Infection Dynamics of Herpesvirus in Gopher Tortoises

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INFECTION DYNAMICS OF HERPESVIRUS IN GOPHER TORTOISES

by

JOANNE SALDANHA

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Biology in the College of Sciences and in the Burnett Honors College at the University of Central Florida Orlando, Florida

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Thesis Chair: Dr. Anna Savage
ABSTRACT

_Gopherus polyphemus_, commonly known as the Gopher Tortoise, is a dryland reptile native to the southeastern United States. It is commonly a resident of longleaf pine and dry oak sand hill habitats. It is considered a keystone species because they dig deep burrows that provide shelter to them as well as many other animals. Habitat loss, fragmentation, and disease are major threats and have caused this species to be federally listed as a threatened species under the Endangered Species Act (ESA). Disease is a major threat to the gopher tortoise’s survival, and with declining populations, the need to investigate pathogens is crucial. Herpesvirus, is known to contribute to upper respiratory tract diseases (URTD) in _G. polyphemus_ and is the primary focus of this project. Due to high mutation rates in the virus, a modified version of PCR, nested PCR, was conducted on eye and nose swabs and blood samples obtained from _G. polyphemus_ to detect the presence of the alpha herpesvirus pathogen. The positive samples were then sent for genetic sequencing to confirm the occurrence of the pathogen. The detectability of Herpesvirus in eye and nose swabs was compared to blood and lymph samples and statistical tests concluded that both sample types had the same detectability.
DEDICATIONS

To my parents, for supporting and inspiring me to follow through with my vision to make the world a better place.

To my friends, especially Irfan Parkar and Net Galt for encouraging and believing in me every step of the way.

To my thesis chair, Dr. Anna Savage, for her dedication to my work.

To everyone trying to protect endangered species and correct the environmental disasters the human race has subjected Mother Earth to.
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I would also like to thank the everyone in the Savage lab who helped train me. Especially, Johnathan Napier who collected samples and worked through the analysis of the samples with me.

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INTRODUCTION

_Gopherus polyphemus_: A keystone species

Native to the diverse, yet fragile long leaf pine habitat in the United States, the gopher tortoise _Gopherus polyphemus_, a dry-land reptile, is an environmental keystone species (Johnson et al., 2017) known to dig burrows that range between 4.6 m long and 2 m deep (Dziadzio et al., 2016). Longleaf pine ecosystems are distinguished by “pine barrens” that dominate the mosaic of forests, woodlands and savannahs they include (Landers et al., 1995). In a habitat dependent on fires for seed dispersal, these burrows provide shelter to their engineers-the gopher tortoise- as well as a plethora of other organisms, including mammals, amphibians, birds, reptiles, insects, etc. Some common occupants of the gopher tortoise burrow comprise of the gopher frog _Rana capito_, indigo snake _Drymarchon couperi_, and cotton mouse _Peromyscus gossypinus_ (Dziadzio et al., 2016). A threat to the survival of the gopher tortoise thus results in a domino effect that threatens the survival of the inhabitants of the gopher tortoise’s burrows.

Behavior and mating patterns of _Gopherus polyphemus_

Adult gopher tortoises have been observed to congregate in breeding colonies in spring, especially in drought prone areas. During the mating season, which ranges from spring to summer, females have been observed to be more sedentary than males, with males actively looking for mates (McRae et al., 1981). Mating also greatly affected the burrow preferences and movements of males, as they would preferably seek burrows occupied by a female and move between mates in different burrows (McRae et al., 1981). On average, males used seven
different burrows and females used four different burrows. Males and females rarely cohabitated in burrows overnight. Juveniles have been observed to typically moved short distances compared to adult, and only used 1 or 2 burrows (McRae et al., 1981). Juveniles usually stay within the vicinity of breeding colonies during their earlier years, but move away from breeding colonies before or during their years as sub-adults. This behavior is an evolutionary adaptation to add young adults to sparsely populated breeding colonies and for the formation of new breeding colonies (McRae et al., 1981). Due to their social behavior, gopher tortoises can easily transmit diseases between populations. Movement patterns of juveniles exposes them to diseases present in adults and their movement away from breeding colonies makes them good carriers of pathogens and provides a larger range for the spread of disease. Gopher tortoises are a late-maturing, long-lived species and the effect of diseases like upper respiratory tract disease (URTD) reduces adult survival (Ozgul et al., 2009). Coupled with environmental stressors, disease can severely influence the population dynamics. (Jacobson, 1994).

