THE BEHAVIORAL ECOLOGY AND CHEMICAL ANALYSIS OF MENTAL GLAND SECRETIONS OF BOTH SEXES OF GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*)

by

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VITA

Meghan Doris Kelley, daughter of Timothy E. Kelley and Marilee C. Kelley and wife to John W. Finger Jr., was born May 17, 1988 in Wyandotte, Michigan. Meghan, sister to Robert, David, and Jeffery Prueitt and Kevin and Wendy Kelley, was the youngest of six and was a first generation college attendee and graduate. In high school, she was third in her graduating class from Gabriel Richard in Riverview, Michigan, from which she graduated in 2006. She attended Michigan State University (MSU) immediately after high school and graduated in both 2010 and 2011 with the credits for three undergraduate Bachelor of Science Degrees (General Zoology, Evolutionary Ecology and Organismal Biology, and History Philosophy and Sociology of Science) and a specialization in Bioethics and Humanities in Society (BHS) from Lyman Briggs College (LBC) of MSU. During her time at MSU, she also originated an LBC-Chapter Women in Science Organization for undergraduates with Dr. Cori Fata-Hartley, for which Meghan wrote the constitution still in MSU's bylaws and was the first President of the group. She also served as a Public Relations Officer for the MSU Zoological Student Association (ZSA). From MSU, she went on to have a productive internship working for African Safari Wildlife Park in Port Clinton, Ohio, before taking a year off from her academic education to become certified through CVS Pharmacy's online program as a Pharmacy Technician, while applying to graduate school. She then went on to receive a Master of Science Degree in Biology under the tutelage of Dr. Chris Sheil from John Carroll University (JCU) in University Heights, Ohio in 2014. From JCU, she accepted her position with Dr. Mary Mendonça in the Doctor of Philosophy Program in Biological Sciences at Auburn University and moved to Auburn, Alabama shortly after her M.S. graduation.

ABSTRACT

All living chelonian species (e.g., turtles and tortoises) are experiencing population declines, causing a new imperative to maintain the longevity of adults and to facilitate reproduction. One strategy in facilitating reproduction is to focus on sensory signaling in social interactions that could lead to mating. For example, olfaction and chemical recognition of pheromones represents the oldest use of senses, and yet, little is known about the chemical ecology of many vertebrates, especially chelonians, which are known to use chemosensory cues despite habitats that are becoming increasingly fragmented. This is especially true for threatened species like gopher tortoises (*Gopherus polyphemus*), which are endemic longleaf pine specialists in a rapidly declining habitat due to deforestation, urbanization, and poor land management (e.g., lack of fire maintenance). With these studies, I aimed to examine the behavioral ecology of the gopher tortoise through chemical signals found in chin or mental gland (MG) secretions.

Chemical bioassays with paired treatments (MG secretion variants vs. controls) revealed MG secretions were used as pheromones in both males and females. Multimodal presentations with 3D tortoise models treated with MG secretions indicated MG secretions are chemical signals required to maintain social behaviors even with visual signals, leading to courtship or mating. To further understand MG secretion use in terrestrial habitats, I found that male tortoises were able to chemically discriminate visually identical models treated with 20X diluted MG secretions, supporting the ecological utility of MG secretions despite breakdown. In chemical analyses of MGs in both sexes from different locations, we identified several chemical classes in MG secretions not previously known in tortoises, with some differing by sex, body size, or

location, including short-chained carboxylic-carbohydrate acids, aromatics, and amide compounds. All chemical compounds found may be signals used to identify conspecific characteristics. My studies are the first documented use of MGs as a source of pheromones in this species and the first thorough chemical composition analysis of MG secretions in both male and female gopher tortoises.

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I appreciate the African proverb that “it takes a village” because it implies that more than one person, more than parents, more than friends, but rather a large community (even across several states) were involved in the ‘raising’ of a person to become the person they are, to fulfill the dreams they have pursued. With that broad understanding, I know that I will never be able to convey my sincere gratitude to all the individuals that helped me on this journey to the completion of this program. But, first and foremost, I would like to thank the best parents anyone could ever have asked for, Timothy and Marilee Kelley, combined we called ourselves “the three musketeers.” My parents have been tirelessly devoted to my education from start to finish, helping me calculate in elementary school “how many years I have left until I graduate from college,” pulling all-nighters with me to finish projects before I learned time management, quizzing me for every test, and supporting me even when I did poorly on an occasional assignment/test. Even with the loss of my father at age 14, I never wanted to give up the challenge of always trying to make both of my parents proud of me for doing something with my life that serves others, both people and animals, and in this, I have devoted my lifetime pursuit of always striving for a bigger picture, the way my dad taught me during his life and after death, as I more recently learned how he received his multiple purple hearts in the Vietnam War as a Marine by trying to save other soldiers with his own body (how he received the shrapnel scars in his back that I never before understood). I will treasure those life lessons forever.

