Figure 13). Average intensity of endoparasite infection was higher in the High Island population than in the Hog Island population ($p=0.026$). Average intensity of nematode infection was higher in the High Island population than the Garden Island population ($p=0.007$) and the Hog Island population ($p=0.001$).

Blood Parasite Loads

Blood borne parasites detected were *Plasmodium*, *Babesia* and *Trypanasoma* species. All island populations had individuals infected with at least one of these species except on High Island. Only plasmodium species were detected in the High Island population. Only trypanosome and plasmodium species were detected on Hog Island. The prevalence of plasmodium in the Garden Island, High Island and Hog Island populations was 13%, 46% and 18.1% respectively. The prevalence of trypanosome infection in the Garden Island and Hog Island populations was 26% and 9.1% respectively. The prevalence of *Babesia* inn the Garden Island population was 15%. All parasite data are summarized in Table 5.

Mixed Model

Mixed models were run to determine whether measures of diversity or disease could predict immune responses, independent of island effects. The

model that was the best fit for predicting PHA response was Ln(Percent PHA Response)= Island + Ln(Heterozygosity By Locus). Island was used a random factor then as a fixed factor. Log_{10}(Endoparasite oocyte count) was used as a weight and as a random covariate. Heterozygosity By Locus had a positive effect on PHA response- i.e. more inbred individuals had higher responses ($p=0.002$) (Figure 14). Island was a marginally insignificant predictor of PHA response ($p=0.057$). When I ran this model within each population, I found that the trend held true for the Garden Island population ($p=0.006$) and the Hog Island population ($p=0.031$) but not the High Island population. Essentially, the relationship seems to be strongest in the Hog Island, moderate in the Garden Island populations and weakest in the High Island population (Figure 14). Parasite abundance had no effect.

Removal of the loci was then performed to see if the association was due to genome wide effects or if any one locus was driving the relationship between inbreeding and PHA. Removal of any of the loci made this relationship non significant and reduced the parameter effect estimate. However, within each population, only the removal of Pm101 or Pm104 loci made this relationship extremely non significant and reduced the parameter of effect to less than 0.75 (Figure 15, 16). Interestingly, removal of the loci in the Garden Island or High Island populations almost erases the relationship but it does not seem to alter the relationship that much within the Hog Island population. In other words, the positive relationship between inbreeding and PHA response appears to be largely dependent on whether or not specific loci are being considered in the calculation as well as which island population is under consideration.

Generalized Linear Model

Univariate models were run to determine if levels of diversity or immune measures could predict intensity of infection, independent of island and other variables. Observed heterozyogosity was not a significant indicator of intensity but looking within populations, some patterns emerge (Figure 17). There was a positive relationship between heterozygosity and intensity of

infection in the Garden Island population. This relationship is reversed in the High Island population; there was a negative relationship between heterozygosity and intensity of infection.

Across the sample, neutrophil:lymphocyte ratio was a significant predictor of intensity of nematode but not cestode infection. Individuals that were moderately infected with nematodes had lower neutrophil:lymphocyte ratios compared to individuals that had heavier loads ($p<.001$).

With respect to inbreeding and intensity of infection within these groups, some interesting patterns emerge. Infected, outbred individuals tended to have higher neutrophil:lymphocyte ratios on Garden Island though there is little difference; most individuals had fairly low ratios regardless of infection or genetics (Figure 18). Inbred, uninfected individuals on Hog Island have the highest neutrophil:lymphocyte ratios, while on High Island the most inbred infected individuals have the highest neutrophil:lymphocyte ratios (Figure 18). These relationships are more obvious in the High Island population when infection by blood parasites is considered (Figure 19).

Chapter 4: Discussion

Endangered and threatened species are more likely to experience lower levels of genetic diversity and inbreeding so as such, I wanted to know if inbreeding depression and loss of heterozygosity lowered immune response and resistance to disease. I predicted that populations with higher levels of inbreeding would have lower immune responses, but the results showed the opposite: populations with higher levels of inbreeding actually had increased PHA responses. This relationship within the Garden Island population and the Hog Island population was diminished when certain loci were removed from the calculation. This implies that the PHA-inbreeding relationship I observed may be due to linkages between these loci and functional immune genes.

Further evidence for this is found in the lack of relationship between an individual's level of heterozygosity and their PHA response. If a general loss of diversity and inbreeding was driving the PHA response, more homozygous individuals in every island should have had a high PHA response. However, the positive association between inbreeding and PHA response did not hold true for the High Island population, indicating either that a functional gene linkage was not present in this population or there was some other factor, likely environmental, affecting the immune response.

My second prediction was that inbred populations would have higher proportions of infected individuals and experience more severe infections. No relationship was found for inbreeding and infection status or intensity of infection. However, high neutrophil:lymphocyte ratios are considered a sign of stress and stress has been shown to increase morbidity and susceptibility to disease.[95] In general, I found that extremely homozygous individuals experienced more intense infections. Further, I observed that infected individuals had lower neutrophil:lymphocyte ratios except for inbred, infected individuals on High Island: they had higher ratios. This finding could indicate that a combination of stress and high inbreeding is leading to a higher probability of being infected.

On the other hand, in the Garden and Hog Island populations, inbred,

infected individuals had lower neutrophil:lymphocyte ratios. Thus my results have some indirect evidence for increased susceptibility in inbred populations and for increased intensity in less heterozygous individuals but do not support the hypothesis that inbreeding depression inevitably increases the risk of disease.

The basis for the predictions was the notion that the general loss of fitness from genome-wide inbreeding would decrease the ability of the individual to muster an immune response. Further, the predicted lower abundance and richness of parasites on smaller islands would lead to smaller investment in immune response due to an increased cost/benefit ratio. In the case of Hog and High Island populations, the lower prevalence of parasites appeared to cause a shift in immune strategy. Investing in more complex and slower immune responses may be more beneficial in an environment where parasite abundance and richness is high; a quick, rapid response is less costly when pathogenic challenge is rarer.[108] This hypothesis is borne out by the results. High and Hog Island populations had fairly similar levels of genetic diversity but the High Island population had the higher parasite abundance and a lower PHA response compared to the Hog Island population. The Garden Island population had the lowest PHA response, the most species of parasites, and the highest prevalence of endoparasite infection. That is, this population seemed to invest in immune responses more suited to an environment with more parasites and more diverse pathogen pressures.

An alternative explanation would be that the PHA response in my study was not accurately assessing immune response. Phytohemagglutinin is a red kidney bean extract that promotes division and transformation of T cell lymphocytes and has been used as measure of immunocompetence in rodents.[109] Exogenous immunosuppressive drugs and physiological stress responses suppress or decrease the reaction in many vertebrates including rodents, swine and birds.[76,96,105] Therefore, using PHA as an indicator of immune response is based on the assumption that the early PHA swelling is caused by the cellular immune response. However, PHA also causes swelling

through tissue damage and irritation.[110] PHA measurements are ideally done at multiple points (6, 12, 24 and 48 hours) to capture the full trend of the immune response.[109] Nevertheless, histological and clinical pathology studies have found leukocytes present in the swelling at 6 hours and confirmed the swelling as representing the cellular immune response with more precise immune function tests.[109, 111] Because I did not measure acquired immunity, I cannot say with absolute certainty that my results show that these populations are investing more in their cellular immune response. However, the patterns of higher inbreeding and increased immune response in my study are consistent with other studies that detected trade-offs between the two types of immune systems.

My results are consistent with other studies that detected parasites and genetics as drivers behind differential immune investments. Non parasitized jungle fowl had higher cellular immune responses than parasitized fowl and both evidenced a negative relationship between PHA swelling and circulating immunoglobulins.[112] Cellular response decreased and antibody titers increased with increasing parasite prevalence in insular populations of Darwin's finches.[113] Although not conclusive, my study provides further evidence that immune responses may be down regulated or up regulated depending on pathogen pressures.

However, pathogen pressures do not completely explain the relationship between PHA response and genetic diversity that I observed. When the parasite abundance was accounted for, there was still a pattern of more inbred individuals having higher PHA responses. Further, the relationship was weakened by the removal of markers. It should be noted that the removal of any marker reduced the relationship likely due to the low number of loci that I examined. However, only two markers decreased the parameter's effect substantially.

Prior to removal of a marker, the estimated effect was over four and after removal of Pm101 or Pm104, the estimated effect of diversity on the PHA response was less than one. Therefore it is possible these microsatellites

are linked to functional genes that are involved in the cellular immune response. Although these primers have been used fairly extensively in this species as independent, neutral markers, this does not preclude stochastic events like drift from causing such a linkage in isolated populations. The PHA-inbreeding relationship was not as strong within the High Island population.

However, inbred individuals on High Island appeared to be more likely to be infected and have a high neutrophil:lymphocyte ratio. Stress, as measured by the high neutrophil:lymphocyte ratio, has the potential to modulate the PHA response. In other words, the inbred individuals on High Island are prevented from exhibiting the higher PHA response due to their infection status and subsequent higher neutrophil:lymphocyte ratio.

Additionally, the differences seen between the island populations in total parasite abundance and intensity of infection may illustrate the importance of environmental factors in inbred individuals' vulnerability. It was expected that the Garden Island population would have the most abundant parasite population followed by the High Island population and then the Hog Island population. Instead, the High Island population had similar levels of parasite abundance to the Garden Island population. High Island and Hog Island populations have similar levels of heterozygosity yet the High Island population has more intense infections and much higher prevalence. The higher prevalence is expected due to the higher abundance of parasites but the Garden Island population, with a similar parasite abundance and density, has less intense infections.

The main differences that I observed in my study between the Garden Island population and the High Island population were levels of inbreeding and level of PHA response. The more inbred population on High Island seems to be less able to control their parasite infections than the Garden Island population despite having similar levels of density and parasite abundance. Moreover, despite having similar levels of inbreeding to the High Island population, the Hog Island population has less parasite abundance and less intense infections. This implies that there is something about High Island that

is leading to inbred individuals to be more susceptible to infection and to suffer more intense infections.

The patterns seen in neutrophil:lymphocyte ratios further support this perception. Increased neutrophil:lymphocyte ratios have been associated with increased mortality and morbidity of infection.[104] Infected individuals on Hog Island and Garden Island exhibit a decrease in neutrophil:lymphocyte ratios while infected individuals on High Island have increased ratios. Further, there is no positive relationship between neutrophil:lymphocyte ratios and intensity of infection except in the High Island population. Moreover, although inbred individuals in the Hog Island population and in the High Island population tend to show high neutrophil:lymphocyte ratios, only ratios of inbred individuals in the High Island population increase as intensity of infection increases. These patterns imply that inbred individuals in both populations experience increased stress but only on High Island, where there is higher pathogen pressure, does increased magnitude of disease result.

