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Behavioral and Disease Ecology of Gopher Tortoises (Gopherus polyphemus) Post Exclusion and Relocation with a Novel Approach to Homing Determination

Johnathan Napier
University of Central Florida

BEHAVIORAL AND DISEASE ECOLOGY OF GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*) POST EXCLUSION AND RELOCATION WITH A NOVEL APPROACH TO HOMING DETERMINATION

by

JOHNATHAN OLIVER HOWARD NAPIER
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ABSTRACT

In the wake of human expansion, relocations and the loss of habitat can be stressful to an organism, plausibly leading to population declines. The gopher tortoise (*Gopherus polyphemus*) is a keystone species that constructs burrows it shares with 362 commensal species. Frequent exclusions and relocations and long generation times have contributed to *G. polyphemus* being State-designated as Threatened in Florida. Prior studies have indicated that *G. polyphemus* may possess homing behavior and thus be able to counteract stressors due to relocation and exclusion. I radiotracked a cohort of *G. polyphemus* for 11 months following excavation, relocation, and exclusion due to a pipeline construction project. In conjunction with analyzing *G. polyphemus* movement patterns post-release, I developed novel statistical methodologies with broad application for movement analysis and compared them to traditional analyses. I evaluated habitat usage, burrowing behavior, movements, growth, and disease signs among control versus relocated and excluded individuals and among sexes and size classes, forming predictors for behavior and disease risk. I found statistical support that my new methodology is superior to previous statistical tests for movement analyses. I also found that *G. polyphemus* engages in homing behavior, but only in males. Behavioral differences were also found between the sexes with respect to burrowing behavior. Overall health, disease prevalence, and immune response were unaffected by relocation and exclusion, nor were they statistically correlated. Signs were unreliable as etiological agents, outperformed by serological detection. I determined that the Sabal Trail

pipeline as a potential stressor did not affect movement behavior, homing, nor the disease/immune profile of *G. polyphemus* in this study.

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CHAPTER 1: A NEW METHOD, ADDRESSING AZIMUTH TESTS AND HOMING

Specific Aim: Develop a novel method for homing determination that solves the deficiencies in traditional methods, with specific emphasis on the Rayleigh z-test and the Watson U2 test.

Introduction

Site Fidelity vs. Homing

Site fidelity (or philopatry) is the tendency for an animal to remain in an area for an extended period of time or to return to a previously occupied region (White and Garrott 1990). A notable example is sea turtles, organisms that travel thousands of kilometers to their feeding grounds and then return to the shores of their birth, particularly females laying eggs (Meylan 1982). However, these definitions of site fidelity are binary and are often used synonymously and confusingly with "homing." It is important to distinguish between an organism's *ability* to remain in or return to an area vs. the end result of whether they return home. For clarity, I will use the term homing to mean the specific ability to navigate towards an original location through unfamiliar areas. Animal navigation can be accomplished using several mechanisms, including the identification of landmarks, celestial navigation, auditory and olfactory cues, or by detecting variations in the Earth's magnetic field (Frost and Mouritsen 2006). The above-mentioned sea turtles are well known for their ability to navigate the oceans using magnetoreception to maintain compass headings. However, several mechanisms such as chemoreception, detection of polarized light, wave intensities, and identification of landmarks are used in tandem to determine the position from a source (Lohmann et al. 2008).

In the absence of other indicators, broad-sense magnetoreception and celestial

navigation alone are insufficient as forms of navigation. Birds and sea turtles are often pushed off course by strong currents and winds and must make corrections along the way (Johnsen and Lohmann 2005). Magnetic north would be perceived by an inherent direction, but it would not indicate an organism's latitude or longitude. A sense of field intensity and inclination (vertical component of a magnetic field) as observed in amphibians, birds, and reptiles is sufficient in solving one's rough latitude (Wiltschko and Wiltschko 2005); longitude would still be somewhat ambiguous. Celestial navigation also only produces a direction relative to the sun, moon, or stars; the addition of a sense of time is necessary to determine a direction. Given that the sun rises in the east and set in the west, an absolute direction could be taken. A sense of yearly patterns and length of day would be necessary to estimate one's latitude. Thus, other indicators such as relative habitat types, landmarks, or sensory clues would be necessary to complete navigation. While broad-sense homing in a species can be determinable, the exact navigational method employed can be somewhat ambiguous, especially when multiple methods are used in tandem. However, if an organism fails to reach its objective (amongst other scenarios), current methods for homing detection are unable to accurately determine homing.

Traditional Methods

Currently, a myriad of methods exist that test hypotheses related to site fidelity and "homing" behavior. One method for determining if an individual's overall location is *changing over time* is to use Hotelling's T2 test (1931) which operates as a multivariate t-test. This can be biased, however, according to the timeframe chosen. An alternative is to use a method by Spencer et al. (1990) which takes an individual's movement vectors and reattaches them at

random angles to determine if the mean squared distance of the center of gravity (MSD) of the original path is statistically different than by random trajectories. These two are good first checks of *binary* decisions: "stayed" vs. "left" a given region. They, however, do not accurately determine if one was "returning." The standard for testing whether a distribution has directionality is the Rayleigh z-test. This test assumes, for the null hypothesis, that a distribution of azimuths (angles) is uniformly distributed and not diametrically bidirectional. The test for determining if two distributions of azimuths are statistically different is Watson's U^2 test. At first glance these tests would seem appropriate for testing travel patterns and the return of an organism to an area. Certainly, the Rayleigh z-test could be used to indicate significant directionality from "one starting point" across multiple individuals. However, in a study where each individual is trying to reach a different original location and have different starting points, this renders this test inappropriate. The next option would be to test if azimuths taken (α_T) are statistically different than "direct" azimuths (α_D) using Watson's U^2 test. This too fails for three major reasons: (1) On a sphere, a path's initial azimuth (α_I) may differ from its final azimuth (α_F), (2) an individual can consistently take indirect azimuths and converge to the correct location of origin, and (3) an individual can head toward a location and then pass by it, meaning homing can be incorrectly inferred if the animal passes by its location of origin by chance.

Calculation of Distance and Azimuths

Addressing the first issue, consider a direct arc path between Capetown and Melbourne (Figure 1). Without altering one's course, the azimuth changes from 141° to 42°. If we were only interested in α_I, this fact would be irrelevant. However, we are interested in if an

individual is making it to a location with consideration to stops or course changes made along the way. Therefore, it is important to consider the average azimuth taken along a path ($\bar{\alpha}_T$) or more importantly the average error along a path with respect to direct azimuths ($\Delta\bar{\alpha}_T$).

Next consider a loxodrome (Figure 2). A loxodrome is a spherical spiral with constant azimuth. While this type of movement (constant azimuth -85°) would be atypical for an individual, it does illustrate how a path can be consistently taking an indirect path and converge to a point (in this case, true north). However, if an individual stops, for example at the equator, it was engaged in homing behavior, but did not successfully reach its destination. Thus, the angle taken to a destination is complimented by the distance taken along a path. The shortest distance between two points on a sphere is an arc along a great circle: a circle on the surface of a sphere whose radius is the same as the sphere e.g. the equator (forgoing the ellipsoid nature of the Earth). Traditionally, the Spherical Law of Cosines is used for determining distances between GPS coordinates, however it is slightly inaccurate for distances less than 1 km and troublesome for distances less than 1 meter. The Vincenty formula corrects this issue, as well as accounts for issues with "antipodal points" along a sphere. The Vincenty formula determines the central angle (Δσ) between two points with respect to latitudes (ϕ) and longitudes (λ). The Vincenty formula is as follows (Vincenty, 1975) (Figure 3):

$$\Delta\sigma = \arctan\frac{\sqrt{(cos\phi_2 \cdot sin\Delta\lambda)^2 + (cos\phi_1 \cdot sin\phi_2 - sin\phi_1 \cdot cos\phi_2 \cdot cos\Delta\lambda)^2}}{sin\phi_1 \cdot sin\phi_2 + cos\phi_1 \cdot cos\phi_2 \cdot cos\Delta\lambda}$$

Then the distance between the two points is the arc length (d) with respect to the radius of

the earth (r).

$$d = r\Delta\sigma = 6{,}371{,}008.8\, m \cdot \Delta\sigma$$

Next, to determine the angle between three points on a sphere it is necessary to use the Atan2 function. Atan2 is a modified version of the arctangent function which returns (properly) the angle (θ) between the positive x-axis and the line segment between the origin (0,0) and a point (x, y). As adapted by Bullock (2007), the Atan2 function uses two sets of coordinates (ϕ_1, λ_1) and (ϕ_2, λ_2) and determines the angle (α_T) between the great-circle containing true north & the 1st point and the great-circle containing both points:

$$\alpha_T = Atan2[y, x] = Atan2[cos\phi_2 \cdot sin\Delta\lambda, cos\phi_1 \cdot sin\phi_2 - sin\phi_1 \cdot cos\phi_2 \cdot cos\Delta\lambda]$$

Incorporating a third point (ϕ_H, λ_H), the angle (α_D) between the great-circle containing true north & the 1st point and the great-circle containing the 1st and 3rd point is

$$\alpha_D = Atan2[y, x] = Atan2[cos\phi_H \cdot sin\Delta\lambda, cos\phi_1 \cdot sin\phi_H - sin\phi_1 \cdot cos\phi_H \cdot cos\Delta\lambda]$$

For convenience, α_D and α_T have been labeled to denote a “direct” azimuth versus a “taken” azimuth” with respect to the “home” destination of ϕ_H, λ_H. The initial error in azimuth taken ($\Delta\alpha_T$) i.e. the angle between these three points, is then the difference between α_D and α_T:

$$\Delta\alpha_{T,1} = \alpha_T - \alpha_D$$

However, a correction is necessary to account for which way around the circle, or in this case the globe, is shorter:

$$\Delta\alpha_{T,2} = \frac{\alpha_{T,1}}{|\alpha_{T,1}|}(\Delta\alpha_{T,1} - 360°)$$

For example, -59.13° is shorter than +300.87°. Whichever has the smaller absolute value, i.e. is less than 180°, is the true $\Delta\alpha_T$. Next, to find the average error in azimuth taken ($\Delta\bar{\alpha}_T$), the "ant-walk" method must be employed. To generate a point (ϕ_G, λ_G), between and along the great circle containing (ϕ_1, λ_1) and (ϕ_2, λ_2) a set of formulae commonly used in the aviation industry must be applied (Williams, 2004):

$$a = -\frac{\sin((1-f)\Delta\sigma)}{\sin\Delta\sigma} \qquad b = -\frac{\sin(f*\Delta\sigma)}{\sin(\Delta\sigma)} \qquad f = [0,1]$$

$$x = a * \cos\phi_1 \cos\lambda_1 + b\cos\phi_2 \cos\lambda_2$$

$$y = a * \cos\phi_1 \sin\lambda_1 + b\cos\phi_2 \sin\lambda_2$$

$$z = a * \sin\phi_1 + b\cos\phi_2$$

$$\phi_G = Atan2[z, \sqrt{x^2 + y^2}\,]$$

$$\lambda_G = Atan2[y, x]$$

The variable f determines the fractional distance from ϕ_1, λ_1 e.g. f = 0: (ϕ_1, λ_1) and f = 1: (ϕ_2, λ_2). Repurposing this equation, we can find $\Delta\bar{\alpha}_T$ by iterative calculation of $\Delta\alpha_T$ between (ϕ_H, λ_H), (ϕ_G, λ_G), and (ϕ_2, λ_2).

$\Delta\bar{\alpha}_T = \frac{1}{n}\sum\Delta\alpha_{T_f}$, where n is the number of different values of f taken.

Methods

Novel Statistical Methods

To account for variable destinations/starting-points and magnitude of movements, I constructed 5 statistical tests: Convergence Test (CT), Direct Test I (DTI), Direct Test II (DTII), Orbit Test I (OTI), Orbit Test II (OTII). The ground-covered (Δd_H) is the difference between the Vincenty distances from a home location (ϕ_H, λ_H) after moving from (ϕ_1, λ_1) to (ϕ_2, λ_2):

$$\Delta d_H = d_{initial} - d_{final}.$$

Using the BSDA package in R (Arnholt and Evans 2017), CT performs a sign-test on Δd_H values, where H_0: the median of Δd_H is not significantly different than 0. Significant p-values with positive median Δd_H are considered converging by distance i.e. approaching the destination point. DTI performs a sign-test on $\Delta\alpha_T$ values, where non-significance indicates taking an initial direct path to the point. DTII similarly evaluates $\Delta\bar{\alpha}_T$ to determine if an individual is taking an average direct path to the point. To evaluate if an individual is moving forward or backwards with respect to a destination, the angles $\Delta\alpha_T$ and $\Delta\bar{\alpha}_T$ where mapped to new domains respectively labeled "orbit vectors" $V_{o,\,I}$ and $V_{o,\,II}$.

$$V_{o,I} = 90° - |\Delta\alpha_T| \quad V_{o,II} = 90° - |\Delta\bar{\alpha}_T|$$

This is reasoned as follows: individuals who consistently move orthogonally to a point will orbit said point, so it is necessary to test whether or not the median $\Delta\alpha_T$ and $\Delta\bar{\alpha}_T$ values are statistically different from 90° (orbiting). Thus, significance in OTI / OTII, with positive median $V_{o,\,I}$ / $V_{o,\,II}$, indicate initial / average forward motion by azimuths taken.

Simulations

To compare traditional methods (Rayleigh-Z and Watson U^2) to my new methods (CT, DTI, DTII, OTI, and OTII) I generated constructed-random walks in R using the University of Central Florida's Stokes High Performance Computing system. The total number of random walks performed was 9,576,000. For a fixed number of movements and space, a random uniform series of latitudes and longitudes were generated: number of movements + 1. A single dataset consisted of 280 individuals: 10 individuals each at 28 movement types (Table 1).

Table 1: Movement types for random walk simulations. Each movement type utilized 4+ subtypes: true random vs. sorted parameters and forward vs. reverse directions.

Movement Type	**Destination**	**Sorted Movement Parameters**	**Note**	**Total**
Point-to-Point	Initial Point / Final Point	None / ϕ only / λ only / ϕ and λ	-	8
Along the Equator ϕ = 0°	Initial / Final λ Random Uniform ϕ = [-90°,90°]	None / λ only	Movement: Constant ϕ Destination: Variable ϕ	4
Along the Equator ϕ = 0°	Initial / Final λ ϕ = 90°	None / λ only	Orthogonal movement about destination	4
Along the Equator ϕ = 0°	Initial / Final λ ϕ = 0°	None / λ only	Destination is confined to direct movements	4
Loxodrome (Rhumb Lines)	True North / True South	None / ϕ only	Constant azimuth to destination	4
Logarithmic Spiral	ϕ = λ = 0°	None / θ only & Forward / Reverse Order: θ	Convergent / Divergent behaviors	4

Datasets were confined to 360 varying confined spaces across a range of 95 movements: 6-100 movements per individual (Table 2).

Table 2: Confined latitude (ϕ) and longitude (λ) spaces for random walk simulations.

Sequential	Min(ϕ)	Max(ϕ)	Min(λ)	Max(λ)	Number of Spaces
Increase: Max ϕ = Max λ	0°	[1°,90°]	0°	[1°,90°]	90
Decrease: Min ϕ	[-90°,0°]	90°	0°	90°	90
Decrease: Min λ	-90°	90°	[-90°,0°]	90°	90
Expand: Min λ = Max λ	-90°	90°	[-180°,-90°]	[-180°,-90°]	90

For spiral movements, angles (θ) were generated instead and vectors were squeezed inward until the latitude was in the confined space. Spirals were in the logarithmic form of $r = e^{\frac{\theta}{b}}$, where $b = -\mathrm{e}^{n}$ for random uniform n = [-4.6, 4.6] and centered about $\varphi = \lambda = 0°$. Loxodromes were constructed by the parameters $x = \cos t \cos c$, $y = \sin t \cos c$, $z = -\sin c$, $c = \tan^{-1} at$, for random uniform a = [0.08, 0.5]. Selection of boundaries for random uniform variables a and n where empirically determined so as to promote spiraling behavior that was smooth and without hard kinks in motion.

For each individual, the Vincenty distances (Δd_H), error in azimuth's taken ($\Delta\alpha_T$ and $\Delta\bar{\alpha}_T$), and subsequent orbit vectors ($V_{o,\,I}$ and $V_{o,\,II}$), where calculated for each movement made. Using the R package *circular* (Agostinelli and Lund 2017), a Rayleigh-Z test was performed, setting $\mu = \Delta\alpha_{T_{initial}}$, to determine if $\Delta\alpha_T$ had significant directionality in the direction of $\Delta\alpha_{T_{initial}}$. Significance was counted as direct motion i.e. homing. Using the R package *CircStats* (Agostinelli 2012), a Watson U^2 test was performed to determine if α_T were statistically different than α_D values. Non-significance was counted as homing. Additionally, the novel methods CT, DTI, DTII, OTI, and OTII were performed on each individual's movements. Utilizing p-values and test statistics, individuals were labeled binarily as

significantly homing/converging/taking direct paths vs. non-significant and significant non-homing/diverging/taking indirect paths as is appropriate for each test. For each dataset a Cohen's (1960) kappa coefficient (κ) was computed to determine the agreement between traditional and new methods, as well as internal agreement between Rayleigh-Z and Watson U2 and between CT and OTII (Table 3).

$$\kappa = \frac{1-p_o}{1-p_e}, \text{ where } p_o = \frac{a+d}{total} \text{ and } p_e = p_{yes} + p_{no} = \frac{(a+b)(a+c)}{total^2} + \frac{(c+d)(b+d)}{total^2}$$

Table 3: Possible outcomes used to determine kappa coefficient's and power.

Count of Individuals that are …		**Test B Determines:**	
		Significant Homing (or equivalent)	Non-homing
Test A Determines:	Significant Homing (or equivalent)	a	b
	Non-homing	c	d

For κ = 1 tests were considered in complete agreement and for κ = -1 complete disagreement. Power was determined by the probability that a second test B did not make type II errors (β) with respect to the assumption that the first test A had accurately described the behavior of individuals in the dataset: $1 - \beta_{B|A} = 1 - \frac{b}{total}$. The reverse scenario, test B accurately describes behaviors in the dataset and the power of test A with respect B was computed similarly: $1 - \beta_{A|B} = 1 - \frac{c}{total}$. The relative power of B with respect to A ($\prod_{B,A}$) was calculated as $\frac{1-\beta_{B|A}}{1-\beta_{A|B}}$. If $\prod_{B,A} > 1$, test B was determined to have superior power with respect to A. $\prod_{B,A} = 1$ and/or κ = 1 was labeled as statistically equivalent testing. Number of κ, $1 - \beta_{B|A}$, $1 - \beta_{A|B}$, and $\prod_{B,A}$ was 12 each per dataset and 410,400 across all datasets. Using

the bootstrap method, means and 95% confidence intervals were obtained by resampling each analysis for a fixed number of movements and variable confined spaces, sampling 200 values at a time, 1000 times, with a trim of 0.05. This process was repeated for the percent surface area of the earth contained by a confined space, allowing for variable movement. Bootstrap confidence intervals of the mean κ, $1 - \beta_{B|A}$, $1 - \beta_{A|B}$, and $\prod_{B,\,A}$ across all movements and confined spaces were recorded (Table 4).