**Major threats to the survival of* Gopherus polyphemus***

Anthropogenic activities have led to changes in the natural environment across several ecosystems. These changes include, but are not limited to, habitat fragmentation, and habitat loss for several organisms across various ecosystems; leading to the endangerment and extinction of a myriad of organisms. Disease coupled with anthropogenic activities has resulted in the classification of the gopher tortoise as a threatened species (Stapleton, 2011) under the Endangered Species Act (ESA) and as a vulnerable species in the International Union for Conservation of Nature (IUCN) Red List (Smith et al., 2009).
Alpha herpesvirus and Upper Respiratory Tract Diseases (URTD)

Amongst the multitude of disease causing organisms that infect the gopher tortoise, alpha herpesvirus (referred to as “herpesvirus” here on) was the focus of this study, which belongs to the order Herpesvirales. The family Herpesviridae is among three families that share structural, genetic, and biological properties, but infect a number of different hosts. Herpesviridae can be further divided into subfamilies alpha herpesvirinae, beta herpesvirinae, and gamma herpesvirinae. Distinct from the evolutionary lineage of the beta herpesviruses and gamma herpesviruses, which can be traced back to mammalian and avian infections, the subfamily alpha herpesvirinae contains enveloped, double-stranded viruses. (Gander et al., 2015). A characteristic feature of these viruses is that they cause necrotizing stomatitis across several tortoise genera, including Gopherus (Gander et al., 2015).

Clinical signs of infection in Gopherus polyphemus include necrotizing lesions of the oral mucosa (Une, et al., 1999), diphtheritic plaques on the dorsal surface of the tongue and hard palate, nasal discharge (Origgi et al., 2004), and high mortality rates (Johnson et al. 2005). Mediterranean tortoises (Testudo spp.) with herpesvirus infections have been reported to show signs of central nervous system disease (Martel et al., 2009).

While a single cause of URTD has not been successfully identified, several known factors have been identified as contributing agents. Environmental factors like stress, decreased food availability, capture, and relocation can result in nasal and oral discharges, palpebral edema (Wendland et al. 2009), which are all symptoms of Herpesvirus infections. Coinfections of Mycoplasma and Herpesvirus can lead to symptoms of URTD symptoms. (Jacobson et al. 2014). A weakened immune system can also lead to infection by Ranavirus which has been linked to
Gopherus polyphemus populations declines (Johnson et al., 2010). Despite this, the need to study the correlation between Herpesvirus infections and symptoms of URTD, is pressing.

**Sample type vs. Detectability**

Reptiles, including Gopherus Polyphemus, have nucleated red blood cells thus making them susceptible to viral infections (Taylor et al., 1982). Acute infections may involve rapid replication of the virus, allowing it to be detected in bodily fluids, however most acute infections show clinical symptoms before the virus can infect the blood and antibodies can be produced. Thus, an individual showing clinical signs of URTD may not have viral presence in the blood (McArthur et al., 2002). While blood can show the history of latent infections, swabs show the presence of active infections (Soares et al., 2004). The detectability (sensitivity and specificity) of swabs and blood samples have been studied and show that detectability of swabs is higher than blood samples, when analyzed using nested PCR, making diagnosis more accurate (Grange et al., 2012). This study involves the analysis of both blood samples as well as swabs and compares the effectiveness of nested PCR for both.