On the other hand, this pattern appears to be particularly driven by two organisms: nematode and *Plasmodium* species. Within the High Island population, individuals infected with *Plasmodium* species and individuals with intense nematode infections tended to have higher neutrophil:lymphocyte ratios. Thus it is possible that either parasite is raising stress levels in infected individuals and impairing their ability to fight off macroparasitic infections. This would also imply that the High Island population is generally more susceptible and sensitive to *Plasmodium* and nematode infections. These findings coincides with other studies. *Plasmodium* infection in lizards altered competition dynamics because one species was more susceptible than the other.[80] Infection with nematodes rendered mice unable to resist infection with a malarial parasite.[116]

However, the Garden Island population had a higher prevalence of nematode infection but a much lower *Plasmodium* prevalence than the High Island population. Again, the main difference seems to be that the High Island population has higher levels of inbreeding. Alternatively, there may be other

pathogens, predators or habitat quality differences that I did not account for that may be leading to these differences in stress and infection. Overall, my study was not able to discern a clear relationship between diversity and disease resistance due to local differences in the population. These findings emphasize the importance of examining multiple pathogens and incorporating life history of the parasite when investigating diversity and disease resistance.

The lack of significant association between diversity and disease resistance in my study may be due to the level of inbreeding. Full sibling white mice experienced decreased resistance to *Salmonella* infection while first cousin inbred mice did not.[117] Many of the studies that have found an association have examined bottleneck populations which have much lower levels of diversity than the populations in my study.

Additionally, my sample size and my primer set for each island was very small. A larger number of microsatellites and wider range of heterozygosity on each island, particularly Garden Island, may very well have enabled me to detect a difference in susceptibility of homozygotes. However, my findings of diversity are consistent with previous studies on the archipelago islands (Zac Taylor, personal communication, August 2010).

Using fecal floatation is a fairly imprecise method of detecting parasites- there is a large chance of either overestimating or underestimating the prevalence of infections. A small sample size and use of the fecal method could result in a large bias in either direction. However, High Island population's prevalence and abundance were always similar to those of the Garden Island population and Hog Island was consistently low no matter which parasite was being examined. Similarly, individuals on High Island had higher intensities for every type of parasite. This consistency of patterns in abundance and intensity leads me to think my counts were reasonably accurate. Further, the prevalence of endoparasitic infection in the High Island population is similar to that found for fluke prevalence in deer mice on High Island in the 1990s.[118]

Another factor that needs to be considered is the tendency of rodent population to fluctuate in 2-5 year cycles; these cycles lead to random changes in the age structure and genotype frequencies in a population.[118] Island rodent populations also cycle but their cycles are less extreme and they reach higher maximum densities unless conditions are prohibitive.[119] The driver of the low peak of the cycle is likely specific to the population; it could be many factors from disease to food availability to genetics. Therefore it is possible that each of the island populations I examined were at different points in their individual cycles. For instance, the High Island population's cycle may be driven by parasites. Nematode infection and acorn availability interacted to cause population declines in populations of *Peromyscus leucopus* and *Peromyscus maniculatus* in Florida.[120] Nematodes appeared to be having a significant impact as measured by the high neutrophil:lymphocyte ratio in the High Island population. An interesting question would be to determine if inbreeding was interacting with parasite infections to accelerate population cycles in this system.

Rodents in dense island populations tend to have smaller home ranges; this then reduces the amount of interactions with other individuals. Hog Island's population's lower prevalence and intensity of infection as compared to the High Island population may have been due to this restriction in movement rather than differences in susceptibility.

Lastly, another consequence of the population cycles would be rapid fixing of genotypes within a population. An isolated environment supplies near perfect condition for evolutionary forces to act on traits ideally suitable to that particular habitat.[121] Therefore, the increased PHA response I observed may be a result of a fixed gene or group of genes that give immunological advantage within these islands. This hypothesis has implications for forecasting what might happen if a new disease is introduced into an isolated population. The PHA response represents a rapid cellular immune response; as I mentioned, individuals exhibiting this type of response tend to trade-off against other components of the immune system. This type of immune

strategy is not suitable for every type of pathogen. If immune defenses become a fixed cost written in the genetic code, these populations may be non-resilient in the face of new diseases due to inflexible strategies.

Conclusion

Understanding factors that contribute to the vulnerability of small, isolated populations to disease is key to their appropriate management and protection. This study contributes to the growing literature on this issue by providing evidence about the relationship among disease, immune response and genetic diversity. First, my results indicate that inbred populations are capable of modulating their immune response but that the capacity is dependent on genotype-environment interactions. Second, my results provide evidence that, while inbred individuals do appear to have more intense infections, their susceptibility to infection appears to depend more on environmental factors than genetic diversity. Thirdly, I did not find that inbreeding, at least at the level of these populations, produced decreased immunocompetence from genome wide effects. Rather than supporting an absolute negative correlation between inbreeding and defense against disease, my results indicate that the combination of genetic diversity and local pressures create specific immunological signatures. Applied to management plans, this means that the genetic vulnerability of each population needs to be assessed within the context of their environment prior to re-introduction of captive populations. Further research, some of which has already been done, should focus on developing models that incorporate both environment and life history and genetics of the host as well as the parasite to try to predict responses to disease.

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APPENDIX I: Tables

Table 1. Trapping results for each island.Trap success is calculated as total caught/100 traps/#trap nights. Density is calculated as number of mice caught per square meter.

	Hog	**High**	**Garden**
Island Area (acres)	2300	3600	4600
Total Caught (n)	33	22	29
Recaptures (n)	n/a	1	0
Sample Size (n)	23	13	18
Trap Success	.35	.23	.21
Density	.12	.11	.31

Table 2 , Morphometrics and sex of the samples on each island. Snout to vent and tail measurements were taken separately except for on High Island. The standard deviation is noted in parenthesis.

	Hog	**High**	**Garden**
Island Area (acres)	2300	3600	4600
Weight (gm)	17 (1.8)	17.5 (2.8)	17.4 (2.23)
Body Length (in)	3.5 (0.28)	---	3.2 (0.27)
Tail Length (in)	3.5 (.31)	---	3.17 (.36)
Ear Length (in)	0.65 (0.08)	0.66 (0.11)	0.56 (.06)
Females (n)	10	4	8
Males (n)	12	9	10

Table 3. Initial set of primers used in PCR and genetic analysis. PO-35 was not used in subsequent data analysis. Base pair size listed reflects the range found in this study.

Name	Forward Primer	Reverse Primer	Size (bp)
Pm101	CATTCAAGACCTGGCTTTTT	TGGGTTTCATCAGTGCTTCT	165-188
Pm 104	CATAAGGTGGCTCGGAATCA	CAGGAAGGGGAAATGACCAT	207-231
PO26	GCTTCAGTGTTGATGTCTGAT	GCCTCTCTGTCTCTGTCTAT	144-216
PO35	AGCACCAGTGACTTCCGTTGT	GCTGCCCTTTCTCAGTCTGT	256-272
PLGT15	GATCAAGTCTCACTATGTAG	GACCTCCACAAATACACTGT	176-268
PLGT62	AGAGCAGTGACTAGAAATAG	GTTCATCAACTGCATTCAGT	132-167

Table 4. Genetic diversity parameters for each island. Observed Heterozygosity; He: Unbiased Expected Heterozygosity; F_{is} Inbreeding coefficient; HL: Heterozygosity By Locus (inbreeding estimate)

	Acres	Allelic Richness	Effective Allelic Diversity	Ho	He	F_{is}	HL
Garden	4600	6.44	4.5	0.676	0.7744	0.19	0.25
High	3600	5.53	4.1	0.492	0.778	0.478	0.59
Hog	2300	4.14	3.3	0.455	0.67	0.33	0.52

Table 5. Individual mice were divided into groups based on their infection, magnitude of PHA response, observed heterozygosity (HO) and level of inbreeding (HL). The groupings were also done within each island.

	Entire Population	Garden	Hog	High
INFECTED	0: n=19 1: n=33	0: n=7 1: n=11	0: n=9 1: n=13	0: n=3 1: n=12
RESPONSE	0: n=26 1: n=26	0: n=12 1: n=6	0: n=7 1: n=15	0: n=7 1: n=5
HL CATEGORY	1: n=12 2: n=24 3: n=14	1: n=12 2: n=5	2: n=5 3: n=8	1: n=5 2: n=11 3: n=6
HO CATEGORY	1: n=6 2: n=24 3: n=9 4: n=4	2: n=6 3: n= 2 4: n=9	1: n=2 2: n=7 3: n=2 4:n=2	1: n=4 2: n=11 3: n=5 4: n=2

Table 6. Prevalence, median intensity and mean intensity of total parasite infection and individual types of parasites for each island population. Values were calculated for total endoparasite infection and separately for each time of endoparasite. Prevalence but not intensity was calculated for each type of hematozoan.

Island	Prevalence	Median Intensity	Mean intensity
Garden-EndoParasites	70.6%	2.5	3.67
High-Endoparasites	53.8%	5	5.43
Hog-Endoparasites	36.4%	1.5	1.63
Garden-Nematodes	47.1%	2	1.88
High Nematodes	23.1%	4	3.67
Hog Nematodes	18.2%	1	1
Garden-Cestodes	35.3%	1.5	1.5
High- Cestodes	46.2%	2.5	4
Hog-Cestodes	13.6%	1	1.33
Garden-Trematodes	17.6%	2	2
Hog-Trematodes	9%	1	1
Garden-*Coccidia*	5.8%	1	1
High- *Coccidia*	15.3%	1	1
Garden-*Giardia*	5.8%	1	1
High-*Giardia*	5.8%	1	1
Garden-*Plasmodium*	13%		
High-*Plasmodium*	46%		
Hog-*Plasmodium*	8.1%		
Garden-*Trypanasome*	26%		
Hog-*Trypanasome*	9.1%		
Garden-*Babesia*	15%		