Results

Greater relative power ($\prod_{B,\,A} > 1$) was observed for all novel methods with respect to traditional methods. Internally, OTII had greater relative power than CT and Rayleigh-Z was greater than Watson U^2 (Table 4). Thus, novel methods were determined to be superior to traditional methods, with the Watson U^2 as the least powerful test. The highest confidence interval of the mean of the mean κ: [0.860, 08.73] was found between CT and OTII, showing a high degree of agreement. Additionally, their confidence interval of the mean of the mean $\prod_{OTII,\,CT}$ was closest to 1: [1.034, 1.036] (Table 4). Thus, CT and OTII were determined to be statistically similar, with OTII as slightly more powerful than CT.

Table 4: Bootstrap 95% confidence intervals about mean agreement ($\kappa_{A,B}$), a given power ($1-\beta_{B|A}$, $1-\beta_{A|B}$) and relative power ($\prod_{B,A}$). Complete agreement and disagreement were κ = 1 and κ = -1, respectively. κ = 0 is considered 50% accurate. A relative power: $\prod_{B,A}$ > 1 concludes B as superior to A. For $0 \leq \prod_{B,A} < 1$, concludes A as superior to B. For $\prod_{B,A} = 1$ and/or κ = 1, tests A and B are statistically equivalent. Statistically superior tests were marked with an asterisk (*).

Test A	**Test B**	$\kappa_{A,B}$	$1-\beta_{A\|B}$	$1-\beta_{B\|A}$	$\prod_{B,A} \geq 1$
Rayleigh-Z *	Watson U^2	[-0.158, -0.148]	[0.867, 0.870]	[0.813, 0.828]	[0.936, 0.955]
CT	OTII *	[0.860, 0.873]	[0.957, 0.961]	[0.992, 0.994]	[1.034, 1.036]
Rayleigh-Z	CT *	[0.502, 0.543]	[0.904, 0.912]	[0.943, 0.947]	[1.036, 1.050]
Rayleigh-Z	DTI *	[-0.145, -0.132]	[0.481, 0.488]	[0.881, 0.890]	[1.818, 1.858]
Rayleigh-Z	DTII *	[-0.124, -0.114]	[0.485, 0.497]	[0.886, 0.895]	[1.801, 1.869]
Rayleigh-Z	OTI *	[0.227, 0.245]	[0.665, 0.686]	[0.959, 0.961]	[1.419, 1.460]
Rayleigh-Z	OTII *	[0.444, 0.479]	[0.870, 0.878]	[0.944, 0.948]	[1.078, 1.091]
Watson U^2	CT *	[-0.008, -0.002]	[0.796, 0.804]	[0.894, 0.896]	[1.113, 1.126]
Watson U^2	DTI *	[-0.021, -0.015]	[0.486, 0.493]	[0.939, 0.940]	[1.910, 1.935]
Watson U^2	DTII *	[-0.008, -0.005]	[0.494, 0.499]	[0.942, 0.944]	[1.894, 1.916]
Watson U^2	OTI *	[0.134, 0.159]	[0.607, 0.637]	[0.961, 0.964]	[1.562, 1.627]
Watson U^2	OTII *	[0.052, 0.058]	[0.779, 0.786]	[0.908, 0.911]	[1.157, 1.170]

Overall, an increase in the number of movements i.e. an increase in the number GPS points for each individual stabilized mean κ and power values leading to exponential decay (Figure 4). Percent confinement space was found to be nonlinear-to-erratic with respect to mean κ and power values with no definitive pattern (Figure 5). Similarly, across movements and confinement spaces, the highest κ agreement and $\prod_{B,A}$ relative power closest to 1 was observed between CT and OTII. Overall, $\prod_{B,A} > 1$ for novel methods (CT, DTI, DTII, OTI, and OTII) with respect to traditional methods (Rayleigh-Z and Watson U2) (Figures 4 & 5). Thus, for a sufficient sample size and any area size, novel methods always outcompeted traditional methods.

The median distance in meters (m) than an individual converged (toward) or diverged (away) from a home location was plotted against the median average bearings (°) taken from a

direct path (Figure 6). Amongst 9,576,000 data-points, 4,043,945 were found to statistically significant with respect to both CT and OTII. Eliminating non-significant points revealed that the remaining were mostly confined to converging & homing or diverging & emigrating behaviors. A few rare instances were found to be converging & emigrating (20,368) or diverging & homing (21,115), accounting for the non-total agreement ($\kappa = 1$) in table 4. However, these points were found at 90±2.794° and 0±5.521 meters at 95% confidence. This indicates that 90° and 0 meters are not necessarily asymptotes, but that combinations outside the two given regions are exceedingly rare (1.026% of the data).

Future Applications & Broader Impacts

As identified by simulation, each of the five novel methods outperformed traditional methods by possessing greater relative power. While any one of these novel tests has greater accuracy than traditional methods, I recommend that they be used in tandem, as they describe different aspects of movement. CT describes how well an individual proceeded toward or away from a destination by distance. DTI and DTII determine if an individual takes biased angles consistent with clockwise or counterclockwise behaviors. OTI and OTII describes homing accuracy with respect to bearings taken by an individual. However, given the accuracy and relative power between CT and OTII, these tests are statistically similar and can used somewhat interchangeably. Recall that DTII and OTII are constructed as ant-walk parallels of DTI and OTI, respectively. As the time between sampling periods decreases, DTI converges to DTII and OTI converges to OTII, i.e. become statistically equivalent. Thus, in the instance where $p_{DTI} \approx p_{DTII}$ and median $\Delta\alpha_T \approx \Delta\bar{\alpha}_T$, or similarly $p_{OTI} \approx p_{OTII}$ and median $V_{o,I} \approx V_{o,II}$, an adequate sampling period was performed in the collection of location data. Given

that these methods where tested on both the local and global level and across various movement types, they have applications across various systems. Significant indirect movements by DTI and DTII have the potential for testing clockwise and counterclockwise behaviors, e.g. sea turtles moving in relation to micro-scale oceanic eddies (Mansfield et al. 2014) or following the overall clockwise flow of the Sargasso Sea (Teal and Teal 1975). The code for these new methods is currently being refined and prepared for R-Package publishing.

A future avenue of research is to reverse the problem: using these methods to identify the home location when it is unknown by the researcher. I propose that two methods be employed, a "density method" and a "simulation method." The density method is as follows: for a given species, the cumulative density function (CDF) for convergence (Δd_H) and the probability density function (PDF) for directness ($|\Delta\alpha_T|$) must first be empirically determined. Then for a set of movements from the same species: the probability (p_H) of a single location being the destination point equals:

$$p_H = \prod_i CDF\left(\Delta d_{H_i}\right) \cdot PDF(|\Delta\alpha_{T_i}|)$$

The process continues, selecting a new destination point with each iteration, and recalculating a new p_H against the same set of movements. In this way, each movement is assumed to be purposeful with respect to homing and compared against the profile of the organism. The point with the highest p_H would be the most likely candidate for a home location. Alternatively, the simulation method employs sampling points at random and

recalculating the p-values for all novel methods. Individual topographies of the p-values are charted and “valleys” of statistical likelihood are located.

In summary, I have created five novel statistical tests for usage in movement ecology which out-compete, but compliment prior statistically analysis: Rayleigh-Z and Watson U^2. Methods have greater statistical power, accuracy, and provide alternative information about the movement behavior of an organism. One unexpected benefit is the determination of whether or not the sampling frequency is adequate for a study. I have provided a proposed method for determining the reverse question “Where is the destination?” and will upload these new statistical methods to CRAN databases for easy of utilization by the scientific community.

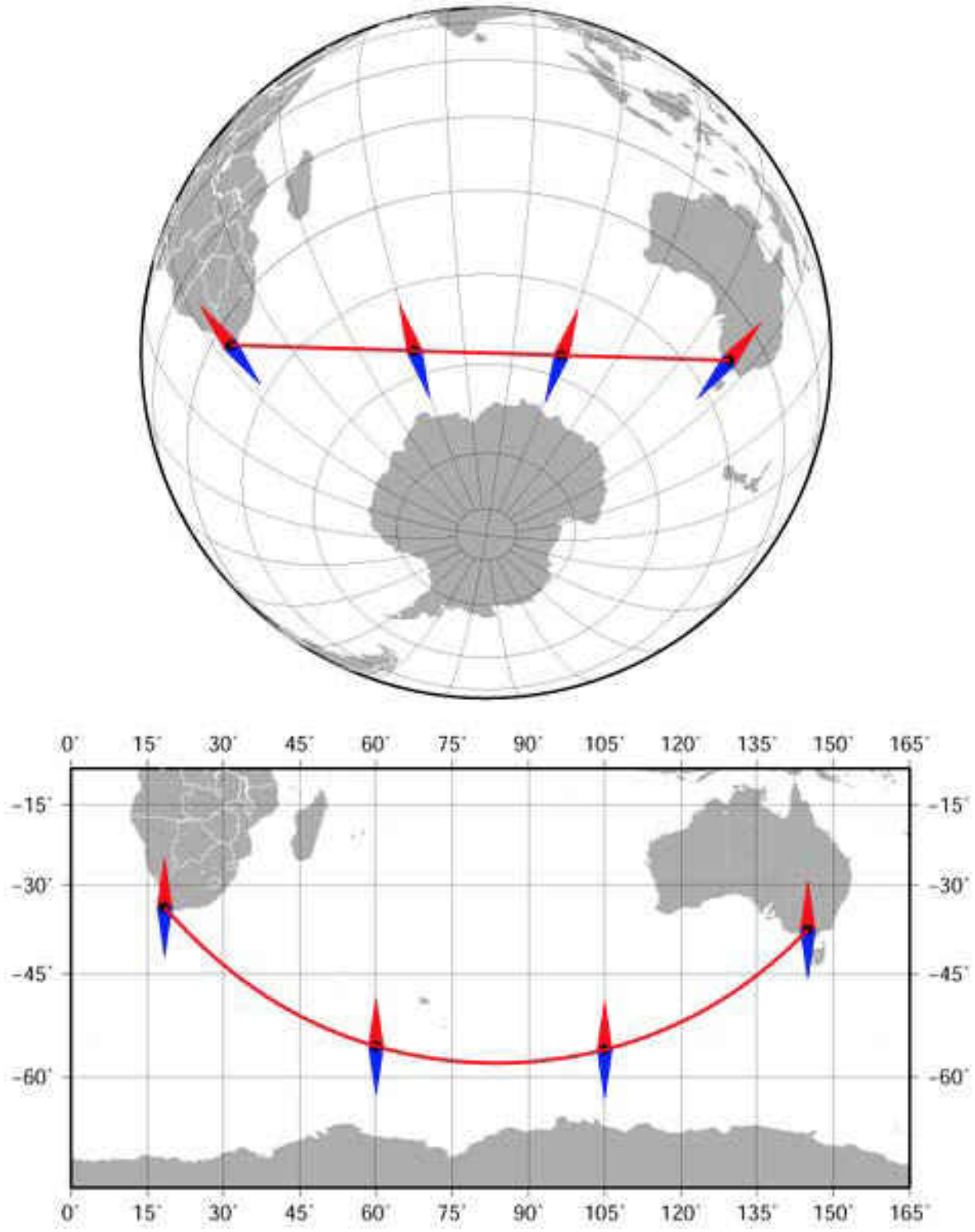

Figure 1: A direct path from Capetown to Melbourne reflecting a change in azimuth from 141° to 42°. (Darekk2 / CC-BY-SA-4.0 2015)

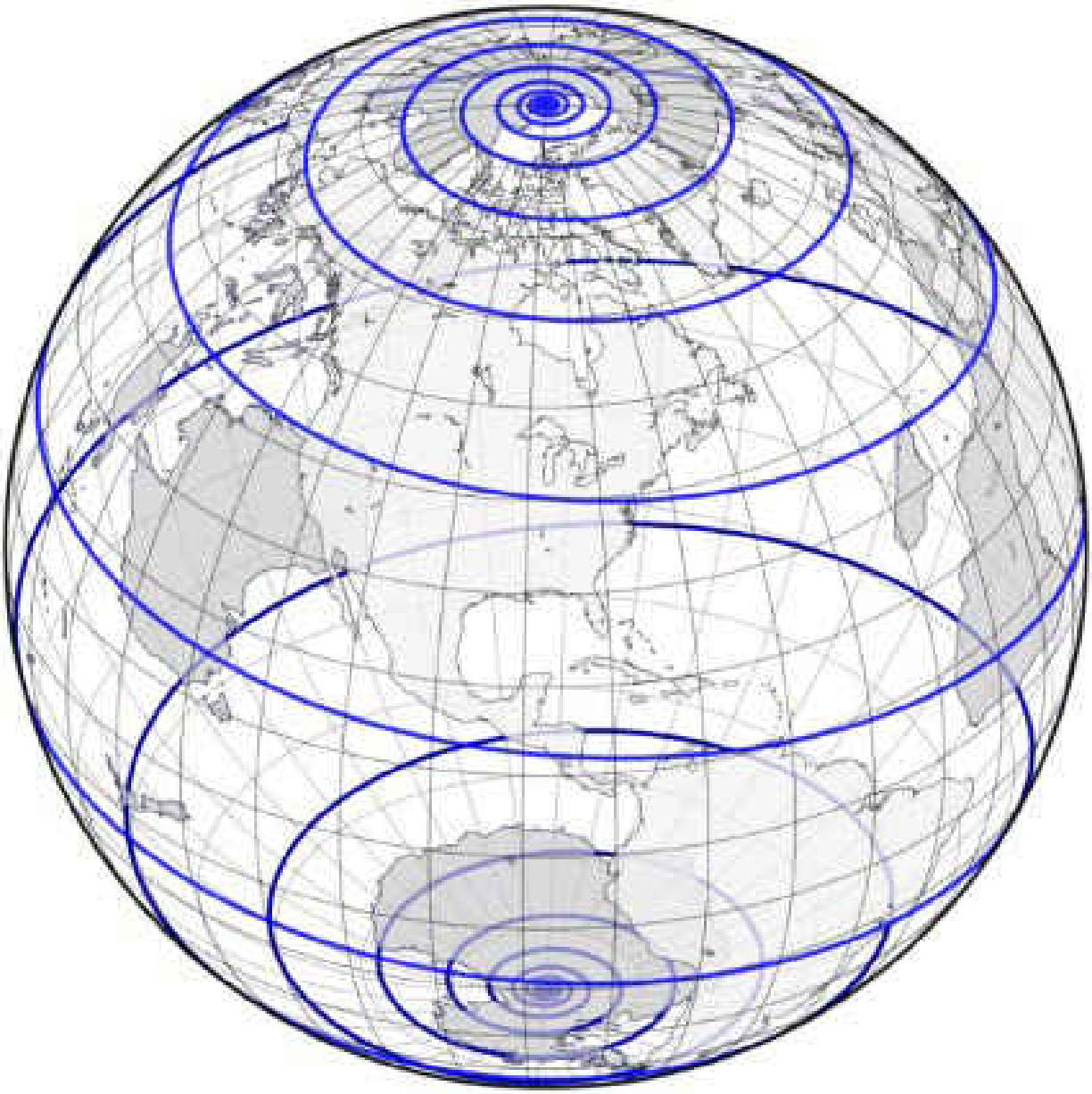

Figure 2: A loxodrome with a constant azimuth of -85°. (Furuti, 2013)

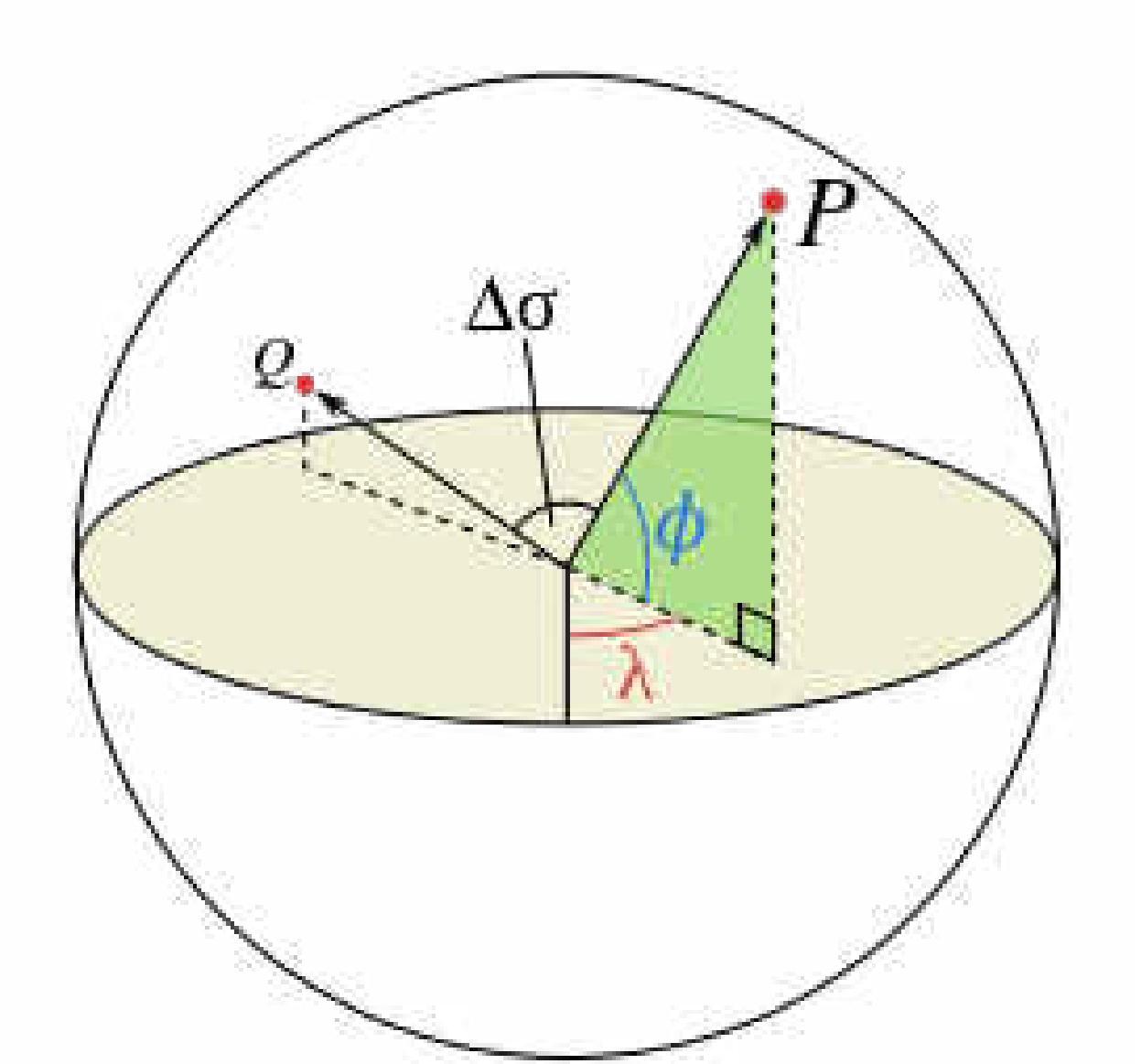

Figure 3: Elements of Vincenty formula for calculating great distances on a sphere.
(CheCheDaWaff / CC-BY-SA-4.0 2016)