**Nested PCR:**

Herpesviruses are known to have high mutation rates and evolve quickly (VanDevanter et al. 1996), making the use of degenerate primers essential in this study. Degenerate primers have binding sites that consists of positions which can be occupied by any nucleotide base pair, allowing the primer to bind to quickly mutating regions of the viral genome (Iserte 2013). However, degenerate primers are equally likely to amplify the host genome. This problem is resolved by using two consecutive rounds of PCR, to reduce the chance of amplifying the host
genome to a statistically insignificant value. Different primer binding sites result in different amplicon sizes produced as a result of different primer combinations. The figure below (VanDevanter et al. 1996) shows the different primer combinations and binding sites of the five primers used in nested PCR (Appendix).

Figure 1: Nested PCR and primer binding sites. VanDevanter et al. 1996
HYPOTHESIS AND OBJECTIVE

Objective

Grange et al., (2012) concluded that nose and eye swabs have higher detectability (sensitivity and specificity) when analyzed using nested PCR compared to blood/lymph samples. Using the blood, lymph, and eye and nose swabs collected by Johnathan Napier, a master’s student in the Savage Lab in the Department of Biology at the University of Central Florida, Orlando, as part of his thesis project, I detected the presence of herpesvirus and compared detectability using swabs and tissue samples.

Hypothesis

I hypothesize that the detectability of Herpesvirus is greater in eye and nose swabs compared to blood and lymph samples, using nested PCR.
MATERIALS AND METHODS

DNA Extraction

Using DNeasy blood and tissue kits (Qiagen), DNA was extracted from blood and nasal/eye swabs using the following protocol.

Blood and/or lymph samples

Twenty μl proteinase K, 190 μl PBS, 10 μl of the sample and 200 μl Buffer AL were added to a 1.5 ml microcentrifuge tube, vortexed and incubated at 56°C for 10 min. 200 μl ethanol (96–100%) was added to the microcentrifuge tube and mixed thoroughly by vortexing. The mixture was added to a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at ≥ 6000 x g (8000 rpm) for 1 minute. The flow through and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 μl Buffer AW2 was added and centrifuged at ≥ 20,000 x g (14000 rpm) for 3 minutes. The flow through and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 200 μl Buffer AE was added and incubated at room temperature for 1 minute and then centrifuged for 1 minute at ≥ 6000 x g (8000 rpm) to elute a final volume of 200μl.

Eye and nose swabs

The cotton swab was cut, so that minimal plastic remained, into a microcentrifuge tube. 180 μl Buffer ATL and 20 μl proteinase K, were added to a 1.5 ml micro centrifuge tube, vortexed and incubated at 56°C until swab was completely lysed. The tube was vortexed for 15 seconds. Two-hundred μl Buffer AL and 200 μl ethanol (96–100%) were added to the micro
centrifuge tube, and mixed thoroughly by vortexing. The mixture was added to a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at ≥ 6000 x g (8000 rpm) for 1 minute. The flow through and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 μl Buffer AW2 was added and centrifuged at ≥ 20,000 x g (14000 rpm) for 3 minutes. The flow through and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 200 μl Buffer AE was added and incubated at room temperature for 5 minute and then centrifuged for 1 minute at ≥ 6000 x g (8000 rpm) to elute a final volume of 200µl.

Nested PCR

Published protocol

The presence of Herpesvirus was detected using published PCR protocols that targeted conserved coding motifs in DNA-dependent DNA polymerases among alpha-herpesvirus (Van Devanter et al. 1996, Ossiboff et al., 2015). The first round of PCR included 2.5 μl of each degenerate primer DFA, ILK and KG1, 3.8 μl of One Taq buffer, 0.25 μl Taq polymerase, 1.6 μl of 2.5 mM dNTPs, 9.85 μl of molecular grade H₂O to create a master mix of 23 μl. 2 μl of template DNA was added to the master mix to generate a final reaction volume of 25 μl. The second round of PCR included 2.5 μl of each degenerate primer TGV and IYG, 3.8 μl of One Taq buffer, 0.25 μl Taq polymerase, 1.6 μl of 2.5 mM dNTPs, 12.35 μl of molecular grade H₂O to obtain a master mix of 23 μl, to which 2 μl of PCR round 1 product was added to obtain a final reaction volume of 25 μl. The reaction conditions for both rounds were: 95° C for 12 m; 45
cycles at 95° C for 20 s, 46° C for 60 s, and 72° C for 60 s; and a final extension step of 72° C for 10 m. The amplicons were analyzed under 2% agarose gel, using a Hi-Lo ladder in the first well and a negative control (2 μl of DNA replaced by 2 μl molecular grade H₂O) in the last well and visualized under ultraviolet (UV) radiation. The positive amplicons were 200 base pairs in size, as suggested by published PCR protocols (Van Devanter et al. 1996, Ossiboff et al., 2015).

