INVESTIGATION OF HEALTH IN TRANSLOCATED GOPHER TORTOISES (GOPHERUS POLYPHEMUS) AT A PROTECTED SITE IN NORTHWEST FLORIDA

by

REBECCA ANNE COZAD

(Under the Direction of Sonia M. Hernandez)

ABSTRACT

Gopher tortoises (*Gopherus polyphemus*) are state listed as threatened throughout most of their range (and federally protected in the western-most portion), but are still facing population declines as a result of habitat loss and fragmentation. Throughout the state of Florida, an estimated 22,000 tortoises are in need of relocation as their habitat is being developed. Nokuse Plantation is a protected conservation preserve that has received nearly 4,500 of these translocated tortoises since 2006. From 2012 until 2015, 90 of these tortoises were found sick or dead at multiple enclosures on the property. Thus far, clinical signs and post-mortem findings suggest starvation as the immediate cause of death, but habitat analyses indicate these sites are suitable habitat with easy access to typical gopher tortoise forage plants. This project investigates the demographic, spatial, temporal, and environmental factors that are contributing to this mortality event and define potential predictors of mortality.

INDEX WORDS: Gopher tortoise, *Gopherus polyphemus*, mortality investigation, thermoregulation, home range, hematology, health

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DEDICATION

To my mom, Peggy, and my brother, Alex, who have always known I would end up playing with animals and have always embraced my weirdness. I bet Dad would have loved this. Also, to the best butts in the world - my doggos, Mizo and Spencer. Their snuggles definitely kept me going on so many occasions.

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CHAPTER 1

HEALTH PROTOCOL FOR TRANSLOCATION OF FREE-RANGING GOPHER TORTOISES

Translocation as Conservation Strategy

One of the tools currently being used for the conservation of many species is the use of translocation, or the purposeful moving of individuals from one location to another to establish, re-establish, augment a population, and other conservation goals (IUCN 1987; Griffith et al. 1989). Although historically translocation has been used successfully to manage game species (e.g. wild turkey, *Melagris gallopavo*, and white-tailed deer, *Odocoileus virginianus*; Downing 1987; Lewis 1987), the practice has also been employed as a conservation strategy for non-game species with varying degrees of success (Seddon et al. 2014; Wolf et al. 2006; Siegel and Dodd 2000; Sullivan et al. 2015; Germano and Bishop 2009). However, there have been extensive debates regarding ethical issues surrounding the practice and the efficacy of translocation, especially for species or populations that are translocated solely to remove them from areas undergoing development (McCoy and Berry 2007), and with regards to the possible movement of pathogens (Griffith et al. 1993; Alberts et al. 1998).

Translocation as a Conservation Tool for Gopher Tortoises

The gopher tortoise (*Gopherus polyphemus*) once ranged throughout most of the Coastal Plain of the southeastern United States, particularly in areas where longleaf pine sandhill was historically present (Auffenberg and Franz 1982). Direct habitat loss, habitat degradation, and overexploitation of tortoises for their meat caused a serious range-wide population decline,

though habitat loss is the single greatest range-wide threat to gopher tortoises (Enge et al. 2006; FWC 2007; USFWS 2009). The gopher tortoise is currently listed as a vulnerable species on the IUCN Red List, is federally protected in the western portion of its range, and is a candidate for Federal listing in the eastern portion of the range because of a significant range-wide population decline and loss of habitat (USFWS 1987; USFWS 2011). The majority of strategies currently in use for gopher tortoises throughout most of their range are directly related to habitat retention and protection, although translocation has been increasing used in the last two decades (Tuberville et al. 2008; Berry and Aresco 2014). In fact, gopher tortoises are currently one of the most widely translocated reptiles in the southeastern United States (Seigel and Dodd 2000). Translocation has been used to facilitate recovery of depleted populations, re-establish populations at sites where habitat has been restored but that do not currently support viable populations, to mitigate conflicts with threatened or endangered species with development, or a combination of these goals. All of these goals have been addressed to some degree with gopher tortoises through translocation efforts throughout the range (Burke 1989; Tuberville et al. 2005; Ashton and Burke 2007; Aresco et al. 2010; Bauder et al. 2014).

Florida comprises the majority of the extant range of gopher tortoises. Due to the relatively high numbers of gopher tortoises and the increased commercial and residential development pressures within the state, Florida has the highest number of gopher tortoise translocations (FWC 2012). With the issuance of Incidental Take permits (ITPs, which caused the legal entombment or killing of gopher tortoises on development sites) from 1991-2007, the current estimates for number of tortoises that were "taken" range from ~84,000 to over 100,000 individuals (Flescher 2005; Mushinsky et al. 2006; Myrkalo et al. 2010). Although FWC stopped issuing new Incidental Take permits in 2007, thousands of tortoises have been translocated to

recipient sites around Florida, and an estimated 180,000 tortoises will be translocated by 2022 under existing ITPs for sites that are slated for future development (Berry and Aresco 2014; FWC 2007). In Florida, translocation of tortoises from lands that will be made uninhabitable for gopher tortoises by development or other activities has become an important conservation tool for minimizing losses of individuals and restoring populations elsewhere. These mitigation-driven translocations prevent the mortality of tortoises that would have otherwise been subjected to incidental take by relocating tortoises to protected and well-managed sites with either depleted or extirpated tortoise populations (USFWS 2009). As such, under the newest Florida Fish and Wildlife Conservation Commission (FWC) management plan and permitting guidelines, translocation and restocking of sites with displaced tortoises became a key objective with more formal methodology to enhance the success of translocations (FWC 2009).

Current approaches for gopher tortoise translocation protocols rely heavily on data collected during previous projects in order to improve the success of translocation efforts, such as implementing on-site penning to increase site fidelity (Tuberville et al. 2005; Aresco et al. 2010). Additional success related topics (e.g. survivorship, habitat selection, mating system) have been studied during translocations of this species (Burke 1989; Ashton and Burke 2007; Tuberville et al. 2008; Bauder et al. 2014; Tuberville et al. 2011), although disease transmission, known to be an inherent risk, has been understudied in a translocation context with gopher tortoises. An additional critical component for enhanced success of translocations is the consideration of the impact on health of both the gopher tortoises being translocated and animals at the recipient site. There are currently no formal guidelines for incorporating pathogen testing for health assessments into translocation protocols for gopher tortoises, although release of animals with clinical signs of disease is discouraged (FWC 2017).

Health Risks Associated with Translocation

Translocation poses a number of potential health risks to both individuals being translocated, as well as conspecifics and potentially individuals of other species at the recipient site. One of the most important negative elements associated with the translocation of animals is the risk of introduction of infectious agents and parasites (Corn and Nettles 2001). Many of these risks have been outlined and described at length for some species in other studies (Corn and Nettles 2001; Kock et al. 2010). In addition, translocated animals, which may be naïve or poorly adapted to their new environment, are exposed to local pathogens or contaminants (Kock et al 2010; Wolff and Seal 1993). Stress, such as that associated with the translocation process, is an additional factor that may lead to increased susceptibility to disease and infection (Jacobson 2014; Aiello et al. 2014; McGuire et al. 2014; Drake et al. 2012; Dickens et al. 2010).

Even though herpetofauna are some of the most commonly translocated species globally, until very recently, their health during translocation events had not received as much attention as many of their mammalian or avian counterparts (Suarez et al. 2017). Yet, infectious diseases have been recognized as an important threat to tortoise conservation, particularly in the context of relocation (Martel et al. 2009; Brown et al. 2002; Jacobson 1993). Health and disease risks may be species-specific. Some of these risks (especially regarding dilemmas that also affect desert tortoises, a federally protected species closely related to the gopher tortoise) have been discussed in previous papers and reports (McCoy and Berry 2007; Brown et al. 2002; USFWS 2015). The objectives of this paper are to 1) synthesize the potential health risks associated with gopher tortoise translocations, 2) classify infectious agents and parasites by risk level, and 3) offer recommendations for translocation protocols for gopher tortoises.

Methods

We conducted a search of databases including Web of Knowledge and reference literature, to determine what infectious agents and parasites have been reported in free-ranging and captive gopher tortoises and taxonomically related species. We also consulted with various experienced gopher tortoise biologists and wildlife disease specialists with experience specific to translocated gopher tortoises. We first evaluated each reported infectious agent and parasite based on 1) a qualitative assessment of the ability of the agent to be introduced to a new geographic area and become established within the southeastern US and 2) if there was a reasonable probability that the infectious agent could become established, we assessed pathological consequences based on disease potential for tortoises, other wildlife, and humans as reported in the literature.

We considered the risk of health consequences for each infectious agent or parasite to be Low, High, or Unknown. This risk assignment was made based on an evaluation of the potential for establishment and pathogenicity. An infectious agent was assigned a low risk status (Low hereafter) if (1) the agent has been reported in wild or captive terrestrial chelonians outside of the US, (2) the agent has been reported in animals other than terrestrial chelonians, (3) the agent has been reported in non-native terrestrial chelonians, (4) the agent has only been reported in non-*Gopherus polyphemus* captive terrestrial chelonian populations within the US, (5) only serological evidence of exposure for the agent exists, (6) only experimental evidence of susceptibility for the agent exists, (7) the agent is not known to be pathogenic to gopher tortoises, other wildlife, or humans, (8) the agent is non-contagious, or (9) the agent is highly pathogenic to gopher tortoises but it is likely that infected animals would be detected during transportation and could be excluded from release. An agent was assigned high risk (High hereafter) if (1) the

agent can cause clinical disease in gopher tortoises, (2) the agent has been reported in either captive or free-ranging gopher tortoises, (3) the agent has been reported in any free-ranging terrestrial chelonian in the US, (4) the agent can be directly transmitted between tortoises without a vector, or (5) the agent may be harbored by any terrestrial chelonian without clinical disease. Infectious agents and ectoparasites were placed in the unknown (Unknown) category when sufficient information was not available (either in terms of establishment potential or pathogenicity potential) to make an assignment of either low or high risk.

Results and Discussion

Low Risk Pathogens

We found reports of 24 infectious agents and parasites in gopher tortoises and other tortoise species, and considered 7 to be Low. To receive a designation of Low, an agent had to satisfy 2 or more of the low risk criteria listed in the Methods section (see Table 1.1), and less than 2 of the high risk criteria. Selected agents based on amount of information available are described below.

Adenoviruses are double-stranded, linear, nonenveloped DNA viruses that have been detected in all tetrapod classes. They have been reported in at least five turtle and tortoise species, of which all except one were associated with individuals that had been in captivity at some point of their lives, but to date have not reported gopher tortoises (Gibbons and Steffes 2013). Adenoviruses can be transmitted between conspecifics (and between some chelonian species) via direct routes (fecal-oral route or direct contact by oronasal secretions), with poor prognosis for infected animals (Gibbons and Steffes 2013; Kim et al. 2002; Schumacher et al. 2012). Adenoviruses are also very environmentally persistent, and disinfection of adenovirus contaminated objects or settings is difficult (Schumacher et al. 2012).

The bacterium *Pasteurella testudinis* has been isolated from the respiratory tract of healthy desert tortoises and desert tortoises with URTD (Snipes et al. 1980). Though transmission studies later showed *Mycoplasma* to be the causative agent of the URTD cases in desert tortoises, *P. testudinis* has been found to be associated with respiratory disease and septicemia in other captive tortoise species (Henton 2003). There was one report of *P. testudinis* in a gopher tortoise, but an account of associated clinical signs was not included (Jang and Biberstein 1991). *Erhlichia ruminantium*, a tick-borne rickettsial pathogen and the causative agent of Heartwater in cattle, has been detected in an exotic African tick species (*Amblyomma sparsum*) on leopard tortoises (*Stigmochelys pardalis)* being imported into Florida (Jacobson 2007; Burridge et al. 2000). Related African tortoise ticks (*A. marmoreum*) were also found on several species of captive tortoises in an outdoor enclosure in central Florida (Allan et al. 1998). Though studies have shown that leopard tortoises are unlikely to introduce Heartwater into new areas (Peter et al. 2001), gopher tortoises are able to host exotic ticks (Corn et al. 2011).

An intranuclear coccidian parasite of Testudines (TINC) was first detected in the US in two captive radiated tortoises and has since been documented in multiple other captive chelonian species in both Europe and Asia representing five families, but not in *Gopherus spp.* (Kolesnik et al. 2017; Gibbons and Steffes 2013). Infection by this parasite causes systemic disease with variable and unspecific clinical signs, including respiratory distress, anorexia, lethargy, subcutaneous edema, and ulceration of cloacal mucosa, commonly followed by death (Kolesnik et al. 2017; Gibbons and Steffes 2013). Antemortem diagnosis of TINC can be done through the use of oral, conjunctival, or cloacal swabs, although there is not much known regarding transmission, route of infection, or pathogenicity (Kolesnik et al. 2017).

Table 1.1. Criteria used to assign low risk level for pathogens associated with translocation in tortoises with the results for the seven Low agents. Agents represented with a " * " are High agents that met multiple low risk criteria.

		Low Risk Criteria								
		agent reported as pathogenic in animals other than terrestrial chelonians in the USA	agent reported in non-*Gopherus polyphemus* captive terrestrial chelonians in the USA	agent reported in captive and wild terrestrial chelonians outside the USA	non-contagious	reported in non-native terrestrial chelonians	only serological evidence of exposure reported	only experimental evidence of susceptibility exists	not known to be pathogenic to *Gopherus polyphemus*, other wildlife, or humans	highly pathogenic to *Gopherus polyphemus*, likely to be detected during transport
Viruses	herpesvirus*	X	X	X						
	adenovirus	X		X		X				
	ranavirus*	X	X	X						
	poxvirus	X	X			X				
	reovirus			X		X				
Bacteria	*Mycoplasma spp.**	X	X	X						
	Salmonella *	X	X							
	Pasteurella		X	X						
	Chlyamydia and *Chlamydophilia*	X	X			X				
	Ehrlichia ruminantium	X				X				
Parasites	Intranuclear Coccidiosis (TINC)	X	X							

High Risk Pathogens

Infectious agents were designated as "high risk" if they met more than 2 of the criteria listed in the Methods section (Table 1.2), even if they also met criteria from the low risk category. We identified six High agents.

Ranavirus, a large double-stranded DNA virus in the Iridoviridae family, is a highly infectious pathogen of fish, amphibians, and reptiles that has been associated with multiple mortality events in amphibians and chelonians (McGuire and Miller 2012; Johnson et al. 2008; Jacobson 2014). This virus is a multi-host pathogen that can be transmitted between classes of ectothermic vertebrates (e.g. from turtles to amphibians) through direct contact, ingestion, or shared water source, and in multiple species has been associated with very high mortality rates for infected populations (McGuire and Miller 2012; Johnson et al. 2008; Jacobson 2014; Allender et al. 2011; Gray and Miller 2013). There is only one report of ranavirus causing clinical disease in gopher tortoises (Westhouse et al. 1996), but serologic surveillance studies indicate the pathogen appears to occur at relatively low prevalence (Johnson et al. 2010). Ranaviruses have been documented in wild gopher tortoises in Florida and Georgia at relatively low prevalence serologically, but samples from tortoises through the remainder of the range were all seronegative (Johnson et al. 2010). Ranavirus DNA has also been detected through qPCR in both oral/cloacal swabs and tissue samples of translocated (Cozad unpubl. data) and in blood of wild (Brooks 2018) gopher tortoises in Florida.

Infections have been reported from two strains of herpesvirus (tortoise herpesvirus-1 and tortoise herpesvirus-2), which can cause respiratory disease and are associated with high mortality rates. Although they have not yet been documented in gopher tortoises, they have been reported in a number of other tortoise species through antibody testing and PCR virus

amplification in captive settings, including desert tortoises (Salinas et al. 2011; Johnson et al. 2005; Jacobson 1993).

The threat of infections with *Mycoplasma agassizii* and *Mycoplasma testudineum*, causative agents of Upper Respiratory Tract Disease (URTD), to wild gopher tortoise populations has been debated extensively after the discovery of exposed, symptomatic, or dead gopher tortoises in Florida and exposed and symptomatic tortoises in Georgia and Mississippi (McGuire et al. 2014; Berish et al. 2010; Seigel et al. 2003; Smith et al. 1998). URTD has been characterized as a chronic disease with high morbidity but low mortality, and *Mycoplasma* spp. can be transmitted through direct contact with an infected tortoise (Berish et al. 2010). Although the disease can cause morbidity or mortality of individual tortoises, little is known of population-level effects on gopher tortoises and to what extent URTD contributes to overall population declines (Mushinsky et al. 2006; USFWS 2009). As *Mycoplasma* was originally thought to have been introduced into wild desert tortoise populations by the release of infected captive tortoises, it was suspected the same occurred in gopher tortoise populations; however, more intensive surveillance has demonstrated that these pathogens are widespread through much of the gopher tortoise's range (McGuire et al. 2014; Berish et al. 2010; Seigel et al. 2003; Smith et al. 1998; Karlin 1998).