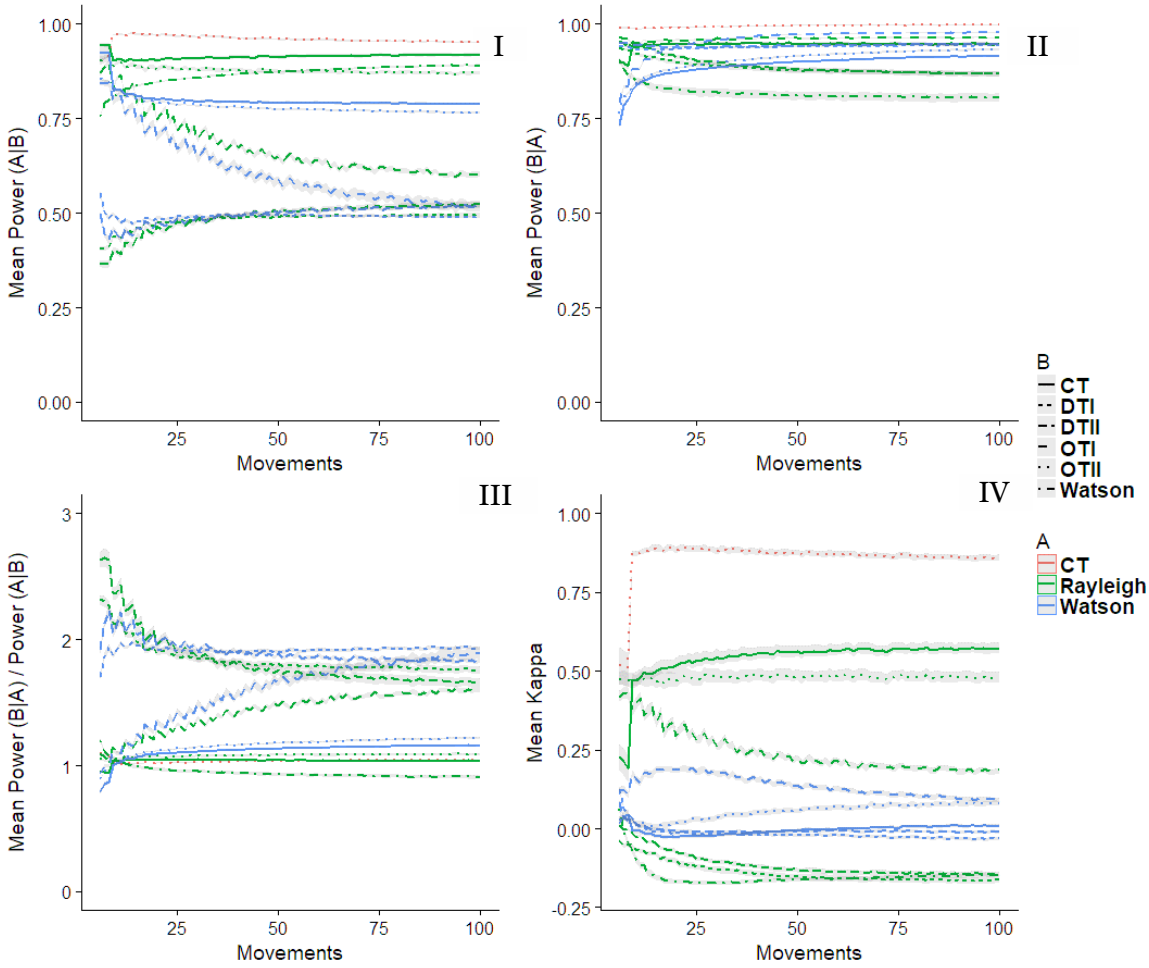

Figure 4: Graphs of bootstrap 95% confidence intervals about the pairwise comparisons of mean power of test A over B ($1-\beta_{A|B}$) (I), mean power of test B over A ($1-\beta_{B|A}$) (II), mean relative power of test B over A ($\prod_{B,A}$) (III), and the mean agreement between test A and B ($\kappa_{A,B}$)(IV) for a fixed number of movements made by each individual within variable confinement spaces. Comparisons were made between all tests and the Rayleigh-Z and the Watson U^2. An additional analysis was performed between CT and OTII. Power analyses were performed by determining how often a given test did not make a type II error (β) after accepting the second test as the ground-truth. Figure 4III is the result of dividing figure 4II by figure 4I. Kappa values ranged from -1 (100% disagreement) and 1 (100% agreement) for corresponding conclusions between tests. Stabilization increased (exponential decay) with the number of movements. Highest κ agreement and $\prod_{B,A}$ relative power closest to 1 was observed between CT and OTII. Overall, $\prod_{B,A} > 1$ for novel methods (CT, DTI, DTII, OTI, and OTII) with respect to traditional methods (Rayleigh-Z and Watson U^2).

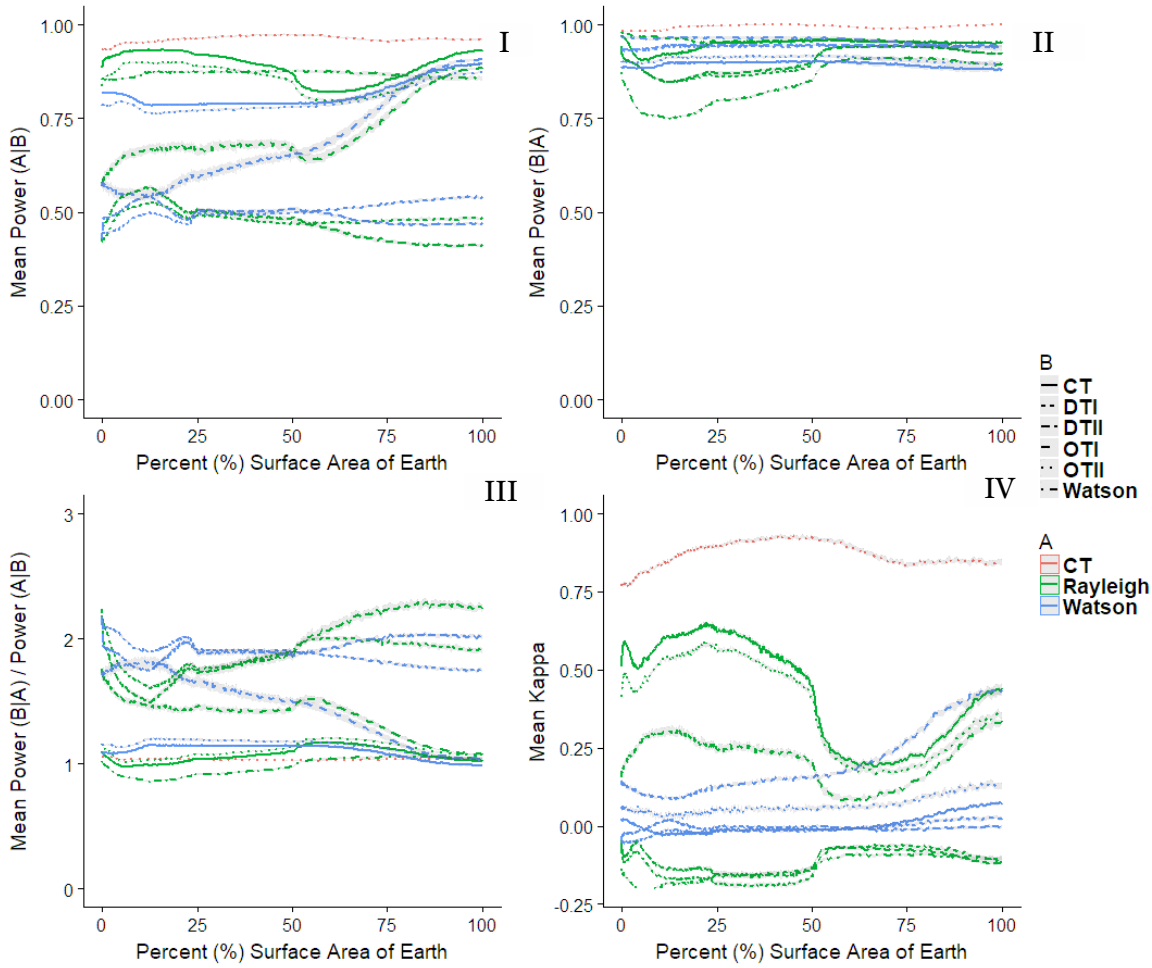

Figure 5: Graphs of bootstrap 95% confidence intervals about the pairwise comparisons of mean power of test A over B ($1 - \beta_{A|B}$) (I), mean power of test B over A ($1 - \beta_{B|A}$) (II), mean relative power of test B over A ($\Pi_{B,A}$) (III), and the mean agreement between test A and B ($\kappa_{A,B}$)(IV) within fixed confinement spaces with variable movements made by each individual. Comparisons were made between all tests and the Rayleigh-Z and the Watson U^2. An additional analysis was performed between CT and OTII. Power analyses were performed by determining how often a given test did not make a type II error (β) after accepting the second test as the ground-truth. Figure 5III is the result of dividing figure 5II by figure 5I. Kappa values ranged from -1 (100% disagreement) and 1 (100% agreement) for corresponding conclusions between tests. Confinement space seemingly acted as a nonlinear-to-erratic variable. Highest κ agreement and $\Pi_{B,A}$ relative power closest to 1 was observed between CT and OTII. Overall, $\Pi_{B,A} > 1$ for novel methods (CT, DTI, DTII, OTI, and OTII) with respect to traditional methods (Rayleigh-Z and Watson U^2).

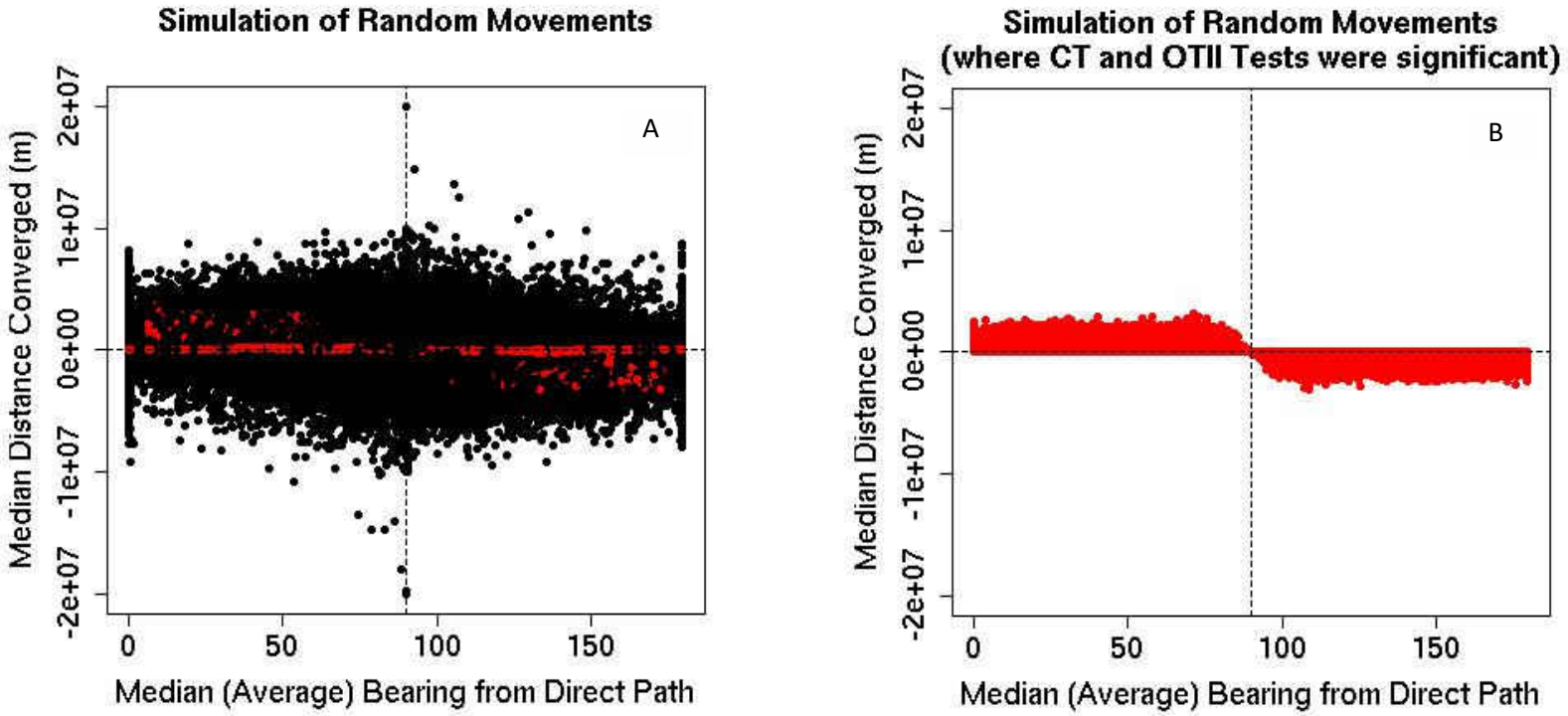

Figure 6: Graphs of median distance in meters (m) versus median average bearings taken by an individual with respect to a home location. Figure 6A displays a total 9,576,000 points with 4,043,945 of the data-points being statistically significant with respect to CT and OTII tests (red). Figure 6B was reduced to only those points which were significant. Median distance > 0: convergence. Median distance < 0: divergence. Bearings < 90° were in a decaying orbit or "homing". Bearings > 90° were in a non-orbit or "emigrating." Bearings = 90° were orbiting the home location.

CHAPTER 2: BEHAVIORAL ECOLOGY OF GOPHER TORTOISES (GOPHERUS POLYPHEMUS) POST-EXCLUSION AND RELOCATION

Introduction

As human populations continue to grow and expand, the amount of urban sprawl is expanding with them, placing regions of protected status at high risk due to pollution, resource consumption, or direct conversion (McDonald et al. 2008). Currently, a third of the world's animal and plant species are under threat of extinction; biodiversity as a whole is threatened by habitat loss/degradation/fragmentation, invasive species, pollution, overexploitation, and climate change (Stein et al. 2000; Wilcove et al. 1998). Urban development alone is the causative agent in the listing of 8% of the vertebrate species on the International Union for Conservation of Nature (IUCN) Red List (McDonald et al. 2008). The level of biodiversity continues to decline, with emphasis on curtailing this loss being placed on overturning bad policies, incorporating biodiversity into land-use planning and economic decision making, and the development of new biodiversity-specific policies (Butchart et al. 2010). However, developing biodiversity policies for all species in a region is impractical. Rather, a push toward identifying conservation targets are encouraged for which planning will indirectly conserve a majority of those species for that region (Groves et al. 2002). Frequent targets are "keystone species" for which the biodiversity and ecology are vitally maintained by said organism's presence whose importance is exceptional relative to the rest of the community. A bulk of conservation funds are provided for those keystone species that are threatened with extinction (Mills et al. 1993). After identification of a conservation target, the next major step towards

conservation planning is understanding the organism through information gathering and ecological assessments (Groves et al. 2002).

Movement is a key behavioral component that affects the vitality of several organisms, whether it be through habitat selection, finding resources, seeking mates, avoiding predators, or a variety of other behaviors that impact health and survival. Movement ecology is the collective framework which seeks to understand the mechanisms of movements, their causes and effects, as well as its cost and benefits to the organism (Nathan et al. 2008). As research in movement ecology has grown, it has been shown to be well complimented by biodiversity research, as an organism's movement affects genetic diversity, habitat usage, and community dynamics (Jeltsch et al. 2013). Amongst one of the strongest factors which influences movement patterns, and subsequently population dynamics, is that of habitat loss and fragmentation. However, these risks are often very dependent upon which movement strategy is employed by the organism, which can either offset or exacerbate the effects of anthropogenic influences (Niebuhr et al. 2015). Thus, research and documentation of organisms' movement strategies have begun to be incorporated into management and conservation planning through facilitation of migration, avoiding areas of risk, and/or maintaining connectivity between important regions (Allen and Singh 2016).

Animal relocations have become a popular method of dealing with resident organisms in the wake of human expansion. While this approach can temporarily generate conservation funds, positive publicity, and heightened awareness for an organism's status, its effectiveness as a conservation strategy is often unclear (Dodd and Seigel 1991). Amphibian and reptile

projects are often less successful than bird and mammal projects, and yet turtles and tortoises are amongst the most frequently relocated organisms (Griffith et al. 1989). In the southeastern United States, relocations of Gopher Tortoise (*Gopherus polyphemus*) populations are more numerous than any other species, with thousands of individuals sometimes being relocated simultaneously (Dodd and Seigel 1991). The Eastern Box Turtle (*Terrapene carolina*), another frequently relocated reptile, shows increased home range size, wandering activity, and mortality rates post-relocation. The suggested mechanism of this behavioral alteration is due to organism's unfamiliarity with the relocation site (Hester et al. 2008). A similar pattern of range expansion and wandering was observed by Hinderle et al. (2015) in Desert Tortoises (*Gopherus agassizii*). However, in this instance all animals were able to "home" back to their capture point, eventually erasing the impact of translocations with no resulting mortality. Efforts to counteract this homing behavior is to install exclusion fences. However, this has the negative effect of reintroducing risk of mortality, as well as thermal stress and predation as tortoises engage in "fence-pacing" (Hinderle et al. 2015, Farnsworth et al. 2015). Due to the high levels of turtle/tortoise relocations coupled with frequent exclusion fencing, it is plausible that these methods pose great risk to chelonian populations, especially to those that are already threatened. What is unclear, however is to what effect prolonged usage and their distance from the relocation site has on those with homing mechanisms.

Gopherus polyphemus is a terrestrial tortoise native to the southeastern United States that is well known for its burrowing and commensal behavior. Common in regions of dry sandy uplands, especially within longleaf pine (*Pinus palustris*) savannas, *G. polyphemus*

grazing behavior is important to the dispersal and fertilization of several plant species (Jose et al. 2006). *G. polyphemus* individuals seek out and rely on regions of sparse canopy cover produced by periodic fires and burrows dug for protection from fires, predators, and other environmental conditions (Brown et al. 1999; McCoy et al. 2013). An estimated 362 commensal species share these burrows, ranging from regular to opportunistic use, with examples including crawfish frogs (*Rana areolatus*), the eastern indigo snake (*Drymarchon couperi*), pine snake (*Pituophis melanoleucus*), and oldfield mice (*Peromyscus polionotus*) (Lips 1991). *Gopherus polyphemus* is considered a keystone species for which anthropogenic expansion will result in extirpation and declines of other species due to the commensalist nature of its burrows (Smith et al. 2005). *G. polyphemus* is listed as Threatened in Florida and vulnerable (VU) according to the IUCN, but has not been properly assessed since 1996 ("Species Profile for Gopher tortoise (*Gopherus polyphemus*)" Web; "The IUCN Red List of Threatened Species: *Gopherus polyphemus*" Web). Current estimates of population size are unclear. Historical threats to *G. polyphemus* have been largely anthropogenic through human development, habitat loss, fragmentation, predation, and relocation leading to population disruption and disease (Hudson 2007, Guyer and Bailey 1993). Non-random restoration efforts such as reseeding have traditionally been used to mitigate the effects of habitat loss and fragmentation (Huxel and Hastings 1999).