**Revised protocol I**

Round 1 of published PCR protocols (Van Devanter et al. 1996, Ossiboff et al., 2015) was modified to round 1A and included 2.5 μl of each degenerate primer, DFA and KG1, 3.8 μl of One Taq buffer, 0.25 μl Taq polymerase, 1.6 μl of 2.5 mM dNTPs, 12.35 μl of molecular grade H₂O to obtain a master mix of volume 23 μl. 2 μl of template DNA was added to the master mix to obtain a final reaction volume of 25 μl. 2 μl of amplicons from round 1A were used to set up round 2A containing 2.5 μl of degenerate primers DFA and IYG, round 2B containing degenerate primers ILK and KG1 and round 2C containing TGV and KG1. The volume of all other reagents remained the same and the final reaction volume was 25 μl.

Round 1 of published PCR protocols (Van Devanter et al. 1996, Ossiboff et al., 2015) was also modified to round 1B and included 2.5 μl of each degenerate primer, ILK and IYG 3.8 μl of One Taq buffer, 0.25 μl Taq polymerase, 1.6 μl of 2.5 mM dNTPs, 12.35 μl of molecular grade H₂O to obtain a volume of 23 μl, to which 2 μl of template DNA was added. Amplicons from round 1B were used to set up round 2D containing 2.5 μl of degenerate primers DFA and IYG and the same volume of other reagents used in round 1B.
Extension times for each round were also modified to 43 seconds for round 1A, 30 seconds for round 2A, 28 seconds for round 2B, 25 seconds for round 2C, 15 seconds for round 1B and 13 seconds for round 2D. The number of cycles were reduced from 45 cycles to 30 cycles. The amplicons were analyzed under 2% agarose gel, using a Hi-Lo ladder in the first well and a negative control (2 μl of DNA replaced by 2 μl molecular grade H₂O) in the last well and visualized under ultraviolet radiation. The positive amplicons for round 1A were ~700 bp, for round 2A were ~500 bp, for round 2B were ~450 bp, for round 2C were ~ 400 bp, for round 1B were ~250 bp and for round 2D were ~ 200 bp. Due to large smears found in round 2C and 2D, these steps were omitted from further protocols.

Revised protocol II

A temperature gradient ranging from 46°C to 56°C was done for round 1A, 2A and 2B to determine a more accurate annealing temperature. An average of the best two results were used to determine a more accurate result. The annealing temperature was determined to be 52.5° C for round 1A, 55° C for round 2A and 54° C for round 2B. Using this method, the number of multiple bands reduced drastically, but faint double bands were still visible.

Revised protocol III

The high GC contents of the primers was assumed to be an issue, thus various combinations of DMSO (0-0.2 μl), G+C enhanced buffer and high GC Enhancer ranging from 10-20% of the final volume (25 μl) were added. No bands were produced and gels appeared clean.
**Final protocol**

A more precise temperature gradient varying by 0.1° C was run for each round. The final annealing temperatures were determined to be 52.2° C for round 1A, 55.2° C for round 2A and 54.3° C for round 2B. The volume of the master mix and all other protocols remained the same.

**Statistical tests**

*Table 1:* Positives and negatives determined by nested PCR. A tabular representation of all positive and negative samples determined by nested PCR.