I would also like to thank my brothers, Rob, Dave, and Jeff, who have also over the years been among some of my best teachers. Sometimes by best teachers, I mean that they taught me things by example that I appreciated—however, as being the little sister by 20+ years to all of them, they were the ones (with my parents) that gave me all the lessons I needed to love and appreciate nature at home downriver in the marshes and at our property Up North in Gladwin, Michigan that encouraged me to pursue this field professionally. My brother Rob is also the first person to introduce me to my first gopher tortoise ‘accidentally’ when I was 8 years old on a vacation visiting him in Florida. I also would not be the person I am without a number of other important family members and friends from back home, including my Grandma Priscilla, my Godfather Uncle Pat, nieces and nephews, great aunts and uncles, aunts and uncles, cousins, neighbors, and friends (including my two best friends Jessica Caton-Diefenbach and Stephanie Mikulski) that have been continuous cheerleaders and a wonderful support system in this process.

During this program, I would be remiss not to address the current sorrows of the COVID-19 pandemic taking place while I have been finishing this dissertation, but also some of the personal life-changing joys that I have experienced while at Auburn. First, during the time that I have lived in Auburn, for many reasons including the pandemic, I have lost some very important people to me that would have been delighted to share in the joy of my completion of this program. Recently, my brother Kevin Kelley, passed away suddenly and unexpectedly during the pandemic, too young for it to have been his time. After years of being apart from the family for various reasons including his service in the U.S. Army, Kevin more recently became a strong shoulder for me in this program to seek interpersonal advice, stories about our dad, and comfort in times of mutual need. For that, I will only ever be able to thank him in these acknowledgements and in my prayers. During this program, I have also lost my 23 year old nephew Daniel Saucier (also too young and before his time), who shared my love of herping and the great outdoors; my Uncle Colin “Beeps” Lane, who was a brave sky-diving retired Paratrooper always excited and proud during every phone call to find out “the status” of the program; my cousin Jim Nocella, a chemistry teacher with a child-like heart that always connected to students/kids including me; my great aunt Dolores, the remaining matriarch of my maternal grandma's side of the family; an extended family member Rich Antolovic, who I had not seen in a few years but always made time to find out how everything was going with me; and a beloved family pet, my brother Rob's dog Poe, who was the only family pet to attend one of my college graduations and was one of the sweetest dogs in the whole world. To all these loved ones, I am grateful for our time together, the support you gave me, and hope that wherever you are after death, that you are still able to somehow share with us or at least know how much you helped me in this process and during the course of my life.

On a positive note, I would also like to dedicate this dissertation to my husband John W. Finger Jr. and our new baby boy on the way as I write this. I first met John in Rouse Life Sciences Building at Auburn University during his post-doc job interview in 2015, when I was a second year PhD student. John was giving a talk on crocodilians for his research experience interview, and while I was impressed at his knowledge of the innate immune systems of crocodilians being "cool," John was simultaneously being interrogated by microbiology professors on "why he couldn't just cut limbs off alligators to measure stress and the immune response." It all came together for us with my coming to his defense in that talk to remember an example with "*Spinifera apalone,*" and flash forward once he got the job and moved to Auburn himself, the rest was history and we were married in 2017, also during this program. John has been invaluable to me as a life partner and as a full-time colleague and often a co-author as we have often banded together over the last few years on projects with tortoises and alligators and sometimes turtles, writing and editing papers together (especially him editing my papers because he is an editing wizard), job interview preparation, presentation timing and critiques, and mutual interests, including hiking, road trips, and always herping, including the big herps, working together to capture live alligators with permission in Alabama, sharing our love of the “bigger picture.” Even now as I am working on this dissertation pregnant, before John, I would have seen this pregnancy as an obstacle that would prolong my time in this program, but instead, I am invigorated and somehow immediately knew when learning the news that the time to finish this program is now. I felt this way because I wanted to one day be able to tell our baby that you can truly do whatever you set your mind to and work towards. Therefore, I want to thank John and our little one for the motivation I needed to seize the day and complete this work. I also want to impart to our baby that despite the difficulties John’s and both sides of my family have endured as immigrants coming to this country (my Grandma Doris namesake and Grandpa Tom coming from England and Ireland, respectively through Ellis Island, around World War II; my Grandma Priscilla’s family coming from Belgium, and my Grandpa Fred’s family coming from Hungary), we have strong family attributes that have kept us all going to a hopefully bright future. Because John and I have both had the privilege to obtain higher education, an opportunity that many people in our families were not afforded, we have also both worked hard to pursue doctoral degrees, thus on some level, allowing our little one to be grateful as we are for our roots, never forgetting the challenges that many other people had to endure that led us to this place in our lives, and importantly, to appreciate that hard work can affect change.