Gopherus polyphemus activity levels are directly correlated to temperature and during colder months (November to February) and *G. polyphemus* exhibits overall sedentary behavior interrupted by spikes in warmer weather. However, females remain sedentary throughout

spring and summer months (McRae et al. 1981a), plausibly due to nest building activities in mid-May to mid-June (Diemer 1986). On average, burrow usage is found to be doubled in males (10 burrows annually) as compared to females (Eubanks et al. 2003). Males tend to maintain a home range twice that of females (Diemer 1992). Males tend to be active during the day, coming out earlier and returning later than females, indicating differences in temperature-dependent activity. Males also engage in patrolling behavior, waiting outside female burrows for hours on end (Douglass and Layne 1978). Males are also demarcated from females with respect to movement patterns. Males tend to be the more active/aggressive sex, taking on multiple female mates, whereas females are more sedentary, passively waiting for males (McRae et al. 1981a). Of particular note, however, is that McRae et al. (1981a) found a generalized homing behavior in males when displaced, and it remains unclear whether this suggests males are better wanderers or have true homing ability.

Site fidelity is defined as the tendency for an animal to remain in area for an extended period of time or to return to a previously occupied region (White and Garrott, 1990). While long term exclusion (9+ months) and relocation (200+ km) by penning has successfully inhibited overall movement and increased site fidelity of *G. polyphemus* to the recipient site (Tuberville et al. 2005), it is unclear as to what effect this has on the homing process. Individuals in this extreme example were likely unable to make the journey toward home, even in the event of true homing ability. Plausibly, males and females may also be impacted differently by translocation. Males, as the more active sex, may either wander until suitable habitat is discovered or home back to the original location (if that region is reasonably obtainable).

Females, as the more sedentary sex, are predicted to remain in the translocated area. A corollary to this is the availability of suitable habitat. Individuals relocated to open habitats and experiencing long term exclusion (10+ months) showed increased site fidelity (Bauder et al. 2014). While ordinally longer exclusion periods alone result in increased site fidelity to the relocation site (Tuberville et al. 2005), no study has fully evaluated exclusion and relocation on the short and continuous scale with respect to homing ability of *G. polyphemus*. Further, given that *G. polyphemus* individuals seek open regions with sparse canopy, the effect of habitat type between the relocation site and the site of removal remains unclear. Here, I compared males to females and control to excluded/relocated individuals to address the following specific aims: (i) Determine the effect of relocation and exclusion on *G. polyphemus* homing behavior, habitat usage, and rhythmic daily and seasonal activity, and (ii) Predict *G. polyphemus* behavior with respect to habitat usage, rhythmic activity, and burrow site-selection. To address these aims, I asked and answered the following five research questions. (1) Is *G. polyphemus* homing behavior affected by time excluded or distance relocated? (2) What habitat type(s) will *G. polyphemus* migrate to after release? (3) Which metric best predicts burrow occupancy: temperature, humidity, time of day, or time of year? (4) What is the distribution of *G. polyphemus* burrows with respect to habitat type? (5) What is the migration rate and period between burrow changes?

Methods

Study Area

The construction project known as the Sabal Trail (ST) is a 515-mile instate natural gas pipeline extending from Alabama, through Georgia, and to mid-Florida. The right-

of-way (ROW) for the project cut through several areas of pristine *G. polyphemus* habitat (6 miles long, 75 ft wide) of the Halpata Tastanaki Preserve and the adjacent Marjorie Carr Cross Florida Greenway, both in Marion County, Florida, USA. Prior to construction, all *G. polyphemus* required excavation and relocation from the ROW and temporary exclusion to prevent reentering the construction zone. This was predicted to potentially disturb, disorient, and stress the penned *G. polyphemus*. Additional predicted stressors stemmed from the temporary loss of habitat, habitat restructuring, and the destruction of the original burrow. To mitigate habitat disturbance, plans to reseed the ROW were initiated after construction concluded. The reseeding mixture consisted of wire grass (*Aristida stricta*), anise-scent goldenrod (*Solidago odora*), silkgrass (*Pityopsis graminifolia*), summer farewell (*Dalea pinnata*), *Liatris* spp., splitbeard bluestem (*Andropogon ternarius*), little bluestem (*Schizachyrium scoparium*), roundhead lespedeza (*Lespedeza capitate*), and various Asteraceae species. Seed mixtures were determined by x-ray analysis by the United States Department of Agriculture Forest Service, National Seed Laboratory, Dry Branch, GA.

Experimental & Control Cohorts

Adult G. polyphemus individuals located directly along the pipeline were removed in October 2016 prior to fencing off the ROW and housed in two four-acre silt-fence pens within the Halpata Tastanaki Preserve (Figure 7, red circles). These individuals comprised our experimental cohort and were fully excluded within pens for a period of 10-21 days, depending upon date of excavation. Sites for the two pens were selected based on the following criteria: (a) both sites had been recently burned (north site November 2014; south site January 2016) and plenty of forage was available, (b) both were located away from major recreation trails to

minimize human interference, (c) both sites were improved/semi-improved pasture with only scattered trees, which is comparable or superior to sites where G. polyphemus were removed and facilitated construction and maintenance of pens while providing some shade for G. polyphemus, and (d) preliminary assessment indicated low numbers of resident tortoises. After ROW fencing was complete, pen silt-fences were partially removed in November 2016 (openings were created to allow tortoises to leave the fenced area) and fully removed (complete removal of all fencing material) in December 2016. Full removal of silt-fencing along the ROW occurred at the end of March 2017.

Animals were evenly and randomly distributed between the two pens and no animal was penned more than 4 miles from where they were collected. Individuals in the temporary pens were not manipulated in any way and, after the partial fence removal, were free to move outside of the area where they had been penned. Three exclusion periods were considered: the initial time of placement in the pens until (a) partial removal of the pens, (b) full removal of the pens, and (c) removal of silt-fencing along the ROW, when animals had the potential to return to their original site of removal.

Additional nearby *G. polyphemus* individuals that were not impacted by the ST project and were not excluded or relocated were included in the study as a control cohort. Control *G. polyphemus* were captured either using flap traps (Enge et al. 2012) or chance encounters from within regions of Halpata Tastanaki Preserve and the Marjorie Carr Cross Florida Greenway that were not impacted by the pipeline construction.

Marking-Method/Identification

Each *G. polyphemus* that was trapped, excavated, or encountered in the open received a systematic identification through marginal scute drilling (Appendix 5 of the FWC Gopher Tortoise Permitting Guidelines, February 2015). This drilling pattern represents the numerical ID of the organism by assigning values to each of the marginal scutes. Combinations of drilled scutes is additive. Counting outwards from the supracaudal (SC) scutes, Left-Posterior- Marginal (LPM) scutes 1-4 represent 10, 20, 40, 70 and Right-Posterior-Marginal (RPM) scutes 1-4 represent 1, 2, 4 and 7; respectively. Counting outwards from the nuchal (NS) scute, Left-Anterior-Marginal (LAM) scutes 1-3 represent 400, 700, and 2000 and Right-Anterior-Marginal (RAM) scutes 1-3 represent 100, 200, and 1000; respectively. Note: RAM3 and LAM3 were notched instead of drilled. Juveniles too small to be drilled were notched instead.

Morphological Traits & Environmental Metrics

All *G. polyphemus* were weighed (in grams), sexed, and two metrics of their shell were recorded (in mm): straight-carapace length (SCL) and plastron length (PL). SCL was measured from the anterior most point of the NS and the posterior most point of the SC. PL was measured from the posterior most point of the gular notch (females) or the gular projection (males) to the anterior most point of the anal notch. For all *G. polyphemus* encountered (excluded, control, or random encounter), the ambient temperature, humidity, location of the individual (in direct sunlight, shade, or burrow), GPS coordinates, and the time and date was recorded. For all burrows encountered, a GPS marker was saved for later analysis. Prior to release of the experimental cohort into the pens, all pre-existing burrows were also marked.

Radiotelemetry, camera-traps, and resurveys

To complement identification by scute drilling, as well as track movements of *G. polyphemus*, experimental and control individuals over 230mm in SCL were outfitted with radio transmitters (American Wildlife Enterprises, Monticello, Florida, USA) attached to the right anterior costal scutes with the use of epoxy putty (West Marine, Watsonville, CA). All *G. polyphemus* were tracked weekly for the first 8 weeks of the study and twice per month thereafter for a total of 11 months. Morphological traits (when out-of-burrow) and environmental metrics were recorded for each subsequent tracking event. When a *G. polyphemus* individual was radiotracked to a burrow, I confirmed presence of the individual in the burrow using a burrow scope (Environmental Management Systems, Canton, GA, USA). Twelve infrared camera traps (Wildgame Innovations, Grand Prairie, TX, USA) were placed outside the burrow apron of radiotracked individuals, starting with six in February 2017 and adding an additional six in May 2017. Camera traps were placed at active burrow entrances and used to reconfirm the presence of a *G. polyphemus* in the interim between radio-tracking events. Camera traps were placed such that an even number of males and females and an even number of controls and experimental *G. polyphemus* were being monitored at a time. As individuals moved, camera traps were uprooted and moved to the new burrow. Each camera trap image was counted as a single voucher for an individual *G. polyphemus* at the GPS location for which the camera trap had been placed. Individuals were cross-referenced to the list of radio-tracked individuals by presence/absence of a transmitter, epoxy pattern around transmitter, identifiable markings/notching, overall morphology, and sex-specific behaviors. Any non-tracked individuals were noted and sexed by behavior (where able).

At the conclusion of the study, 11 months from the first capture, radio-tracked *G. polyphemus* (controls and experimental) were recaptured using flap traps and had their transmitters removed. Morphological traits and environmental metrics were rerecorded. Camera traps were cycled forward to remaining individuals as captures and releases occurred. Environmental metrics were calculated for each camera trap entry using weather data from the National Oceanic and Atmospheric Administration (NOAA), as they were not recorded by the camera traps. Dry bulb (T_{DB}) (in °C) and dew point temperatures (T_{DP}) (in °C) were downloaded from for the six closest weather stations: Apalachicola, Daytona Beach, Jacksonville, Orlando, Tallahassee, and Tampa. Temperatures between sample times at each station were calculated as smooth transitions between sampled temperatures. The weighted dry bulb and dew points for a given sample time were calculated for Halpata Tastanaki as a function of the inverse Vincenty distance-squared (d^2) from each weather station.

$$T = \frac{\sum_i \frac{T_i}{d_i^2}}{\sum_i \frac{1}{d_i^2}}$$

The relative humidity (RH) was calculated using the weighted dry bulb temperature (in K), dew point temperature (in K), and the Clausius–Clapeyron relation:

$$E = 0.611e^{\left(\frac{2{,}453{,}000}{461}\right)\left(\frac{1}{273.15}-\frac{1}{T_{DP}}\right)} \qquad E_s = 0.611e^{\left(\frac{2{,}453{,}000}{461}\right)\left(\frac{1}{273.15}-\frac{1}{T_{DB}}\right)}$$

$$RH = 100\% \cdot \frac{E}{E_S}$$

Average daily humidities were also calculated, after first calculating average weighted dry bulb and dew point temperatures.

Homing Behavior Statistical Analyses: Chapter 1 Novel Method

Utilizing the GPS coordinates for the last known location (ϕ_1, λ_1), the new location (ϕ_2, λ_2), and the (home) burrow of excavation (ϕ_H, λ_H) for each *G. polyphemus*; the distance traveled (d_T), distance from "home" pre-movement ($d_{H,1}$), distance from "home" post-movement ($d_{H,2}$) were calculated using the Vincenty Formula (Vincenty, 1975). Ground-covered (Δd_H) was calculated as the difference between the initial and final distance to the home location, for each movement. Direct azimuths (α_D), azimuths taken (α_T), and error in azimuths taken ($\Delta\alpha_T$) with respect to the home burrow were calculated using the Atan2 function. Using the "ant-walk" method, the effective or "average error" in azimuth taken ($\Delta\bar{\alpha}_T$) was calculated by taking 10,000 even sub-steps between initial and final locations. $\Delta\alpha_T$ and $\Delta\bar{\alpha}_T$ were then remapped to their "vector orbit" counterparts: $V_{o,I}$ and $V_{o,II}$, respectively; by taking the difference between 90 degrees and the absolute value of azimuthal error. Using the R-package BSDA (Arnholt and Evans 2017), novel statistical methods CT, DTI, DTII, OTI and OTII (outlined in Chapter 1) were performed on all movements. Movements where the ground-covered was statistically positive i.e. getting closer to the destination where considered "converging." Negative values i.e. moving away from the destination were considered "diverging." Movements where the azimuthal error was not statistically different than 0 degrees i.e. neither clockwise nor counterclockwise where considered "direct." Movements where the absolute azimuthal error was statistically less than 90 degrees i.e. forward facing were considered "homing." Movements above 90 degrees i.e. facing away

were considered “emigrating.” Finally, those movements not statistically different than 90 degrees about the destination point i.e. moving tangentially were considered “orbiting.”. Only movements post opening of the silt-fence pens and prior to the end of the study (i.e., the period when animals had the opportunity to move freely) were considered. Additionally, movements were logistically compared against time excluded in the pens and relocation distance to identify any significant effects. Ground covered and vector orbits were binarily divided between positive and negative values for logistic comparisons.

Release Behavior Statistical Analyses: Habitat Usage & Predicted Behavior

Following the pen openings, logistic regressions were used to predict the probability of a *G. polyphemus* leaving the originally penned area or reaching the ROW (within 100m) over time and between sexes. Using environmental metrics (temperature and relative humidity as calculated from NOAA weather data), binomial smoothed predictions (coupled with logistic regression) were used to predict burrow occupancy: “in” or “out” of a burrow, separated by sex. Using time of day and date, gamma smoothed predictions were used to predict burrow occupancy, separated by sex. Daily and seasonal occupancy of *G. polyphemus* were estimated and fitted by cosine regression using the R-packages: cosinor (Sachs 2014) and season (Barnett et al. 2014). Using the Florida Wildlife Commission Cooperative Land Cover (CLC) 3.1, habitats were determined for all GPS locations. Across all movements, the number of movements between habitat types were calculated and separated between controls and experimental individuals. Movement counts were displayed using the R-package circlize (Gu et al. 2014). Relative distances between burrows were calculated using the Vincenty formula. Any coburrowing (two or more *G. polyphemus* in one burrow) in the pens,

prior to pens being opened and thereafter, were noted along with the number of new burrows created during the penning period. An ANOVA was conducted to determine the relationship between sex and group (experimental vs. control) on days that a *G. polyphemus* remained in a location as well as distances traveled.

Results

A total of 34 experimental individuals (24 adult, 10 juvenile/sub-adult) were excavated. The 24 adults (11 male, 13 female) were affixed with radio-transmitters and placed into pens for an initial exclusion period of 10-14 days before partial fence removal, depending upon date of excavation. After 37 days, the pens were fully removed but the ROW remained fenced, creating a secondary exclusion period of 47-51 days. Silt-fencing along the ROW was removed 99 days after the silt-fencing comprising the pens were removed, creating a third exclusion period of 146-150 days. Using flap-traps and chance encounters, 13 control individuals (4 males, 9 females) were captured throughout the first 247 days of the study and affixed with radio-transmitters. Experimental individuals were successfully tracked for a period of 64-339 days and control individuals were tracked for 164-362 days. A total of 5 individuals (3 controls, 2 experimental) were not recovered at the end of the study due to 3 radio-transmitters that had come off the shell, 1 loss-of-signal, and 1 collapsed burrow. The 10 untracked individuals removed from the ROW that were under 230mm (4 male, 6 female) were classified as juvenile/sub-adult and not encountered again after the pens were opened. Across all radio-tracking, 1,179 GPS locations (experimental: 775 locations; controls: 404 locations; males: 513 locations; females: 646 locations) were recorded. Complimenting radio-telemetry data, a total of 21,206 camera trap images containing *G. polyphemus* (6,111

images: controls, 13,129 images: experimental) (7,499 images: males, 13,117 images: females) were obtained. Across individual *G. polyphemus*, an average of 30 ± 2 photos was taken daily at 95% confidence. Each image was treated equally to a radio-tracking event to the GPS location after positive identification of the individual. Examples of camera trap imagery have been provided (Figure 9).

Immediately after the pens were opened, individual *G. polyphemus* began migrating out of the penned regions. Migrations continued gradually throughout the study with only 2 females remaining in the regions previously defined by the pens at the study conclusion. Males had a significantly lower logistic probability of remaining in the penned region and left sooner than females ($p \sim 2 \times 10^{-16}$) (Figure 10A). According to logistic modeling, an estimated 50% of *G. polyphemus* individuals are predicted to remain in the penned regions after 30 - 51 days (males), 113 - 121days (females), and 95 - 101 days (overall) after the pens are opened, at 95% confidence. Males also had a significantly higher logistic probability of reaching the ROW and arrived sooner than females ($p \sim 2 \times 10^{-16}$) (Figure 10B). According to logistic modeling, an estimated 50% of *G. polyphemus* individuals are predicted to reach the ROW 51 - 87 days (males), 274 - 279days (females), and 249 - 249 days (overall) after pens are opened at 95% confidence. Among radio-tracked experimental individuals, 8 of 13 females and 2 of 11 males were never found within 100m of the ROW.

Consistent with diurnal *G. polyphemus* behavior, both males and females displayed rhythmic daily patterns, with peak activity (outside of the burrow) during the day (cosinor: $p \sim 2 \times 10^{-16}$) (Figure 11A). *G. polyphemus* also displayed rhythmic behavior with respect to season

(season: $p = 3.29 \times 10^{-12}$) (Figure 12A). According to the cosinor model, peak daily activity from female *G. polyphemus* is predicted at 777 (12:57 PM) ± 3 minutes with male activity peaking at 768 (12:48 PM) ± 3 minutes at 95% confidence ($p \sim 2 \times 10^{-16}$). Similarly, according to the season model, peak seasonal activity of females is predicted at April 19th ± 3 days with male activity peaking at April 23rd ± 4 days at 95% confidence ($p \sim 2 \times 10^{-16}$). Females showed a statistically significant ($p \sim 2 \times 10^{-16}$) logistic trend of being in the burrow during higher humidities, as opposed to males which were found in burrows at lower humidities ($p = 1.4 \times 10^{-9}$) (Figure 11B). Logistically, both males ($p = 0.00026$) and females ($p \sim 2 \times 10^{-16}$) were found predominantly in burrows at lower temperatures (Figure 11C). Temperature and relative humidity followed roughly inverse trends across the day (Figure 11D). Thus, time of day and humidity were found to be good predictors of burrow behavior. Females logistically remained inside burrows significantly more often at both higher daily humidities ($p \sim 2 \times 10^{-16}$) and higher daily temperatures ($p \sim 2 \times 10^{-16}$) as compared to males which were found inside the burrow at lower daily humidities ($p = 0.02913$) and lower daily temperatures ($p = 8.65 \times 10^{-7}$) (Figures 13B and 13C). As was observed with immediate temperatures and relative humidities (Figure 11D), daily temperatures and daily relative humidities did not seem to follow concurrent trends across seasons (Figure 12D). Thus, season, daily temperature, and daily relative humidity were found to be good predictors of burrow behavior. Given the above logistic predictions, females have a greater probability of being in the burrow as real-time humidity, daily humidity, and daily temperatures increase. The opposite effect, while much more shallow, was observed for males (Figures 12B, 13B, and 13C). Cutoff values at which the sex-ratio shifted logistically were at 49.61% - 53.62% real-time humidity, 60.55% - 64.85%

daily humidity, and a daily temperature of 22.33°C - 23.47°C at 95% confidence. Both males and females were predicted to be in burrows at lower real-time temperatures.