Appendix II: Figures

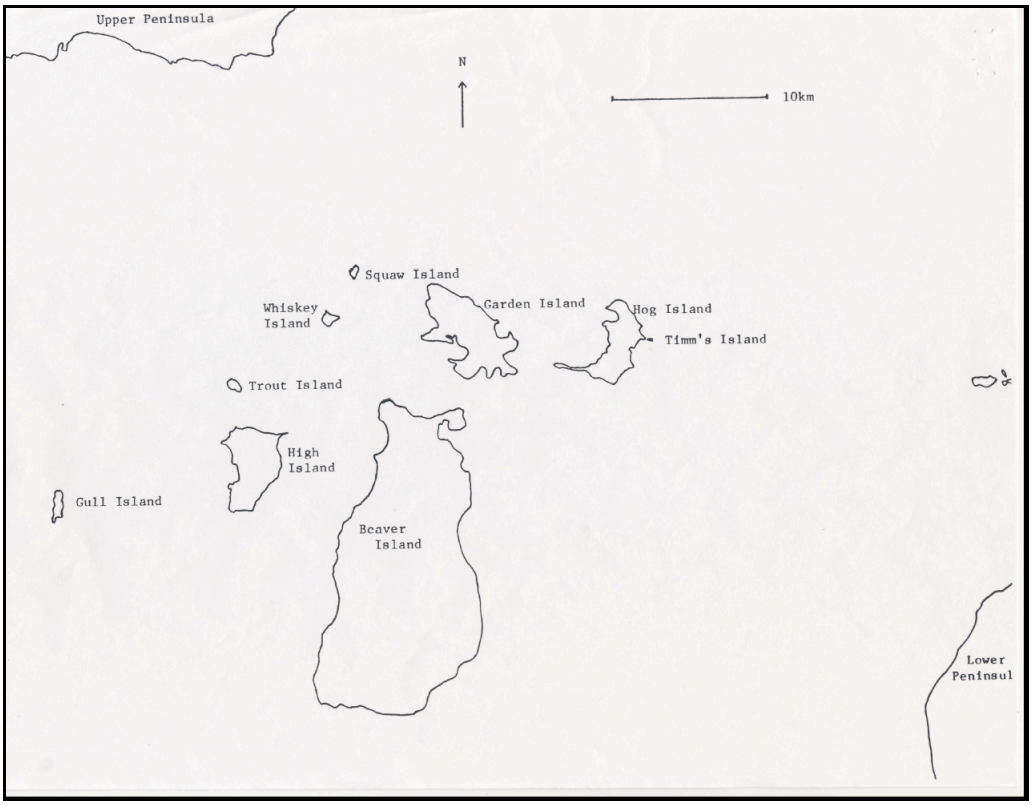

Figure 1. The Beaver Island Archipelago is located in the northeastern tip of Lake Michigan. All islands are approximately the same distance from Beaver Island and from the shore. Garden Island is 4600 acres, High Island is 3600 acres and Hog Island is 2300 acres. The figure is taken from an unpublished appendix prepared by Dr. Philip Myers.

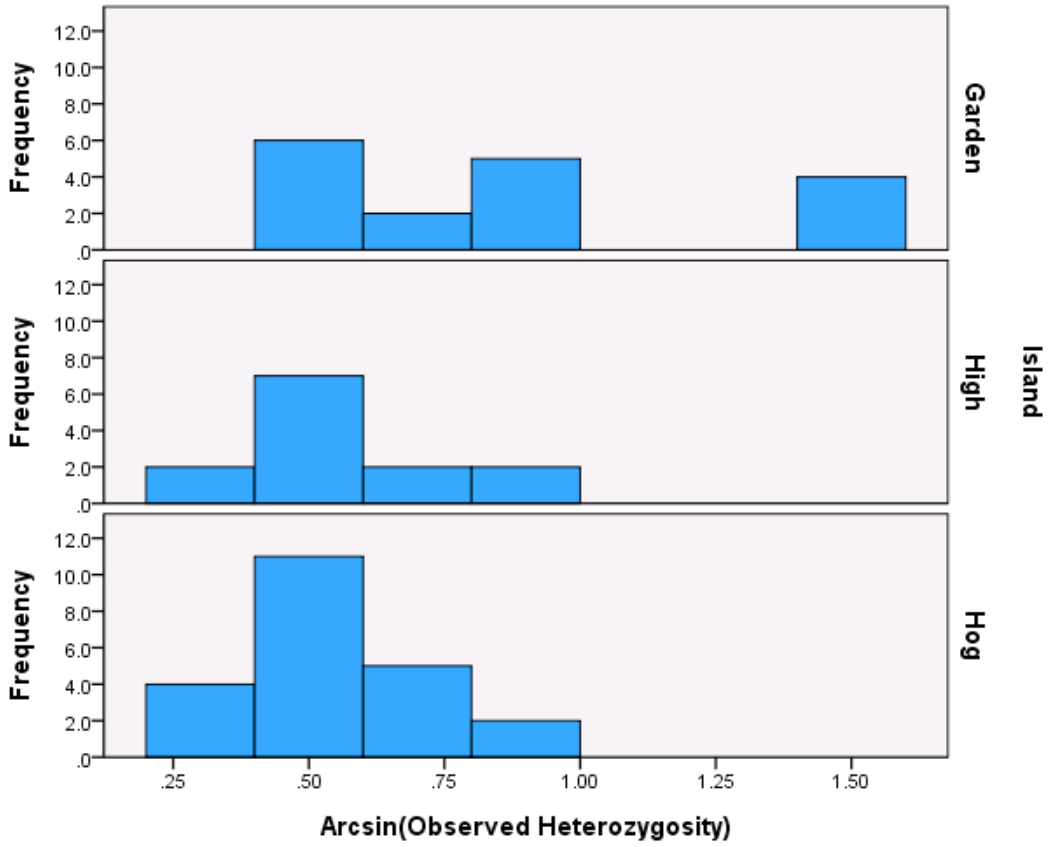

Figure 1. The distribution of diversity within each island population. Larger values represent individuals that have higher proportions of heterozygous loci. Note that the Garden Island population has wider distribution than the other two populations. Additionally, the High and Hog Island populations had higher frequencies of homozygous individuals than the Garden Island population

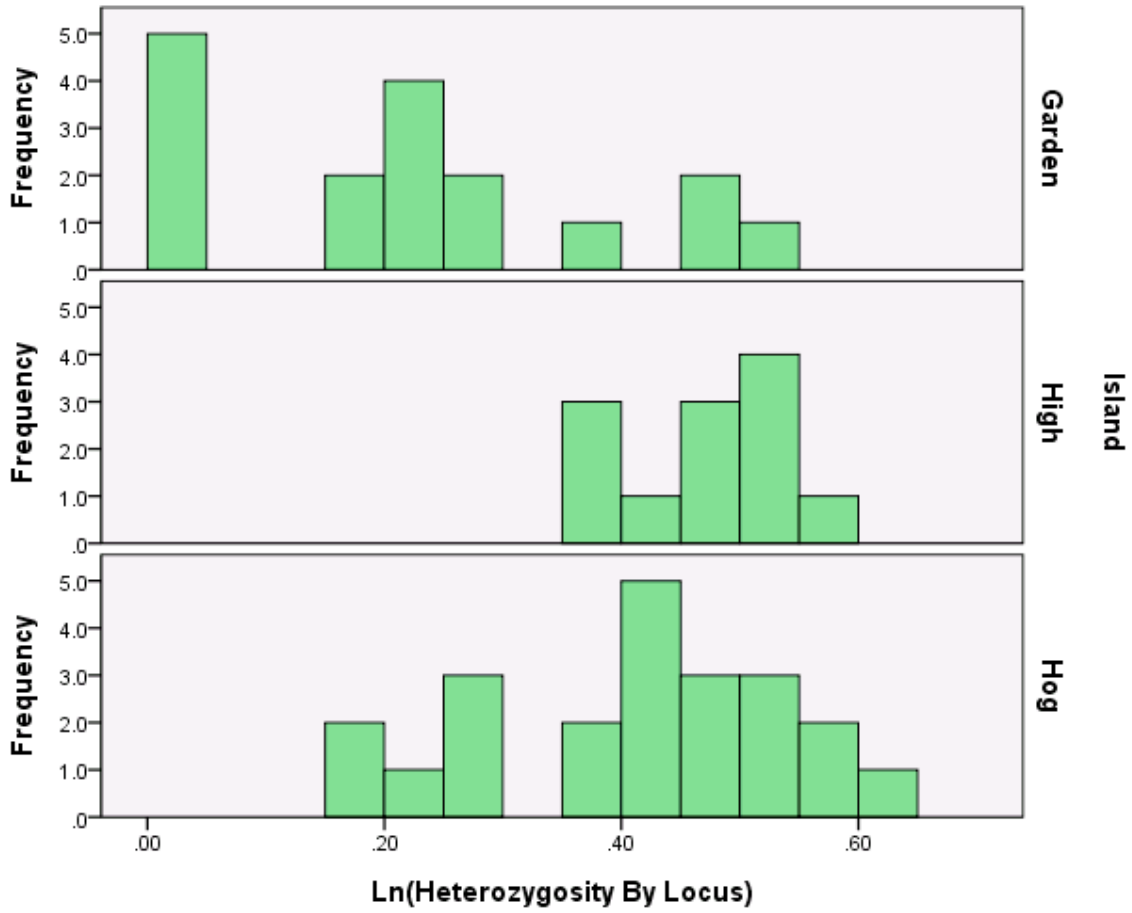

Figure 2. Distribution of inbreeding levels within each island population. Values of Heterozygosity By Locus closer to one indicate higher levels of inbreeding. Note that the Garden and Hog Island populations have a range of individuals exhibiting varying degrees of inbreeding while the majority of High individuals are highly inbred.

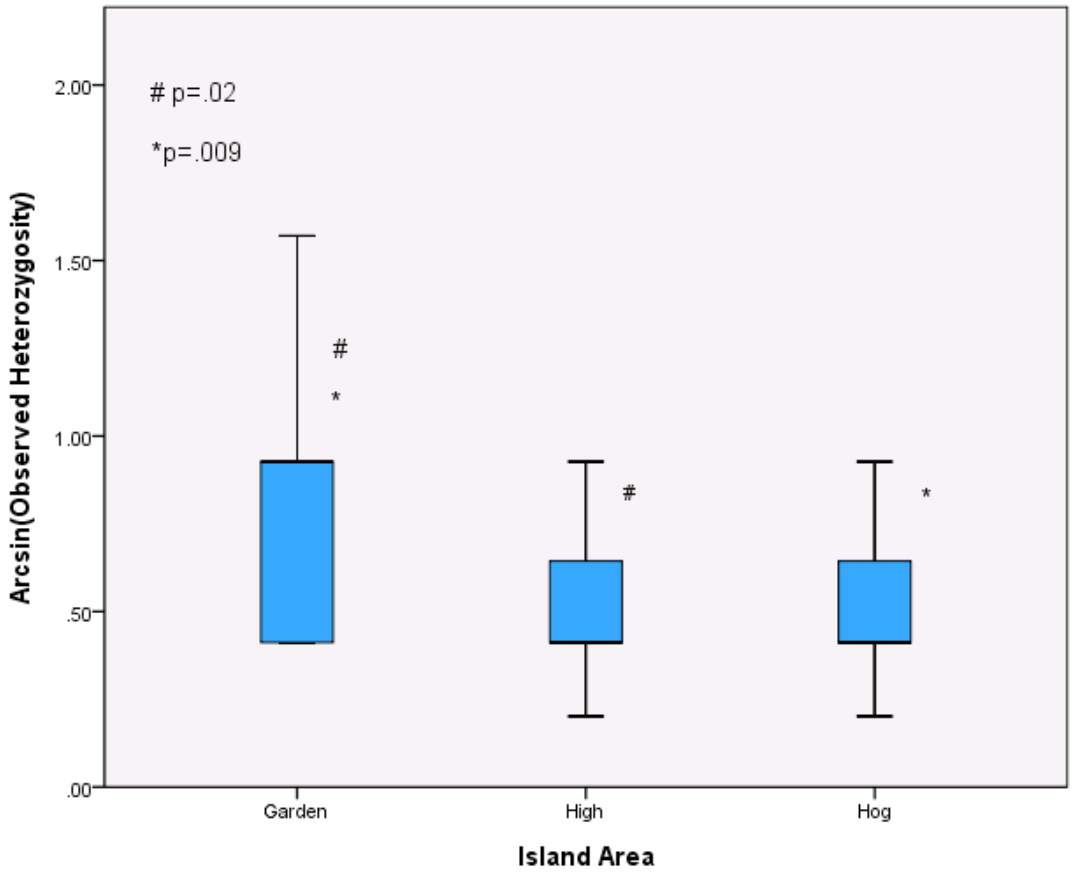

Figure 3. The range of genetic diversity within each island population. As expected, genetic diversity generally decreased with island area but there was only a small difference in observed heterozygosity between the High and Hog Island populations.