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Lastly, for my general emotional health and well-being throughout the last several years, I need to thank my scaled, feathered, and fur buddies that have never failed yet to lift my spirits and keep my sights focused on what I am working towards. For all the tortoises that “let” me catch them and patiently allowed me to observe their behaviors, take their blood and mental gland secretions occasionally, and were good sports generally in all their transportations to and from their burrow abodes, thank you for allowing me to study you. During this project, I was also fortunate to observe a nesting female gopher tortoise creating a functional nest of five eggs in one of our pens in time to be able to extract and successfully incubate the eggs in Mary's lab, thus bringing three new baby gopher tortoises (Butterscotch, Marmalade, and Bumble; an 80% hatching efficiency) into the world and later releasing them to their mother's burrow at Fort Rucker. This was by far one of the most rewarding experiences of my project, and I hope we were able to give these babies a bit of a head-start so that they can successfully survive to adulthood. Additionally, thanks to John, my last few years have also been filled with alligators, both juveniles (our research juveniles and our runt alligators, Munchkin and MuShu) and adults, that have also brought a number of unique experiences to my life (e.g., alligator juveniles in our bathtub during hurricane Irma warnings, saving a 9ft mama alligator from a drainage pipe, encountering a 15ft behemoth male alligator in basically a puddle, and many, many more). And, last but not least, thank you to my fur-baby Sweet Pea, our sweet kitten that we rescued in the ditch in front of our house at 10 days old and raised up to adulthood of 16 pounds; my Mama's kitty Kikiya, who I named and who made every Christmas and trip home to Michigan more snuggly with "Kiki-cuddles;" our chickens (Henrietta, Lady Cluck, Ruby, Henny Penny, Peep, Lucy, and Ethel) that we love dearly and have given us lots of protein-rich eggs; and our turtles (Noodle and Dumpling) and tortoise (Mowsey) that have us trained to feed them with just a look. We love you all, and you make life worth living every day.

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MG	Mental gland or mental gland secretions
DI	Distilled water
s	Seconds (usually for behavior duration)
m/z	Chemical charge
min	Minutes for retention time
RT or RI	Retention time or retention index

INTRODUCTION TO DISSERTATION

Importance of Olfaction to Social Behavior

Olfaction is the oldest and furthest reaching sense, not to mention the most highly conserved sensory pathway among vertebrates (Taniguchi and Taniguchi, 2014). Both known olfactory systems (i.e., main olfactory pathway and vomeronasal organ/accessory olfactory pathway) ultimately lead to nuclei of the brain (e.g., medial preoptic area (MPOA), the amygdala, and the hippocampus) that are involved in memory, recognition, and social behavior (Dulac and Torello, 2003; Yoon et al., 2005), especially in regards to finding mates and engaging in courtship or reproductive behaviors (Nelson and Kriegsfield, 2017). Blocked olfaction or elimination of either olfactory bulb (e.g., main olfactory or accessory olfactory bulb) can result in the elimination of sexual behavior, not to mention the inability to receive scent cues (Powers and Winans, 1975; Baxi et al., 2006). One major way in which anosmia, or the lack of or blocked olfaction, can halt sexual behavior is through the inability to stimulate sex hormone production (e.g. to simultaneously block gonadotropin-releasing hormone production through parallel hypothalamic pathways to olfaction; Wray et al., 1994; Yoshida et al., 1995; Forni and Wray, 2015) through pheromone-related cues. Furthermore, without olfactory cues, receptivity to courtship may also not occur, such as shown in Plethodontid female salamanders that are activated by PMF (i.e., pheromone-mediating factors in mental glands) and red-sided garter snake methyl ketones (*Plethodon shermani*, or red-legged salamanders; Wiersig-Weichmann et al., 2002; Houck et al., 2007; *Thamnophis sirtalis*; Huang et al., 2006). These are just a few examples that demonstrate the interconnectivity between the olfactory pathway and the hypothalamo-pituitary-gonadal axis (HPG), which directly results in modulating the production of sex hormones and social behaviors.