A total of 53 movements were recorded for control individuals and 214 movements were recorded for experimental individuals. Controls moved among three habitat types: mesic hammock (MH), sandhills (Sn), and rural open (RO) habitats (Table 5A). Experimental individuals moved between six habitat types: coniferous plantations (CP), depression marshes (DM), mesic hammock (MH), sandhills (Sn), and rural open (RO) habitats (Table 5B). Internal movements were characterized as movements where the source and destination were the same habitat type. Movements between different habitats were classified as external movements. Both controls and experimental individuals most often migrated internally within (73.58% and 63.55%, respectively) and externally between (24.52% and 8.41%, respectively) sandhills and rural open (Tables 5A and 5B) (Figures 14A and 14B). A total of 613 unique burrows were marked by GPS (Figure 14), ranging across 10 unique habitat types (Table 6). Rural open, sandhills, and coniferous plantations were the most common placement of burrows, as coupled with previous movement preferences amongst these habitats. Rare burrow placements were also found among five habitats, to which no individual was radio-tracked: basin marshes (BM), mesic flatwoods (MF), xeric hammock (XH), improved pastures, and transportation. Mean Vincenty distances between burrows in applicable habitats were 170.21 ± 8.94m (BM), 81.18 ± 5.08m (CP), 35.25 ± 4.24 (MF), 252.06 ± 8.49m (MH), 58.26 ± 5.31m (RO), 93.42 ± 5.99m (Sn), and 55.99 ± 2.36m (XH) at 95% confidence. Some habitats e.g. mesic flatwoods had low burrow-to-burrow distance and low burrow counts indicating

single-patch distributions. In the pens (which were rural open), 3 and 4 resident burrows were present prior to penning, respectively. Post-release of excavated individuals, the number burrows present increased to 15 and 16, respectively. Prior to the pens being opened 12 single, 4 double, 4 triple, and 1 case of quadruple resident occupancies were detected. After the pens had been opened, 116 single and 87 double occupancies were detected. Given the increase in burrow density, increase in burrow occupancies, and rate at which individuals left the penned region (despite preferable habitat), pens were likely oversaturated with *G. polyphemus*.

Amongst *G. polyphemus* that changed locations, independent t-tests revealed that males and females ($p = 0.762$) as well as controls and experimentals ($p = 0.799$) did not differ in number of days that they remained at any one location. On average all *G. polyphemus*, regardless of status, remained at each individual burrow 17.45 ± 2.68 days at 95% confidence. Distances moved did not differ between males and females ($p = 0.422$) and were slightly non-significant between controls and experimentals, although experimentals tended to move farther ($p = 0.057$). For any one movement made, the distance traveled was 78.11 ± 3.34m at 95% confidence. Overall, amongst experimental individuals, *G. polyphemus* were not converging toward the burrow of excavation ($p_{CT} = 0.098$), and not using indirect paths, either initially ($p_{DTI} = 0.771$) or on average ($p_{DTII} = 0.771$). However, significant homing (forward-motion toward home) was found with respect to initial angle taken ($p_{OTI} = 0.001$), but not on average when the ant-walk method was considered ($p_{OTII} = 0.098$). Overall, *G. polyphemus* moved -0.78m - 29.29m, took initial bearings of 35.00° - 75.04°, and average bearings of

56.15° - 91.33° at 95% confidence with respect to the burrow of excavation. Separating by sex, males significantly converged (p_{CT} = 0.036), significantly homed towards the burrow of excavation (p_{OTI} = 0.002, p_{OTII} = 0.036), and did not use indirect movements (p_{DTI} = 0.902, p_{DTII} = 0.902). Males converged 1.17m - 34.37m (Figure 15A), taking initial bearings of 23.37° - 62.47° (Figure16B), and average bearings of 42.02° - 86.20° (Figure 15C) at 95% confidence. In contrast, females did not converge (p_{CT} ~ 1.000), did not home towards the burrow of excavation (p_{OTI} = 0.268, p_{OTII} ~ 1.000), and did not use indirect movements (p_{DTI} = 0.430, p_{DTII} = 0.430). Females moved -15.72m to - 34.58m (Figure 15A), taking initial bearings of 60.20° - 99.22° (Figure 15B), and average bearings of 71.54° - 107.58° (Figure16C) at 95% confidence. Thus, males were significantly different than females with respect to homing/converging behavior while maintaining non-significance in the number of days between movements and the magnitude of any one movement made. Removal distance was not found to be logistically significant (for males and females, respectively) with respect to convergence (p = 0.721 and 0.434), initial bearing (p = 0.095 and 0.648), or average bearings taken (p = 0.721 and 0.434). Similarly, exclusion period was not logistically significant (for males and females, respectively) with respect to convergence (p = 0.565 and 0.619), initial bearing (p = 0.687 and 0.28), or average bearings taken (p = 0.565 and 0.619).

Table 5A: Total number of movements amongst control individuals between habitat types according to the CLC 3.1.

Movements amongst Controls		**Destination Habitat**			
		Mesic Hammock	Rural Open	Sandhills	**Total**
Originating Habitat	Rural Open	1	18	6	**25**
	Sandhills	0	7	21	**28**
	Total	1	25	27	**53**

Table 5B: Total number of movements amongst experimental individuals between habitat types according to the CLC 3.1.

Movements amongst Experimental		**Destination Habitat**					
		Coniferous Plantations	Depression Marshes	Mesic Hammock	Rural Open	Sandhills	**Total**
Originating Habitat	Coniferous Plantations	41	0	2	0	0	**43**
	Depression Marshes	0	0	0	0	1	**1**
	Mesic Hammock	3	0	0	0	0	**3**
	Rural Open	10	1	1	99	11	**122**
	Sandhills	1	0	0	7	37	**45**
	Total	55	1	3	106	49	**214**

Table 6: Total number of unique burrows recorded (613 total) among habitat types according to the CLC 3.1.

Habitat	**Burrow Count**	**Habitat**	**Burrow Count**
Basin Marsh	9	Mesic Hammock	14
Coniferous Plantations	104	Rural Open	356
Depression Marshes	2	Sandhills	115
Improved Pastures	2	Transportation	1
Mesic Flatwoods	6	Xeric Hammock	4

Discussion & Future Research

Previous studies have indicated that penning increases site fidelity and reduces dispersion among *G. polyphemus* post-translocation. However, this effect is time-dependent, often requiring 9-12 months to be effective (Tuberville et al. 2005). Given that our individuals were only truly penned for 10-14 days, it was unlikely to produce a statistically significant effect. While the opened pens remained for a total of 47-51 days with respect to translocated individuals, most gopher tortoises crossed the previous silt-fence threshold the moment the pens were opened, preventing any short-term and partial effects that the remaining pens could have provided. Given that males are the more aggressive and active

sex (McRae et al. 1981a) and that they maintain wider home-ranges (Diemer 1992), it is not surprising that they left the penned regions and reached the ROW sooner than females.

As noted but unconfirmed by McRae et al. (1981a), I found statistically supported evidence of homing behavior by *G. polyphemus* males in this study. Whether or not females possess this ability, but do not engage in the behavior, is unclear. Future research into the mechanisms enabling homing behavior in *G. polyphemus* could utilize methods that identified magnetoreception in green sea turtles (*Chelonia mydas*), another long-lived chelonid, such as scanning for magnetite deposits (Perry et al. 1985) or attaching magnets to the plastrons (Baldwin 1972) of *G. polyphemus* and radiotracking their movements. In a few rare cases, gopher tortoises were found on the ROW, nearly on top of the exact GPS coordinates of the previous burrow from which they had been excavated. While promising for magnetoreception as a potential method, this may be confounded by other mechanisms that could have aided in site familiarity, such as chemical cues or surrounding landmarks (Lohmann et al. 2008).

A pressing concern within any translocation event is the success of the individuals thereafter. In a 2011 study by Tuberville and colleagues, male and female *G. polyphemus* were translocated 110km and released to a region containing resident *G. polyphemus*. Regardless of female status (resident or translocated), resident males were preferentially selected in mating events and clutch production. However, within this study males were able to and did home back to the region that they had been excavated from, whereas females remained in their newly established region. What is unclear is whether homing males will be

perceived as residents or introduced individuals to the originating location. Future research should provide information on reproductive success on translocated individuals with respect to homing behavior and whether it is a concern in *G. polyphemus* conservation.

Consistent with the findings of McRae et al. (1981a), *G. polyphemus* activities levels were highly correlated with mean seasonal temperatures (decreased in colder months) and diurnal behaviors. However, I quantified activity by logistic probability rather than relative frequency. An important difference in this study was that camera traps provided the bulk of activity information (which were pointed directly at burrows), as opposed to chance encounters, and used real-time humidity (previously unstudied) and temperatures values. This allowed for fine-scale analysis of *G. polyphemus* behavior. Initially, status of burrow occupancy was cryptic with radio-telemetry consistently leading to gopher tortoises in burrows. After the addition of camera traps, I confirmed that individuals were highly conservative, sometimes staying for hours within the camera's frame, and ducking into burrows on approach of the research team.

Camera traps with high sensitivity can be cumbersome to process as they are easily falsely triggered (Swann et al. 2011). However, infrared camera traps provided additional levels of information. As opposed to plate-triggered camera traps previously used on *G. polyphemus* (Guyer et al. 1997), infrared-triggered traps provided information on behaviors past the burrow mouth (where applicable) and among the surround areas. *G. polyphemus* crossed the threshold of the burrow mouth several times a day (from both forward and reverse directions) and in many cases remained at the burrow mouth for prolonged periods

(hours). An additional advantage was the capturing of crepuscular and nocturnal activities with occasional long-term observations of gopher tortoises sleeping near the mouth of the burrow. Finally, camera traps provided accountability and additional sampling in the interim to and well complimented radio-tracking events.

In summary, site fidelity and dispersion of *G. polyphemus* was unaffected by relocation and penning due to the Sabal Trail construction pipeline, with exclusion time being too short to affect movement ecology. Unconfirmed in prior studies, I have shown that male *G. polyphemus* engage in significant homing behavior. The disparity between males and females as well as the underlying method is unknown, requiring further research. Consistent with previous assessments, gopher tortoises are well characterized as rhythmically diurnal and seasonally active in the summer season. However, temperature and humidity both on the micro- and macro-scale play important (and often opposite) roles in activity levels between males and females. Point metrics for these analyses, as well as for habitat usage have been provided for greater predictability and location of G. polyphemus for future conservation efforts.

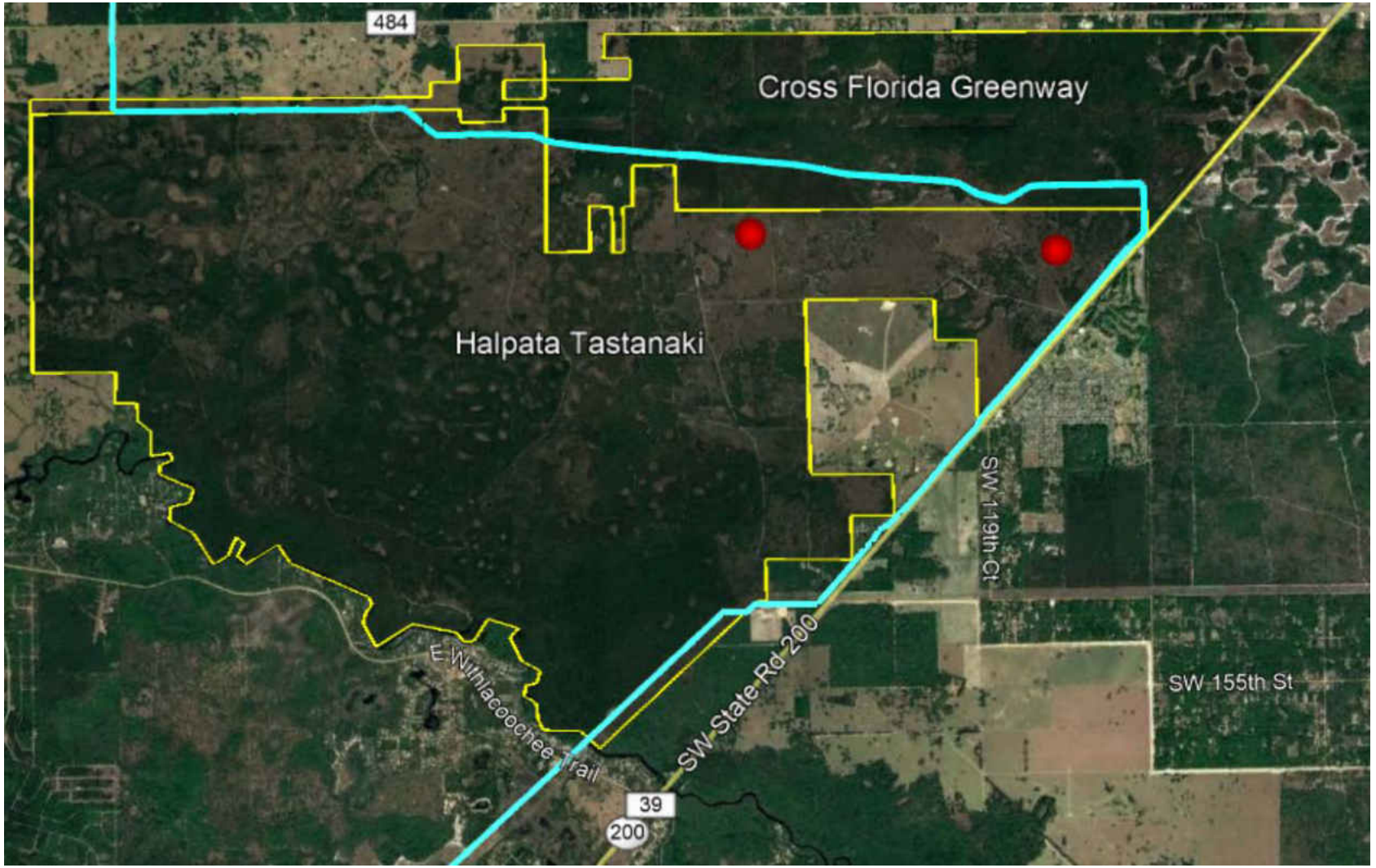

Figure 7: Map of study sites: Halpata Tastanaki Preserve and the adjacent Cross Florida Greenway for which the Sabal Trail construction project took place. Pen locations for which all relocated *G. polyphemus* were relocated are depicted as red dots. Outlines of the preserve are depicted in yellow. Path of the Sabal Trail pipeline is depicted as a cyan line.

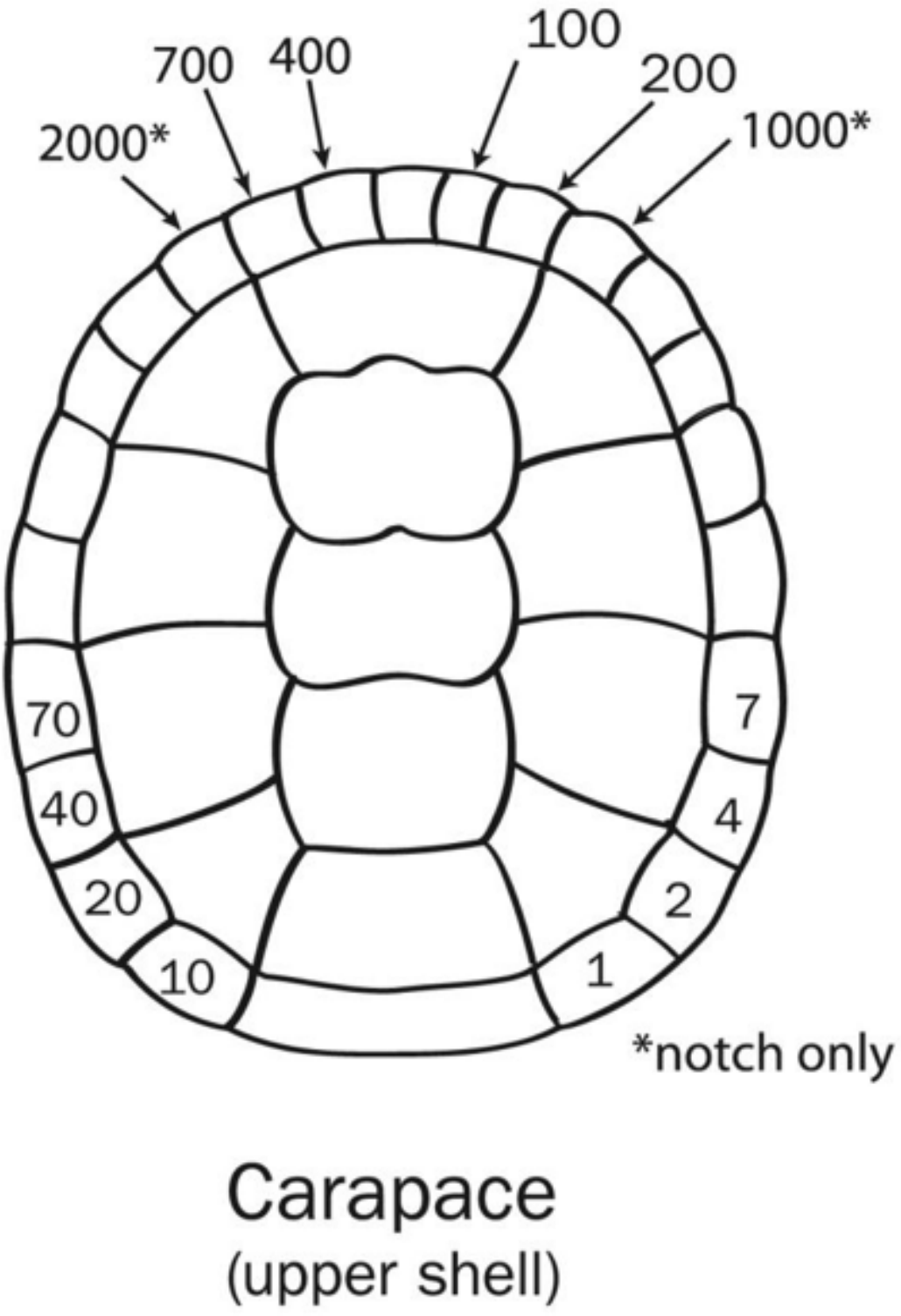

Figure 8: Drill marking method, as developed by FWC (Appendix 5 of the FWC Gopher Tortoise Permitting Guidelines, February 2015).

Figure 9: Examples of camera trap imagery for *G. polyphemus*. Included are instances of a female emerging from her burrow (A), male-to-male combat (B), a male returning to a burrow obscured by long-leaf pine and a commensalist snake (C), and a female sleeping near the mouth of the burrow along with a commensalist mouse (D).