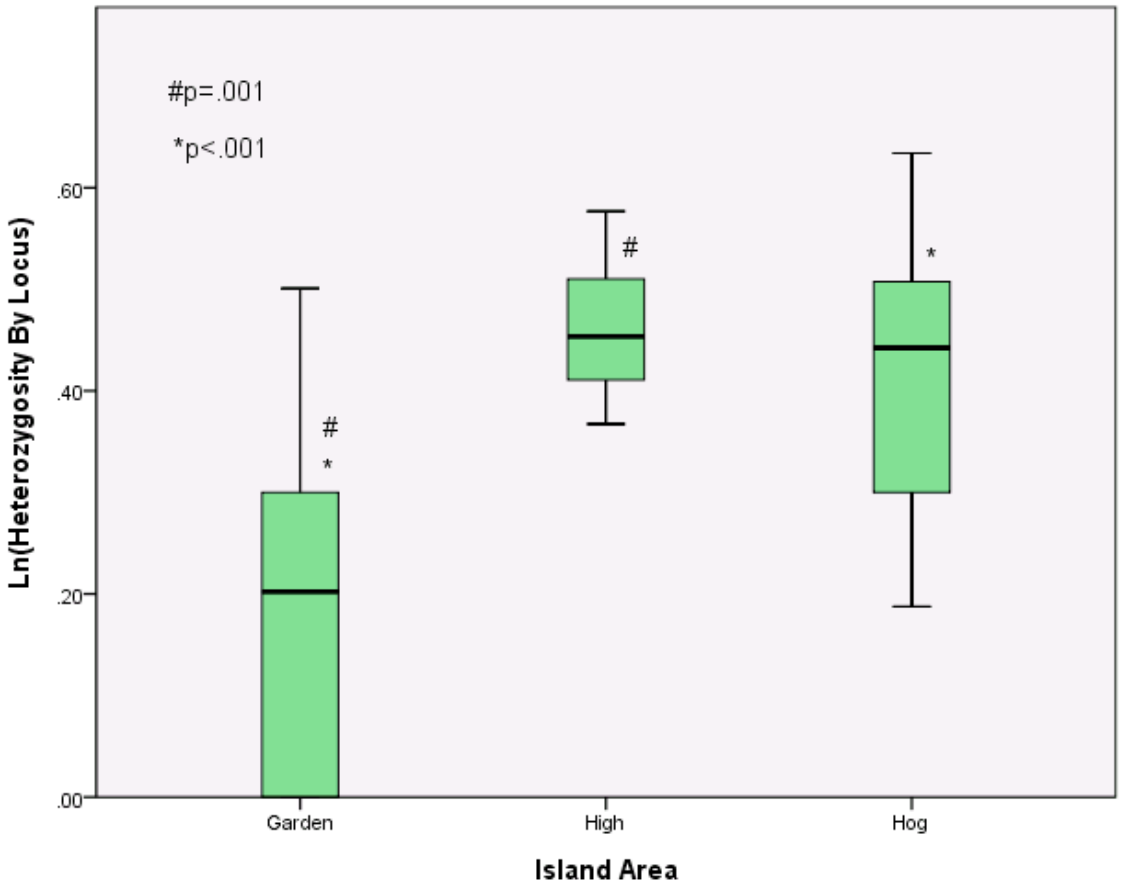

Figure 4. The range of inbreeding levels within each population. The High Island population has much less variation around the mean than the other two populations; the Hog and Garden Island populations have a range of individuals with varying degrees of inbreeding while the High Island population has a more narrow range.

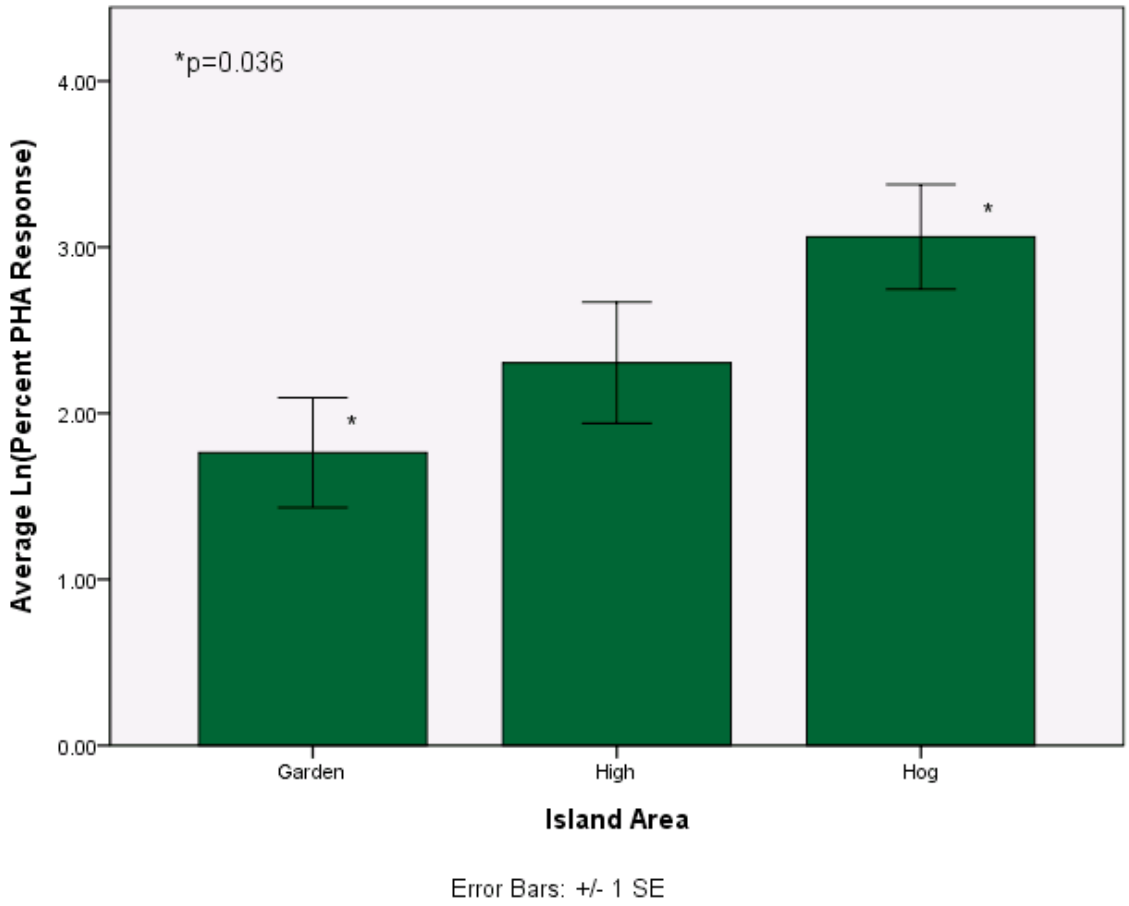

Figure 5. On average, the magnitude of response to PHA decreased as island size decreased. However, only the Garden Island population and the Hog Island population were significantly different (p=0.036).

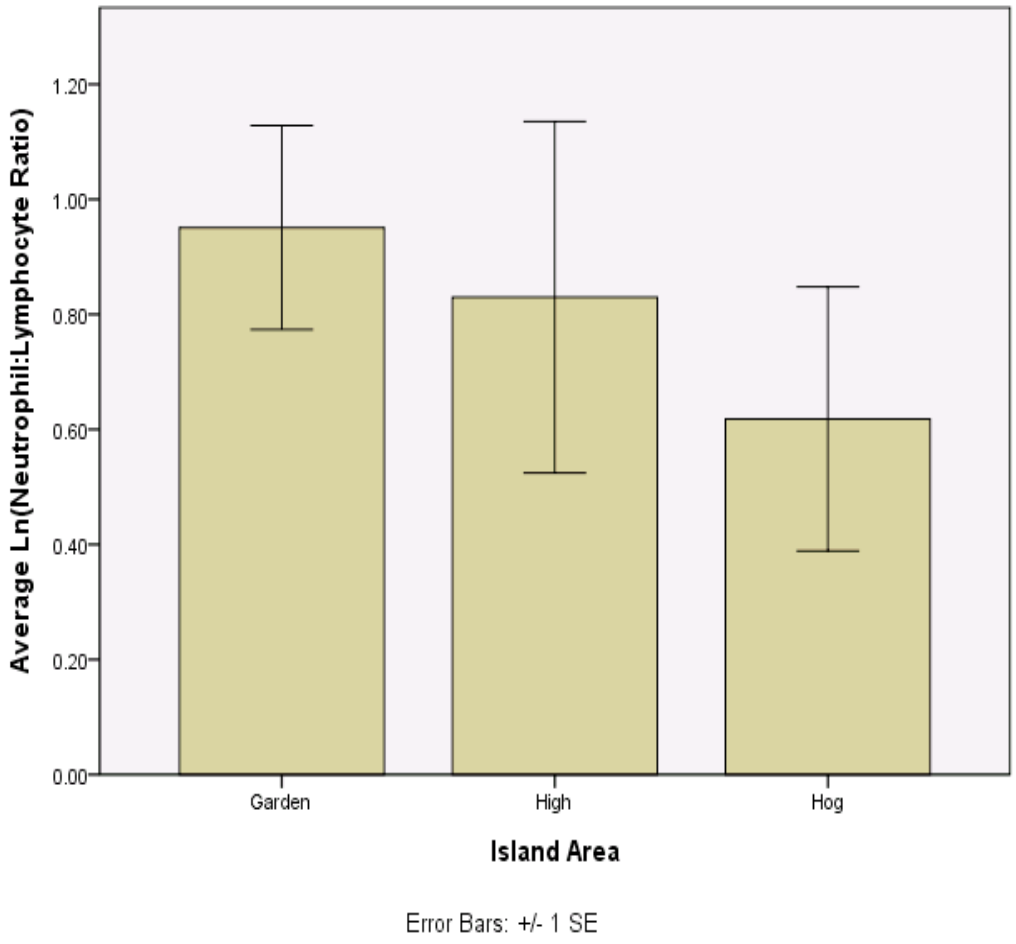

Figure 6. Neutrophil:lymphocyte ratios, which are a measure of stress, generally decreased with island size. The Garden Island and High Island populations were closer in value than the Hog Island population.