<table>
<thead>
<tr>
<th></th>
<th>Blood/ Lymph</th>
<th>Eye / Nose Swabs</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>31</td>
<td>20</td>
<td>51</td>
</tr>
<tr>
<td>Negative</td>
<td>42</td>
<td>28</td>
<td>70</td>
</tr>
<tr>
<td>Total</td>
<td>73</td>
<td>48</td>
<td>121</td>
</tr>
</tbody>
</table>

*Table 2:* Error matrix. A tabular representation of all blood/lymph and eye and nose swabs showing the true positives, true negatives, false positives and false negatives.

<table>
<thead>
<tr>
<th>Sampling method assumed to represent true state.</th>
<th>True positives</th>
<th>True negatives</th>
<th>False positives</th>
<th>False negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood/ Lymph</td>
<td>7</td>
<td>28</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>Eye/ Nose Swabs</td>
<td>7</td>
<td>28</td>
<td>18</td>
<td>8</td>
</tr>
</tbody>
</table>

**Sensitivity**

\[
\text{Sensitivity} = \frac{\text{True Positives}}{\text{True positives} + \text{False negatives}}
\]

Assuming that blood and lymph samples report the true state of the sample, i.e. positive or negative, the sensitivity of eye and nose swabs was calculated using table 2. The reverse assumption was also made, i.e. that eye and nose swabs report the true state of the
sample (positive or negative). The sensitivity of blood and lymph was calculated using table 2.

**Specificity**

\[
Specificity = \frac{True \ Negatives}{True \ Negatives + False \ Positives}
\]

Assuming that blood and lymph samples report the true state of the sample i.e., positive or negative, the specificity of eye and nose swabs was calculated using table 2. The reverse assumption was also made, i.e. that eye and nose swabs report the true state of the sample (positive or negative), thus, the specificity of blood and lymph was calculated using table 2.

**Chi-Square test**

Using Table 1, a Chi- square test was conducted using the formula below:

\[
\chi^2 = \sum \frac{(observed - expected)^2}{Expected}
\]

Using a 95% confidence interval and degree of freedom as 1, the chi square critical-value obtained was 3.841
RESULTS

Determining an efficient protocol for nested PCR

The initial published method (Ossiboff et al., 2015) was used as a starting point for molecular analysis in this project. After two rounds of PCR, following the exact published protocol, the gel images showed large smears and multiple bands (Figure 1), indicating the need to revise the protocol for more accurate results.

![Image](https://example.com/figure1.png)

*Figure 1: Gel image showing smears and multiple bands.*

Modifying extension times and primer combinations (Revised Protocol I) resulted in significantly less smearing (Figure 2). However, the presence of multiple bands still posed an issue for genetic sequencing to confirm the presence of the virus and needed further modifications of the annealing temperatures.

![Image](https://example.com/figure2.png)

*Figure 2: Results of published PCR protocols. PCR amplicon sizes of ~200 bp.*
Figure 3: Results of revised protocol I. PCR amplicon sizes of ~500 bp produced in round 2A (Fig. 2A) and ~450 bp in round 2B (Fig. 2B).

A temperature gradient from 46°C to 56°C helped determine the most likely annealing temperature to be an average of the two best results obtained from each round (Figure 3). The most likely annealing temperature was mathematically determined to be 52.5°C for round 1A, 55°C for round 2A, and 54°C for round 2B.

Figure 4: Results of revised protocol II. PCR amplicon sizes of ~500 bp in round 2A.

Due to the annealing temperature having exceeded the calculated melting point of the degenerate primer DFA and the high G+C contents of all other primers, the protocol was readjusted using various combinations of DMSO (0-0.2 μl) and G+C enhanced buffer and GC
ranging from 10-20% of the final volume (25 μl). However, the gel images produced (Figure 4) as a result showed no bands and this method was omitted from future use.

Figure 5: Results of revised protocol III. No PCR amplicons produced in round 2A (Figure 4A) and round 2B (Figure 4B).