A novel species of *Anaplasma spp* bacteria has also been recently documented in gopher tortoises that presented with anemia and intracytoplasmic inclusions in red blood cells (Brown et al. 2017; Wellehan 2016; Cozad unpubl. data). Anaplasma are arthropod-borne obligate intracellular bacteria in the order Rickettsiales, although none of the documented cases in tortoises reported the presence of ticks positive for *Anaplasma spp* DNA (Wellehan 2016; Cozad unpubl data). Infections with *Salmonella spp* in chelonians are common and have been widely

documented, especially in regards to freshwater turtles in the pet trade (Jacobson 2007). *Salmonella* has also been detected in both wild (Lockhart et al. 2008) and asymptomatic relocated gopher tortoises (Charles-Smith et al. 2009). Though not generally associated with clinical disease in reptiles, asymptomatic individuals shedding *Salmonella* or move salmonellae serotypes from geographically distinct sites represent a concern for both public and livestock health (Jacobson 2007).

Cryptosporidium is a coccidial parasite that has been documented in multiple tortoise species which typically manifests with clinical signs of gastrointestinal disease such as diarrhea, decreased appetite, weight loss, and decreased growth (Jacobson 2014; Gibbons and Steffes 2013). This parasite has been reported with increasing frequency in many chelonian species, including the gopher tortoise (Gibbons and Steffes 2013). Although no evidence of infection-related mortality for the species currently exists, cryptosporidiosis may cause a population level effect with the potential for decreased reproductive output or success as a result of decreased eating and growth. The fecal-oral transmission of this pathogen may also be more important in terms of infection for juvenile tortoises potentially participating in coprophagy (Bishop et al. 2017) and for other species that are susceptible to cryptosporidiosis and use the same habitat, such as snakes and lizards (Graczyk and Cranfield 1997).

Unknown Pathogens

Infectious agents were deemed "unknown risk" if they did not meet the criteria specified for either Low or High risk. We determined the remaining 11 agents to be Unknown, primarily due to lack of available information. These agents included viruses (Togaviridae – equine encephalitis; flavivirus; paramyxovirus; "virus X"), bacteria (*Aeromonas spp.*; *Serratia spp.*; *Heliobacter spp.*; *Dermatophilus spp.*), fungi (fungal shell disease - *Fusarium incarnatum*;

fungal pneumonia – *Aspergillus spp.* and *Candida spp.*), and parasites (*Amblyomma tuberculatum* and *Ornithodoros turicata*).

Table 1.2. Criteria used to assign high risk level for pathogens associated with translocation in tortoises with results for the six High agents.

High Risk Criteria							
		can be harbored by any native terrestrial chelonian species without clinical disease	transmitted directly between native terrestrial chelonians (no vector)	if vector required, known/potential vectors or intermediate hosts present in release area	agent reported to cause clinical disease in *Gopherus polyphemus*	agent reported in either captive or free-ranging *Gopherus polyphemus* tortoises	agent reported in any free-ranging terrestrial chelonian in the USA
Viruses	herpesvirus	X	X				X
	ranavirus	X	X		X	X	X
Bacteria	*Mycoplasma spp.*	X	X		X	X	X
	Anaplasma spp.	X			X	X	
	Salmonella	X				X	X
Parasites	Cryptosporidium	X	X		X	X	

Factors Affecting Risk Level

Temporary Captivity Prior to Translocation

Some tortoises slated for translocation go through a temporary period of captivity before they are moved, such as a holding period while waiting for the capture of other individuals or during health screenings. This temporary captive period could increase risk for pathogen transmission by allowing contact with other, potentially infected individuals. This is especially true of individuals that may be housed near exotic chelonian species that they would not encounter in the wild, which should be avoided at all costs.

Other Health Effects of Translocation

In addition to potential spread of infectious agents, stress from translocation may result in additional health effects for moved individuals. Sources of stress for translocated tortoises can be either direct, such as capture and handling or repeated monitoring, examinations, and relocation, or less direct factors, such as introduction to a novel environment, and disruption of established social structures (Aiello et al. 2014; Berry 1986; Dickens et al. 2010). Stress experienced by animals during translocation increases the vulnerability of those individuals and that population to factors, such as predation, starvation, or disease (Dickens et al. 2010).These stressors can lead to behavioral shifts, which may change the risk of exposure to pathogens, and/or expression of otherwise subclinical infections (Jacobson 2014; McGuire et al. 2014; Kahn 2006). Stress may be particularly important for species with social structures, such as the gopher tortoise, as translocation may disrupt existing social bonds. Gopher tortoises have hierarchical social networks that can be disrupted by relocating a population (Guyer et al. 2014; Tuberville et al. 2011; Riedl 2006). One effect of this social disruption is demonstrated in the closely related desert tortoise, where one study found that males had no paternal genetic integration following

translocation although translocated females at the same site were successfully reproducing with the resident males (Mulder et al. 2017). The introduction of new potential mates from different translocation groups can lead to increased competition, visitation of higher numbers of burrows, and increased social interactions, all of which would provide additional opportunities for pathogen transmission through direct contact (Guyer et al. 2014; Tuberville et al. 2011). The combination of stressors may lead to the mounting of a stress response that becomes sustained at a chronic level. This type of chronic stress may inhibit immune function and result in increased susceptibility to infection and disease (Dickens et al. 2010). These effects and the potential for disease expression and pathogen dispersal may also be density-related, and therefore be affected by increased or otherwise altered densities that occur during translocation efforts (Aiello et al. 2014).

Risks Specific to Waif Tortoises

Although there are risks associated with any translocation, waif tortoises—animals that have been held in captivity for extended periods or have unknown origins—warrant additional consideration. Waif tortoises are predominately comprised of 1) animals that have been kept illegally as pets that are surrendered to or confiscated by the authorities, 2) animals that may have been picked up on roadways by well-intentioned individuals and dropped off at preserves/parks/conservation facilities and 3) animals that have been held in wildlife rehabilitation settings for extended periods. Many of the concerns with waif tortoises originate from the pathogen transmission potential in captive settings. Because captivity often results in the concentration of individuals of the same species at higher densities and in potentially stressful environments, it provides an increased opportunity for the transmission of pathogens (Ippen and Zwart 1996). Additionally, because captive facilities and private collections are rarely

comprised of a single species, there is also an enhanced opportunity for the introduction of novel pathogens (Snyder et al. 1996). For reptiles specifically, numerous epizootic outbreaks have occurred in captive collections, such as infections with herpesvirus in recently imported Argentine tortoises (*Geochelone chilensis*), captive spur-thighed tortoises (*Testudo graeca*) and captive Hermann's tortoises (*T. hermanni*) (Jacobson 1993). In the case of desert tortoises, the unauthorized release of pet tortoises is thought to have played an important role in the introduction of URTD into wild populations (Brown et al. 1993; Jacobson et al. 1995). Additionally, some hypothesize that the ongoing release of captive pets might partially explain the high prevalence of *Mycoplasma* observed in tortoises in close proximity to human-populated areas (Berry et al. 2015). Based on indirect enzyme linked immunosorbent assay (ELISA) measuring plasma antibodies, captive desert tortoises have documented high levels of exposure to *Mycoplasma agassizii* and tortoise herpesvirus (Johnson et al. 2006).

Although these examples primarily come from desert tortoises, similar concerns for the health of gopher tortoises have resulted in at least one state precluding the release of waif tortoises into stable tortoise populations (FWC 2012). The safest course for managing disease would be to avoid translocating waif animals entirely. However, disease risks must be holistically weighed against the declining status of the species (Smith et al. 2006) and the potential value of each reproductive adult. Thus, as the number of waif tortoises in captive facilities has grown, geographically isolated sites spatially isolated from viable populations have been designated as waif tortoise recipient sites (FWC 2012). One of the largest waif translocation efforts to date has involved the introduction of gopher tortoises to a state preserve in South Carolina (Buhlmann and Tuberville 2015). Although this was a high-risk management action, the dire need of the resident population ($n<10$) for augmentation, and its isolation from other viable

tortoise populations (>80 km) outweighed the potential risks of pathogen introduction. Despite the large scale of this project (moving >150 adult tortoises), due to a lack of funding, no pre-release pathogen screenings were conducted and post-release population monitoring has only recently been initiated.

Management Implications

Existing Translocation Procedures

In comparison to the closely related and federally listed desert tortoise, translocation permitting guidelines for gopher tortoises, especially in regards to health considerations, are not as stringent. As their range spans over six states (LA, MS, AL, GA, FL, SC), specific regulations may differ dependent on locality and regulatory agency responsible (USFWS 2013). Florida currently is the only state with specific guidelines with regards to the relocation and translocation of gopher tortoises, due to the high rate of commercial and residential development in gopher tortoise habitats in the state.

Current protocols according to FWC permitting guidelines dictate that pre-translocation health assessments only require a cursory physical examination including posture/behavior, eyes, nares, shell, skin, and muscles. Authorized agents can collect samples to test for URTD if requested by the recipient site, but are not required or recommended. In addition, if testing is done, it must be performed by the Mycoplasma Laboratory at the University of Florida, with the authorized agent held responsible for all costs associated with the testing and results reported to FWC. Tortoises with obvious health abnormalities must be isolated and reported to FWC, and should be sent to a rehabilitation facility instead of being released. Testing for other pathogens besides *Mycoplasma* spp. is not suggested, recommended, or required by these guidelines. The current disinfection procedure states that the disinfection of equipment and hands between

individual tortoises is recommended but not required, and the cleaning and disinfection of bins/equipment between donor sites is required to reduce cross-contamination between populations.

Management Guidelines Specific to Waif Tortoises

Public education campaigns designed to discourage the illegal collection of wild tortoises and the unauthorized release of waif animals have been suggested as an important management strategy (FWC 2012). Although these education campaigns should be pursued, it is likely that waif animals resulting from misguided citizen actions will remain an ongoing management dilemma. As such, Florida has listed specific guidelines characterizing the translocation of waifs in its updated "Gopher Tortoise Management Plan" (FWC 2012). Central to this management plan is the distinction between releasable or non-releasable waif tortoises. Waifs are considered non-releasable if they exhibit clinical signs of disease, require ongoing medical intervention or are unable to forage/burrow independently, have been held in captivity for greater than six months, or have been exposed to diseased tortoises (FWC 2012). Because even waifs classified as "releasable" carry higher risks, the plan also recommends that waif tortoises be released only at sites without a viable resident population (FWC 2012). Although Florida's plan provides important guidelines to prevent waif tortoises from exposing viable populations to novel diseases, further research is required to evaluate the risks posed by waif animals and to identify additional management strategies.

Proposed Recommendations to Translocation Protocols

We recognize that management protocols for transporting and receiving gopher tortoises may vary depending on recipient site resources. We will describe recommendations for

suggested procedures in ideal conditions, followed by other options based on resources available to managers and recipient sites.

Evaluation of Source Population Health Status

If tortoises are being translocated to an area with an existing population of gopher tortoises, best management practices would include a preliminary health assessment survey of the donor population prior to translocation, including surveillance for high risk pathogens through biological sample collection (especially whole blood, oral and cloacal swabs), with a similar assessment to also be completed for the recipient or pre-existing population. Ideally, this pathogen testing would occur pre-translocation, while the tortoises are still at the donor site, to avoid the need for quarantine at the recipient site. However, as excavations and translocations of tortoises can be a very time-sensitive activity and thus not easily allow for testing pre-translocation, an alternative would be to conduct the sampling and pathogen testing while the tortoises are held for quarantine (if permissible and feasible). If sampling of all tortoises being released is restricted due to financial constraints, selection of a representative subset of tortoises for sampling would be the next best option. Pre-release pathogen testing without a quarantine period would still be an important step for monitoring health of the translocated population, as the results may give insight to potential disease outbreaks or mortality events. Alternatively, for managers that do not have funding for this screening, we still recommend collection and appropriate storage of these samples to send for testing at a later date. Archived samples may also become important resources at a later date as new pathogens are discovered and testing procedures developed.

As mentioned previously, disease testing is not currently required or recommended by USFWS or State agencies prior to translocation. Though infection with *Mycoplasma* spp, and

URTD have been widely studied in gopher tortoises and desert tortoises (McGuire et al. 2014; McLaughlin et al. 2000; Hernandez et al. 2010; Jacobson 2014; Berish et al. 2010; Berry and Christopher 2001), other potentially significant pathogens (e.g. ranavirus, herpesvirus, adenovirus) have not been as thoroughly investigated in either wild or translocated populations. As a result, most health assessment studies for gopher tortoises only focus on clinical signs associated with URTD and *Mycoplasma* (e.g. nasal occlusion or discharge, asymmetric or eroded nares, and ocular discharge or periocular swelling). However, some of these clinical signs are not limited to *Mycoplasma* spp. and may have other potential causes, such as infections with other emergent pathogens including ranavirus and herpesvirus (Johnson 2006; Wendland 2009). This may be an issue if wildlife managers assume that *Mycoplasma* spp. alone are responsible for these clinical signs and the tortoises are not tested for other pathogens as well. For this reason, we suggest screening for a panel of pathogens, based on risk level and resource availability.

Quarantine

During movement of animals in captive populations such as zoos and aquaria, incoming individuals are required to go through a period of quarantine in order to eliminate the potential spread of pathogens to the already-present populations (Miller 1996). These quarantine periods may vary for classes and species of animals, with the ideal quarantine length being the longest incubation period for known pathogens for the species, but the minimum recommended quarantine period is 30 days (Miller 1996; IUCN 2000). For reptile species, the IUCN suggested quarantine period is three months, although this may be an arbitrary length, given that not much known about many pathogens (IUCN 2000). A quarantine period of this length may not be feasible in terms of time or financial resources for many potential recipient sites, especially as translocation of gopher tortoises off of development sites is a very time-sensitive action.

Additionally, quarantine procedures may cause unacceptable stress, and therefore should be assessed on a case-by-case basis (IUCN 2012).

An alternative to strict quarantine procedures is the use of on-site quarantine through penning for tortoises. In this case, we recommend temporary penning of the group of translocated individuals within the larger planned release enclosure. Groups should be assigned with potential disease risk in mind, e.g. tortoises from the same donor site as a group. This type of quarantine would negate the need for a specialized quarantine facility, while allowing for tortoises to already begin rehoming to the area, thus avoiding the stress of multiple new environments and the acclimation to those environments. These internal pens could be maintained for the quarantine period while waiting for pathogen testing results. If quarantine is feasible, we suggest collecting biological samples at beginning and end time points to also monitor potential changes in infection status.

Potential Restrictions on Origin

There may be potential health concerns for moving tortoises from specific areas or populations. One of the threats relating to origin of translocated tortoises related to the gopher tortoise's potential as host for exotic ectoparasites (Corn el al 2011). South Florida, especially near and around Miami, is a hub for numerous imported exotic species for the pet trade. Exotic species may carry ectoparasites such as ticks that could potentially use the gopher tortoise as a host and be inadvertently transported to other areas in Florida if proper precautions are not taken. Non-native tick species have already been documented at captive reptile facilities in Florida (Allan et al. 1998). If tortoises are moved from south Florida, it is especially important to ensure that all ticks are removed prior to release to prevent the spread of exotic ticks and potential tick-borne pathogens.

Conclusion

Translocation of gopher tortoises has become established as a conservation strategy in the past two decades, and will likely persist as a conservation method for many years. As such, it is necessary to ensure that current and future protocols take into account the importance of health and disease risks for both the gopher tortoises themselves and for other species they may encounter. The potential for release of clinically healthy tortoises, or untested but infected tortoises should be taken into account during relocation planning (Martel et al. 2009), though the protocol enacted may depend on the specific goals for a particular recipient site or population. Samples for pathogen surveillance should at least be taken before release and archived, even if lack of funding precludes testing at the time. These samples should be stored appropriately to allow for future analyses should funding become available, if health issues in the translocated population emerge, or as new testing procedures become available. Oral and cloacal swabs are sufficient samples that can be run against a suite of many of the pathogens outlined here, which can be especially useful with regards the higher risk pathogens.

The chronic stress that translocated animals inevitably experience makes them more vulnerable to external factors, such as disease and predation, but could be reduced by adjusting translocation procedures to decrease the amount and magnitude of exposure to those stressors (Dickens et al. 2010). The precautions and recommendations that we have outlined in this paper are based solely on pathogens, though there are many additional factors such as social structure and habitat that may affect infection status of translocated tortoises that should be taken into account when planning and enacting a translocation project with gopher tortoises.