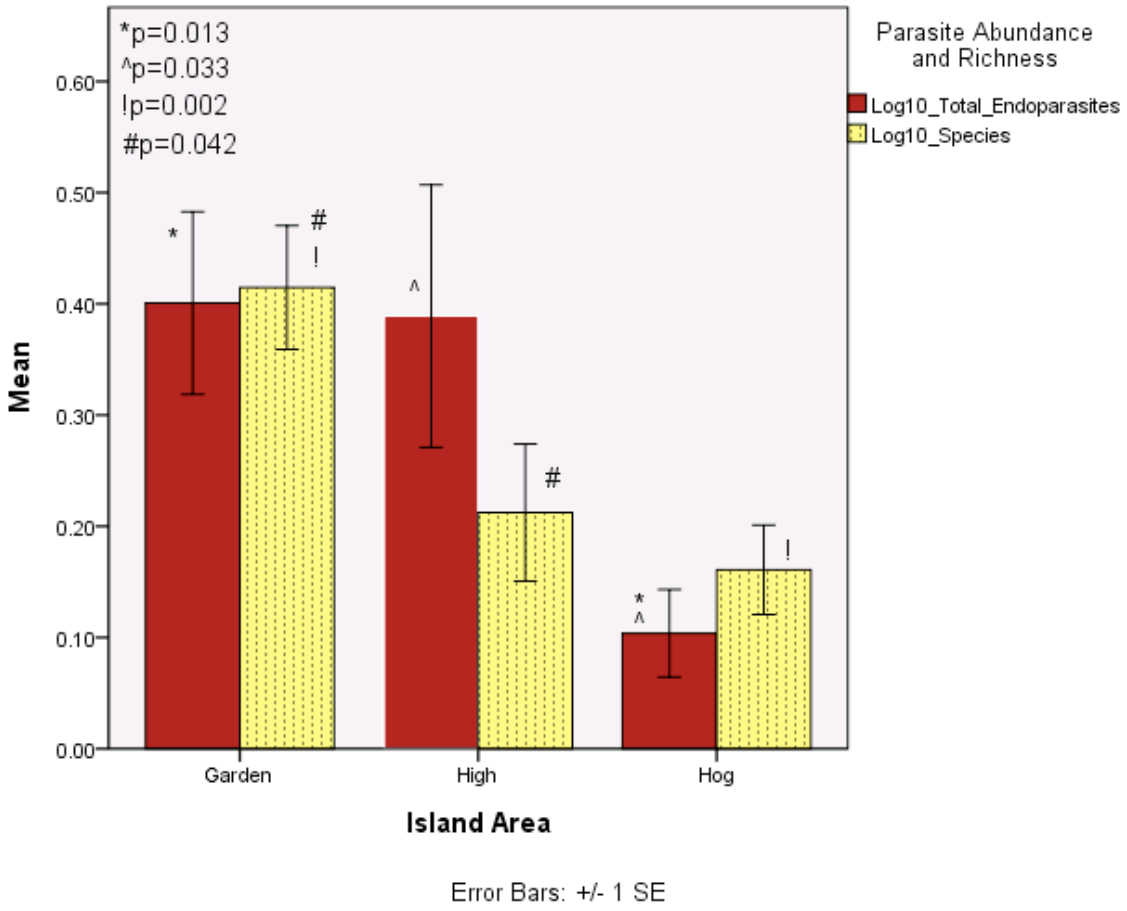

Figure 7. As expected, parasite species richness (yellow, spotted bar) decreased with island size. Unexpectedly, the High Island population had similar parasite abundance (red bar) to the Garden Island population. Please note that parasite species richness refers to both blood parasite and endoparasite species while parasite abundance only refers to endoparasites.

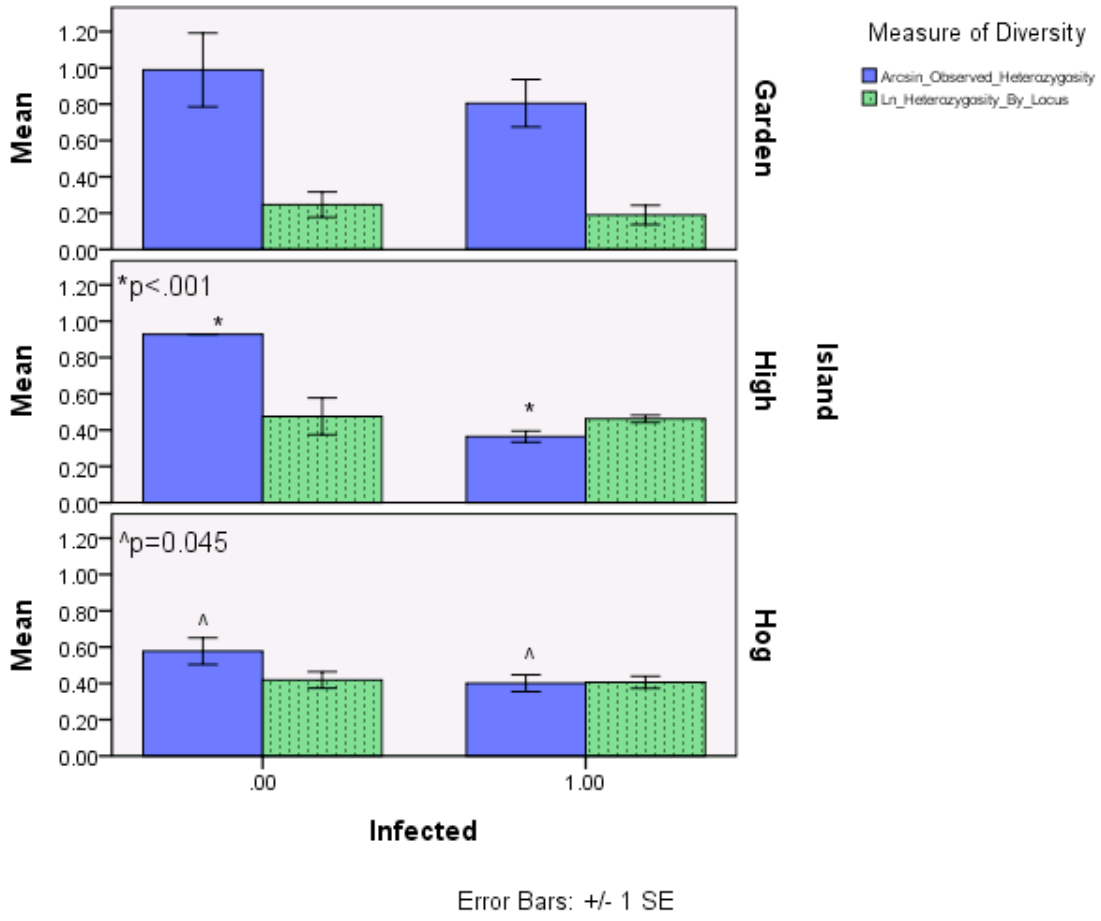

Figure 8. There were no differences between infected and non-infected individuals in level of inbreeding (green, spotted bar) within or across populations. However, more homozygous (blue bar) individuals tended to be infected and this trend was observable within populations as well. One denotes infected individuals and zero denotes uninfected individuals.

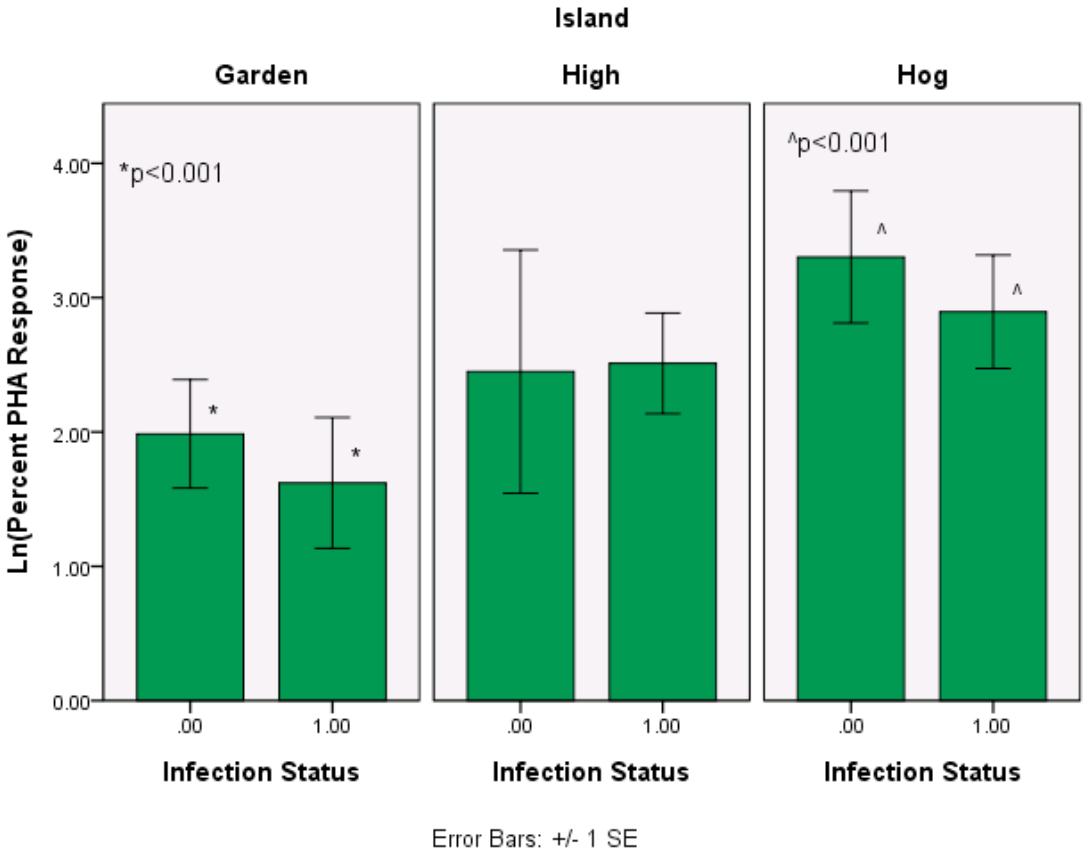

Figure 9. Magnitude of PHA response was decreased in parasite infected individuals compared to uninfected individuals on Garden Island and Hog Island but there was no difference within High Island. One denotes a response to PHA greater than 15% and a zero denotes a response less than 15%.