Importance of Reptilian Skin in Pheromone Production

Although less traditionally studied than other systems (e.g., mammals and insects are the most widely explored taxa for pheromone-induced social behaviors; LeMaster and Mason, 2003; Wyatt, 2014), reptiles have become increasingly important for the study of chemical ecology, pheromone production, and the integration of olfactory signals with social behaviors. The reason for this recent transition to focus on chemical signaling in reptiles is because they are acute to olfaction in every aspect of their life histories (e.g., foraging, predation, and mate-choice) and also because in continuously sloughing old skin, they exude a diversity of chemical compounds directly from their skin that could play a role in chemical signaling (Weldon et al., 2008; Apps et al., 2015). For example, reptilian skin, an evolutionary inherited trait meant to reduce water loss through several dermal layers, (Roberts and Lillywhite, 1980; Weldon et al., 2008) are also rich with lipids specifically exuded to the external environment, providing other integral functions, including antimicrobial properties, thermoregulation, production of alarm cues from predators, and the production of pheromones that attract conspecifics (Mason et al., 1989; Lillwhite, 2006; Shawkey et al., 2003; Weldon et al., 2008). Because it is generally accepted that any sloughed skin or lipid exudate from the dermis can be considered a scent cue, or potential pheromone, the diversity of lipids from structure to function can allow for a variety of signals (e.g., sex, body size, immune condition of the "signaler," etc.) to be conveyed to conspecifics (e.g., the "receivers;" Mason, 1992; Weldon et al., 2008). Particularly in the case of pheromones (attractant chemicals used to communicate between conspecifics), unsaturated free fatty acids, triacylglycerols, methyl ketones, and methyl esters are just a few types of lipids and lipid derivatives that have been reported with pheromone function in a variety of reptilian species, such as snakes, lizards, crocodylians, and turtles (Weldon et al., 2008; Apps et al., 2015).

Aside from the unique stratification of the skin, reptiles also possess a number of glands, a few of which with unknown function, such as the mental gland in *Gopherus spp.* tortoises (Quay, 1972; Rose et al., 1969). Other secreting glands, or exocrine glands, include cloacal glands in snakes, femoral glands in lizards, and Rathke's glands in freshwater and seawater turtles (Weldon et al., 2008). Paired snake cloacal glands are some of the most widely studied reptilian glandular structures that have been found to produce species specific long-chain fatty acids and cholesterol derivatives. For example, one study found primarily C_{15}-C_{18} fatty acid chains in vipers but longer chains in crotalids (Razakov and Sadykov, 1986). However, recent studies have shown that integumentary secretions of snakes are more widely used in conspecific attraction (Mason, 1992). For example, saturated and unsaturated methyl ketones in the epidermis of two sympatric garter snake species were also found to differ by species and can occur in greater concentrations of female garter snakes with a larger body size (Mason et al., 1989; LeMaster and Mason, 2003; Mason and Parker, 2010). Complex long-chain hydrocarbons and lipids are nonvolatile and can persist in scent trails for days after the depositer has departed from the site, as shown in snakes and lizards (Alberts, 1992; Mason, 1992). Even in hot desert climates, such as that of the spiny-tailed lizards (*Uromastyx aegyptia*), nonvolatile scent marks from femoral glands (e.g., pore-like glands on the underside of the femur) persist in territories, using underground burrows as "scent stations" when sun-exposed pheromones degrade too quickly (Martín et al., 2016). These persistent signals in an environment are thought to indicate information about the depositer, such as body condition, size, and health status. Yet another example is oleic acid found in femoral pores of Iberian rock lizards, which is shown to indicate better male body condition (Martín et al., 2016). These derived-olfactory cues have also been suggested to convey honest signals of overall body condition and health, depending on how costly they are to physiologically produce (Penn & Potts, 1998; Zala et al., 2004; López and Martín, 2005; Ibáñez et al., 2012). One current example of nonrandom olfactory discrimination of health status was shown in Spanish terrapins, in which lipopolysaccharide (LPS; a component of bacterial cell walls that is recognized by and stimulates the immune system) was injected or sham-injected into males, causing female conspecifics to preferentially display behaviors towards sham, or control males that did not have a stimulated immune response (Ibáñez et al., 2014).