A more accurate temperature gradient was conducted using the annealing temperatures calculated in revised protocol II, varying by ±0.1°C. This protocol allowed for the determination of the final annealing temperatures as 52.2°C for round 1A, 55.2°C for round 2A, and 54.3°C for round 2B. As seen in Figure 5, the gels appeared to be free of smears and showed clean, single bands of the correct expected size range of ~500 bp for round 2A and ~450 bp for round 2B.
Figure 6: Results of final protocol. PCR amplicon sizes of ~500 bp in round 2A (Figure 5A) and ~450 bp in round 2B (Figure 5B), with Track-it DNA Marker in the first lane (Figure 5A and 5B), a negative control in the second lane (Figure 5B) and samples in all other lanes (Figure 5A and 5B).

Statistical tests:

The sensitivity of eye and nose swabs was calculated to be 28%, while the sensitivity of blood and lymph was calculated to be 46.67%. The specificity of eye and nose swabs was calculated to be 77.78%, while the specificity of blood and lymph was calculated to be 60.87%.

Using a 95% confidence interval and degree of freedom as 1, the chi square critical-value obtained was 3.841. The Chi-square value obtained was 0.075. Since 0.075 < 3.841, sensitivity and specificity are independent of each other.

The results of the statistical tests show that eye and nose swabs (77.78%) are comparatively more specific than blood and lymph (60.87%), but blood and lymph samples are comparatively more sensitive (46.67%) than eye and nose swabs (28%), when analyzed using
nested PCR. To settle conflicting results, a Chi-square test was conducted to detect whether or not the difference in detectability was significant, thus concluding whether or not nested PCR works better with eye and nose swabs or blood and/or lymph samples. Using a 95% confidence interval and degrees of freedom of 1, the Chi-square value (0.075) was observed to be lower than the Chi-square cut off (3.841). Thus, the difference is not statistically significant, and the hypothesis that there is a difference in detectability of blood and lymph vs. eye and nose swabs using nested PCR can be rejected.
DISCUSSION

Chelonian herpesviruses mutate at exceptionally fast rates and so the need to use degenerate primers surfaces to allow detection of viral pathogens that may have mutated. However, the downside of using degenerate primers is that while pairing sequentially to anneal to different regions of the viral genome, they could possibly also amplify the host genome and skew results. Nested PCR allows us to solve this issue because the probability of non-target amplification in two successful rounds of PCR is incredibly low. Thus, it is statistically more likely that amplicons produced after two rounds of PCR belong to the viral genome. While the error of non-target amplification is significantly reduced due to nested PCR, there is still a possibility for non-target amplification. However, the target genome shows greater representation in volume compared to the host genome. The published PCR protocols (Van Devanter et al. 1996, Ossiboff et al., 2015) use degenerate primers DFA, IYG, TGV, KG1, and ILK. By using two different sets of primers, as done in revised protocol I, the amplification of non-target DNA in round 1 is greatly reduced due to different binding sites of the primers used in round 2. The need for two round two amplifications as done in revised protocol I, allows us to ensure that host amplification doesn’t occur.

The G+C content of the primers are as listed: DFA (46%), IYG (50%), KG1 (55%), TGV (50%) and ILK (55%). This implies that DFA, having the lowest G+C content has a lower melting temperature than primers at 50% G+C (IYG, TGV) and 55% (KG1, ILK). Due to the presence of more than one primer in each round of PCR, the annealing temperatures had to be modified while paying attention to the melting point of the primers used and increasing annealing temperatures beyond the melting point of DFA resulted in more smearing across gels,
calling for the use of G+C enhancer buffer. However, no bands were produced and the protocol appeared to produce no amplicons, thus this method was discarded.

Sequencing results appeared to have multiple signals, however consistency in the state of the sample (positive or negative) across three replicate (with the exception of two blood/lymph samples) allowed me to conclude with fair certainty than the amplified pathogen was indeed herpesvirus. Low signal to noise ratios were also observed, and is characteristic of protocols that use degenerate primers. To increase the occurrence of single bands in the correct size range, allow for more specific binding of the primers and reduce smearing in the gels, the annealing temperatures were raised according to a temperature gradient (Revised protocol II) and then a more specific temperature gradient (Revised protocol IV) which ultimately produced clean bands. However, due to lack of clean sequence data, there is a relative uncertainty that the amplicons were indeed representative of Herpesvirus. At the same time, the consistency of data through multiple protocol revisions, across three replicates (using the final protocol) and appearance of expected fragment sizes, allows me to conclude that the presence of herpesvirus is fairly certain.
CONCLUSIONS

Although the lack of clean sequences poses an uncertainty to the results, the constant detection of predicted fragment sizes across the exact same samples over the course of multiple revisions in protocol and three replicates (using the final protocol) allowed us to conclude with fair certainty that Herpesvirus was indeed present in the sample.