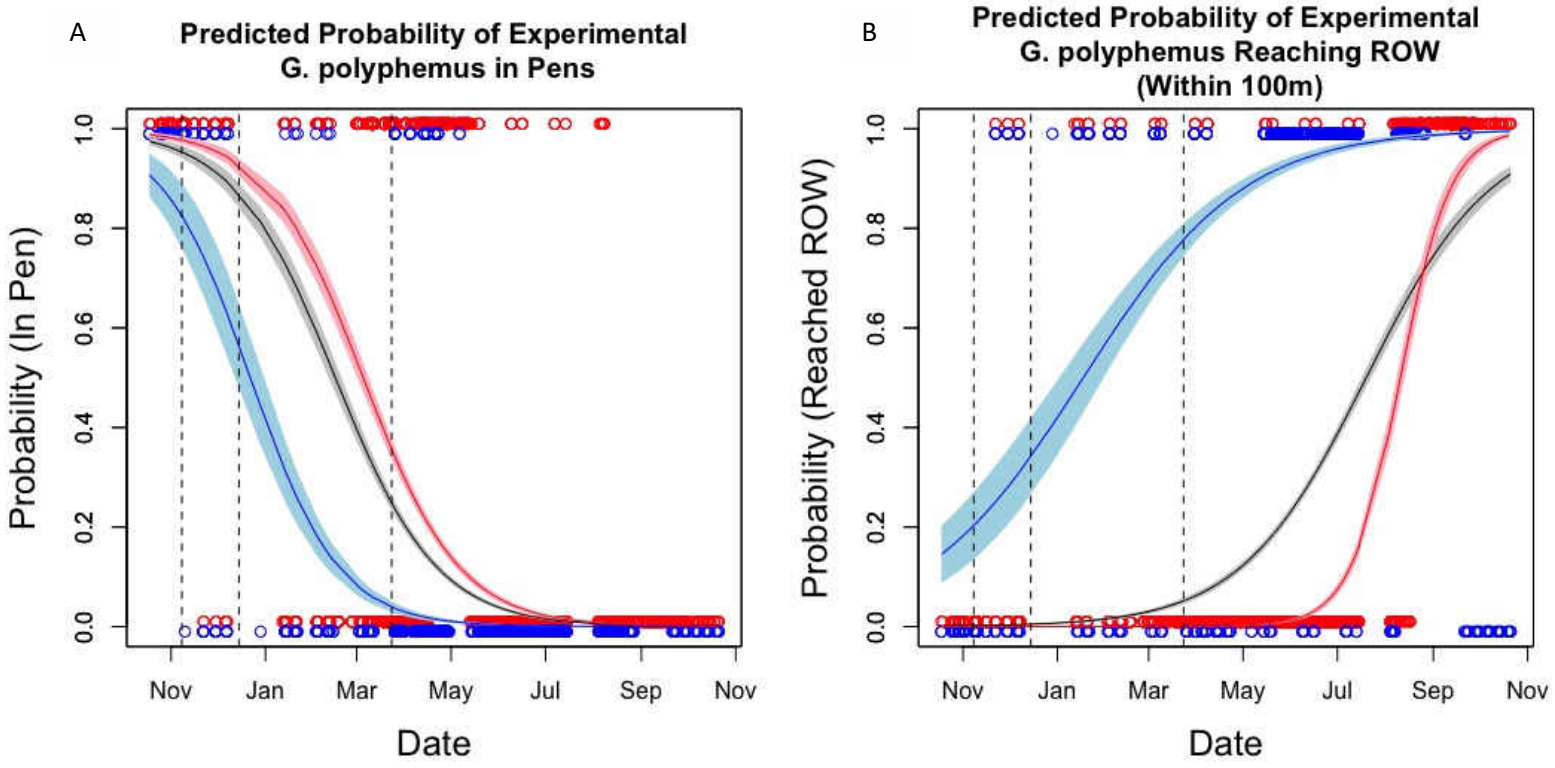

Figure 10: Logistic predictions with 95% confidence intervals of *G. polyphemus* leaving the penned region (A) or reaching the ROW (B) (within 100m) over time. Predictions were conducted on *G. polyphemus* experimental individuals overall (black), males (blue), and females (red). Three relevant exclusion dates (pens opened, pens removed, and silt-fence along the ROW removed; vertical dashed lines) are shown for comparison. Circles denote point measurements of males (blue) and females (red) located in and out of burrows via radio-tracking (left panel) and at the ROW (site of home) or not at the ROW (right panel).

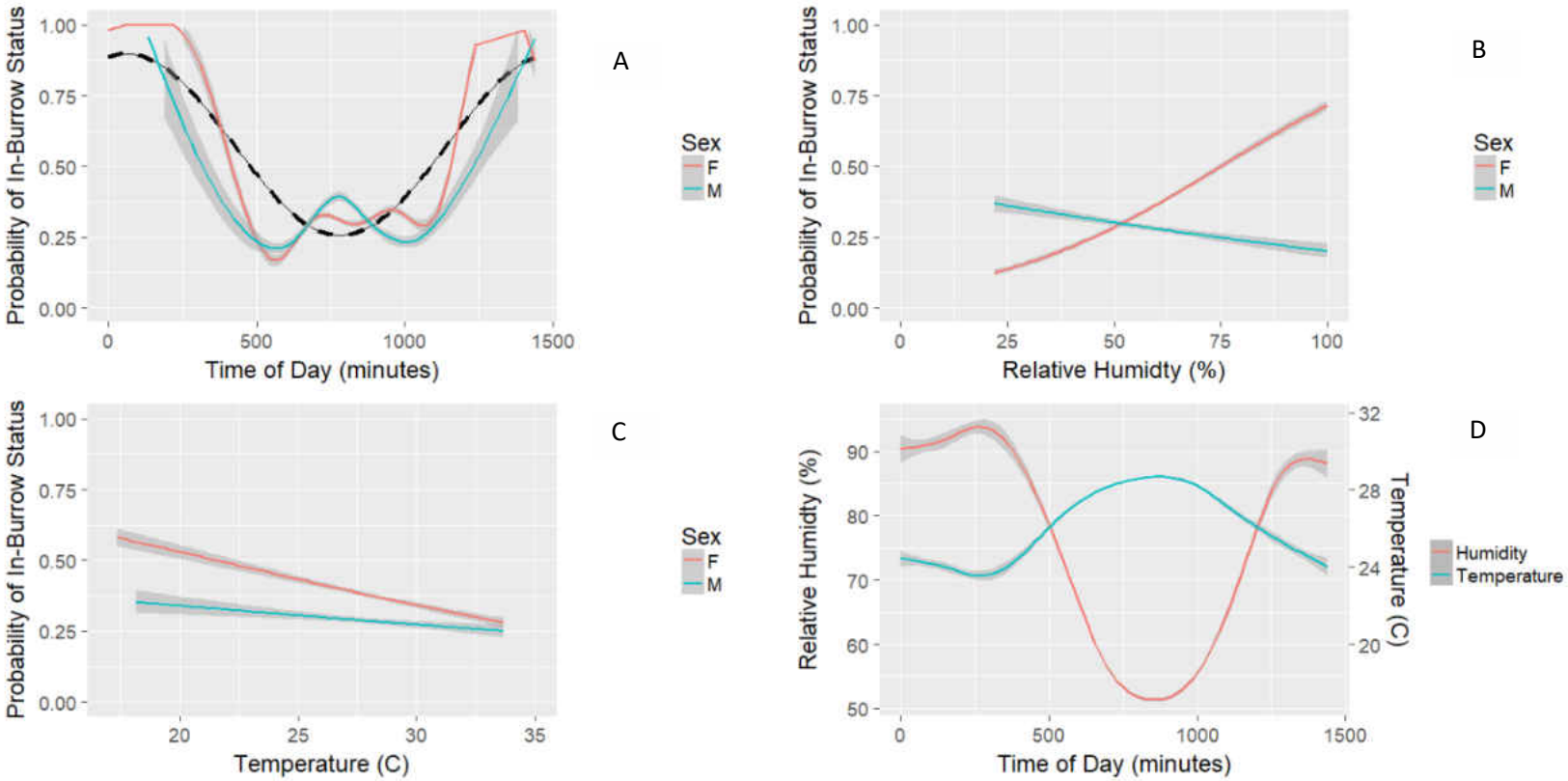

Figure 11: (A) Gamma-smoothed predicted probability of *G. polyphemus* in burrows with respect to sex and time of day (minutes). A cosinor fitted prediction (dashed black line) is also shown for all *G. polyphemus* individuals. Binomial smoothed predicted probability of *G. polyphemus* in burrows with respect to (B) relative humidity (%) and (C) temperature (°C). (D) Gamma smoothed predicted relative humidity (%) and temperature (C) with respect to time of day.

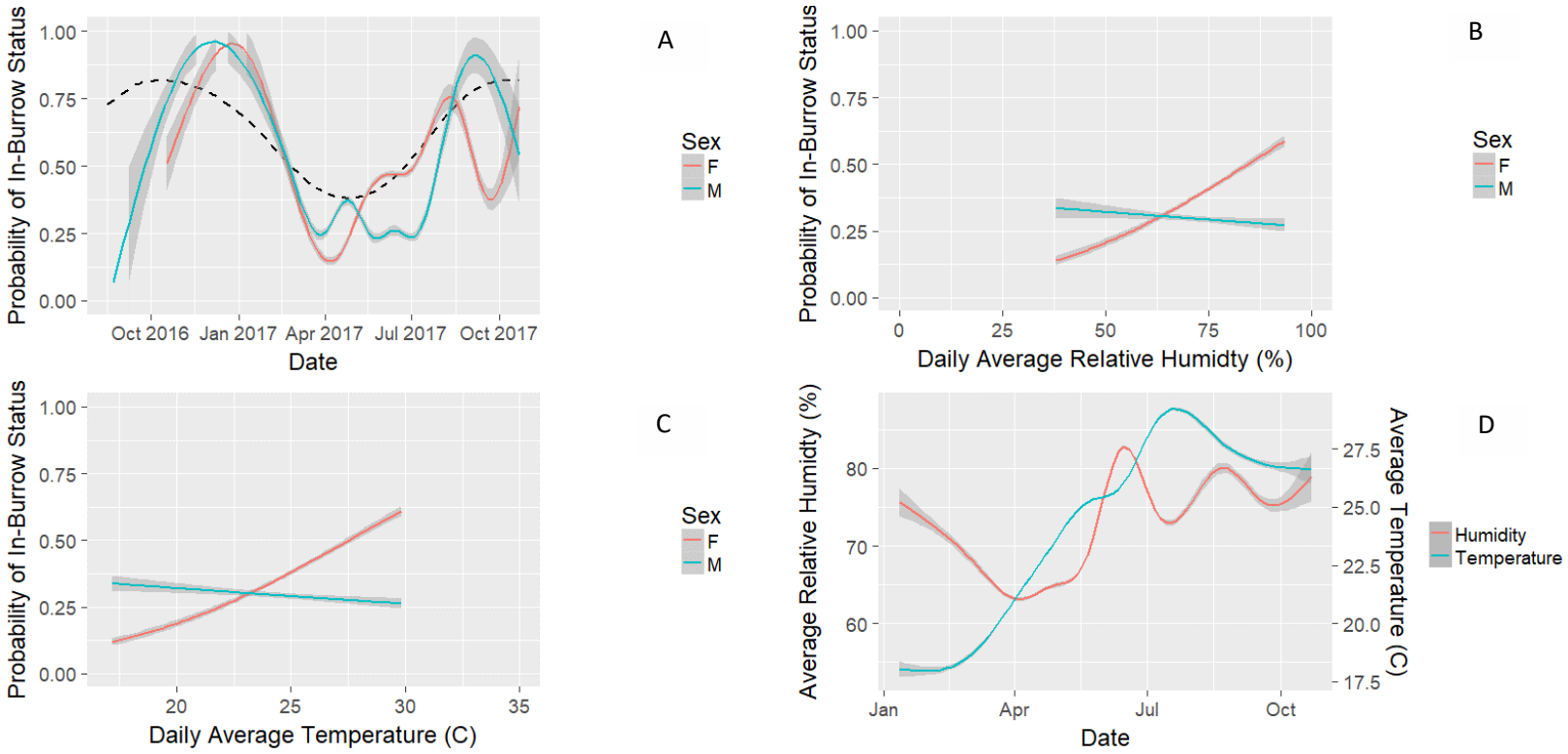

Figure 12: (A) Gamma-smoothed predicted probability of *G. polyphemus* in burrows with respect to sex and date. A cosinor fitted prediction (dashed black line) is also shown for all *G. polyphemus* individuals. Binomial smoothed predicted probability of *G. polyphemus* in burrows with respect to (B) relative humidity (%) and (C) temperature (°C). (D) Gamma smoothed predicted relative humidity (%) and temperature (C) with respect to time of day.

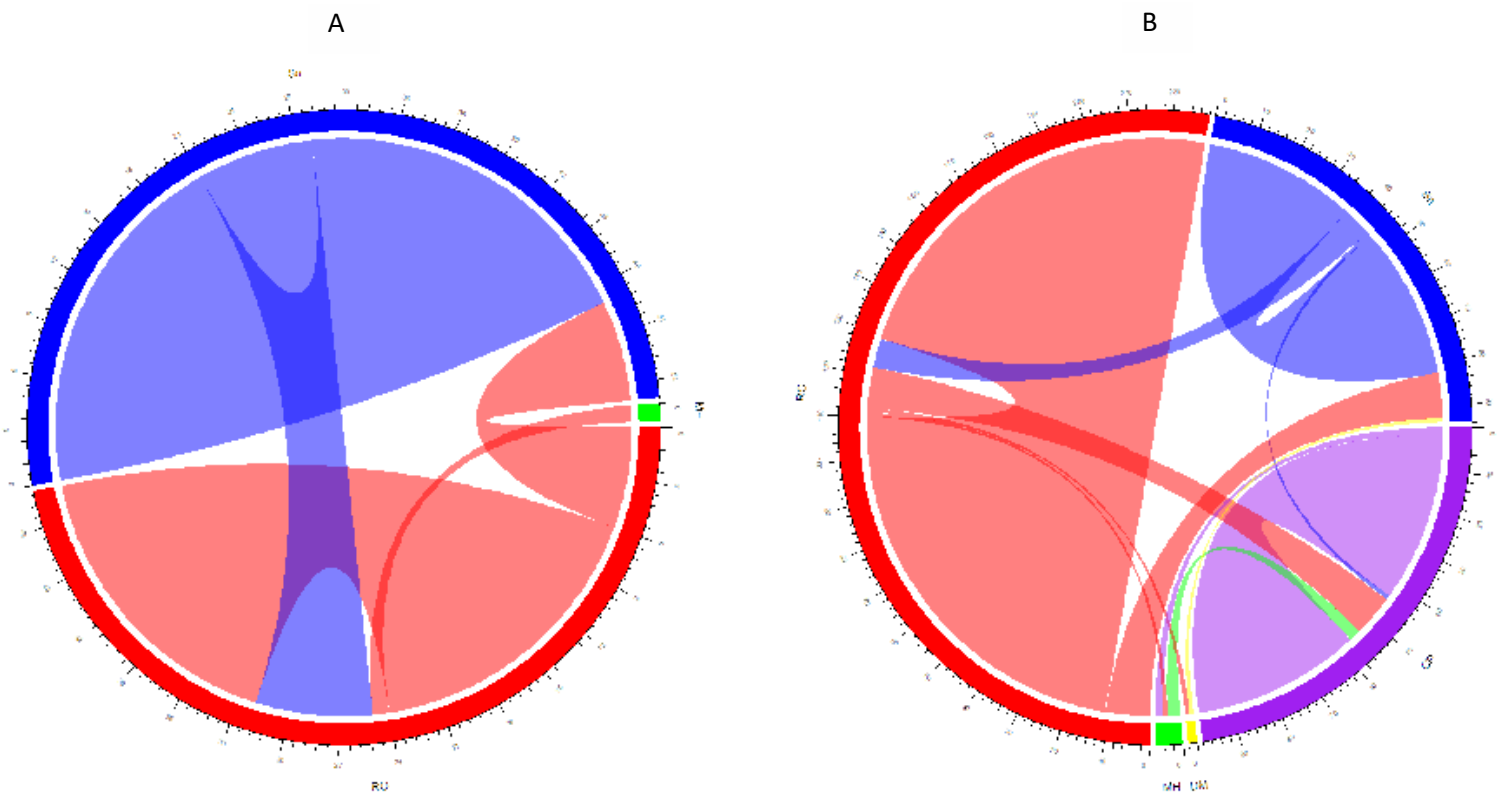

Figure 13: Chord diagrams of movements among habitat types, as determined by the CLC 3.1, for (A) control and (B) experimental individuals. Habitats ranged from rural open (RO; red), mesic hammock (MH; green), sandhills (Sn; blue), coniferous plantations (CP; purple), and depression marshes (DM; yellow). Colors are shown by originating habitat.

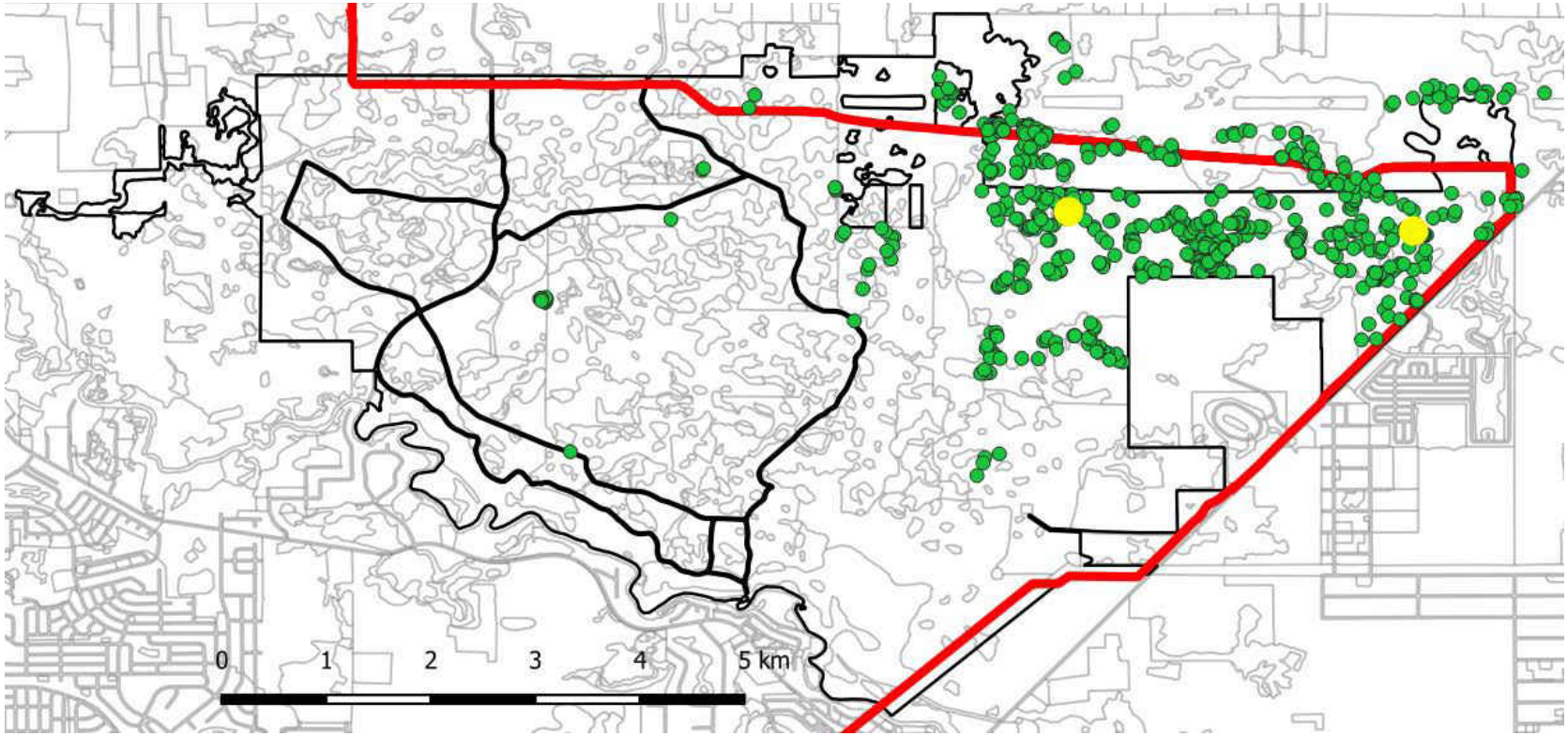

Figure 14: Map of Halpata Tastanaki region against CLC 3.1. Burrows discovered throughout the study (green) are shown in relation to the two pens (yellow) and the ROW (red). A total of 613 unique burrows were discovered across 10 unique habitat types. The contiguous boundary of all habitats within Halpata is depicted in black. Individual habitat boundaries are depicted in gray.