Here, we discuss the importance of olfactory cues in the social interactions of the Gopher tortoise (*Gopherus polyphemus*) as a result of secretions from mental glands, which we have recently found support to call a source of pheromones (Kelley and Mendonça, 2020; Kelley et al., 2021). Past studies of other *Gopherus spp.* tortoises have indicated that male tortoises will behaviorally investigate burrows longer if mental gland secretions are present than if there are cloacal scents or no scent application (Bulova, 1997). Studies also show that conspecifics can recognize familiar from unfamiliar males due to mental gland secretions (Alberts et al., 1994; Tuberville et al., 2011), or that certain components of mental gland secretions may cause aggressive displays like combat behaviors (e.g., flipping and ramming; Auffenberg, 1977; Rose, 1970). Because mental glands are seasonally enlarged only during the breeding season and are larger in males than females (Winokur and Legler, 1975; McRae et al., 1981), their enlargement is presumably controlled in part by endogenous testosterone (T) concentrations, but other factors such as chemical components present, effects of seasonal day length, etc. may also contribute to secondary sexual gland growth in the breeding season (Weaver, 1970).

Rose (1969) and Rose et al. (1970) were some of the first studies to determine that tortoise mental glands are composed of mostly fatty acid methyl esters (i.e., caprylic, myristic, stearic, oleic, linoleic acids, etc.) and proteins, in which female tortoises of all four *Gopherus spp.* exhibited a band in electrophoresis not seen in male mental gland composition, which means that secretions are sexually dimorphic. However, to date, no other study has compared mental gland secretion composition in both male and female gopher tortoises, nor further examined them for other chemical classes with modern techniques (gas chromatography-mass spectroscopy). The dimorphic nature of these secretions suggests that mental glands are used for predominately courtship purposes, similar to analogous mental gland secretions of other species such as rabbits, Plethodontid salamanders, and alligators (Mykytowycz, 1968; Weldon and Sampson, 1988). However, through behavioral responses, male Texas tortoises (*Gopherus berlandieri*) with active vs. inactive mental glands have differing abilities to differentiate sex of conspecifics, suggesting a complicated relationship with secretion and olfactory discrimination for competition vs. courtship at certain points during the active mating season that has also yet to be explored in current research (Weaver, 1970).

As an exocrine-secreting integumental gland, it is interesting to note that peak glandular activity also corresponds to peak plasma lipid content and peak testosterone in the desert tortoise (*Gopherus agassizzii*), in which there is an interaction between high lipid content (June) and high testosterone content (August-September; Lance et al., 2002). Plasma lipids usually increase during the period of peak vitellogenesis in females (e.g., which occurs in May and June in gopher tortoises; Ott et al., 2000) in order to produce yolk lipids before eggs are deposited (Lance et al., 2002). Therefore, it seems logical that male tortoises would have a larger amount of circulating free fatty acids in their plasma when compared with females, but males also have larger mental glands that secrete more [fatty acids and proteins] than females as well, which suggests blood plasma lipid cycling may be closely related to mental gland composition and seasonality (Winokur and Legler, 1975; Lance et al., 2002). According to Ott et al. (2000), the only comprehensive study of the hormone cycles in the gopher tortoise, there are two important peaks of mating behavior in gopher tortoises, which are during the spring (June) and late summer/early fall (August to October). These peaks correspond with both the peak plasma-lipid content and peak testosterone times of the active season, if the Lance et al. (2002) results were to be repeated in gopher tortoises. Gopher tortoises and desert tortoises do have comparable sexual hormonal cycles and breeding seasons, so there is a foundation for this work in future studies (Niblick et al., 1994; Ott et al, 2000).