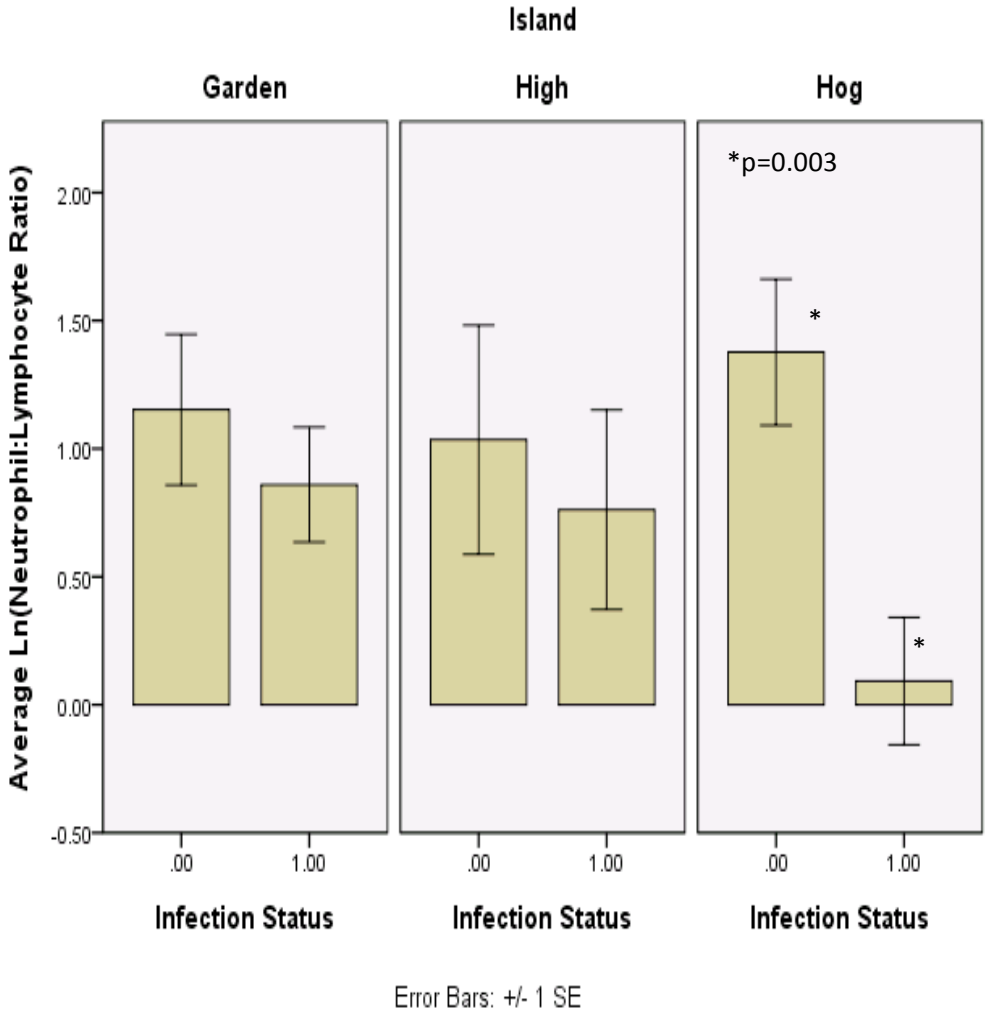

Figure 10. Infected individuals tended to have lower neutrophil:lymphocyte ratios within each island population. Zero denotes an uninfected individual and one denotes an infected individual.

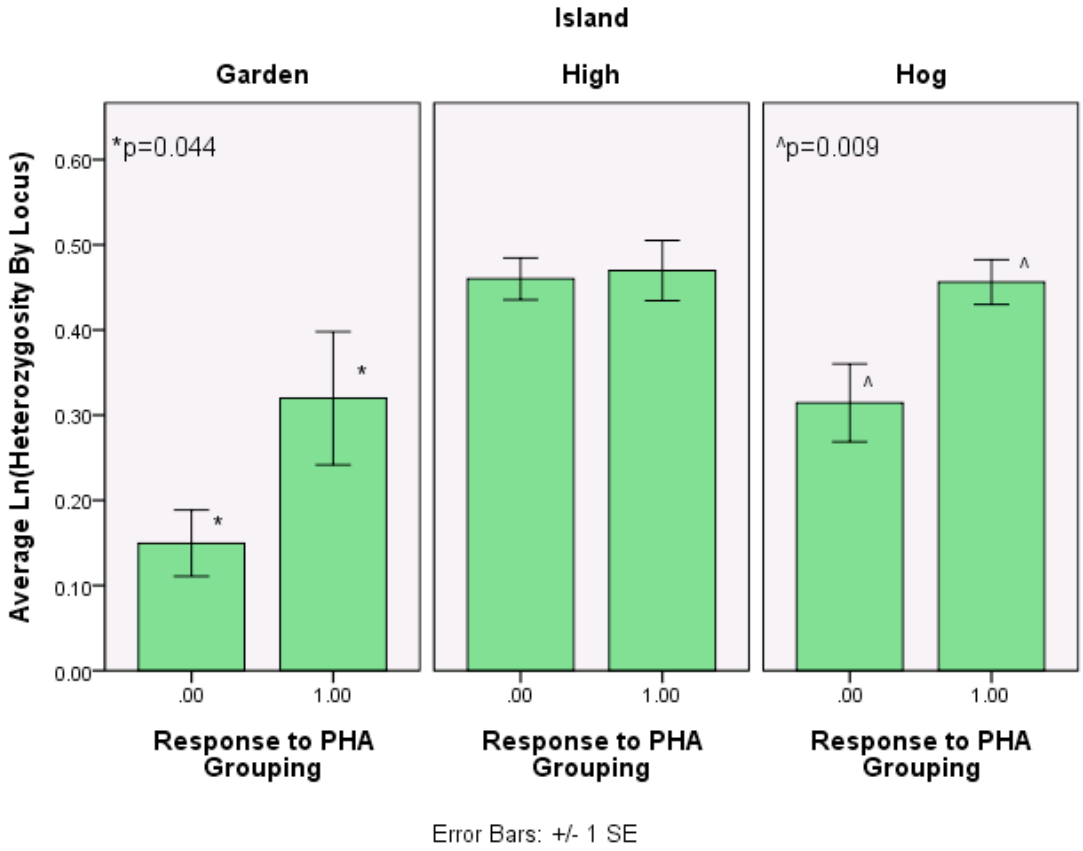

Figure 11. Within the Garden and Hog Island populations, individuals that had a high response to PHA were more likely to be inbred. No such pattern existed within the High Island population. One denotes a response to PHA greater than 15% and a zero denotes a response less than 15%.

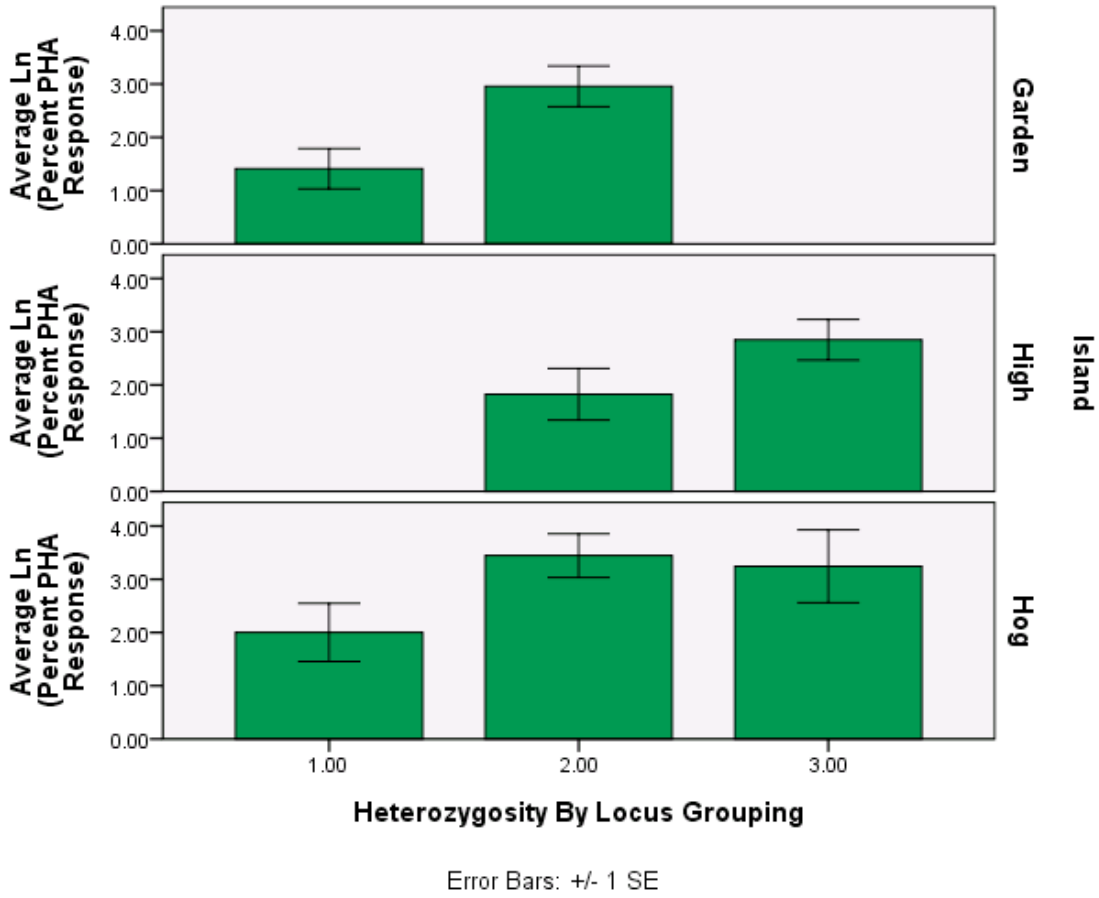

Figure 12. Inbred individuals had higher PHA responses within every island population. However, the difference was less extreme within the High Island population. The Heterozygosity Locus grouping categorized individuals on their level of inbreeding. One denotes less inbred individuals with Heterozygosity By Locus values from 0-.3, two denotes individuals with Heterozygosity By Locus values from 0.33-0.66 and three denotes highly inbred individuals with Heterozygosity By Locus values from 0.66-1.