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CHAPTER 2

EPIDEMIOLOGICAL INVESTIGATION OF MORTALITY EVENT IN A TRANSLOCATED GOPHER TORTOISE POPULATION IN NORTHWEST FLORIDA

Introduction

The gopher tortoise (*Gopherus polyphemus*) once spanned throughout most of the southeastern United States, particularly in areas where longleaf pine savannas were historically present (Auffenberg and Franz 1982). Direct habitat loss, habitat degradation, and overexploitation of tortoises for their meat caused a serious range-wide population decline, though habitat loss is the single greatest range-wide threat to gopher tortoises (Enge et al. 2006; FWC 2007; USFWS 2009). Currently listed as a vulnerable species on the IUCN Red List, the gopher tortoise is federally protected in the western portion of its range, and is a candidate for Federal listing in the eastern portion of the range (USFWS 1987; USFWS 2011). The majority of management strategies currently in use for gopher tortoises through most of the range are directly related to habitat retention and protection, although translocation has been increasingly used in the last two decades (Tuberville et al. 2008; Berry and Aresco 2014). Translocation, or the purposeful moving of individuals from one location to another to establish, re-establish, or augment a population, is one of the tools currently being used in the conservation efforts for many species (IUCN 1987). Previous reviews on the success of translocation programs in general have reported various levels of failure and success (Wolf et al. 2006; Seigel and Dodd 2000; Sullivan et al. 2015; Germano and Bishop 2009). Gopher tortoises remain one of the most widely translocated reptiles in the southeastern United States (Seigel and Dodd 2000). However,

there have been extensive debates regarding ethical issues surrounding the practice, especially in terms of species or populations that are translocated solely to remove them from areas of development (McCoy and Berry 2008; Germano et al. 2015).

Translocation can be used to facilitate recovery of depleted populations, re-establish populations at sites where habitat has been restored but that do not currently support viable populations, to mitigate conflicts with threatened or endangered species with development, or a combination of these goals. In Florida, the state which comprises the majority of the extant range of gopher tortoises, translocation is used to meet all of these goals. Due to the relatively high numbers of gopher tortoises and the increased commercial and residential development pressures within the state, Florida has the highest number of gopher tortoise translocations (FWC 2012). With the issuance of Incidental Take permits (ITPs, for the legal entombment or killing of gopher tortoise on development sites) from 1991-2007, estimates for number of tortoises "taken" range from ~84,000 to over 100,000 individuals (Flescher 2005; Mushinsky et al. 2006; Myrkalo et al. 2010). Since FWC stopped issuing Incidental Take permits in 2007, thousands of tortoises have been translocated to recipient sites around Florida, and an estimated 180,000 tortoises will be translocated by 2022, many of which are still located in areas with existing ITPs that are slated for future development (Berry and Aresco 2014; FWC 2007). Translocation of tortoises from lands that will be destroyed by development or other activities has become an important conservation tool for minimizing losses of individuals and restoring populations elsewhere. These mitigation-driven translocations prevent mortality of tortoises that would have otherwise been subjected to incidental take by relocating tortoises to protected and well-managed sites with either depleted or extirpated tortoise populations (USFWS 2009).

Post-translocation monitoring studies are important and determine whether properly planned and executed translocations can successfully augment or reestablish populations for gopher tortoises. Translocation success in slow-growing, long-lived species such as the gopher tortoise is especially difficult to assess (Riedl et al. 2008). However, researchers rely on other metrics (such as site fidelity, survivorship, and evidence of reproduction) as signs that a translocation has been a success. Current approaches for gopher tortoise translocation protocols rely on previous studies to improve short-term translocation success, such as implementing on-site penning to increase site fidelity (Tuberville et al. 2005; Aresco et al. 2010). An additional critical component for enhanced success is the inclusion of health assessments and disease screenings. In some cases, health problems that may arise after a translocation event are not a result of the translocated animals introducing diseases or pathogens, but rather that these naïve or poorly adapted animals are exposed to local pathogens or contaminants (Kock et al. 2010; Wolff and Seal 1993). Stress, such as that associated with the translocation process, is an additional factor that may lead to increased susceptibility to disease and infection in tortoise species (Jacobson 2014; Aiello et al. 2014; McGuire et al. 2014; Drake et al. 2012; Dickens et al. 2010). Elevated disease prevalence or mortality in a translocated population suggests that translocation practices merit review and modification. In this paper, we investigate the epidemiology of a mortality event that occurred in a population of translocated gopher tortoises in northwest Florida with the objectives of 1) determine cause of mortality event through analysis of necropsy and histology results from dead tortoises, diagnostics and rehabilitation of live tortoises, and infectious pathogen testing and 2) use modelling to evaluate variables for use as predictors for detection of disease in this tortoise population.

Methods

Study Site

Nokuse Plantation, a 22,040-hectare private preserve in the Florida Panhandle, has embarked on a large-scale private effort to rescue and relocate gopher tortoises to many areas of the property starting in 2006. Tortoise density on Nokuse property prior to serving as a recipient site for translocated gopher tortoises was 0.004 per hectare due to past habitat conversion and heavy predation by humans. From 2006–2016, 4,521 gopher tortoises were translocated from development sites across Florida to Nokuse. Within Nokuse, there are 19 large (10-136-hectare) enclosures contained by 1 meter tall silt fences buried 0.3 m into the ground to prevent escape. These pens are also surrounded by 1.5-meter high electric fences to prevent trespass by predators. From 2006 – 2013, mortality of translocated tortoises at Nokuse Plantation was 5.3% (n = 180/3366), with ~67% of that mortality due to predation, most of which was attributed to coyotes. In early 2014, electric fences were installed around the perimeters of five relocation sites, which virtually eliminated coyote predation. Release sites are located in sandhill areas with Lakeland soil, and the predominate overstory is longleaf pine (*Pinus palustris*) followed by turkey oak (*Quercus laevis*). The understory is primarily grasses at the sites, dominated by *Andropogon* spp. grasses and Bahia grass. Although Nokuse tries to maintain a burn rotation of 3-5 years to reduce presence of a hardwood midstory and prevent succession into a mixed hardwood forest, one of these study sites (SR1) had not been burned for approximately ten years prior to the initiation of this study but there was not significant hardwood encroachment.

The Immediate Problem at Nokuse

In early 2013, biologists at Nokuse began finding sick and dead tortoises within certain translocation enclosures while conducting routine monitoring. When found alive, tortoises

appeared emaciated, lethargic, behaved abnormally (frequently found outside of burrows in cold weather), and had developed redness under the scutes on the plastron. The majority of these sick tortoises were found pacing or stationary along the inside edge of the silt fence, many of which were found during the cooler months. Between April 2013 and April 2017, 90 tortoises were found sick or dead but an additional, unknown number are suspected to have died within their burrows. Of these tortoises, 19 were suspected or confirmed to have been predated, 50 tortoises were suspected to be diseased, and the remaining died from unknown causes. Many of the tortoises in the latter category were found with an intact shell, suggesting that predation was not involved. However, their remains were found too late to investigate cause of death because there was little to no soft tissue remaining.

Mortality Surveillance and Investigation

Surveys for tortoise mortality at Nokuse were conducted opportunistically during routine monitoring of the five release enclosures between 2011 and 2017. Routine monitoring included walking the interior side of the silt fence around the complete enclosure (daily for newly constructed and filled pens and weekly for others), sporadic transects through portions of the pens, and walking the exterior of the enclosure for electric fence maintenance. When a dead tortoise was found, a GPS location was taken and the carcass or shell was recovered and brought to the field lab for individual identification (via shell notching and morphometric measurements) and processing. Tortoises found within approximately 24-48 hours of death were sent to a licensed wildlife veterinarian who conducted a full gross necropsy (T. Norton, DVM, Georgia Sea Turtle Center). All live tortoises found during monitoring were captured if possible and subjected to a physical exam to determine health status. If abnormalities were found during the exam, it was brought to the lab for monitoring or transported to a rehabilitation center for

diagnostics and treatment (Georgia Sea Turtle Center, Jekyll Island, GA). Mortality and health monitoring occurred throughout the year.

Diagnostics and Rehabilitation of Live Tortoises

Tortoises found alive but sick were transported to the Georgia Sea Turtle Center for diagnostics and rehabilitation treatment. Standard morphometric measurements including weight and straight carapace length (SCL) were recorded (McRae et al. 1981). Each tortoise received a complete health assessment by the same trained individual, including a thorough physical exam, a subjective body condition assessment, and disease surveillance. Body condition scoring (BCS) on a scale of 1-5 was determined through a visual assessment of muscle mass and fat deposits (modified from Lamberski 2013). Due to the subjective nature of this scoring, BCS was decided by a single licensed wildlife veterinarian (T. Norton, DVM). Ectoparasites were documented and collected, if present, and stored in alcohol.

Biological samples were collected for pathogen surveillance and hematological analyses. Blood was collected from either the jugular or brachial vein using a sodium heparinized syringe for a suite of biochemical and nutritional testing to assess both general and nutritional health. Volume of blood collected was < 5% of the tortoise's body weight. A small amount of heparinized whole blood was transferred to a microhematocrit tube and centrifuged to measure packed cell volume (PCV), and a refractometer was used to measure plasma total protein (TP). A small amount of heparinized whole blood was used to make blood smears for a Complete Blood Cell (CBC) count and differential white blood cell (WBC) count, performed by a clinical pathologist with chelonian expertise (N. Stacy, DVM, University of Florida, Gainsville, FL). A small amount of whole blood was used for infectious pathogen testing, and the remaining whole blood was centrifuged for collection of plasma. A complete biochemical panel was performed on

all plasma samples using standard dry slide determinations with a Kodak 700XR chemical analyzer by IDEXX Laboratories (Westbrook, ME). The following values were measured: aspartate aminotransferase (AST), creatine kinase (CK), albumin, total protein, globulin, blood urea nitrogen (BUN), cholesterol, glucose, calcium, phosphorus, chloride, potassium, sodium, uric acid, and triglyceride. A cotton-tipped swab was also used to collect mucosal epithelial cells from the choana and the cloaca for pathogen testing.

During rehabilitation, tortoises were kept in housing maintained between 24-27 degrees Celsius. All tortoises received antimicrobial therapy with enrofloxicin and fluid therapy with Lactated Ringers Solution. Dependent on initial diagnostics, tortoises also received supplementation with B vitamins and/or iron. Tortoises with gastric ulcers were treated with sucralfate, and tortoises with ocular discharge or palpepral edema were treated with chloramphenicol or tetracycline ointment. All tortoises were intensely monitored for eating and defecation, and were soaked in shallow water frequently to promote hydration. Tortoises that did not eat on their own were provided nutritional support through tube feeding with a reptile herbivore complete diet.

Necropsy and Histology

A full gross necropsy was completed on all dead tortoises. Histopathology was only conducted on tortoises that were not autolyzed. All tissues were examined microscopically by a boarded pathologist with considerable experience with chelonians (N. Stedman, DVM, Busch Gardens, Tampa, FL). Additional representative tissue samples were also collected and frozen for further testing, such as infectious disease analyses or toxicology.

Infectious Pathogen Testing

Tissues from six dead tortoises were submitted to the University of Florida ZooMed Diagnostic Laboratory for screening of select infectious agents (Adenovirus, Ranavirus, Herpesvirus, and *Mycoplasma* spp.) using quantitative PCR. Tissues from an additional thirteen dead tortoises were submitted to University of Illinois and screened against a panel of nine pathogens using Fluidigm quantitative PCR. The DNA in oral and cloacal swab samples was extracted with a QIAmp Blood mini Kit (QIAGEN Inc., Redwood City, CA, 94063 USA), following the manufacture protocol. DNA in tissues and whole blood was extracted using the DNAeasy Blood and Tissue Kit (Qiagen) following manufacturers protocol with the following exception: 50μL of whole blood was used instead of the recommended 200μL due to the presence of nucleated RBCs. Quantity (ng/μL) and quality (a260:A280 ratio) of DNA was evaluated using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, 02451 USA). Quantitative PCR was performed in a multiplex format to evaluate nine pathogens simultaneously using published or in house primer-probe assays (Table 1). Initially, Specific Target Amplification was performed on each sample with pooled pathogen Taqman assays and preamp mastermix (Thermo-Fisher, Waltham, MA 02454 USA). Each reaction was performed under the following cycling program on an MJ Tetrad thermocycler: 95°C (10 min), 14 cycles of 95°C (15 sec) and 60°C (4 min). The qPCR assay was then performed in triplicate using 2.25 μL of amplified DNA from the first reaction on a Fluidigm 96.96 Gene Expression IFC and amplified on the Fluidigm Biomark HD Real Time PCR thermacycler (Fluidigm, South San Francisco, CA 94080 USA) using the following cycling protocol: 70°C (30 min), 25°C (10 min), 95°C (1 min), followed by 35 cycles at 96°C (5 sec) and 60°C (20 sec). Serial dilutions of positive controls for FV3-like ranavirus, *Mycoplasma agassizii*, and *M. testudineum* were

prepared from 107 to 101 copies per reaction. A non-template control was included on each plate. All reactions were then analyzed using Fluidigm Real Time PCR analysis software (Fluidigm, South San Francisco, CA 94080 USA). Following Fluidigm analysis, all positive samples were verified in a simplex reaction. Briefly, qPCR was performed in triplicate on a QuantStudio3 real time thermal cycler for validation. Samples were considered positive if all three replicates had a lower cycle threshold (Ct) value than the lowest detected standard dilution.

Toxicants in Liver Tissues

Liver samples from dead tortoises (n=6) were also submitted to test for presence of toxicants. This panel included arsenic, cadmium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, selenium, and thalium, in addition to >100 common agricultural pesticides and herbicides evaluated by GMS mass spectrometry (Michigan State University, East Lansing, MI).

Forage and Soil Sampling and Testing

Forage and soil samples were collected at active gopher tortoise burrows in two enclosures, DB1 and SR1 during July and August of 2017. DB1 was a site of high disease-related mortality, while SR1 had no documented disease-related mortality and was chosen as a control site. At each selected burrow, an angle (0-180°) was assigned randomly. A 1 m X 1 m quadrat was placed approximately 5 meters out from the mouth of the burrow at the randomly selected angle. If the randomized angle resulted in placement around the base of a tree or shrub, the next angle was chosen instead.

Above-ground vegetation within the quadrat was cut at the soil line, collected, and transported to the lab for processing. Vegetation samples from each enclosure was separated into two types, 1) grasses and grass-like plants and 2) non-grass mixed forbs and legumes. For each enclosure, individual burrow samples of each vegetation type were thoroughly mixed by group to

create a composite mixture (e.g. DB1 mixed grasses, DB1 mixed forbs and legumes, etc.). Samples of these mixtures were then submitted for analysis through wet chemistry procedures (Dairy One Forage Laboratory, Ithaca, NY, see Appendix A for procedure details and results).

Soil cores of 30 cm in depth were collected from the center of each quadrat using a soil auger. Each soil core sample was then separated into a top (depth = 0-15 cm) and bottom (depth = 16-30 cm) layer. Samples were then pooled by enclosure and depth layer and thoroughly mixed to create a composite sample: SR1_0-15, SR1_16-30, DB1_0-15, and DB1_16-30. Soil total elemental analysis was performed for samples from these sites by the University of Georgia soils lab (Athens, GA), where levels of the following elements were measured through acid digestion: phosphorus (P), potassium (K), Calcium (Ca), Iron (Fe), Magnesium (Mg), Manganese (Mn), Molybdenum (Mo), Boron (B), Copper (Cu), Nickel (Ni), Sodium (Na), Sulfur (S), Zinc (Zn), Aluminum (Al) Arsenic (As), Cadmium (Cd), Chromium (Cr), and Lead (Pb).

Modeling for Predictor Variables for Disease

In order to determine potential predictor variables for tortoises at Nokuse detected with disease (e.g. tortoises that were identified in the mortality surveillance as disease-related mortality or transported for rehabilitation), we compiled all known data for translocated tortoises in five of the release enclosures (including enclosures with known disease-related mortalities). These data included information regarding their donor site location and characteristics, recipient site (release enclosure) characteristics, and individual tortoise data (such as sex, weight, morphometric measurements).

We performed logistic regression with fixed effects on sets of candidate models to determine which variables were most important for the detection of disease. We started with three sets of candidate models to determine whether any of these sets of characteristics were

most important in the prediction of disease. Each set of candidate models was focused on a set of characteristics – e.g. donor site, recipient site, or host (tortoise). Highly correlated variables (such as static categorical variables pertaining to site) were excluded from use in the same model. We also compared the results from these models with a null model (intercept only). A global model was not included, as there were a number of correlated variables that could not be run in the same model.