Gopher Tortoise Biology, Social Behavior, and Olfaction

Gopher tortoises (*Gopherus polyphemus*) are state and federally listed species of concern in the western part of their range, recommended for endangered species status upgrades throughout the southeastern United States (U.S. Fish and Wildlife Service 1986; TESII 1995). Tortoise decline has been shown to be mostly due to habitat fragmentation and deforestation, but also recently, due to the prevalence and unknown population effects of Upper Respiratory Tract Disease syndrome (URTDs), caused by the bacterial agent *Mycoplasma spp* (Auffenberg and Franz, 1982; Aresco and Guyer, 1999). URTDs is of growing concern in tortoises because the symptoms of nasal discharge and lesions, nasal epithelial erosion, plus a weakened immune response can cause unspecified population declines and possible risks to reproduction, particularly from the onset of anosmia in many affected individuals (Jacobsen et al., 1991). Gopher tortoises are also socially aggregating animals that associate within their populations through colonies of burrows closely arranged near one another (Hansen, 1963; Auffenberg, 1977; Diemer, 1986; Guyer et al., 2016). As a keystone species that helps to support more than

300 other species (i.e., Eastern Diamondback Rattlesnake, the Eastern Indigo Snake, and the Gopher Frog, to name a few; Jackson and Milstrey, 1989) and the species diversity of the endangered longleaf pine landscape that it inhabits (Frost, 1993; Noss et al, 1995; Ashton and Burke 2005), much of the social behavior of gopher tortoises is understood through their movement patterns tracked by locations of burrows, which serve as critical shelters to many species, including sometimes multiple tortoises at one time (Auffenburg and Franz, 1982; Bulova, 1997).

Burrows and burrow aprons (the flat, disturbed ground at the entrance to the burrow) are the sites of tortoise courtship and mating, but may also be the site of URTDs transmission through characteristic tortoise nose-to-nose interactions in assessing mates (Auffenburg, 1977). Male and female tortoises also do frequently share burrows of other tortoises, both of the same or different sex with males being more likely to share burrows than females (Johnson et al., 2009). Both sexes of tortoises also differ in which sex they associate with in burrows (i.e., females more likely to associate with males, and males will share burrows with either sex), which they assess at the apron through extensively documented sniffing behavior, characterized by head extension with nose taps to substrate, dust disturbance, and the sounds tortoises often make when actively sniffing (Johnson et al., 2009; Bulova, 1997).

However, in past observations of Gopher tortoise courtship, both visual and olfactory cues have been emphasized when tortoises locate and assess burrows of conspecifics for mate selection (Auffenberg, 1966; 1977). For example, the common visual displays of courtship in tortoises include the characteristic head bob, aligning of carapaces, biting of the limbs and face, mounting, and most importantly, the nose-to-nose interaction, some of which could serve both a visual or olfactory function in tortoise social behavior (Auffenberg, 1977; Weaver, 1970). On the other hand, past sensory modality work with tortoises has mostly focused on these visual behavioral assessments with discriminating individuals at aprons or in close range of their assessed conspecific (Weaver, 1970; Niblick et al., 1994). Often, when in a natural habitat, tortoises may be separated by great distances in a highly fragmented landscape (Ashton and Burke, 2005), which complicates their ability to find and choose mates, especially without the use of olfaction, as in URTDs-affected tortoises.

A few studies have examined field mating success of tortoises in natural populations, which could lead to speculation about olfactory use in finding conspecifics. For example, through game-cam observations, Boglioli et al. (2003) showed that isolation of active tortoise burrows was not related to the amount of courtship behaviors displayed at those burrows. This means that tortoises do commute longer distances to find conspecifics for mating, sometimes at distances between 8.8-124.9 meters apart (based on three nearest neighboring burrow averages; Boglioli et al., 2003). Although isolated females had overall less visitations by either sex, they still had similar opportunities to mate as other, closer females (with up to 3 males on average courting each female; Boglioli et al., 2003). In addition, although the tortoise mating system is obviously highly polygynous (i.e., female tortoises may experience anywhere from 6-14 courting males), it is unclear how males are finding females or how females are choosing mates, if not for some kind of a dependence olfaction (Moon et al., 2006; Johnson et al., 2007).