According to the tests conducted for sensitivity and specificity, eye and nose swabs are comparatively more specific than blood and lymph, but blood and lymph samples are comparatively more sensitive. Due to conflicting results in specificity and sensitivity, a Chi-square test was conducted to detect whether or not the difference in detectability was significant. Using a 95% confidence interval and degrees of freedom of 1, the Chi-square value was observed to be lower than the Chi-square cut off. Thus, the difference is not statistically significant, and the hypothesis that there is a difference in detectability of blood and lymph vs. eye and nose swabs using nested PCR can be rejected.

This result implies that both the tests work equally well in detecting the prevalence of Herpesvirus when analyzed using nested PCR. Rules enforced by government agencies restrict the number of attempts of blood drawl and limit invasive techniques. Due to the fact that both sample types are equally detectable, the two tests can be used in lieu of each other as alternate or back up methods in case the researcher’s preferred method is not feasible. Another implication of the result is that both tests report the same amount of bias (false positives and false negatives) collectively, even though their individual biases may differ.
The presence of Herpesvirus could cause infection and disease, that can be tracked by the occurrence of clinical symptoms like oral plaques, conjunctivitis, etc. and threaten the health of *G. polyphemus*. Any further threats to an already dwindling population can be catastrophic and could lead to the extinction of this keystone species, thus measures must be taken to diagnose and treat herpesvirus infections in *G. polyphemus*. 
APPENDIX

PCR

PCR is a molecular diagnostic technique that relies on the complementary nature of DNA replication. The first step is denaturation in which the two strands of DNA are separated due to exposure to high temperature. The forward primer attaches to the start codon of the template DNA while the reverse primer attaches to the stop codon of the complementary strand of the DNA. This is called the annealing step. DNA polymerase can then begin synthesizing complementary strands to the primer DNA. This is called the extension step. As these three steps are repeated for 30 cycles, billions of copies of the region of interest are produced. These are called amplicons. The amplicons are then compared to positive and negative controls to determine the status of infection.

Nested PCR

Nested PCR is a modification of PCR that provides higher sensitivity and specificity. It involves two successive PCR reactions conducted with two different sets of degenerate primers. Degenerate primers are “A mix of oligonucleotide sequences in which some positions contain a number of possible bases, giving a population of primers with similar sequences that cover all possible nucleotide combinations for a given protein sequence” (Iserte 2013). The first round of nested PCR follows the standard protocol observed in PCR, while using the blood, lymph of swab DNA samples as template DNA. It includes a set of primers in which one primer anneals to the DNA sequence upstream while the other primer anneals to the sequence downstream. The
first round of PCR results in amplicons which are used as template DNA for the second round of PCR. The second round of PCR greatly increases the sensitivity and specificity of DNA amplification. The amplicons generated can then be sent for sequencing to confirm the presence of the herpesvirus and to eliminate the possibility of a false positive that could’ve been generated due to over amplification of the DNA during the second round of PCR. (Carr et al., 2010).

Forward primers:
DFA: 5’GAYT TYGCNAGYYTNTAYCC 3’
ILK: 5’ TCCTGGACAAGCAGCARNYS GCNMTNAA 3’
TGV: 5’ TGTAACCTCGGTGTAYGGNTTYACNGGNGT 3’

Reverse primers:
KG1: 5’ GTCTTGCTCACC AGNTCNACNCCYTT 3’
IYG 5’ CACAGAGTCCGTRTCNCCRTADAT 3’
REFERENCES


8. Stapleton C: Gopher tortoise's status change could take years. *The Palm Beach Post* (West Palm Beach, FL) 2011.