Variables included in the donor site characteristics (when available) included donor site, donor county, number of tortoises in cohort (in this study defined as tortoises collected from the same donor site and moved at the same time), donor site latitude, change in latitude from donor site to recipient site, presence of a "sick" cohort member, and number of sick tortoises in the cohort. Recipient site variables included initial stocking density (tortoises/acre), number of donor populations in enclosure, and days since last burn. Individual host variables included sex, straight carapace length (SCL, as a proxy for age), initial body condition index (BCI, mass/SCL^3), days in pen, and month/season found. Initial BCS was not determined before release, thus BCI using morphometric data collected prior to release was used as the body condition modeling variable.

We then used variables included in the top model(s) for each candidate set to create a new set of combination models. Models that were tested in this set only included variables that made biological sense when combined. Model fit results were compared using Akaike Information Criterion (AIC) for top model selection.

Results

Overview of Mortality Event

We defined the mortality event as the period between April 2013 and August 2015, although there were additional mortalities between August 2016 and April 2017 that are also

included in analyses. All accessible carcasses were collected and either submitted immediately for necropsy and diagnostics (n=21), or frozen (if too autolyzed for histology) for later analyses (n=4). The other tortoises found (n=25) were too autolyzed for appropriate diagnostics including gross necropsy. Tortoises were found sick or dead in all months except for February, and the majority (48/50) were adults. There was no significant difference in mortality rates between males (n=25) and females (n=25). The time the tortoise since introduction to Nokuse was not an accurate predictor of mortality, so that both tortoises recently introduced and those that have been there for years have died. The mortalities were initially confined to one site (Doe Branch 1; 37 acres; April-Dec 2013) but several tortoises were later found sick or dead in an adjacent site, Doe Branch 2 (60 acres; Dec 2013) and sporadic mortalities have occurred elsewhere in other release sites, sometimes several miles away from the Doe Branch sites.

Tortoises in Rehabilitation

Nineteen tortoises were found sick, but still alive between August – October 2014, January - August 2015, and August 2016 - April 2017 and submitted for medical care to the Georgia Sea Turtle Center (GSTC). Physical exams for all tortoises indicated poor body condition, lethargy, and severe weakness. Some also had obstructed nares, nasal discharge, pale mucous membranes, and varying degrees of hemorrhage under the scutes of the plastron, but no other consistent abnormalities (see Figure 2.1). The neurologic function of the tortoises was assessed frequently and was considered normal. In fact, when tortoises were treated with supportive care (fluids, warmth, vitamins and other supplements), nutritional support, and antibiotics, some of the tortoises improved and showed strong appetites and normal food consumption. Seven animals (37%) died or were ultimately euthanized because they failed to show any improvement, while the other twelve (63%) recovered at the GSTC. Recovered

tortoises were returned to Nokuse, where they were released into a separate, smaller enclosure and provided with supplemental feeding. After 11-15 months, seven of the 12 recovered tortoises (58%) were successfully recaptured, reassessed, and released in a new larger enclosure. Two tortoises rehabilitated and released were later found dead in or near their burrows.

Figure 2.1. Clinical signs documented in gopher tortoises undergoing rehabilitation at the Georgia Sea Turtle Center included emaciation (left) and hemmorhaging under the scutes on the plastron (right). Photos provided by T. Norton.

Gross Necropsy and Histology

Of the animals that have been submitted for necropsy, histology, and ancillary testing (n=21) we found: 1) lesions consistent with inanition or malnutrition (e.g., poor body condition (n=17), hepatic (n=4) and skeletal muscle (n=14) atrophy, atrophy of adipose tissue within the bone marrow (n=1), etc.), 2) mild to severe osteopenia of the plastron, 3) hemosiderosis (accumulation of iron) in the liver (n=13), and 4) no microscopic evidence of infectious disease (n=21). Emaciation, muscle atrophy, empty (or near empty) gastrointestinal tract, and liver pigmentation and hemosiderosis were the most common findings for dead tortoises.

The esophagus, stomach and small intestine of many tortoises that died were typically devoid of forage contents: 33% had completely empty stomachs (n=7), five had distended stomachs filled with gas or clear fluid, and an additional two had abnormal material and mucous present in the stomach. Two tortoises had only tube-feeding formula (from rehabilitation) present, and only one necropsied tortoise was reported to have grass present in the stomach. Two tortoises also had necrotic mucosa or necrotic material present in the GI tract. Some of the tortoises had fecal material in the colon and the GI tract exhibited ileus, or lack of digestive propulsion. None contained unusual plants or foreign objects. An enlarged or distended bladder was also reported in 10 (48%) of the tortoises, which may be indicative of dehydration or starvation.

Infectious Pathogen Testing

Tissue samples (pooled gastrointestinal tract, kidney, liver, spleen and tongue for each individual) submitted to UF were negative for most pathogens. Three individuals were positive for *Mycoplasma* spp. infection. Histopathology of the upper respiratory tract of two tortoises was consistent with mild to severe upper respiratory tract disease (URTD).

Tissues from an additional thirteen tortoises were submitted to University of Illinois for pathogen screening, including gastrointestinal tract (n=12), kidney (n=11), liver (n=11), spleen (n=5), and tongue (n=5). Low levels of ranavirus (Frog Virus 3) DNA (mean= 0.1433 copies/ng DNA, SD ±0.085) were detected in the gastrointestinal tract of three tortoises, while all other samples were negative for all pathogens tested.

Toxicants and Soil Minerals

All levels of toxicants in liver samples fell within reference ranges, when available. There were no significant differences in any of the tested soil minerals between sites. These results

together suggest it is unlikely that these tortoise mortalities were a result of toxicity.

Models Exploring Correlates with Disease

Biologically relevant combinations of variables within each candidate set of models were estimated with logistic regression, and model fit was tested using AIC as a ranking variable in the statistical software program R (version 3.4.2). These were run in a fixed-effects model with a binomial distribution to determine relationship between the variables and the binomial response outcome of presence (1) or absence (0) of disease.

$$model <- glm(disease \sim X_1+X_2..., family=binomial(link="logit"), data=data)$$

Equation 1.

The top model for the Recipient Site candidate set was initial_density + days_since_burn1, with an AIC of 319.2 (Table 2.1). Within the candidate set for Donor Site, the top model included number of tortoises in cohort and presence of disease in the cohort with an AIC value of 303.3. There were four models in the host characteristic set that were relatively close in value (within 5) to the top model of just season as a variable (AIC=191.3, Table 2.2). These models also included initial BCI, SCL, and month detected as predictor variables.

Table 2.1. AIC values of logistic regression models from the recipient site candidate set demonstrating variables for predicting detection of disease in translocated tortoises.

Recipient Site Model Variables	df	AIC
initial_density+days_since_burn	3	319.2
number_of_different_cohorts	2	367.6
initial_density	2	369.2
initial_density+ number_of_different_cohorts	3	369.6
null model (intercept only)	1	413.2

Table 2.2. AIC values of logistic regression models from the host characteristic candidate set demonstrating variables for predicting detection of disease in translocated tortoises.

Host Model Variables	df	AIC
season	5	191.3
initial_bci+season	6	192.6
scl+season	6	193.0
scl+initial_bci+season	7	194.5
month_found	12	196.0
scl	2	412.9
initial_bci	2	415.1
scl+sex	4	415.5
sex	3	416.4
null model (intercept only)	1	413.2
date_relocated	208	656.1

Variables from these top models from each set were then combined in new biologically relevant models to determine if a mixture of variable types was more predictive of disease in an individual. All top models from the combined models considered included the two variables of initial density and season (top AIC=176.9, Table 2.3).

All models that included the categorical variable of season along with one or more other variable fell within 4 AIC, suggesting that the season in which a tortoise was found was an important factor for detection of disease, indicating that there is likely a seasonal component involved either for presence of disease or for detection probability. Disease was detected in tortoises most frequently in the fall (Sept – Nov) and winter (Dec – Feb).

Other common variables in the top models (Table 2.2) include initial tortoise density of the release site, number of tortoises in a cohort, days since last burn, and initial body condition index.

Table 2.3. AIC values and model weights of top logistic regression models demonstrating variables for predicting detection of disease in translocated tortoises.

Model	K	AIC	ΔAIC	Model Weight
initial_density+season	6	176.9	0	0.245
initial_density+season+days_since_burn	7	177.1	0.2	0.216
number_in_cohort+initial_density+season	7	177.7	0.8	0.166
initial_bci+initial_density+season	7	178.6	1.7	0.102
number_in_cohort+initial_density+season+days_since_burn	8	178.7	1.8	0.1
null model (intercept only)	1	413.2	236.3	0

Discussion

Studies have demonstrated that some translocated gopher tortoise populations have very high rates of retention and nearly 98% annual survival (Ashton and Burke 2007; Tuberville et al. 2008). While the mortality event at Nokuse Plantation was abnormal enough to warrant an investigation, the annual mortality rate for the affected enclosures (and Nokuse as a whole) remained well under 1% (Aresco, unpubl. data), which is less than previously published ranges of 1-10% annual mortality for wild (Heppell 1998; Ozgul et al. 2009) and translocated (Ashton and Burke 2007; Tuberville et al. 2008) gopher tortoises. The timing between a tortoise's introduction to Nokuse and when it was found sick or dead varied between 0-5 years, such that at least one tortoise was assessed and determined to be sick upon arrival to Nokuse and was transported for rehabilitation instead of released, and others were found years after release. Affected tortoises were evenly split between males and females, suggesting that detection of

disease was not biased by sex. The majority of tortoises were also found in the cooler months, when they would be expected to be less active, although tortoises with poor health may also display abnormal thermoregulatory behaviors such as spending significantly more time basking or being out of their burrows at odd times (McGuire et al. 2014; Berry and Christopher 2001; Jacobson 2014). This behavior of being out or basking in cooler weather may suggest that the tortoises found in the fall and winter were potentially attempting to induce a behavioral fever, which gopher tortoises have been shown to do when their immune system is challenged (Goessling et al. 2017).

The body condition, physical exam, complete cell counts and plasma biochemistry of tortoises in rehabilitation (hypoproteinemia, hypoalbuminemia, anemia, etc) indicated severe malnutrition. The majority of rehabilitated tortoises that were subsequently released back at Nokuse Plantation remained in apparent good health after monitoring them for nearly a year. The two individuals that were found dead after release were found within their burrows, and cause of death could not be determined.

There were some commonalities among tortoises that were necropsied. They were emaciated, had moderate to severe muscle atrophy, a mostly empty gastrointestinal tract, and hemosiderin accumulation in the liver. The combination of these factors suggests that these animals had not been eating for a long time prior to death, although the cause of which is unknown. When found alive, individuals were very weak (muscle atrophy), had low body condition, subkeratin hemorrhage of the plastron, and bloodwork consistent with chronic inanition or starvation. Although starvation due to lack of adequate forage within the release sites seems highly unlikely given the abundance and diversity of suitable forage plants present in all of the known affected sites, a dysregulated stress response caused by chronic stress can lead to

decreased feeding and a decreased ability to assimilate nutrients (Dickens et al. 2010; Herzog et al. 2009). Based on the toxicity testing on both tortoise tissues, ingestion of toxins due to past agricultural and silviculture practices also seems unlikely as the cause of disease and mortality in these tortoises.

There is scant information on the diseases and pathogens of wild gopher tortoises, other than URTD and *Mycoplasma* spp. However, none of the histopathology findings or infectious pathogens analyses provide evidence to suspect an infectious pathogen as the cause of the mortalities. We did detect *Mycoplasma* spp. DNA in tissue from three tortoises and the presence of FV3 DNA at low levels in the gastrointestinal tracts of three tortoises. However, the severity and the inconsistent frequency of the lesions do not indicate that these mortalities were caused by URTD or by *Mycoplasma* spp. infection. Additionally, due to the lack of ranavirus detected in other organs and the low number of copies present in the three positive samples, it is also unlikely that this was the cause for mortality. These three tortoises instead may have been asymptomatic carriers of ranavirus. Novel pathogens remain a possibility, and continued testing to eliminate infectious agents as the cause of future mortalities is recommended.

There are no readily apparent differences in the quality of habitat, plant community, or soil type and composition among release sites at Nokuse (Aresco, pers. comm.). All sites can be characterized as former xeric sandhill converted to agriculture in the 1960's and presently contain an abundance of herbaceous groundcover plants such as broomsedge and bluestem grasses (both *Andropogon* spp.) and scattered planted longleaf pine. Nokuse Plantation follows a prescribed burning regime every 3-4 years as is recommended to decrease woody debris and stimulate growth of native grasses and herbs (the main food source for tortoises). Examination of the affected enclosures (i.e., those experiencing mortalities) by a botanist did not detected poisonous

exotic or native plants and there is an ongoing investigation on the plant community composition. Forage nutrition testing results suggest that, although it was the site with higher mortality, the forage tested from DB1 had higher amounts of nonfibrous carbohydrates, like starches and sugars, than SR1 which suggests more easily available energy was present in DB1 plants (see Appendix A). No land management practices had recently changed prior to this mortality event. Although the historical farm practices from 1968-1990's (use of center pivot irrigation and length of time phosphate fertilizer was applied) differed among sites, no discernible correlation between farming practice and tortoise mortality has yet to emerge (Aresco, pers. comm.) and no agriculture-related toxicants had elevated levels in soil samples or liver samples. The only habitat management related factor that emerged as important for detection of disease was time since last burn. Generally, tortoises were more likely to be detected with disease within the month or so following a prescribed burn, although this is likely due to the increased visibility following the removal of the understory vegetation and not that fire was related to the disease. There was also a group of tortoises that were detected within the one site that had not been burned for nearly a decade – in the case of these tortoises, it is more likely that the longer time since burn was an indicator of less suitable habitat conditions.

The results of our analyses may serve as more of a cautionary tale for future translocation events, as new pathogens and syndromes continue to be discovered. We did not find a specific etiologic agent to be responsible for the mortalities, and our current most likely explanation for illness and death includes a multitude of factors including density, which may affect social disruption, and stress. Our highest ranked models suggest that there were recipient site, donor site and individual tortoise characteristics that were important in predicting disease status.

Season was an important component of several of the models with the overall lowest AIC, suggesting that there is a seasonal component to the detection of disease at Nokuse. This is not a surprising result, as many diseases have some sort of seasonal component. However, season itself does not give us further information on what factors may predispose an individual tortoise to disease. The other most common variables among the top models included initial tortoise density of the release site, number of tortoises in a cohort, days since last burn, and initial body condition index. All of these variables are biologically relevant to the potential for a tortoise to develop disease, especially when combined. Holding animals at higher densities increases probability of infection and disease, which can be exacerbated in the case of a social species such as the gopher tortoise. We were more likely to detected disease in tortoises held at higher densities. Given the social structure associated with gopher tortoise populations, it is logical that the number of tortoises in a cohort may influence the stress (and health) exhibited by an individual after translocation. We saw that we were more likely to detect disease in larger cohorts sizes than in smaller ones when the effect of density was included, as seen in the top models. Without density as a variable, however, it was more likely to detect disease in tortoises from smaller cohorts. This suggests that tortoises from larger cohorts may not respond as well as those moved as individuals or as part of a small cohort, at least when considering a higher density. Presumably, the social structure may be better established in a larger cohort, and it may be harder for individuals from large cohorts to acclimate to the mixing of cohorts at a high density as opposed to tortoises from small cohorts. For gopher tortoises in longleaf pine habitats, days since burn acts as a measure of habitat quality. Fire is required within this ecosystem in order to maintain an open canopy with little midstory that gopher tortoises prefer, and lack of frequent fire on the landscape can lead to suboptimal habitat and foraging conditions. However,

the probability of finding a sick or dead tortoise is also increased directly following a prescribed burn, as there are less grasses and other understory plants to mask visibility, therefore making them easier to find. When these burns are conducted in the winter months, there is also a higher likelihood of detection for sick tortoises that are exhibit atypical overwintering behavior, such as making larger movements, pacing the fence line, or abnormal basking (see chapter 3). Moving tortoises that have been part of different cohort sizes into a release area with other groups of tortoises, especially in a high density situation, may disrupt existing social structures and hierarchies present in a particular group, and increase stress. Individuals with a low initial body condition index may be more likely to remain at a lower body condition, especially under stressful conditions.