With the understanding of tortoise social interactions from these ecological studies and a few well documented behaviors for both turtles and tortoises in competition, courtship, and mating, the aim of this work was to ethologically examine both male and female gopher tortoise (*Gopherus polyphemus*) behavioral responses to chemical secretions of mental glands. First, I aimed to develop a protocol for behaviorally observing tortoises, utilizing a basic chemical bioassay of only scent cues presented on cotton swabs of male mental gland secretions in a paired-design to ascertain if tortoises of one or either sex can behaviorally discriminate relevant biological cues from negative (e.g., distilled water) or positive (e.g., acetone) controls. Second, with the knowledge of tortoise olfactory discrimination of male mental gland secretions and observation of which behaviors were observed (olfactory behaviors such as sniffing vs. social behaviors such as head bobbing), I wanted to build in complexity with a similar experimental paired design, implementing competing sensory modalities (olfactory presentations of mental gland secretions vs. visual presentations of a tortoise model) to observe how tortoises of either sex utilized multiple senses or if they preferentially chose olfactory or visual cues in directing social behaviors. These first two simple paired design studies were meant to establish proof of concept to be able to develop more ecologically based questions, such as how mental glands might be used in a fragmented environment to locate conspecifics for mating opportunities or how diluted mental gland secretions might be to still elicit olfactory discrimination. Finally, these behavioral works allowed for an ethological and ecological based framework when chemically analyzing mental gland secretions of both male and female tortoises using gas-chromatography-mass spectroscopy for chemical constituents.

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CHAPTER ONE

BEHAVIORAL DISCRIMINATION OF MALE MENTAL GLAND SECRETIONS OF THE GOPHER TORTOISE (*GOPHERUS POLYPHEMUS*) BY BOTH SEXES[1]

Introduction

Chemical communication is the oldest form of sensory signaling in nature (Taniguchi and Taniguchi, 2014; Apps et al., 2015). As such, chemoreception has the longest evolutionary timeline among species to allow for adaptation (i.e., many strategies of use, such as scent trails; Cooper and Vitt, 1986; Cooper et al., 1987) and a high degree of specificity for signature chemical mixtures that indicate sexual status (sagebrush lizards: Ruiz et al., 2008), age (Iberian [1]wall lizard: López et al., 2003), size (European pond turtle: Poschadel et al., 2006; red-sided garter snakes: Shine et al., 2003), health (Spanish terrapins: Ibáñez et al., 2012), or even individual or genetic recognition (zebra finches: Caspers et al., 2015; sagebrush lizards: Martins et al., 2006). Patterns of both the type of chemical emission (e.g., signals), especially among broadly present pheromones, and patterns of the evolutionarily sustained behavioral reactions to specific social cues, including chemical emissions, can be vastly identified throughout the animal world. For example, within the Order Hexapoda, insect species emit singular chemical pheromones (i.e., chemical compounds that supply general information to conspecifics; Wyatt, 2014) that act as species identifying chemicals, whereas within the clade Vertebrata, signature chemical mixtures (i.e., specific compounds that indicate information about a specific individual only; Wyatt, 2014) are more complex and composed of certain chemical compounds at specific

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[1] Kelley, M.D., C. Ka, J.W. Finger Jr., and M.T. Mendonça. 2021. Behavioral discrimination of male mental gland secretions of the gopher tortoise (*Gopherus polyphemus*) by both sexes. *Behavioral Processes* 183:104314.

concentrations that allow specialization in identifying many levels of possible recognition between conspecifics (Martín and López, 2011). However, despite the fact that chemical communication has become so widely studied especially in recent years in many animal taxa, many studies have been unable to demonstrate that certain secretions are actual pheromones due to their failure to incorporate behavioral bioassays with the identified compound (Mason and Parker, 2010; Apps et al., 2015). In fact, both chemical and behavioral components are required to be able to conclude a chemical is used as a pheromone (Mason and Parker, 2010; Apps et al., 2015).

Within Vertebrata, reptiles have provided a model framework for understanding chemical communication and chemoreception through describing chemical compounds found in secretions and defining ecologically-relevant behaviors within chemically-producing species (Baumann, 1927; 1929; Mason and Parker, 2010). The main reason chemoreception has been so widely studied in reptiles is because it is used in all manners of their life histories, including, prey detection, predator awareness and avoidance, and courtship and mating (Mason and Parker, 2010). Another important reason for the wide study of reptilian chemoreception is because both sloughed skin and dermal lipid exudate can contain social scent cues (e.g., methyl ketones identified in the skin of red-sided garter snakes; Baedke et al., 2019; Weldon et al., 2008; Mason and Parker, 2010; femoral pores in lizards; Martín and López, 2011, 2014). Although well-studied in model systems such as garter snakes and lizards, chemoreception is still under-studied in many groups, such as chelonians (Apps et al., 2015).