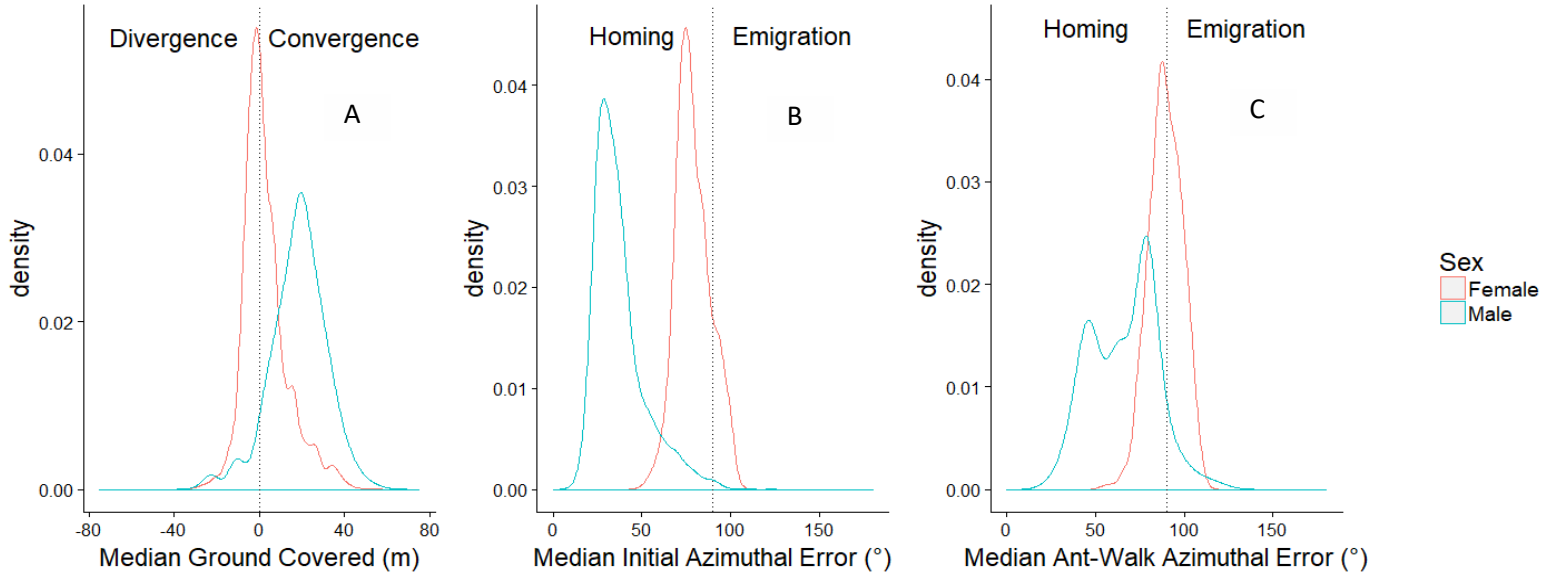

Figure 15: Distribution of Bayesian-resampled movement statistics for male (blue) and female (red) *G. polyphemus*. Median values of ground-covered (A), initial azimuthal error (B), and the "ant-walk" (average) azimuthal error (C) were resampled 10,000 times, with 20 movements per sample (with replacement). Positive ground covered values were labeled as converging towards a destination, whereas negative ground covered was labeled diverging from the destination. Azimuthal error below 90° was labeled as homing, whereas azimuthal error above 90° was labeled as emigration.

CHAPTER 3: DISEASE ECOLOGY OF GOPHER TORTOISES (GOPHERUS POLYPHEMUS) POST-EXCLUSION AND RELOCATION

Introduction

Two North American tortoise species, the gopher tortoise (*Gopherus polyphemus*) and the desert tortoise (*Gopherus agassizii*), are prone to infections by the bacterium *Mycoplasma agassizii*, with disease transmission being directly linked to physical contact and relocations by captive individuals (Dodd and Seigel 1991). Stress alone, be it through relocation, can lead to changes in immune expression, plausibly altering rates of disease infection (Hing et al. 2016). Animals infected with disease often engage in complex habitat usage to clear parasites or fail to migrate following infection, as movement can lead to increased reinfections due to compromised immune function (Altizer et al. 2011). In particular, *G. agassizii* engages in homing behavior to mitigate the stress component of mortality-risk post-relocation (Hinderle et al. 2015). It is unclear, although inferable, if *G. polyphemus* engages in a similar behavior. Paradoxically, though, the movement itself may lead to an individual becoming immunocompromised due to stress and energy expenditure, which may lead to an overall increased risk of pathogen infection. This could be particularly complicated if there is a strong urge to home, regardless of disease status. To counteract homing behavior when tortoises are relocated as part of conservation mitigation strategies, exclusion fencing is often installed. Unfortunately, exclusion can elevate mortality risk and thermal stress, as has been seen in *G. agassizii* due to the behavioral aspect of "fence-pacing" (Hinderle et al. 2015, Farnsworth et al. 2015). Furthermore, ordinally longer exclusion periods produce increased site fidelity and a decrease in overall movement post-release (Tuberville et al. 2005). Thus, longer exclusion

periods likely lead to increased internal pacing and stress within fenced enclosures, as well as greater opportunities for direct contact and pathogen transmission. In contrast, pathogen infection may negatively affect homing and overall movement, which potentially reduces stress and immune function changes associated with extensive movement, but could increase stress and/or alter immune function as a direct result of pathogen burdens. To date, no study adequately has comprehensively evaluated exclusion and relocation on the short and continuous scale with respect to disease development, pathogen infection, immune function, and homing behavior in *G. polyphemus*.

Mycoplasma agassizii and *Mycoplasma testudineum* are bacteria lacking cell-walls, possessing a trilaminar unit membrane, and are amongst the smallest bacteria, typically between 350 to 900 nm in size (Brown et al. 2001). Both pathogens are well known for causing Upper Respiratory Tract Disease (URTD) and have been isolated from many species of tortoises, to include *G. agassizii* and *G. polyphemus*; all tortoises are considered potential susceptible. (Jacobson et al. 2014). Coupled with habitat destruction and other environmental factors, UTRD is associated with population declines in both species (Brown et al. 1994). Outward disease signs are often absent for mild infections, but chronic pathogen loads can lead to rhinitis (stuffy nose), high degrees on nasal exudate, and a demarcated increase in neutrophils in blood serum (Brown et al. 1994). Other signs of URTD include ocular discharge, conjunctivitis (pinkeye), and palpebral edema (eyelid swelling). Tortoises with chronic URTD eventually become emaciated, anorexic, lethargic, and die from wasting away (cachexia) (Brown et al. 1999).

Iridoviruses are icosahedral double-stranded DNA viruses 150-300nm in diameter which

necrotize tissue from excessive membrane budding (Westhouse et al. 1996). In *G. polyphemus*, the genus *Ranavirus* is known to cause URTD by necrotizing the trachea, lungs, pharynx, and esophagus (Westhouse et al. 1996). Typical ocular and nasal discharge and dyspnea (labored breathing) are present in chelonians with intracytoplasmic inclusions observed in three granulocyte types: azurophils, basophils, and heterophils (Allender et al. 2006, Westhouse et al. 1996). Lethargy, anorexia, conjunctivitis, and edema have also been observed across several chelonians infected with *Ranavirus* (Marschang 2011).

Herpesviruses are icosahedral double-stranded DNA viruses characterized by causing necrotizing stomatitis (mouth sores) (Johnson et al. 2005). Infections of herpesviruses occur across tortoise species, including *Gopherus*, causing necrotizing swelling on the tongue, trachea, pharynx and nares; URTD; and high rates of mortality (Johnson et al. 2005). Infected tortoises exhibit nasal and ocular discharge, labored gasping sounds in their breathing, and anorexia (Schumacher 1997). Amassment and infiltration of heterophils, lymphocytes, and macrophages are observed in other tissues with epithelial cells showing inclusion of eosinophilic to amphophilic intranuclear bodies (Johnson et al. 2005).

Narrowing down a single cause to URTD is difficult. *Mycoplasma* is commonly identified as the etiological agent based on anti-*Mycoplasma* antibodies detected in blood (Schumacher et al. 2017), but given a strong overlap in overall signs, *Ranavirus* cannot be discounted as a contributing factor (Jacobson et al. 2014). Coinfection of Herpesvirus and *Mycoplasma* is also believed to have a synergistic effect in the development of outwards signs (Jacobson et al. 2014). Furthermore, *G. polyphemus* displays several behaviors that can falsely identified as disease

signs. During high levels of stress, such as capture, nasal and oral discharges are common along with gaping during breathing; bucket trapping can also lead to palpebral edema (Wendland et al. 2009). Environmental factors can also lead to URTD signs. Wheezing and nasal exudate occur more often during drier parts of the year, linked to stress and decreased food availability (Karlin 2008). To complicate things further, during drought-months higher rates of *Mycoplasma* antibody detection occur as compared to high rainfall-months, leading to the supposition that weather can drive disease outbreaks (Lederle et al. 1997). Thus, the overlapping clinical disease signs associated with these three pathogen groups coupled with environmental stress are confounding, with no exact metric for diagnosis and no clear relationship between pathogen infections and health consequences.

Here, I evaluated *Mycoplasma*, *Ranavirus*, and Herpesvirus infection status, disease signs, and blood leukocyte profiles in adult *G. polyphemus* from central Florida, USA. A cohort of relocated and excluded tortoises were tracked and compared to a control group of unmanipulated tortoises for one year, with repeated samples collected for health and pathogen screening throughout the duration of the study. Using these data, I evaluated the following three research questions: (1) Do disease signs, growth rate, distance traveled, or leukocyte profiles predict infection or co-infection with any of the three focal pathogens? (2) Is the prevalence of *Mycoplasma*, *Ranavirus,* and Herpesvirus higher in relocated and excluded compared to unmanipulated *G. polyphemus*? (3) Does infection status over time change significantly among relocated and excluded compared to unmanipulated *G. polyphemus*?

Methods

Health Conditions

Along with morphological traits and environmental metrics (Chapter 2), the following signs from each *G. polyphemus* were recorded: level of nasal exudate (mild, moderate, or severe), conjunctivitis (+/-), palpebral edema (+/-), oral plaques (+/-), parasites (+/- & type), and severity/type of injuries (superficial, moderate, or debilitating; crushed, laceration, predation, etc.). Common parasites that were identified were Gopher Tortoise Ticks (*Amblyomma tuberculatum*) (At) and Soft-bodied Ticks (*Ornithodoros turicata*) (Ot). These conditions were recorded according to the US Army Corps of Engineer Handbook on Gopher Tortoise (*Gopherus polyphemus*) (Wendland et al. 2009).

Turtle tissue samples

Blood samples were collected from the subcarapacial venous sinus, located under the carapace on the dorsal midline, following established procedures that cause minimal discomfort to the animal (Hernandez-Divers et al., 2002; Dodd, 2010). Using a sterile needle inserted at a 45-degree angle, 0.5-2ml of blood was drawn. If the needle stick was dry, a maximum of 4 needle sticks were attempted. Immediately after blood was drawn, a drop of whole blood was aliquoted onto a glass slide (for immune cell counts) and immediately smeared by placing a cover slip over the blood. The remainder was dispensed into a vacutainer collection tube. Blood was drawn from as many *G. polyphemus* as possible, both from experimental excavated and control individuals during intimal assessment, final captures, and any chance encounters in-between. Blood was not drawn from the same animal within a three-week period, nor was blood drawn from any animal more than 3 times total during the study. Additionally, a swab of any

nasal discharge was collected and stored. In the event of no nasal discharge, an eye swab was collected instead.

Molecular Pathogen Screening

Genomic DNA was extracted from blood and nasal/eye swabs using DNeasy blood and tissue kits (Qiagen), eluting into a volume of 200µl for all samples. Testing for the presence of the three pathogens known to cause URTD in *G. polyphemus* (*Ranavirus*, *Herpesvirus*, and *Mycoplasma*) was performed. The prevalence and intensity of *Ranavirus* was determined using a Taqman quantitative (q)PCR assay, targeting a highly-conserved region of the major capsid protein (Allender et al. 2013). *Ranavirus* qPCR was run on a Bio-Rad CFX96 Real-Time qPCR System and analyzed with Bio-Rad CFX Manager software. All samples were run at least twice, and conflicting results were resolved by a third run. Each qPCR plate was run with positive and negative controls. Prevalence of *Mycoplasma* infections was measured using a PCR assay targeting 16S ribosomal RNA gene sequences specific for *M. agassizii* and *M. testudineum*, the two-bacterial species known to cause tortoise UTRD (Braun et al. 2014). Forward and reverse primers of Braun and colleagues (2014) were used at the following conditions: 5 minutes 95°C, followed by 40 cycles of 15 seconds at 94°C, 15 seconds at 64°C, and 15 seconds at 72°C. Finally, chelonian *Herpesvirus* prevalence was measured using a nested PCR assay, targeting conserved coding motifs present in DNA-dependent DNA polymerases among alpha-herpesvirus (VanDevanter et al. 1996). The PCR protocol by VanDevanter and colleagues (1996) was modified to use primers DFA and KG1 in round 1, with a bifurcation into two second rounds, where round 2a used primers DFA and IYG and round 2b used primers ILK and KG1. PCR was run with the conditions of 5 minutes 95°C, 30 cycles of 30 seconds at 95°C, 1 minute at X°C, and Y

seconds at 72°C, and a final extension of 1 minute at 72°C. Annealing temperatures (X) were 54.2°C, 55.6°C, 55.4°C with extension times (Y) of 43 seconds, 30 seconds, and 38 seconds for rounds 1, 2a, and 2b; respectively. Gel electrophoresis was run on 2% agarose at 75V for 35 minutes for all PCR samples.

White Blood Cell Counts

Immediately after all blood draws, a drop of blood was placed on a glass slide and smeared with a cover slip. Blood slides prepared at the time of blood draws were dried overnight, then fixed in 100% methanol for 5 min, stained with DipQuick (MWI Veterinary Supply) and examined at 1000× magnification using a standard light microscope. Leukocyte profiling, an approach for inferring general cell-mediated immune function of any vertebrate animal (Jain 1993), was then performed for all readable blood slides. Leukocytes comprise five distinct cell types in reptiles. Comparing their relative abundances across treatment groups indicates which branches of the immune system have been activated and to what degree (Allender and Fry 2008). For each slide, 100 leukocytes were counted and identified as neutrophils (heterophils), lymphocytes, eosinophils, monocytes, or basophils (Heatley and Johnson 2009).

Statistical Methods: Pathogen Loads & Health Metrics

For disease screening, a binary status of present (1) or absent was recorded for each pathogen. Blood draws and tissues swabs were analyzed separately. For each pathogen a Cohen's (1960) kappa coefficient (κ) was computed to determine the agreement between detectability in screening between blood and swab samples. Power of each screening method was determined as the probability that a second screening (e.g. PCR on blood samples) did not

make type II errors (β) with respect to the assumption that the first screening (e.g. PCR on swab samples had accurately determined pathogen presence ($1 - \beta_{A|B}$). Power was determined in both directions, swab screening over blood screening and blood screening over swab screening. The relative power of each method was computed as the ratio of power between each screening method ($\Pi_{B,A}$). Synergistic/antagonistic relationships between pathogens was computed using Cohen's kappa coefficients (κ). For each subsequent molecular sample, a status change (or lack thereof) of infection status for all three pathogens was recorded. Binomial proportion of infection (i.e. infection prevalence) and 95% Clopper-Pearson confidence intervals were calculated among four groups of tortoises: all males, all females, all relocated and excluded individuals (=experimental group), and all unmanipulated individuals (=control group). Proportion tests were used to indicate significance between control and experimental individuals, males and females, and between pathogens. Leukocyte profiles were examined by composition analysis using the following R packages: compositions (van den Boogaart et al. 2014) and energy (Rizzo and Szekely 2017). Effects of any one pathogen on any one leukocyte composition were double-checked using t-tests. Chi-squared tests were used to determine whether external signs correlated with pathogen presence using the R package MASS (Venables & Ripley 2002). Rates of growth of SCL and plastrons were calculated for each individual. ANOVAs were used to determine variable rates of growth between males and females, between controls and experimental individuals, and among pathogen infection statuses. Linear models were used to compare growth rate to number of days excluded within the pens and total

distance traveled. Total distance traveled was also compared to disease prevalence, sex, and control/experimental statuses using ANOVAs.

Results

Size Metrics & Growth

Amongst radio-tracked adults, male size metrics were 3.379 ± 2.25kg, 26.67 ± 3.94cm SCL, and 24.69 ± 3.61cm plastron at 95% confidence. Female size metrics were 4.36 ± 1.66kg, 28.22 ± 3.73cm SCL, and 25.12 ± 3.28cm plastron at 95% confidence. Growth rates of SCL were 0.002 ± 0.002 cm/day (males/females), 0.000 ± 0.004 cm/day (experimental), and 0.005 ± 0.008 cm/day (controls) at 95% confidence. Growth rates of plastrons were 0.000 ± 0.002 cm/day (males/females), -0.001 ± 0.002 cm/day (experimental), and 0.002 ± 0.00g cm/day (controls) at 95% confidence. ANOVAs determined that neither sex ($p = 0.12$), experimental/control statuses ($p = 0.097$), nor pathogen infection statuses ($p_{Herpesvirus} = 0.76$, $p_{Ranavirus} = 0.13$, $p_{\textit{Mycoplasma}} = 0.39$) were statistically significant with respect to SCL growth rates. Similarly, ANOVA analysis of plastron growth rates were not statistically affected by sex ($p = 0.56$), experimental/control statuses ($p = 0.20$), nor pathogen infection statuses ($p_{Herpesvirus} = 0.15$, $p_{Ranavirus} = 0.0921$, $p_{\textit{Mycoplasma}} = 0.31$). While non-significant, SCL growth variability was minimally explained by experimental/control status. Likewise, some non-significant variability of plastron growth was explained by the presence of *Ranavirus*. By linear modeling, SCL growth rate was not significantly correlated with days excluded ($p = 0.33$) nor total distance traveled in meters ($p = 0.36$). Linear modeling of plastron growth confirmed non-significance of days excluded ($p = 0.34$) and total distance traveled in meters ($p = 0.21$). However, total distance traveled was significantly linked with sex ($p = 0.026$) and control/experimental status ($p = 0.0051$). Pathogen infection status did

not statistically affect distance traveled ($p_{Herpesvirus} = 0.76$, $p_{Ranavirus} = 0.42$, $p_{\textit{Mycoplasma}} = 0.13$). Experimental individuals traveled 1000m ± 800m further than controls and males traveling 800 ± 700m further than females at 95% confidence.