The scope of this study was limited, however, as many retrospective analyses are. This study has revealed factors that appear to be important correlates in determining probability of disease, and therefore provide guidance about important data to collect as part of translocation and factors to consider when planning releases. As an example, although we were able to look at donor sites on a county and region level, we were unable to look at the finer-scale effect of changes in latitude for many of the tortoises that were found with disease. Additionally, more information regarding donor site, such as habitat or vegetation type and quality, are not available for most of these sites, as these translocations are time-sensitive endeavors and development occurs quickly once the primary goal of removing tortoises has been achieved. This type of information may actually be more important for determining health status at time of translocation and how well individuals cope with stress of translocation, but cannot be analyzed through this study given our data.

When it comes to translocation of gopher tortoises in Florida, the objective is usually to remove individuals from areas slated for development to avoid direct and immediate mortality, with the secondary objective of reintroducing tortoises to an area where they were previously extirpated or augmenting existent populations. While both of these objectives are met with translocation to Nokuse, our site has underscored the importance of monitoring health and disease for years after release. Disease screening (or at least collection of samples for later pathogen testing if an issue arises) may be a particularly important consideration when combining tortoises from multiple source populations. Although gopher tortoises exhibit many characteristics that make them a difficult species to measure post-translocation success (Riedl et al. 2008), we can still learn from continued surveillance and amend future translocation and release protocols to increase likelihood of maintaining a stable population. Since recipient sites and land managers cannot necessarily regulate the size of a particular cohort, an individual's incoming body condition, or the season, it would make more sense to focus on the other variables that could be accounted for and regulated. One of the most important tasks is to maintain frequent fire as a habitat management tool. Increased monitoring during the inactive season, particularly following prescribed burning, may also lead to early detection of tortoises in poor health, which could then receive early treatment. Managers should also ensure that release sites are maintained below a set maximum tortoise density (we recommend no more than 8 tortoises per hectare) to reduce the potential for additional chronic stress associated with social disruption. This is especially important for tortoises coming from large cohort sizes, which may need to either remain separate from other cohorts, be split amongst release sites, or just maintained at an even lower density. This maximum density is higher than many documented wild populations (0.02-3.08 tortoises/hectare) of gopher tortoises (Eubanks et al. 2003; Guyer et

al. 2012; Berish et al. 2012; Bauder et al. 2014; McGuire et al. 2014) and one other translocated population (Tuberville et al. 2011), and may need to be reduced pending further research into the effects of social network disruption and density in translocated gopher tortoises.

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CHAPTER 3

EFFECTS OF HEALTH STATUS AND DENSITY ON MOVEMENT AND THERMOREGULATORY BEHAVIOR OF TRANSLOCATED GOPHER TORTOISES

Introduction

The gopher tortoise (*Gopherus polyphemus*) is currently listed as a vulnerable species on the IUCN Red List, is federally protected in the western part of the range, and is a candidate for Federal listing because of a significant range-wide population decline (USFWS 2016). These dramatic population declines throughout their range have occurred most notably as a result of habitat loss and fragmentation (Enge 2006). Due to the high development pressures in Florida, the Florida Fish and Wildlife Conservation Commission (FWC) has leaned heavily on translocation as a conservation strategy, especially within the last decade (FWC 2009). In addition to habitat-related threats, disease has also emerged as a threat for tortoises, especially in the form of infectious pathogens (Berish 2010; Wendland et al. 2010; Jacobson and Berry 2012).

Thermoregulation in Tortoises

Gopher tortoises, like all ectothermic vertebrates, rely on external temperatures for regulation of their internal temperatures and metabolic processes. Thus, tortoises will regulate their temperatures through behavioral changes (such as retreating into their burrows to escape extreme heat or cold, or basking on the apron of their burrow in order to raise their temperature). Tortoises typically undergo a period of brumation or hibernation during the colder winter months, remaining essentially inactive in their burrows without emerging for up to months at a time, until temperatures are warmer (Nussear et al. 2007; DeGregorio et al. 2012). This

overwintering behavior has been studied within various gopher tortoise populations throughout their range. Adult tortoises in South Carolina at the northern extent of their range demonstrated overwintering periods of 127-128 days on average, although juveniles were found to emerge more frequently during the winter in SC as well as in coastal Georgia, potentially as a result of their increased body surface area to volume ratio and subsequent higher rate of heat loss (DeGregorio et al. 2012; Harris et al. 2015). Meanwhile, tortoises in south Florida can be active through the winter months, at least during the warmest part of the day (Douglass and Layne 1978).

Temperature also plays an important role in tortoise health. Gopher tortoises can induce a fever response to fight off infection or illness through alteration of thermoregulatory behavior (Goessling et al. 2017). This can be accomplished through events such as extensive basking, although this may have trade-offs with vulnerability to predation. Atypical behavior, such as wandering outside of burrows or basking in cold weather, has been noted in both desert and gopher tortoises with clinical signs of disease (Berish et al. 2010; Berry and Christopher, 2001; Diemer-Berish et al. 2000; McLaughlin 2000; Brown et al. 1994). Sick individuals spend more time basking at warmer temperatures, presumably to incite a behavioral fever to increase their body temperature and stimulate an immune response to fight disease (Ouendraogo et al. 2004). A decreased ability to effectively elevate body temperature to boost the immune system may result in increased susceptibility to pathogens and disease.

Effect of Translocation on Movement

Gopher tortoises are one of the most widely relocated reptiles in the southeastern U.S. (Seigel and Dodd 2000). Although home range sizes and burrow usage can vary for this species dependent on specific site and habitat characteristics (McRae et al. 1981b; Aresco and Guyer

1999), a relocated gopher tortoise typically displays altered behavior and movement patterns as its orientation system adjusts to its new environment (Bauder et al. 2014; Morpeth 1997). Most translocation studies on gopher tortoises focus on short-term success (Bauder et al. 2014). Studies examining translocated gopher tortoise populations over 10 years have not analyzed movement patterns, instead relying upon mark-recapture data to measure retention rates and site fidelity (Ashton and Burke 2007; Tuberville et al. 2008). One consistent finding of translocation studies is that relocated gopher tortoises exhibit movement patterns different from wild populations or resident tortoise populations at least during the first year post-release (Bauder et al. 2014; Riedl 2006; Tuberville et al. 2005). No study to date has documented the spatial ecology of translocated tortoises several years after relocation which is an important aspect to consider when determining the long-term success of translocations.

Other Factors Affecting Tortoise Movement and Behavior

Gopher tortoise movement ecology includes a number of density-dependent behaviors. Movements made while searching for mates may increase in either frequency or distance, depending on tortoise density within a given area (Guyer et al. 2012). Tortoises present in higher density areas may also experience a need for increased movement for foraging opportunities as preferred food sources close to the burrow are depleted, although this can also be affected by habitat quality (McRae et al. 1981b).

Several studies have documented changes in gopher and desert tortoise behavior and movement patterns due to disease, particularly with infection with *Mycoplasma* spp. (McGuire et al. 2014; Ozgul et al. 2009; Berry and Christopher 2001; Jacobson 2014). Contrary to the notion that a chronically ill gopher tortoise is less likely to emigrate than its healthy counterpart, one study demonstrated tortoises with severe Upper Respiratory Tract Disease (URTD) clinical signs

had significantly larger home ranges and made longer movements than mild or asymptomatic tortoises (McGuire et al. 2014). Tortoises that are infected with *Mycoplasma* spp. or other infectious pathogens that can be transmitted through direct contact may thus directly increase the risk of disease transmission to conspecifics. Tortoises with poor health may also display abnormal thermoregulatory behaviors such as spending significantly more time basking or being out of their burrows at odd times (McGuire et al. 2014; Berry and Christopher 2001; Jacobson 2014).

Stress is another factor influencing behavior and health. Sources of stress for translocated tortoises can be either direct, such as capture and handling or repeated monitoring, examinations, and relocation, or less direct factors, such as introduction to a novel environment, and disruption of previous social structures (Aiello et al. 2014). Gopher tortoises are social animals with hierarchical social networks that can be disrupted by relocating a population (Guyer et al. 2014; Tuberville et al. 2011; Riedl 2006).These stressors can lead to behavioral shifts, an increase in the risk in exposure to pathogens, and expression of otherwise subclinical diseases (Jacobson 2014; McGuire et al. 2014). Disease transmission risk in particular may be exacerbated in recently translocated tortoises due to increased movements post-release and potential for increased social interactions, particularly when tortoises from multiple sources are combined.

This study explores the relationship between density, health status, and behavior in translocated tortoises at two translocation release pens that varied in stocking density within a protected conservation area in northwest Florida. The higher density release pen was also a site where a previous disease-related mortality event was initially documented (see Chapter 2), while the lower density pen did not experience any disease-related mortality. However, a proportion of tortoises in both pens were deemed “at risk” for developing disease. Given previous research that

suggests that both tortoise density and individual tortoise health can influence behavior in tortoises, we hypothesized that 1) tortoises in the high-density site would have larger home range sizes than tortoises in the lower density site; and 2) tortoises at risk for disease would exhibit abnormal overwintering behaviors in both sites.

Methods

Study Site

This study was performed at Nokuse Plantation, which consists of approximately 22,040 hectares of privately owned and managed conservation lands located in Walton County, Florida (30.4454° N, 85.9619° W). Much of this land is dedicated to longleaf pine restoration efforts, including ~11,020 hectares of xeric sandhill habitat suitable for gopher tortoises. Since 2006, Nokuse has been the recipient site for over 4,500 tortoises displaced by development across Florida though Incidental Take Permits issued by the Florida Fish and Wildlife Conservation Commission (FFWCC). Tortoises in this study had been previously translocated to Nokuse between 2011-2015 and were either released in a high density site (Doe Branch 1 (DB1); 15 ha, ~18 tortoises/ha) or a medium density site (Seven Runs 1 (SR1); 13.75 ha, ~7.7 tortoises/ha). The release sites are both located in sandhill areas with Lakeland soil, and the predominate overstory is longleaf pine (*Pinus palustris*) followed by turkey oak (*Quercus laevis*). The understory is primarily grasses at both sites, dominated by *Andropogon* spp. grasses and Bahia grass. Although Nokuse tries to maintain a burn rotation of 3-5 years to reduce presence of a hardwood midstory and prevent succession into a mixed hardwood forest, one of our study sites (SR1) had not been burned for approximately ten years prior to the initiation of this study but there was not significant hardwood encroachment.

Tortoises Handling Procedures

During June-August of 2016, both sites were surveyed for gopher tortoise burrows. Active burrows were scoped to determine tortoise occupancy, and tortoises from occupied burrows were trapped by placing live wire traps (Hav-a-Hart) at the mouth of the burrow. Traps were shaded using shade cloth and checked twice daily (between 10-11am and 4-6pm). Successfully trapped tortoises were transported to the Nokuse Plantation field lab for processing. Tortoises were identified based on their unique permanent marks to find initial release information. Standard morphometric measurements including weight, straight carapace length (SCL), carapace width, carapace height, and plastron concavity were recorded (McRae et al. 1981a). Each tortoise received a complete health assessment by the same trained individual, including a thorough physical exam and a subjective body condition assessment. Blood was collected from the brachial vein with a heparinized syringe. A small amount of this blood was transferred to a microhematocrit tube and centrifuged to collect Packed Cell Volume (PCV) and Total Protein (TP), which was used to assign health status. The remaining blood was used for additional health and nutritional analyses (see Chapter 4).

As part of a larger study in which we were investigating the health status of translocated individuals, adult tortoises (Carapace Length > 180mm) were assigned to one of two health status groups ("Healthy" and "At Risk") based on PCV, Body Condition Score, and overall behavior. A tortoise was assigned to the "At Risk" group (AR) if at least one of the following criteria was met: PCV $\leq$ 25 (indicative of anemia), low body condition (BCS < 3 on scale of 1-5), or mild to moderate lethargy. Tortoises were assigned as "Healthy" (H) if they did not meet any of the criteria listed above. A selected subset from each site (consisting of 7 "At Risk" and 7 "Healthy" per site; n=28 total) were outfitted with VHF radio telemetry transmitters (ATS model

R1860, 27 x 40 x 10mm), modified GPS units (i-gotU GPS unit with extended battery), and temperature data loggers (Thermochron iButton DS1922L) set to record a temperature every hour. These devices were attached to the anterior carapace of each individual tortoise with quick-drying epoxy (Waterweld). Total weight of hardware and epoxy did not exceed 5% of body weight (Burke et al. 1994).

Home Range Estimation

Tortoises were tracked approximately once a week between when transmitters were affixed to tortoises and when tortoises were recaptured for hardware removal (average 277 days, n=27). Each time a tortoise was located, information regarding burrow use and activity (below ground vs. above ground, whether actively foraging or moving on the landscape) was also recorded. Movement differences were evaluated primarily through home range size based on data collected from 11 July 2016 – 16 July 2017. We calculated 50% core home range and 95% core activity area using Kernel Density Estimation (KDE) using the adehabitatHR package in the program R (version 3.4.2). We compared both 50% and 95% home range (HR) size estimates between sex, release sites, and health groups using analysis of variance (ANOVA).

Overwintering Behavior

We examined the temperature fluctuation patterns from the temperature loggers during the cooler months (November 2016 – March 2017) to detect differences in overwintering thermoregulatory behavior. We determined overwintering onset and termination by visual inspection of the resultant temperature graph. . Following Nussear et al. 2007 and DeGregorio et al. 2012, onset was defined as the date where 7 consecutive days showed no significant temperature spike (indicating an emergence). Termination was designated as the date where the following 14 days showed daily emergences. Duration was calculated as date of termination

minus date of onset, even if there were emergences for periods of less than seven days during the intervening time period. Tortoises that did not have an onset as defined above were deemed as not experiencing an overwintering period. We compared mean overwintering duration by site and health group using a two way ANOVA.

Results

Movement and Home Ranges

Twenty-eight tortoises were outfitted with transmitters and data loggers and released between Jun-Aug 2016. Of those, 27 tortoises were recaptured or recovered during the 2017 active season (Jun-Aug). Data from the GPS loggers were not analyzed due to inconsistent data recovery from the devices. Five tortoises (all from SR1) were excluded from the analysis because we had less than 5 unique locations, the minimum number of points to create a KDE home range estimate, including four tortoises that were removed from the study due to declining health or death. Reported here are data from the remaining 23 tortoises (DB1: 7 H, 7 AR tortoises; SR1: 4 H, 5 AR tortoises).

The average home range size (based on 95% Kernel Density) for tortoises in DB1 was 3.651 ha (range 0.314 – 13.656 ha), and for tortoises in SR1 was 1.569 ha (range 0.034 – 3.984 ha) (Table 3.1). Home ranges in DB1, were larger than in SR1 on average, although not statistically significant ($p<0.08$). The home range size of males and females across both sites was 3.943 ha (range 0.138 – 13.656 ha), and 1.823 ha (range 0.034 – 5.706 ha) respectively. When tortoises were divided by health group, overall H tortoise home range size averaged 2.708 ha (range 0.133 – 13.656 ha) whereas AR tortoises had an average home range size of 2.955 ha (range 0.034 – 9.042 ha), which were not significantly different ($p=0.8$). Both 50% and 95% KDEs showed similar trends when tortoises were split by site, health group, and sex (Figure 3.1).

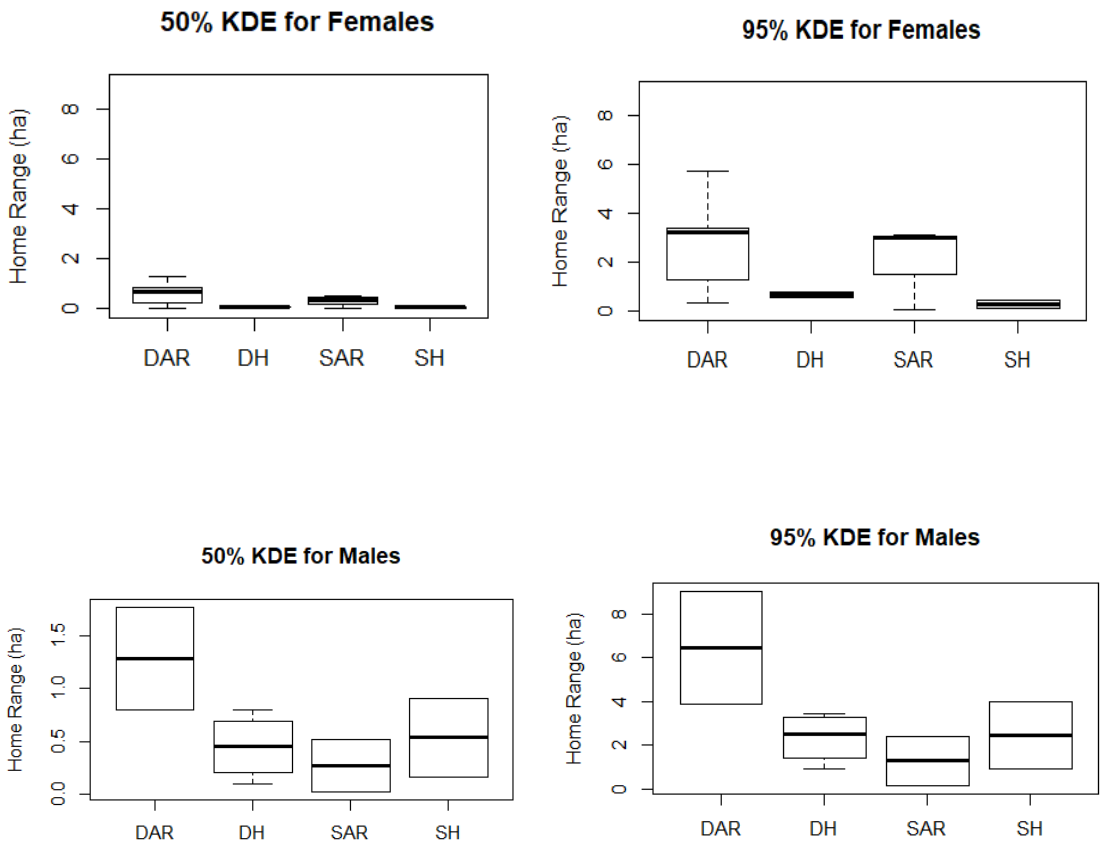

Figure 3.1. Differences in 50% and 95% Kernel Density Estimates for females and males, when separated by site (D v S) and health group (AR v H). One outlier for males in the DH group was removed.