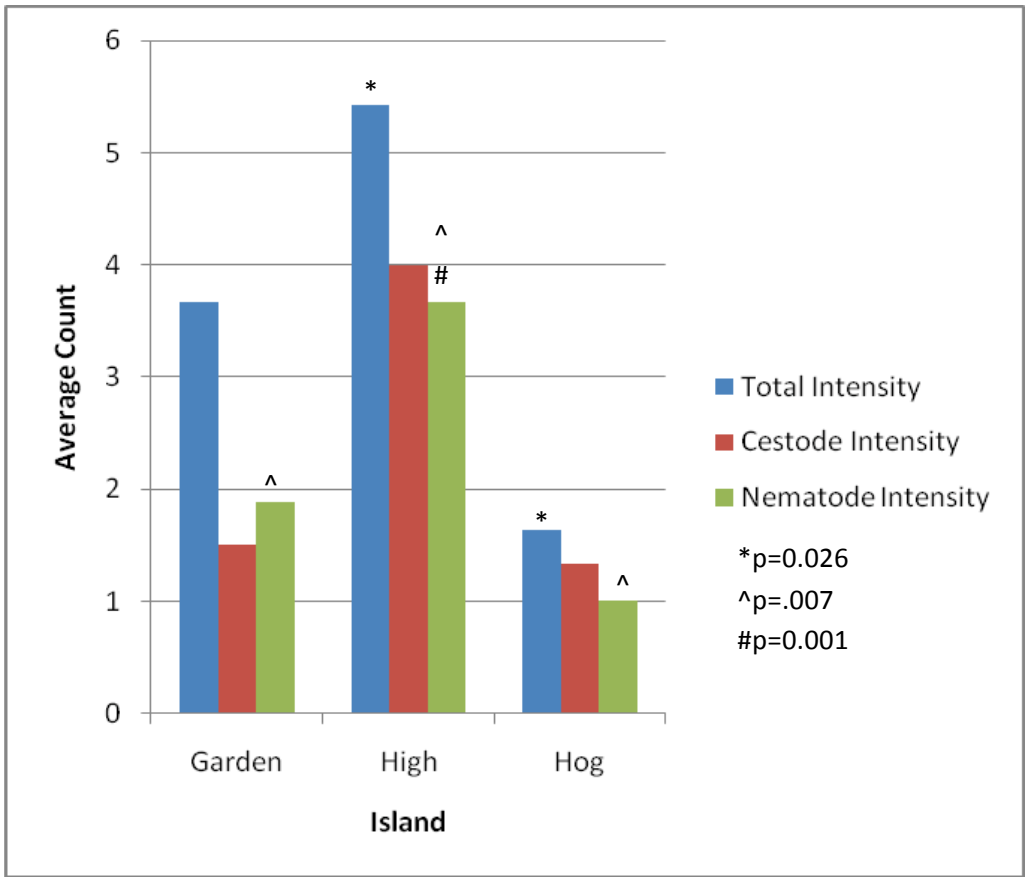

Figure 13. Endoparasite intensity of infection was highest in the High Island population and lowest in the Hog Island population. These trends were reflected in the average intensity of cestode and nematode infections though only the difference in nematode infection was significant.

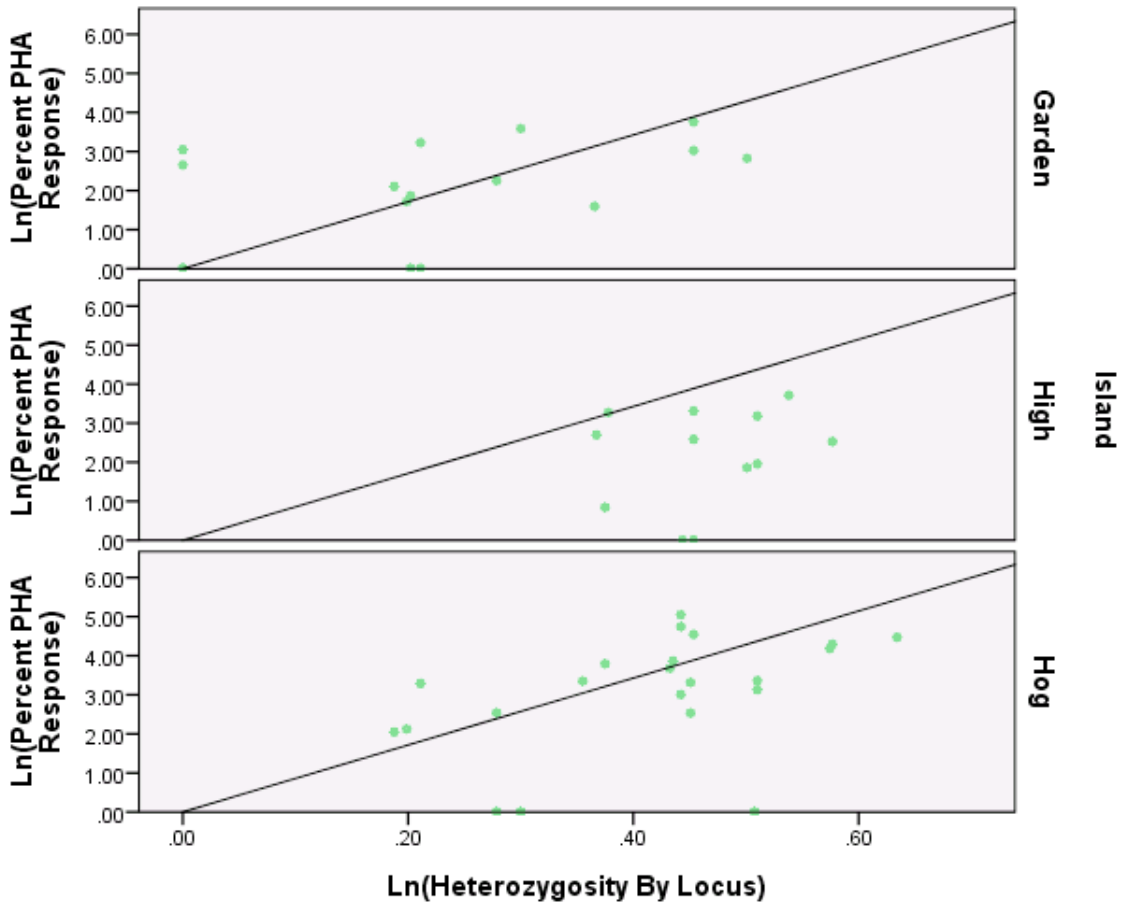

Figure 14. When all genetic markers are used in calculations of Heterozygosity By Locus, there is a positive relationship between degree of inbreeding and magnitude of PHA response. The strength of this relationship is strongest in the Hog Island and the Garden Island population and weakest in the High Island population

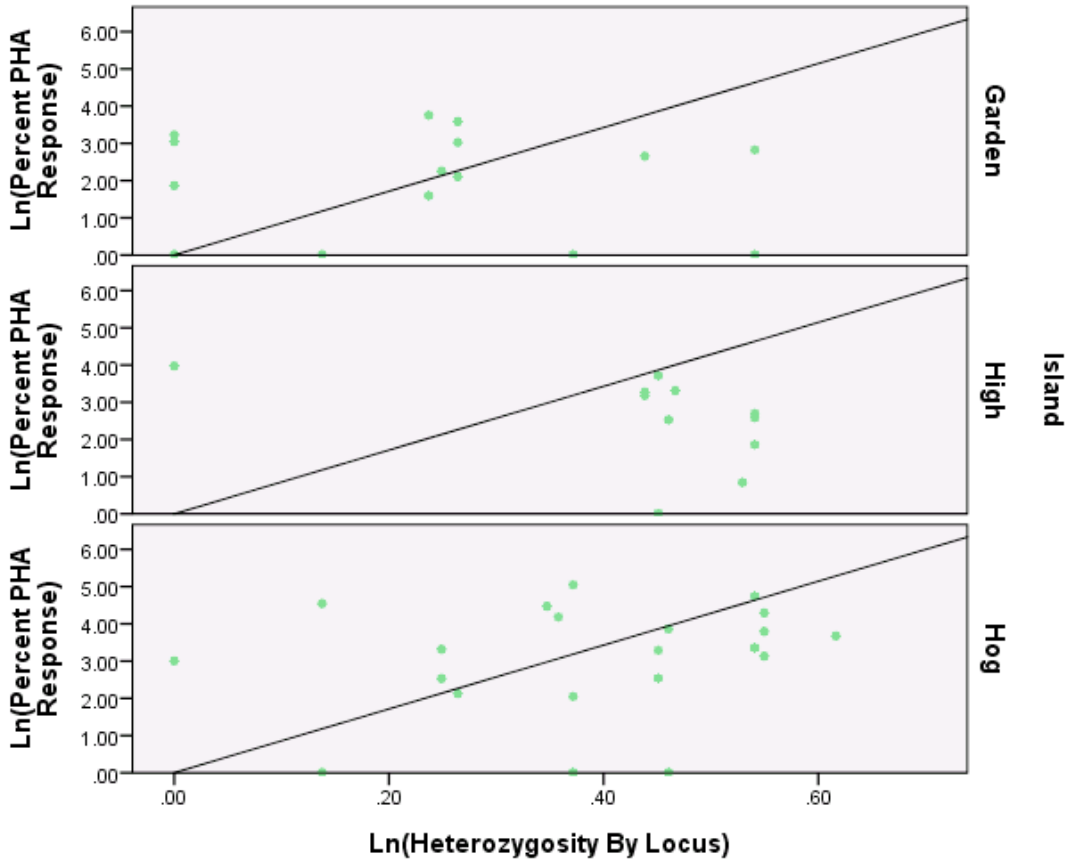

Figure 15. Calculation of Heterozygosity By Locus excluding Pm101 reduces the positive relationship between inbreeding and PHA response. The change in relationship is most extreme in the Garden Island population and least extreme in the Hog Island population.

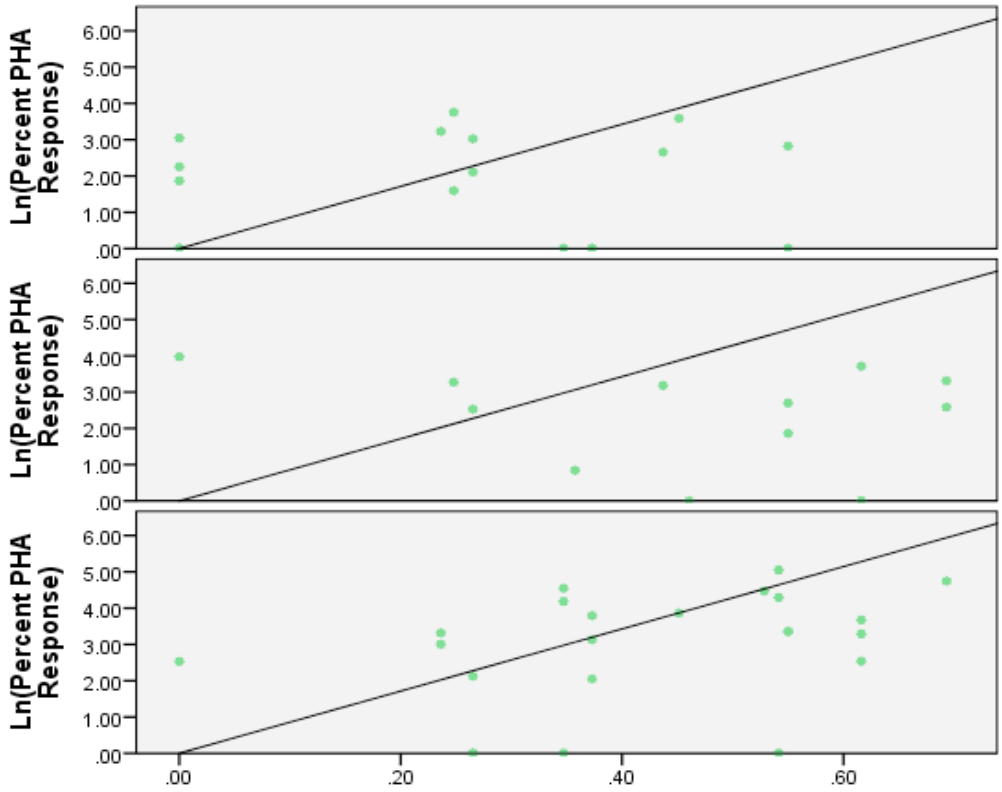

Figure 16. Calculation of Heterozygosity By Locus excluding Pm104 reduces the positive correlation between degree of inbreeding and PHA response within each population.