Pathogen prevalence and disease signs

A total of 73 blood samples, 87 swabs, and 54 microscope slides were obtained across 51 individuals (34 experimental and 17 control, 25 male and 26 female). Cases where clinical signs were present (+) or absent (-) were as follows: 3+/93- nasal exudate, 18+/64- eye-froth, 23+/74- conjunctivitis, 9+/88- palpebral edema, 1+/23- oral plaques, 37+/62- ticks, and 15+/77- injuries. *Ranavirus* viral loads among samples were determined to be highly erratic. Using only positive samples, Shapiro-Wilk tests determined that *Ranavirus* loads in samples were non-normal for blood ($p = 2.684 \times 10^{-7}$) and swabs ($p = 1.891 \times 10^{-4}$). Log-transformed *Ranavirus* loads were also non-normal for blood ($p = 2.225 \times 10^{-3}$) and swabs ($p = 0.0145$). Excess mass tests were conducted using the R package: multimode (Ameijeiras-Alonso et al. 2018). Tests of skew were performed using R package: e1071 (Meyer et al. 2017). Log-transformed *Ranavirus* loads were found to be multimodal for blood (excess mass statistic = 0.159, p-value = 0) and swabs (excess mass statistic = 0.2271, p-value = 0). Log-transformed *Ranavirus* loads were also found to be right-skewed for both blood (skew = 0.433) and swabs (skew = 0.404). Using Bayesian resampling (average of 5 samples, 1000 iterations) the 95% confidence interval for mean *Ranavirus* loads (per 2.5µL sample) were $10^{4.08 \pm 0.02}$ in blood and $10^{3.74 \pm 0.02}$ in swabs. Thus, we were unable to use *Ranavirus* loads to compare infection intensity across groups, and only used prevalence metrics for subsequent statistical testing.

Overall infection prevalence across all samples was 0.48 for *Herpesvirus*, 0.39 for *Ranavirus*, and 0.72 for *Mycoplasma* (Table 7). External disease signs were not statistically linked to infection with Herpesvirus (Chi-squared = 2.88; p = 0.94), *Ranavirus* (Chi-squared = 8.77; p = 0.36), or *Mycoplasma* (Chi-squared = 5.71; p = 0.68). External disease signs were also not significantly linked to infection with any of the three pathogens (Chi-square = 0.87; p = 0.999).

Table 7: Mean agreement ($\kappa_{A,B}$), power ($1-\beta_{B|A}$, $1-\beta_{A|B}$) and relative power ($\prod_{B,A}$) of pathogen screening. Comparisons were made between PCR assays of blood and swab samples of *G. polyphemus* for the pathogens: Herpesvirus, Ranavirus, and *Mycoplasma agassizii*. Additional kappa agreements were computed between any two pathogens to determine synergistic/antagonistic relationships. Complete co-occurrence versus mutually exclusivity were $\kappa = 1$ and $\kappa = -1$, respectively. A relative power: $\prod_{B,A} > 1$ concludes B as having superior screening detection than A. For $0 \leq \prod_{B,A} < 1$, concludes A as superior to B.

Pathogen comparison	**Count A**	**Count B**	**$K_{A,B}$**	$\mathbf{1-\beta_{A\|B}}$	$\mathbf{1-\beta_{B\|A}}$	$\mathbf{\prod_{B,A} \geq 1}$
Herpesvirus: Swabs/Blood	20+/54-	31+/40-	0.0615	0.869	0.705	1.233
Ranavirus: Swabs/Blood	13+/61-	26+/45-	0.0329	0.885	0.672	1.317
Mycoplasma: Swabs/Blood	39+/34-	46+/25-	0.3413	0.902	0.770	1.170
Herpesvirus / Ranavirus	44+/40-	33+/51-	-0.0606	-	-	-
Ranavirus / Mycoplasma	33+/51-	60+/23-	-0.0819	-	-	-
Mycoplasma / Herpesvirus	60+/23-	44+/40-	0.2898	-	-	-

Overall, detectability for all three pathogens was greater ($\prod B,A > 1$) in blood screening assays, as opposed to screening on swab samples. Both Herpesvirus and *Mycoplasma* displayed slightly antagonistic relationships ($\kappa < 1$) with *Ranavirus*, but showed a synergistic relationship with each other ($\kappa > 1$). Infection prevalence varied across pathogens, time, and cohorts of tortoises (Figure 16). Order of infection status from most common to least common was no

infections (U to U), infected throughout study (I to I), cleared infection (I to U), and gained infection (U to I) of any pathogen ($p \sim 2 \times 10^{-16}$). However, neither identity of pathogen ($p = 0.830$) nor group status (male/female, control/experimental) ($p = 0.984$) were found to be statistically significant. Therefore, the most common result was no change in overall infection status across all *G. polyphemus* regardless of relocation or sex.

Of the 54 slides, 17 were countable for leukocytes. Average leukocyte profile was 46.22% ± 6.46% lymphocytes, 12.91% ± 2.31% monocytes, 14.30% ± 6.80% heterophils, 3.70% ± 1.71% eosinophils, and 2.95% ± 1.00% basophils (95% confidence). A density distribution of leukocyte composition has been included in Figure 17. Compositional analysis indicated that leukocyte profiles were not significantly different across Herpesvirus ($p = 0.66$), *Ranavirus* ($p = 0.31$), or *Mycoplasma* ($p = 0.31$) infection statuses. Likewise, leukocyte profiles were not significantly different between control and experimental groups ($p = 0.54$) or between males and females ($p = 0.59$). Additional t-test analyses on individual leukocyte types were statistically non-significant across infection statuses and group statuses. To illustrate this, the closest non-significant effects (minimum p-value) of per status on individual leukocytes were of Herpesvirus-monocytes ($p = 0.238$), Ranavirus-heterophils ($p = 0.241$), *Mycoplasma*-monocytes ($p = 0.415$), experimental-monocytes ($p = 0.187$) and sex-basophils ($p = 0.415$). Thus, overall *G. polyphemus* white blood cell immune metrics were not explained by relocation events, sex, nor infection with any of the three focal pathogens.

Discussion & Future Research

Overall male *G. polyphemus* traditionally tend to be larger (240 mm at maturity) than females (255 mm at maturity) (McRae et al. 1981b). While the females in this study were larger and weighed more than males, age was unknown for these individuals, thus I could not assign individuals to age classes. Prior studies have also indicated that *G. agassizii* engages in indeterminate, but limited, growth (Nafus 2015). Thus, any significant factors affecting growth were likely masked due to the brevity of this study and individuals that were likely in various stages of development. Future research into growth rate as a function of age would plausibly deconvolute age as a random factor. Some rare individuals in this study did have a reduction in SCL and plastron lengths. However, these were not determined to be statistically correlated with presence of any pathogen. Of particular note was individuals infected with *Mycoplasma*, commonly linked with URTD and characterized by lethargy, mortality and cachexia (wasting away) (Brown et al. 1999). By the end of the study, I recorded no instance of mortality and minimal signs of disease. *Mycoplasma* infected individuals did not show a reduction in size nor a reduction in mobility (distance traveled). This trend was equally observed across all three pathogens. While commonly believed to reduce survivability, prior studies have indicated that the survival rate of *G. polyphemus* with respect to *Mycoplasma* is near 100% (Ozgul et al. 2009) with infections being chronic and long-lasting in chelonians (Jacobson et al 1991). This is in stark contrast to Herpesvirus which appears to cause higher rates of morbidity and mortality across chelonians (Marschang and Ruemenapf 2007). Long-term analysis coinfection and survivability with respect to relocations is merited, especially given that I found high rates of infection but no evidence of disease consequences over a one-year sampling period.

Throughout this study, I had a low incidence rate and found no significant relationship between outward signs and infection with all three pathogens. URTD has been linked to both *Mycoplasma* (Brown et al. 2001) and to *Ranavirus* (Westhouse et al. 1996). However, I found a slightly antagonistic relationship in prevalence between these two pathogens and no relationship with disease signs, for each pathogen separately and for co-infection. Mycoplasmosis diagnosis if also often complicated by overlapping signs produced by Herpesvirus (Salinas et al. 2011). It was also suggested by Salinas and colleagues (2011) that these pathogens may work synergistically. While I did find a minor synergistic relationship between *Mycoplasma* and Herpesvirus prevalence, I found no relationship with disease signs, for each pathogen separately and for co-infection. Clearly, the relationship between these pathogens is complex with outward signs possibly being unreliable for etiological identification, emphasizing the need for molecular identification and pathogen quantification to link pathogen burdens with disease states.

A common method for serological detection of these pathogens is diagnosis by antibody response, however this is found to be hyper-variable and dependent upon the pathogen as well as the host (Marschang et al. 2003). An alternative method employed by this study was molecular pathogen detection combined with leukocyte profiles to test for a relationship between infection and immunity. Prior studies have identified *Ranavirus* as being linked to heterophil and basophil (and azurophils) inclusions (Westhouse et al. 1996). Similarly, Herpesvirus has been linked to amassments/infiltration of heterophils and lymphocytes (and macrophages) (Johnson et al. 2005) with *Mycoplasma* as linked to demarcated increases in

heterophils (which are functionally similar to neutrophils) (Brown et al. 1994). While this study found no relationship between pathogen infection and leukocyte profiles with respect to basophils, eosinophils, heterophils, monocytes, and lymphocytes, small sample sizes limited my ability to infer major trends. While 54 slides were collected, only 17 were properly smeared and able to be stained and read. It was determined retrospectively that the placement of a cover slip prior to staining led to a high degree of cell lysis. Of the 17 slides, counts were only feasible on cells along the rim of the cover slip. I believe that this method still has value, but was not feasible within this study due to high degree of sample loss. The most successful method employed was the three molecular analyses for pathogen detection. Unsurprisingly, blood analysis vastly outperformed swab detection. However, it was initially hypothesized that swabs would yield higher results given a high degree of detectability and transmissibility of pathogens from nasal mucosa (Brown et al. 1999). One possible reasoning for this is the sheer comparative volume of blood collected in comparison to swabbing, or that I did not have any visibly sick tortoises in my study. Future investigations that include tortoises displaying major disease signs should compare pathogen prevalence from blood versus mucosal swabs to determine whether sick individuals shed more pathogen particles in mucosa.

Overall, I detected very low rates of pathogen infection status changes. Of particular interest was a low rate of change from uninfected to infected following relocations. Although highest for *Ranavirus*, none of the three pathogens showed significant changes in status for relocated and excluded animals compared to unmanipulated controls. The act of relocation (and the subsequent penning) increased the potential for contact transmission (Dodd and Seigel

1991) and may have induced stress-mediated immunosuppression (Hing et al. 2016), suggesting that experimental animals would show higher rates of acquiring infections. Paradoxically, this was not observed despite multiple coburrowing events and rare triple and quadruple occupancies detected in the pens (see Chapter 2). Given that *G. agassizii* engages in homing to mitigate stress-levels post-relocation (Hinderle et al. 2015), it reasonable that males in this study were not statistically affected given a confirmed homing behavior. Alternatively, given that females did not engage in homing behavior and largely remained within the transplanted location, the likely reduced the risk of thermal stressors and encounters with infected individuals commonly associated with translocations (Farnsworth et al. 2015).

In summary, it is my determination that relocation and exclusion due to the Sabal Trail construction project did not statistically influence immune response, rates of disease transmission or incidence, nor the overall health of *G. polyphemus* in this study. To detect pathogen presence, molecular methods should be used over clinical signs, and blood samples offer better detectability compared to mucosal swabbing as well as enabling leukocyte profiling. While no significant pathogen effects were detected in this study, the one-year duration was likely insufficient to detect possible long-term effects of relocation, stress, and pathogen infections. Long-term follow up studies should be used to determine the possible effects on growth rates, health, and survivability of translocated organisms.

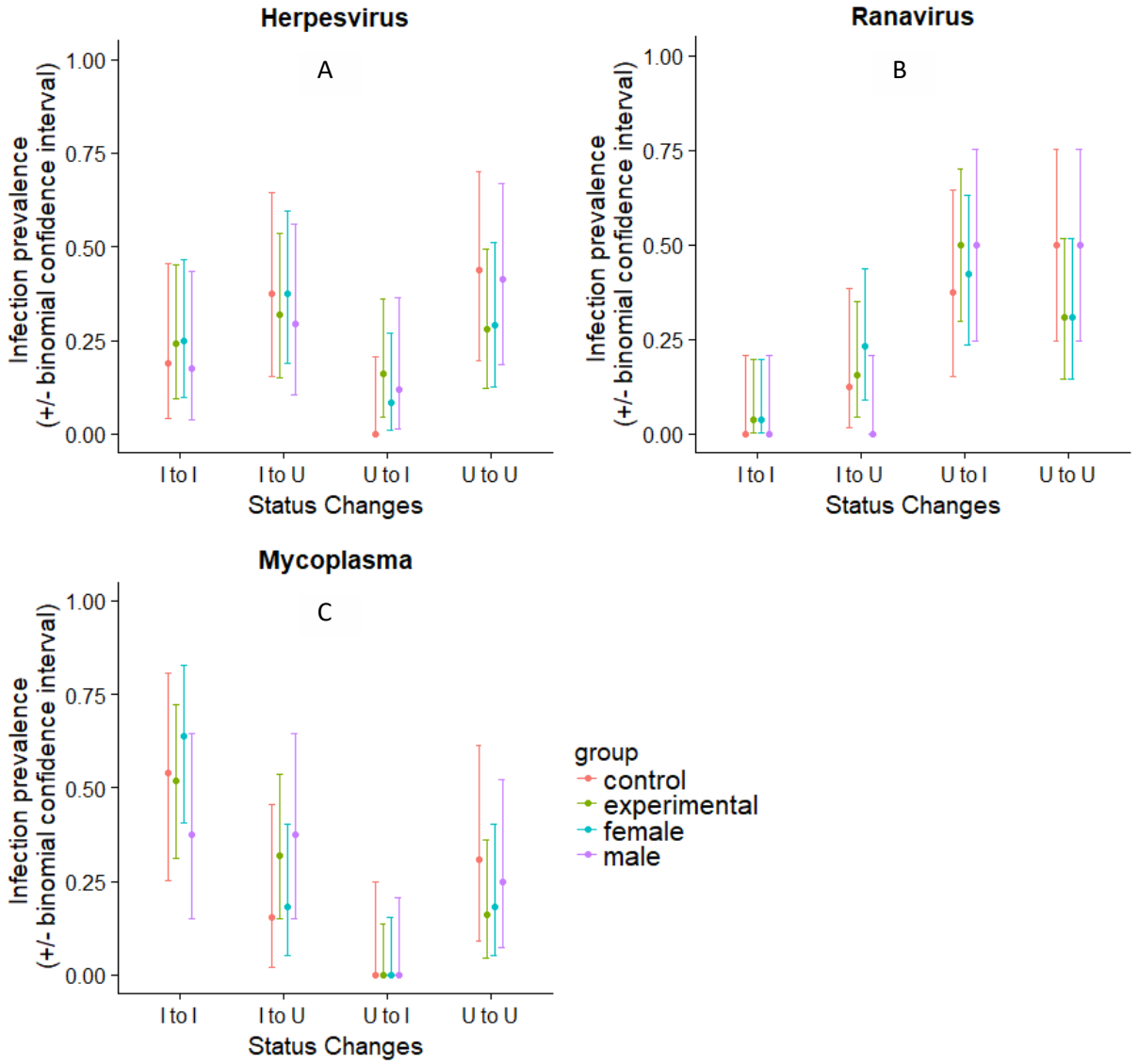

Figure 16: Infection prevalence (± 95% Clopper Pearson confidence interval) for (A) Herpesvirus, (B) *Ranavirus*, and (C)) *Mycoplasma*. Statuses were compared across control (orange) and experimental (green) *G. polyphemus* individuals, as well as across males (purple) vs. females (blue). Infection status changes (or lack thereof) between subsequent samples from the same individual were examined with respect to infected (I) or uninfected (U) pathogen states.

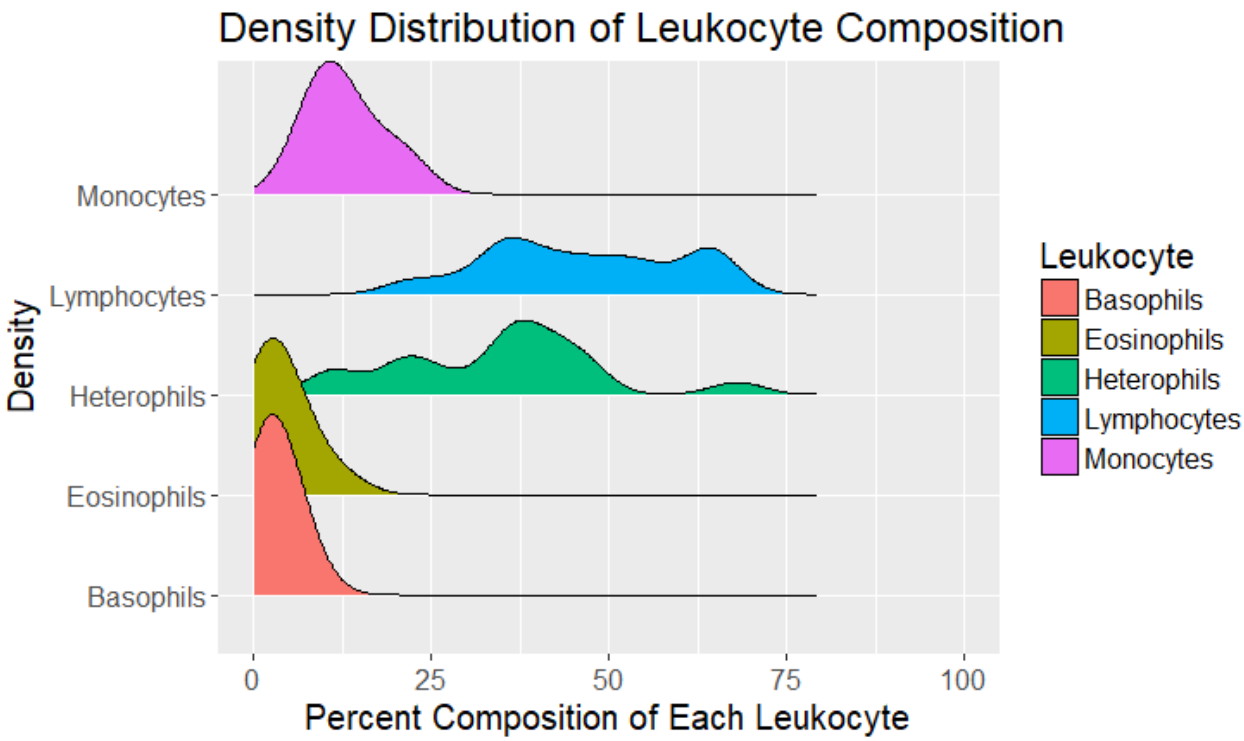

Figure 17: Density distribution of leukocyte composition for *G. polyphemus* blood slides. Blood was primarily heterophil and lymphocyte dominant with low levels of basophils and eosinophils.

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