Table 3.1. 50% and 95% Kernel Density Estimates for home range sizes of individual translocated tortoises by site (DB1 vs SR1), health group (AR vs H), and sex based on weekly tracking data collected from Jul 2016 – Aug 2017.

Site	Tortoise ID	Group	Sex	50%Area (ha)	95% Area (ha)
DB1	2007	AR	F	0.217	1.277
DB1	2133	AR	F	0.008	0.314
DB1	2018	AR	F	0.649	3.367
DB1	2213	AR	F	1.257	5.706
DB1	1954	AR	F	0.812	3.204
DB1	2012	AR	M	1.769	9.042

DB1	2063	AR	M	0.802	3.865
DB1	2006	H	F	0.075	0.753
DB1	2052	H	F	0.037	0.536
DB1	2025	H	M	0.591	3.456
DB1	2230	H	M	0.314	1.967
DB1	2206	H	M	0.796	3.087
DB1	2280	H	M	2.991	13.656
DB1	2148	H	M	0.093	0.894
SR1	1935	AR	F	0.503	3.133
SR1	1868	AR	F	0.006	0.034
SR1	1862	AR	F	0.361	2.975
SR1	1896	AR	M	0.517	2.402
SR1	1883	AR	M	0.022	0.138
SR1	1876	H	F	0.080	0.438
SR1	1873	H	F	0.022	0.133
SR1	1878	H	M	0.166	0.883
SR1	1894	H	M	0.904	3.984

Within the high density site (DB1), the average home range for H tortoises and AR tortoises was 3.478 ha (range 0.536 – 13.656 ha) and 3.825 ha (range 0.314 – 9.042 ha) respectively. In the medium density site (SR1), the average home range for H tortoises was 1.359 ha (range 0.133 – 3.984 ha), and AR tortoises was 1.736 ha (range 0.034 – 3.133 ha). Though there were differences in home range sizes based on the combination of sex, site, and health group, they were not statistically significant ($p=0.5$).

Female tortoises in both sites showed a difference in home range size based on health group for both 50% KDE (average of 0.477 ha for AR and 0.054 ha for H) and 95% KDE (average of 2.50 ha for AR and 0.47 ha for H), with AR tortoises having larger areas. Males showed the same trend on average for 50% KDE (average of 0.778 ha for AR and 0.477 ha for H) and 95% KDE (average of 3.86 ha for AR and 2.378 ha for H), with one outlier male removed. Male AR tortoises in DB1 however, had larger home range size than any of the other

male site x group combinations for both 50% (1.29 ha vs < 0.54 ha) and 95% (6.45 ha vs <2.4 ha) KDES, as well as compared to all female site x group combinations.

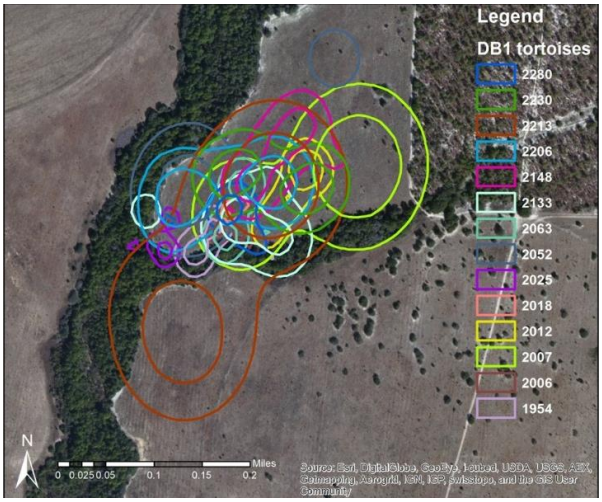

Figure 3.2. Home range sizes (95% and 50% KDE) for tortoises in high density site (DB1).

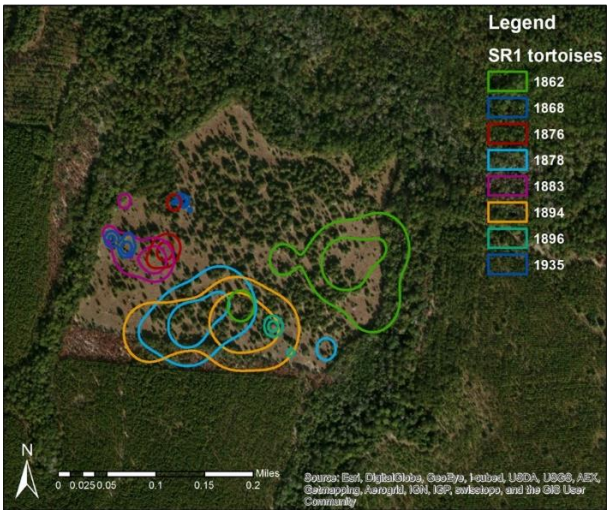

Figure 3.3. Home range sizes (95% and 50% KDE) for tortoises in medium density site (SR1).

Overwintering Behavior

We recovered temperature loggers from 27 out of 28 individuals. We analyzed overwintering onset, duration, and termination for 23 tortoises in total (Table 3.2). The

remaining four tortoises were removed from analysis due to their early removal from the study (e.g. found sick or dead before or during the period of analysis). The earliest onset of overwintering behavior occurred on 12 November 2016, while the latest onset occurred nearly six weeks later, on 25 January 2017. Termination occurred between 18 February and 20 March 2017. Ten tortoises (3 H, 7 AR) did not display overwintering behaviors. The average overwintering period overall for those individuals that did overwinter was 97.4 days (+/- 26.4).

Table 3.2. Overwintering onset, termination, and duration of previously translocated tortoises within Doe Branch 1 and Seven Runs 1. Tortoises were also separated by health group status ("Group"), as determined during health assessments.

ID	Pen	Sex	Group	onset	termination	duration
1954	DB1	F	AR	none	none	0
2133	DB1	F	AR	none	none	0
2213	DB1	F	AR	none	none	0
2063	DB1	M	AR	1/3/2017	3/4/2017	60
2006	DB1	F	H	none	none	0
2025	DB1	M	H	none	none	0
2206	DB1	M	H	none	none	0
2018	DB1	F	H	1/25/2017	3/21/2017	55
2230	DB1	M	H	1/3/2017	2/28/2017	56
2280	DB1	M	H	12/6/2016	3/19/2017	103
2148	DB1	M	H	12/2/2016	3/17/2017	105
2052	DB1	F	H	11/17/2016	3/17/2017	120
1868	SR1	F	AR	none	none	0
1896	SR1	M	AR	none	none	0
NM77	SR1	M	AR	none	none	0
1883	SR1	M	AR	none	none	0
1862	SR1	F	AR	12/5/2016	2/18/2017	75
1935	SR1	F	AR	11/26/2016	3/20/2017	114
1894	SR1	M	H	12/7/2016	3/17/2017	100
1873	SR1	F	H	11/12/2016	3/4/2017	112
1878	SR1	M	H	11/17/2016	3/17/2017	120
1876	SR1	F	H	11/15/2016	3/17/2017	122
1884	SR1	F	H	11/15/2016	3/19/2017	124

When divided by site, tortoises in the high density site experienced shorter overwintering durations ($p<0.03$). The mean duration including individuals that did not overwinter (duration=0)

for DB1 (high density site) was 42.667 days, while the mean for SR1 was 70.91 days. When we examined the overwintering period of tortoises by health group alone, H tortoises at both sites on average spent more time overwintering in their burrows than did AR tortoises (79.23 days vs 15 days; p=0.006).

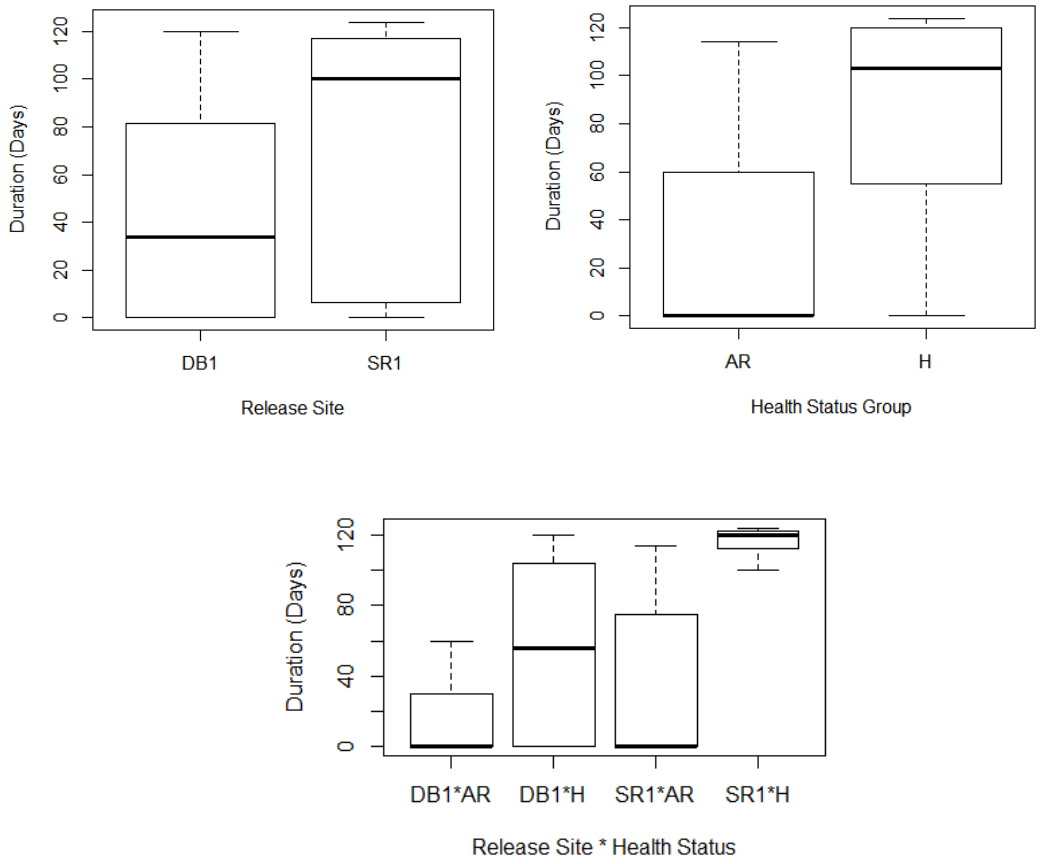

Figure 3.4. Overwintering duration of translocated gopher tortoises at Nokuse Plantation, separated by release site (Doe Branch 1 v Seven Runs 1; A), by health status (At Risk v Healthy; B), and by both site and health status (C).

Discussion

Movement behavior in tortoises is influenced by a number of factors, including sex, translocation, density, and health. Translocated tortoises are presumably acclimated and settled into their release site within 1-2 years of release, especially if penning was used to promote site

fidelity (Tuberville et al. 2005; Aresco et al. 2010). The time that elapsed between tortoise release and the initiation of our tracking study was 2-5 years post-release, suggesting that the tortoises should be settled in at the site. The enclosure surrounding DB1 was removed in 2015, two years after the last tortoise was released in that site and one year before our tracking study began, while the SR1 enclosure is still intact. In our high density site, we saw a trend towards larger home ranges in tortoises that were in our "At Risk" health group, for males and females, although there were a couple of healthy tortoises that also had relatively large home range areas in the site. Conversely, the trend in Seven Runs 1 was that "At Risk" tortoises used smaller areas (two of the tortoises excluded for having less than 5 distinct locations were in the AR group), a difference which was particularly exacerbated in males. This might suggest that tortoises in our high density site may be forced to make longer movements or search out new areas when they are in poorer health, potentially to forage or search for nutrients they may be lacking, or that the necessity for longer movements for adequate levels of forage material may result in declining health (White 2009). Another possibility is that these tortoises are experiencing negative interactions with their conspecifics, either through direct competition for resources or other social interactions.

Although there are clear visual differences in KDE areas between AR tortoises based on sex and site density, there was a lack of statistical significance. This could be due to the tortoises that were excluded reducing the sample size and losing their small home range areas in the analysis, thus skewing the results towards a larger average home range size with the remaining individuals. McGuire et al. (2014) found that asymptomatic tortoises or those with only mild clinical signs of URTD did not have significantly different home range sizes, but animals with severe clinical signs were found to have larger home ranges than healthy animals. Thus, it is

possible some of the tortoises that we assigned to the "At Risk" groups, but were on the margins of the requirements for this group, may be behaving similarly to tortoises with mild clinical signs of URTD in that study, thus skewing our home range averages. The most likely explanation for lack of statistical significance is a combination of these two factors.

Larger home range sizes for individuals in poorer condition and at risk for disease may also increase the potential for pathogen transmission, as those individuals will likely have increased interactions with other conspecifics (McGuire et al. 2014). Sick tortoises making longer movements while potentially shedding bacteria or viruses could also transmit pathogens to other chelonian species (Feldman et al. 2006). This is especially important to think about in terms of translocated animals, as they are exposed to additional sources of stress, may be more susceptible to disease and infection, and typically display dispersal movement patterns shortly following translocation (Aiello et al. 2014; Tuberville et al. 2005).

Further analyses for movement data collected in these tortoises related to this idea include looking at straight line distance moved between tracking points, number of burrows used, frequency of burrow switching, and burrow sharing, which will give a more complete picture of the effect of density and health on movement behavior, especially in translocated tortoises. Conclusions from these analyses may also help explain the potential for increased disease transmission as a result of altered movements.

In contrast, there was a clear and significant difference in thermoregulatory behaviors between tortoises due to health status as well as site. Duration of overwintering behavior between tortoises of different health statuses were significantly different, and we also saw differences between the two sites. Average duration for tortoises designated as "Healthy" was over five times longer than average overwintering period for tortoises in the "At Risk" group, as

many AR tortoises did not demonstrate strong overwintering behavior. One potential explanation is that many tortoises that we designated as "At Risk" were likely emerging from their burrows throughout the cooler months at a higher rate in order to bask and induce a behavioral fever to instigate an immune response (Goessling et al. 2017). As these were also tortoises that had lower body condition, another potential explanation for their increased activity during the cooler months is that they could have emerged in order to forage or attempt to reconcile a dietary or nutritional deficiency, although this seems unlikely.

An additional variable not considered in these analyses that may be particularly important is the location of the individual tortoise's donor site and its latitude, which may additionally affect overwintering behavior. Since overwintering behavior and duration can vary by location and region, it may be that this variation in behavior may be maintained by a tortoise after translocation. Individuals from southern Florida, where tortoises remain active through the cooler months (Douglass and Layne 1978), which are moved to latitudes further north may be more inclined to try and remain active through the winter, which may result in reduced fitness.

There is a need for future research regarding these topics of movement and behavior of translocated gopher tortoises, especially as they relate to health. Some of these needs include 1) determining how larger home range sizes and movements of sick or at risk individuals may play a role in pathogen persistence and transmission, 2) if density or other translocation-related stressors are driving changes in health status of individuals, and 3) determining if donor site latitude has an effect on overwintering durations after translocation.