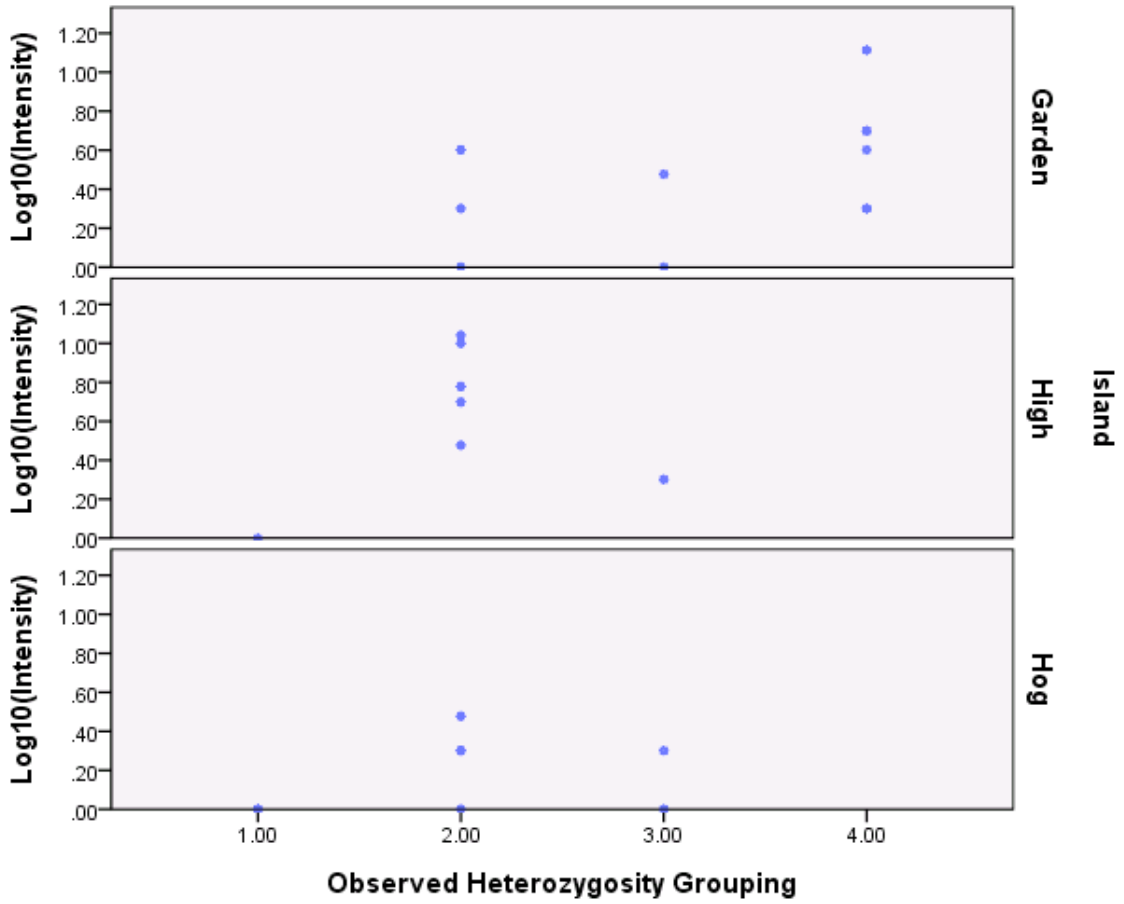

Figure 17. Intensity of endoparasite infection was higher in heterozygotes within the Garden Island population. Intensity of endoparasite infection was higher in homozygotes in the High and Hog Island populations. The observed heterozygosity grouping categorized individuals on the basis of their heterozygous loci proportions. One denotes highly heterozygous individuals with observed heterozygosity ranging from 0-0.25, two denotes individuals with observed heterozygosity ranging from .26-0.5, three denotes individuals with observed heterozygosity ranging from 0.51-.0.75 and four denotes highly homozygous individuals with observed heterozygosity ranging from 0.76-1.

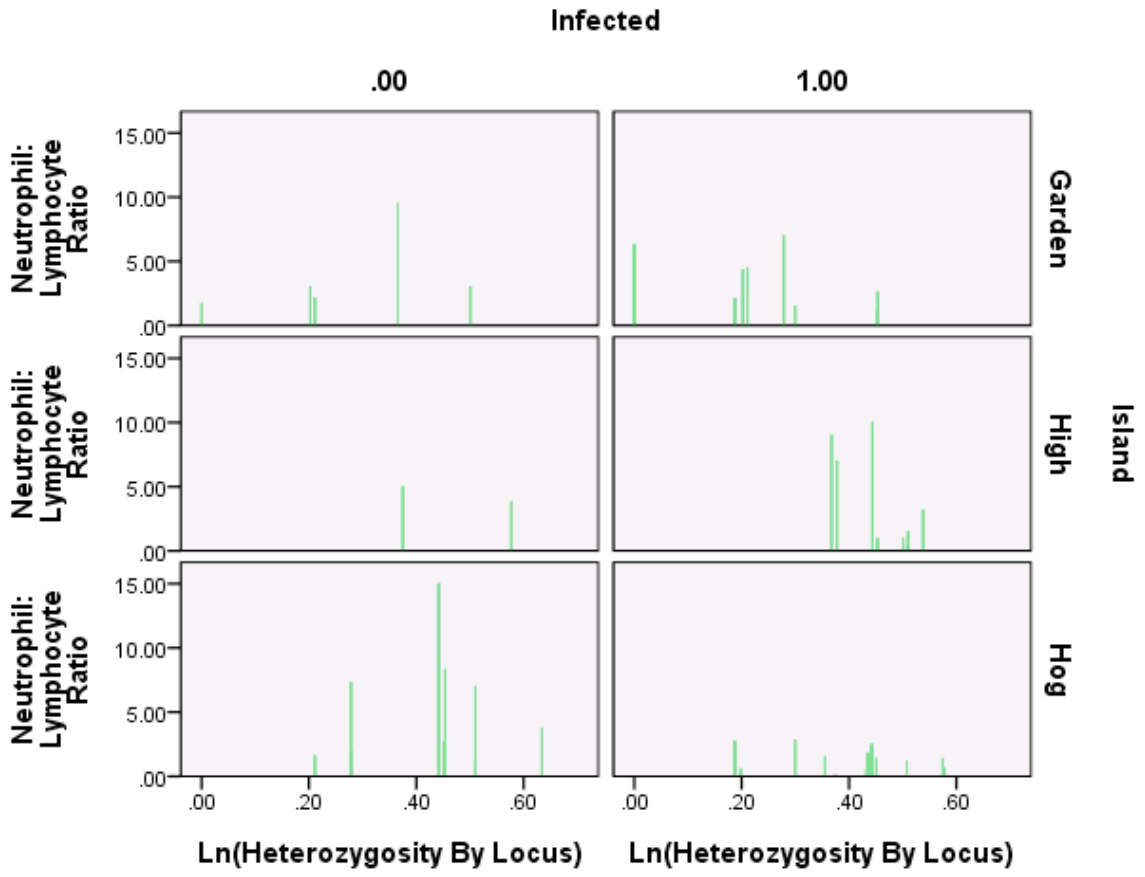

Figure 18. Within the Garden Island population, there is little relationship between inbreeding, infection status and neutrophil:lymphocyte ratios. Within the High Island population, inbred infected individuals tended to have higher neutrophil:lymphocyte ratios. Within the Hog Island population, inbred, uninfected individuals had higher neutrophil:lymphocyte ratios. Zero denotes an uninfected individual and one denotes an infected individual.

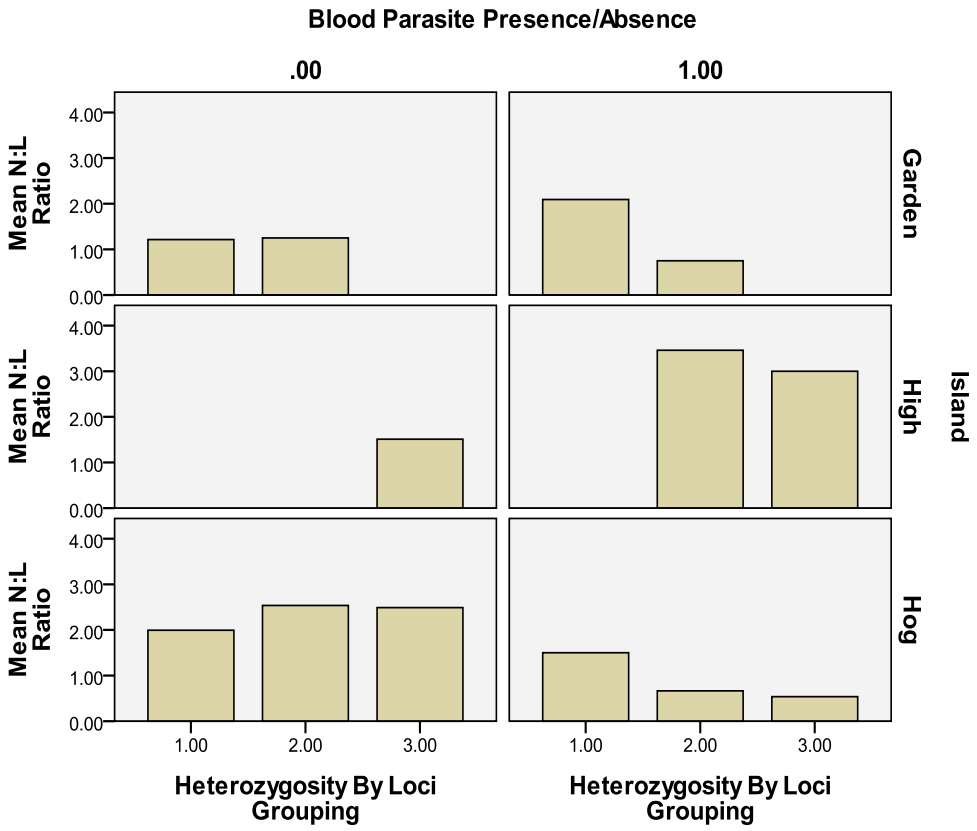

Figure 19. There is little relationship between inbreeding, infection with a hematozoan and neutrophil:lymphocyte ratio within the Garden Island population. Within the High Island population, inbred individuals infected with a hematozoan tended to have higher neutrophil:lymphocyte ratios. Within the Hog Island population, there was little relationship with inbreeding and neutrophil ratios but individuals infected with hematozoan had lower neutrophil:lymphocyte ratios.