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CHAPTER 4

HEALTH ASSESSMENTS OF TRANSLOCATED GOPHER TORTOISES IN NORTHWEST FLORIDA

Introduction

Habitat loss and degradation is the single greatest range-wide threat to gopher tortoises (Enge et al. 2006; FWC 2007; USFWS 2009). The gopher tortoise (*Gopherus polyphemus*) is currently listed as a vulnerable species on the IUCN Red List, is federally protected in the western portion of its range, and is a candidate for Federal listing in the eastern portion of the range (IUCN/SSC 1987; USFWS 1987; USFWS 2016). In Florida, an estimated a 50–80% population decline was inferred from habitat reduction between the 1960s and the mid-2000s, caused by urbanization, development, agriculture, and phosphate mining (FWC 2001a, 2006; Mushinsky et al. 2006).To help combat losses as a direct result of development in areas of the state, the Florida Fish and Wildlife Conservation Commission (FWC) has leaned heavily on translocation as a conservation strategy, especially within the last decade (FWC 2009). Translocation, or the deliberate movement of an individual from one place to another in order to establish, re-establish, or augment a population (IUCN/SSC 1987), has been used for numerous herpetofaunal species over the years, with variable success (Siegel and Dodd 2000; Sullivan et al. 2015; Germano and Bishop 2009). Translocation can be used to facilitate recovery of depleted populations, re-establish populations at sites where habitat has been restored but that do not currently support viable populations, to mitigate conflicts with threatened or endangered species with development, or a combination of these goals. For gopher tortoises, this strategy has been

increasingly used, and gopher tortoises remain one of the most widely relocated reptiles in the United States (Seigel and Dodd 2000; Tuberville et al. 2008; Berry and Aresco 2014).

Yet, translocation comes with risks. The risk of disease transmission is of notable concern in translocated animals, as translocation can promote unintentional movement of pathogens from one region to another, or expose naïve conspecifics or other wildlife at the recipient site (Corn and Nettles 2001). Additionally, other activities associated with translocation (such as capture and handling, holding at higher densities, confinement, or transport) may facilitate pathogen transmission and act as novel stressors for translocated individuals (Aiello et al. 2014). These stressors can affect host immune response and pathogen transmission dynamics, as well as potentially lead to increased pathogen exposure or expression of otherwise subclinical diseases (Jacobson 2014; McGuire et al. 2014; Hernandez et al. 2010). As a result, pre-translocation health and disease risk assessments and monitoring are recommended to minimize these effects (Woodford/IUCN 2000).

Clinical Techniques to Assess Health

Physical exams are routinely performed as part of the health assessment of an individual, and many of the health guidelines for gopher tortoises are based on literature from desert tortoises (Wendland et al. 2009). A Body Condition Index (BCI) based on morphometric data comparing size to weight ratio (calculated two ways: 1) using just Straight Carapace Length, SCL, (SCL^3/weight); and 2) using total body volume, SCL x Straight Carapace Width x Height divided by weight) has been used to assess condition in tortoises (Jacobson 2014; Wendland et al. 2009; Riedl 2006; Nagy 2002). However, because the relationship between body size and weight vary due to sex, reproductive status, and degree of hydration (Berry and Christopher 2001; Lamberski 2013), morphometrics alone may not accurately reflect individual condition. As

an alternative approach, a body condition score (BCS) can be determined based on the evaluation of muscle mass and fat deposits in relation to skeletal features and has been adapted for use with desert tortoises (Lamberski 2013).

Hematology and blood biochemistry are frequently used in health assessments and disease diagnostics of reptiles. Hematological evaluation includes a complete blood count (CBC), which includes packed cell volume (PCV), total protein (TP), estimated white blood cell (WBC) count, and a differential WBC count. CBCs are used to detect conditions such as inflammatory diseases, anemia (regenerative vs. non-regenerative), or parasitemia. Biochemical assays performed on plasma that appear most useful for reptiles are TP, glucose, uric acid, aspartate aminotransferase (AST), creatine kinase (CK), calcium, phosphorus, blood urea nitrogen (BUN), and electrolytes. Vitamin and mineral assays are also common and important components of complete health assessments (USFWS 2011). Clinical pathology values have been reported for healthy and translocated gopher tortoises (Taylor and Jacobson 1981; Hernandez et al. 2010) and free-ranging desert tortoises (Christopher et al. 1999).

The objectives of our study were to 1) compare health assessments results between two designated health groups, and 2) compare health assessment results between a high density site with known previous disease-related mortality and a control site. We hypothesized that there would be a difference in hematological values of translocated tortoises based on health status, and that this difference would be exacerbated in tortoises held at higher densities.

Materials and Methods

This study was conducted at Nokuse Plantation, which consists of approximately 22,040 hectares of privately owned and managed conservation lands located in Walton County, Florida. Much of this land is dedicated to longleaf pine restoration efforts, including ~11,020 hectares of

xeric sandhill habitat suitable for gopher tortoises. Since 2006, Nokuse has been the recipient site for over 4,500 tortoises displaced by development around the state of Florida though Incidental Take Permits issued by the Florida Fish and Wildlife Conservation Commission (FFWCC). Tortoises in this study had been previously translocated to Nokuse between 2011-2015 and released in Doe Branch 1 (15 ha, 275 adult tortoises, ~18 tortoises/ha) or Seven Runs 1 (13.75 ha, 106 adult tortoises, ~7.7 tortoises/ha). Doe Branch 1 (DB1) was a release site where a disease related mortality event was initially documented (see Chapter 2), whereas Seven Runs 1 (SR1) had no documented disease related mortality, and was therefore chosen as a control site.

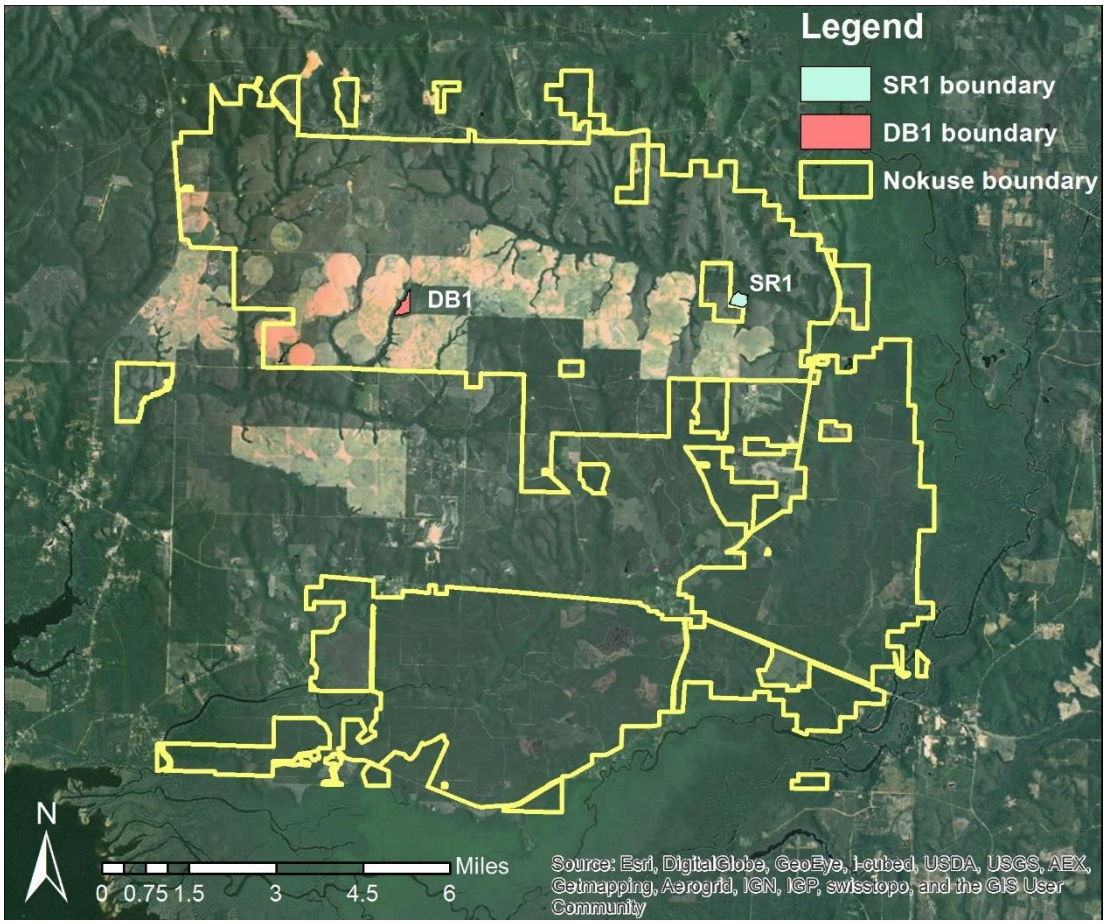

Figure 4.1. Map of Nokuse Plantation in Walton County, FL. The study sites, Doe Branch 1 and Seven Runs 1, are highlighted.

During May-August of 2016 and 2017, DB1 and SR1 were surveyed for gopher tortoise burrows Active burrows were scoped to determine tortoise occupancy, and tortoises from occupied burrows were trapped by placing live wire traps (Hav-a-Hart) at the mouth of the burrow. Traps were shaded using shade cloth and checked twice daily (between 10-11am and 4-6pm) until the tortoise was captured.

Successfully trapped tortoises were transported to the Nokuse Plantation field lab for processing. Tortoises were identified based on their unique permanent carapacial notches which were made at the initial release. Standard morphometric measurements including weight, straight carapace length, carapace width, carapace height, and plastron concavity were recorded (McRae et al. 1981). Dorsal, ventral, and anterior pictures were taken for documentation and potential future comparison. Each tortoise received a complete health assessment by the same trained individual, including a thorough physical exam, a subjective body condition assessment, and collection of biological samples. BCS was determined through a visual assessment of muscle mass and fat deposits. Due to the subjective nature of this scoring, BCS was decided by a single individual after training by a licensed wildlife veterinarian (T. Norton, DVM, Georgia Sea Turtle Center). In addition, inguinal, pectoral, and cervical regions were surveyed for presence of ectoparasites. If present, ectoparasites were documented and collected and stored in alcohol for future analyses.

Biological samples were collected for pathogen surveillance and clinicopathological analyses. Blood was collected from the brachial vein using a sodium heparinized syringe for a suite of biochemical and nutritional testing to assess both general and nutritional health. Volume of blood collected was < 1% of the tortoise's body weight. A small amount of heparinized whole blood was transferred to a microhematocrit tube and centrifuged to measure packed cell volume

(PCV), and plasma total protein (TP) were measured in-house with a refractometer. A small amount of heparinized whole blood was used to make blood smears for a Complete Blood Cell (CBC) count and differential white blood cell (WBC) count, performed by a clinical pathologist with chelonian expertise (Dr. N. Stacy, DVM, University of Florida, Gainsville, FL). A complete biochemical panel was performed on all plasma samples using standard dry slide determinations with a Kodak 700XR chemical analyzer by IDEXX Laboratories (Westbrook, ME). The following analytes were measured: aspartate aminotransferase (AST), creatine kinase (CK), albumin, total protein, globulin, blood urea nitrogen (BUN), cholesterol, glucose, calcium, phosphorus, chloride, potassium, sodium, uric acid, and triglyceride. Whole blood and plasma were also stored in cryovials in liquid nitrogen until transport to various labs for additional nutritional parameters.

Plasma vitamin D (25-hydroxyvitamin D) and ionized calcium (iCa) were measured by radioimmunoassay at the Michigan State University Diagnostic Center for Population & Animal Health (Lansing, Michigan 48910, USA). Protein fractions (pre-albumin, albumin, alpha-1 globulins, alpha-2 globulins, beta globulins, and gamma globulins) and lipoprotein panels (cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL)) were completed at the University of Miami (Coral Gables, Florida 33124, USA). Trace mineral panels (calcium, copper, iron, magnesium, phosphorus, potassium, selenium, and zinc) were conducted by inductively coupled plasma-mass spectroscopy at the University of Pennsylvania Animal Diagnostic Laboratory System, New Bolton Center Toxicology Laboratory (Kennett Square, Pennsylvania 19348, USA). Values less than the limit of detection (LOD) were substituted with LOD/sqrt(2), a method shown to have

the least error (Verbovšek 2011). Due to blood sample volume on some animals, not all of the tortoises were tested for all parameters.

After measurements and in-house assessment of PCV, BCS, and overall behavior, adult tortoises (SCL > 180mm) were assigned to one of two health status groups ("Healthy" and "At Risk"). A tortoise was assigned to the "At Risk" group (AR) if at least one of the following criteria was met: PCV ≤ 25 (indicative of possible anemia), BCS < 3 on scale of 1-5, or mild to moderate lethargy. Tortoises were assigned as "Healthy" (H) if they did not meet any of the AR criteria listed above. We also calculated BCI using both shell length and shell volume methods as mentioned previously to compare our health scoring.

Morphometric data and clinicopathological data were analyzed using Analysis of Variance (ANOVA) in the program R (version 3.4.2) to test for the main effects of health group (H or AR), site (DB1 or SR1), and for a health group*site interaction. We additionally compared calcium, phosphorus, and the calcium:phosphorus ratio between sexes as those values are known to vary during the reproductively active season.

Results

Tortoises

We collected 91 samples from 68 individual tortoises (35 male, 33 females). Mean SCL for DB1 tortoises was 26.76 cm n=36; range 20.2 – 31.1 cm), while mean SCL for SR1 tortoises was 28.47 cm (n=32; range 22.6 – 31.7 cm). Twenty-three tortoises were captured both years, but only the initial sample was used for protein fractions, lipids, trace minerals, and vitamin analyses. Between the two sites, 30 tortoises were assigned to the AR group and 38 tortoises were assigned to the H group.

Both methods of calculating BCI (using weight and SCL alone versus using weight and total shell volume) yielded results that were significantly lower in AR tortoises than in H tortoises ($p<0.007$ for both) (Table 4.1). The majority of blood chemistry and hematological values of the tortoises did not significantly differ based on either health group status, by site, or by a combination of the two. The following results focus only on statistically different values.

Table 4.1. Comparison of in-house hematology and body condition indices between health groups of translocated gopher tortoises.

	At Risk			Healthy			p value
	Average	Min	Max	Average	Min	Max	
PCV	21.6	12	25	30.7	25	43	<0.001
TP (g/dL)	3.34	2	4.5	4.17	1.3	6.4	<0.001
BCI SCL^3	0.171	0.141	0.199	0.179	0.155	0.22	0.007
BCI SCLxWxH	0.547	0.482	0.624	0.564	0.512	0.631	0.004

Hematology

In-house PCV and TP were much lower in AR tortoises ($p<0.001$ for both, Table 4.1). The only value that was different in the leukocyte differentials was the number of absolute basophils. This number was significantly lower in AR tortoises when compared with H tortoises ($p=0.005$), and significantly lower in tortoises from SR1 compared with those from DB1 ($p<0.05$).

Plasma Biochemistry

Albumin and globulin were significantly lower in AR tortoises ($p<0.001$ for both). Globulin was higher in tortoises in SR1 than DB1 ($p<0.05$), but there was no significant interaction between site and group. Cholesterol was also higher in SR1 than DB1 ($p=0.002$).

Triglycerides were significantly higher in H tortoises, as well as for tortoises in SR1 ($p=0.01$, $p=0.028$, respectively). There was also a significant effect on triglycerides from a site:group interaction ($p<0.05$), such that H tortoises in SR1 had much higher tricglyeride levels than the other three subgroups, while AR tortoises in DB1 had the lowest levels on average.

Protein Electrophoresis

The albumin to globulin (A/G) ratio was higher in DB1 tortoises than SR1 ($p<0.0001$). Total protein, pre-albumin, and all globulin fractions (Alpha-1, Alpha-2, Beta, and Gamma globulins) were significantly lower in AR tortoises (all $p<0.01$, Table 4.2). Alpha-2 and Beta globulins were also lower in DB1 than SR1 ($p<0.0003$ for both).

Lipids

Triglycerides, HDL and VLDL were all significantly lower in AR tortoises that H tortoises ($p=0.04$, $p=0.01$, and $p=0.03$ respectively, Table 4.2), as well as lower overall in tortoises in DB1 than SR1 ($p=0.03$, $p<0.05$, and $p<0.03$). Cholesterol and LDL were also significantly lower in DB1 tortoises ($p<0.002$, $p<0.02$, respectively).

Table 4.2. Significant differences in protein electrophoresis and lipid values between health groups of translocated gopher tortoises.

	At Risk			Healthy			p value
	average	Min	Max	average	Min	Max	
Total Protein	3.47	2.2	4.4	4.22	3	6.1	0.000003
pre-albumin	0.49	0.24	0.73	0.59	0.35	0.81	0.0007
Alpha 1 globulins	0.18	0.09	0.28	0.2	0.15	0.28	0.02
Alpha 2 globulins	0.42	0.22	0.79	0.53	0.3	0.94	0.0008
Beta globulins	1.28	0.79	1.84	1.66	0.91	2.56	0.00002

Gamma globulins	0.66	0.39	1.04	0.74	0.45	1.34	0.04
Triglycerides	15.17	6.36	32	39.3	2	318	0.04
HDL	7.5	4	11	9.1	6	19	0.01
VLDL	2.47	0	6	7.56	0	64	0.03

Vitamins A, D, and E panels

The only variable that was significantly different on the three vitamin panels was ionized calcium. Ionized calcium was not different by group or by site alone, but there was a significant effect with a site:group interaction ($p=0.005$).

Trace Minerals

Calcium, copper, magnesium, and phosphorus were all lower in AR tortoises ($p<0.006$ for Ca, Cu and Mg, $p<0.04$ for P). Calcium was higher in females than in males ($p=0.01$), but the calcium:phosphorus ratio was lower in females than in males ($p=0.01$). Potassium was significantly higher in tortoises from DB1 than SR1 ($p<0.0001$).

Table 4.3. Comparative blood chemistry values from the two health groups in this study (At Risk, AR; Healthy, H), two other previous studies, and the International Species Information System (ISIS) physiological reference values (Teare et al. 2013).

	Nokuse AR 2018 (this study)	Nokuse H 2018 (this study)	Hernandez et al. 2010	Taylor & Jacobson 1982	Teare et al. 2013
	average (min-max)	average (min-max)	average (min-max)	average (min-max)	average (min-max)
PCV	21.8 (12 - 25)	31 (25 - 43)	25.14 (14 - 29)	22.7 (15 - 30)	27.1 (3.5 - 45)
AST (SGOT) (U/L)	103 (36 - 202)	90.4 (11 - 212)	162.8 (87 - 252)	136 (57 - 392)	80 (4 - 228)

CK (U/L)	1578.8 (63 - 8341)	1283.5 (42 - 7782)	1407.5 (55 - 5705)	160 (32 - 628)	131 (0 - 644)
Albumin (g/dL)	0.69 (0.3 - 1.1)	0.9 (0.1 - 1.6)	1.2 (1 - 1.5)	1.5 (0.5 - 2.6)	1 (0 - 2.1)
TP (g/dL)	3.4 (2 - 4.5)	4.2 (1.3 - 6.4)	4.5 (3.5 - 5.8)	3.1 (1.3 - 4.6)	3.8 (1 - 6.5)
Globulin (g/dL)	2.75 (1.7 - 3.7)	3.3 (1.2 - 5)	-	-	2.5 (0 - 4.5)
BUN (mg/dL)	6.7 (1 - 70)	2.2 (0 - 21)	-	30.12 (1 - 130)	12.6 (1.96 - 29.1)
Cholesterol (mg/dL)	48.3 (5.8 - 139)	57 (7 - 124)	120.8 (10- 282)	76 (19- 150)	98.98 (13.2 - 301.6)
Glu (mg/dL)	117 (22 - 212)	117.6 (32 - 264)	131 (81 - 197)	75 (55 - 128)	86.12 (11.9 - 197.3)
Ca (mg/dL)	10.1 (8.2 - 13.6)	10.9 (0 - 18.1)	15.7 (11.4 - 23.2)	12 (10 - 14)	11.58 (7.33 - 20.6)
Phos (mg/dL)	2.2 (0.8 - 3.2)	2.5 (0.7 - 4.3)	2.8 (1.7 - 6.1)	2.1 (1 - 3.1)	2.51 (0.99 - 4.4)
Chloride (mEq/L)	104 (91 - 219)	104.2 (35 - 270)	91.7 (88 - 98)	102 (35 - 128)	103 (88 - 122)
K (mEq/L)	5.2 (4.4 - 7.5)	5.5 (2.3 - 9.9)	4.2 (3.3 - 5.3)	5 (2.9 - 7)	5.7 (2.6 - 11.9)
Na (mEq/L)	138.7 (111 - 246)	136.6 (49 - 276)	126 (121 - 130)	138 (127 - 148)	133 (119 - 154)
Uric Acid (mg/dL)	3.5 (1 - 8.7)	3.6 (1.5 - 9.9)	2.6 (1.4 - 4.8)	3.5 (0.9 - 8.5)	3.03 (0.1 - 8.8)
TRI (mg/dL)	13.2 (3 - 28)	34.2 (2 - 252)	177.4 (29 - 523)	-	-

Discussion

Hematological and biochemistry values are commonly used to assess the health of animals. For wild animals, for which reference ranges have not been established for every stage of life, or accounting for important seasonal differences, it is often difficult to utilize these values as predictors of declining health. Although many of the values from Nokuse would generally fall within or close to previously reported (Hernandez et al. 2010; Taylor and Jacobson 1982) or reference values for gopher tortoises (Teare et al. 2013) when the two health groups are combined, many of the values for the AR tortoises fall outside of these previously reported ranges when compared to H tortoises and other studies (see Table 4.3). We also saw a significant difference in some of the hematology, biochemistry, and nutritional values between healthy and at risk tortoises and between our two study sites with different histories of disease-related mortality.

Lower protein and protein fraction levels, such as those seen in the AR tortoises, can be indicative of malnutrition or malabsorption of nutrients. Hypoproteinemia, as well as depressed protein fraction levels, is commonly associated with chronic malnutrition, although additional causes can include malabsorption, maldigestion, or chronic hepatic or renal disease. Hypoalbuminemia can also be associated with other mineral and ion deficiencies. Overall, the low protein levels observed in AR tortoises were most likely associated with malnutrition or cachexia (general physical wasting that may be associated with chronic disease).

Lower amounts of specific minerals (such as calcium, phosphorus, and magnesium) in the AR tortoises is likely also related to malnutrition. Hypocalcemia, hypophosphatemia, and hypomagnesemia can all be caused by starvation or nutritional deficiencies, or by losses through malabsorption. Decreased levels of all of these minerals can also be associated with

hypoalbuminemia, which was also seen in the AR tortoises. Together these results support that the AR tortoises are either not eating sufficient amounts of forage, are eating nutritionally deficient forage, or are unable to properly absorb nutrients from the forage.

Cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) were all lower in tortoises in DB1 than SR1, although only triglycerides, HDL and VLDL were significantly lower in AR tortoises that H tortoises. Individuals that develop protein losing enteropathy may have concurrent hypocholesterolemia and hypoalbuminemia due to loss of both albumin and lipoproteins. Herbivores synthesize cholesterol in the liver, so decreased synthesis can be related to increased fasting or anorexia, or just associated with the decreased cholesterol-containing lipoproteins. Hypotriglyceridemia may also be indicative of nutritional state and can be related to starvation or malnutrition secondary to malabsorption and/or maldigestion.

The decreased protein and lipid values from tortoises in DB1 compared with SR1 suggest that these tortoises are not receiving as many of the nutrients from their forage, or that the forage in that site may be of lower quality. Forage nutrition analysis from both of the sites (see Chapter 2 and Appendix A) show that the forage in DB1 actually had higher percent dry matter of nonfibrous carbohydrates, such as starches and sugars, which has higher amounts of rapidly available energy (carbohydrates). Lower magnesium levels seen in AR tortoises may also be exacerbated within the DB1 site, which also had increased levels of potassium, as elevated potassium ingestion can block normal magnesium absorption. These decreases in nutrient levels within DB1 may be influenced by the density of tortoises within the site, if access to forage is restricted due to competition with conspecifics. Higher densities may also exacerbate stress that

translocated tortoises are subjected to due to the disruption of their social networks and/or increased interactions with other individuals.

In summary, all of these differences in blood analyte levels suggest poorer nutritional status of AR relative to H tortoises, due either to a lack of sufficient quality (or quantity) of forage, decreased foraging behavior by the tortoises, or a depressed ability to absorb. Although starvation due to lack of adequate forage within the release sites seems highly unlikely, given the abundance and diversity of plants typically consumed by gopher tortoises present in all of the known affected sites, a dysregulated stress response caused by chronic stress can lead to decreased feeding and a decreased ability to take in or absorb nutrients (Dickens et al. 2010; Herzog et al. 2009). There may be an additional relationship between these analytes with individual tortoise behavior, such as increased above-ground movements or, especially for AR tortoises, prioritization of basking behaviors over foraging. Further analyses should be directed towards relationships between specific analytes and tortoise behavior, as well as the exploration of whether these differences in blood analytes are the result of starvation versus malabsorption.

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APPENDIX A

FORAGE NUTRITION ANALYSIS PROCEDURES AND RESULTS

Methods

During July and August of 2017, burrows where tortoises had been trapped were selected for forage sampling. At these burrows, an angle was assigned randomly. Approximately 5 meters out from the mouth of the burrow at the randomly selected angle, a 1 m X 1 m quadrat was placed. If the randomized angle resulted in placement around the base of a tree or shrub, the next angle was chosen instead.

Forage samples were photographed for identification and separated into grass/grass-like plants and forbs/legumes. For each site, individual burrow samples were thoroughly mixed by group to create a composite mixture. This composite was then separated into quart-sized bags (size needed for submission). Two composite samples from each forage group from each site were submitted to Dairy One Forage Lab in Ithaca, NY for analysis. Results were compared using Analysis of Variance (ANOVA) tests using statistical program R (version 3.4.2) for differences between site, forage type, and a site:type interaction.

Each sample was weighed and oven dried for four hours at 60 degrees Celcius to measure percent moisture and percent dry matter. The following values were measured as a percentage of the dry matter in each sample: Crude Protein (CP), Soluble Protein (SP), Acid Detergent Insoluble Crude Protein (ADICP), Neutral Detergent Insoluble Crude Protein (NDICP), Acid Digested Fiber (ADF), amylase and sodium sulfite treated Neutral Detergent Fiber on organic matter (aNDFom), lignin, crude fat, ash, starch, Ethanol Soluble Carbohydrates (ESC, simple

sugars), Nonfibrous Carbohydrates (NFC), Relative Feed Value (RFV), Total Digestible Nutrients (TDN), Net Energy for lactation (NEl), Net Energy for Maintenence (NEm), Net Energy for gain (NEg), Metabolizable energy (ME), Digestible Energy (DE), Calcium (Ca), Phosphorus (P), Magnesium (Mg), Potassium (K), Sodium (Na), Iron (Fe), Zinc (Zn), Copper (Cu), Manganese (Mn), Molybdenum (Mo), Sulfur (S), Chloride ion (Cl), and Dietary Cation-Anion Difference (DCAD).

Specific methods for the following analyses were taken from the Dairy One Forage Lab Analytical Procedures Manual.

ADICP: ADF residue analyzed using a Leco TruMac N Macro Determinator to determine the protein fraction bound to the acid detergent fiber.

Crude Fat: Extraction by Soxtec HT6 System using anhydrous diethyl ether. Crude fat residue determined gravimetrically after drying.

ESC: Samples shaken for 4 hours at 180 epm with 80% ethanol to extract ethanol soluble carbohydrates comprised of simple sugars. ESC determined using a Thermo Scientific Genesys 10S Vis Spectrophotometer after a colorimetric phenol-sulfuric acid reaction.

ADF: Solutions as in AOAC 973.18 – Fiber (Acid Detergent) and Lignin (H_2SO_4) in Animal Feed. Samples individually weighed at 0.5g into filter bags and digested for 75 minutes as a group of 24 in 2L of ADF solution in ANKOM A200 Digestion Unit. Samples are rinsed three times with boiling water for 5 minutes in filter bags followed by a 3 minute acetone soak and drying at 105ºC for 2 hours.

Lignin: Solution as in AOAC 973.18 – Fiber (Acid Detergent) and Lignin (H_2SO_4) in Animal Feed. ADF performed as above and residue digested as a group of 24 in 72% w/w sulfuric acid for 3 hours in ANKOM DaisyII Incubator at ambient temperature.

aNDFom: (amylase and sodium sulfite treated Neutral Detergent Fiber on an organic matter (ash free) basis) Samples individually weighed at 0.5g into filter bags and digested for 75 minutes as a group of 24 in 2L of NDF solution in ANKOM A200 Digestion Unit. Four ml of Alpha Amylase and 20g sodium sulfite are added at the start of digestion. Samples are rinsed three times with boiling water for 5 minutes. Alpha Amylase is added to the first 2 rinses. Water rinses are followed by a 3 minute acetone soak and drying at 105ºC for 2 hours. aNDF analyzed as above but with the addition of an ashing step to remove inorganic materials such as minerals, soil, and sand by burning the fibrous residue at 550C for 2 hours.

All minerals: Samples digested using CEM Microwave Accelerated Reaction System (MARS6) with MarsXpress Temperature Control using 50ml calibrated Xpress Teflon PFA vessels with Kevlar/fiberglass insulating sleeves then analyzed by ICP using a Thermo iCAP 6300 Inductively Coupled Plasma Radial Spectrometer. Samples first pre-digested at ambient temperature 10 minutes with 8ml nitric acid (HNO3) and 2ml hydrochloric acid (HCl) and then an additional 10 minutes with 1ml 30% hydrogen peroxide (H2O2). After pre-digestion complete, samples ramped to 200ºC in 15 minutes and finally held at digestion temperature of 200ºC for 15 minutes at 1600W. Vessels brought to 50-ml volume, aliquot used for analysis.

Chloride ion: 0.5g dried, ground sample or 5g wet sample extracted for 15 minutes in 50ml 0.1N HNO3, followed by potentiometric titration with AgNO3 using Brinkmann Metrohm 716 Titrino Titration Unit with silver electrode. For water samples, 25ml of 0.2N HNO3 added to 25ml of sample then analyzed.

NDICP: aNDF performed without sodium sulfite then residue analyzed using a Leco TruMac N Macro Determinator to determine the protein fraction bound to the neutral detergent fiber.

SP: Cornell Sodium Borate-Sodium Phosphate Buffer Procedure. Soy products incubated at 39°C. All other samples incubated at ambient temperature. Residue containing insoluble protein analyzed using Leco TruMac N Macro Determinator.

Starch: Samples are pre-extracted for sugar by incubation in 40ºC water bath and filtration on Whatman 41 filter paper. Residues are thermally solubilized using an autoclave, then incubated with glucoamylase enzyme to hydrolyze starch to produce dextrose (glucose). Prepared samples injected into sample chamber of YSI Analyzer where dextrose diffuses into a membrane containing glucose oxidase. The dextrose is immediately oxidized to hydrogen peroxide and D-glucono-4-lactone. The hydrogen peroxide is detected amperometrically at the platinum electrode surface. The current flow at the electrode is directly proportional to the hydrogen peroxide concentration, and hence to the dextrose concentration. Starch is determined by multiplying dextrose by 0.9.

Results

Here we report only differences between the two study sites. Values for DM, CP, SP, ADICP, NDICP, ADF, aNDFom, lignin, ash, NFC, RFV, TDN, NEl, NEm, NEg, ME, DE, Ca, Na, Fe, Zn, Cu, and Mo were not significantly different by site ($p>0.05$ for all). Starch ($p<0.03$), ESC ($p=0.007$), crude fat ($p<0.03$), potassium ($p<0.001$), manganese ($p=0.03$), and DCAD ($p=0.001$) were all higher in the Doe Branch 1 site. Phosphorus ($p<0.02$), magnesium ($p<0.02$), sulfur ($p=0.007$), and chloride ion ($p<0.001$) values were higher in the Seven Runs 1 site.

Table A.1. Forage nutrient parameter differences between two release sites for translocated gopher tortoises. Mean value for each site is reported.

Parameter	DB1 mean	SR1 mean	p value
Starch	1.525	0.65	<0.03
ESC	6.35	5.075	0.007
CF	3.475	2.475	<0.03

Phosphorus	0.09	0.125	0.02
Magnesium	0.218	0.358	0.02
Potassium	1.15	0.55	<0.001
Manganese	153	86.25	0.03
Sulfur	0.117	0.155	0.007
Chloride ion	0.26	0.43	<0.001
DCAD	16.5	-3.5	0.001

Discussion

Generally, forage mineral content has low bearing on forage quality evaluation, which is where we saw the bulk of the differences in values. The more biologically significant differences are with the amounts of nonfibrous carbohydrates, like the starches and sugars. Higher starch and ESC values in DB1 suggest that the forage in that site has higher amounts of rapidly available carbohydrates (energy) than the forage in SR1. The triglycerides of fatty acids are a high density source of energy for animals, and contain more energy than found in carbohydrates. Higher crude fat amounts in DB1 also supports that the forage in that site is of high quality, and contains more rapidly available energy than that of SR1.