Lizards are one group of reptiles in which many social behaviors have been more proximally and ultimately explored. In addition to a broader understanding of lizard behaviors, classic and recent methodologies have also provided a clear definition for behavioral “choice,” or preference. For example, using paired-choice experiments (e.g., with scented-filter paper, swabs, or in a Y-maze; Cooper, 1994; Hews et al., 2011; Pruett et al., 2016), chemical discrimination or preference has been shown between self and non-self (blue-tongued skinks: Graves and Halpern, 1991; desert iguana: Alberts, 1992), familiar from unfamiliar conspecifics (Bull et al., 1999), and male from female conspecifics (Cooper et al., 1994; López and Martín, 2009). Furthermore, using total time observed in ethological observations towards a particular treatment or the change in rate of a particular behavior (e.g., tongue flick rate or head bobbing in lizards, for example) towards a treatment, preference has been repeatedly quantified for some of these species. While chelonians (turtles, tortoises) can also chemically discriminate self from non-self (Lewis et al. 2007), male from female (Munoz, 2004), and possibly other aspects of conspecific health (Pellitteri-Rosa et al., 2011), relatively less is known, necessitating further study into how chemical communication impacts behavior.

The gopher tortoise (*Gopherus polyphemus*) is a threatened species found in the southeastern United States. Tortoises encounter many risks of mortality generally associated with habitat depletion of longleaf pine forest (their specialized habitat type; Aresco and Guyer, 1999). However, despite obvious fragmentation among populations, isolated individuals do not seem to ecologically suffer from missed mating opportunities (within 30 m of nearest neighbors in one study; Boglioli et al., 2003), indicating that long-distance olfactory cues may drive mate choice for this species. Previous work has also shown gopher tortoises are a social species through sharing burrows and in fact appear to form small aggregations in burrows in which tortoises prefer repeated interactions with specific individuals (Johnson et al., 2009; Guyer et al., 2016). One suggested source of pheromone cues in tortoises are the paired mental glands, for which members of *Gopherus spp.* are uniquely identified (Winoker and Legler, 1975). Recent work has also shown that with the presence of visual cues (e.g., a tortoise model), gopher tortoises will socially respond with up to 16 different behaviors favorably towards mental gland secretions, including courtship behaviors (e.g., head bobbing and possible mounting) relative to a negative control model (Kelley and Mendonça, 2020). However, more work needs to be done on ecological use of mental gland secretions to understand their importance in conspecific locating within a complex environment and chemical individual recognition for this species.

Because work in other *Gopherus spp.* has indicated scent cues from submandibular mental glands play an important role in chemical recognition and behavior (Bulova, 1997; Alberts et al., 1994; Tuberville et al., 2011; Rose, 1970), in this study, we utilized previously defined methodologies to investigate the importance of mental gland (hereafter referred to as MG) secretions in mate choice of gopher tortoises. The goals of our study were twofold. First, we aimed to document tortoise recognition of male MG secretions on cotton swabs by both sexes. Second, we sought to identify specific behaviors that could be chemo-olfactory-driven in function. To accomplish these goals, we used two, simple choice experiments of pooled chemical cues from male mental glands versus: 1) a neutral control of distilled water and 2) a pungent control of acetone, in two separate paired-choice trials. We also wanted to examine possible sex differences in response to male MG secretions. We hypothesized that sexually mature male tortoises would spend the greatest amount of time with and perform the most active and potentially social behaviors (i.e., head bobbing, etc., if any) towards the tortoise-derived MG secretion rather than either control. We hypothesized male-biased time and behaviors to MG secretions because male tortoises have been thought to be more adept at olfactory recognition than females in other tortoise species (*Testudo hermanni*; Galeotti et al., 2007). We further hypothesized that tortoises of both sex would demonstrate increased olfactory behaviors (i.e., sniffing, head extension, etc.) in particular towards the MG secretion treatment as a mechanism for investigating conspecifics. To our knowledge, this is the first study to utilize a true chemical-behavior bioassay (i.e., an assay only presenting chemical cues) to investigate the effects of MG secretions on gopher tortoise social behavioral responses during their mating season.

Materials and Methods

Study Site, Trapping & Housing

All tortoises used in this study were captured from Fort Rucker U.S. Army and Aviation Base (31.342901N, 85.713983W). Fort Rucker is located below the fall-line (i.e., the geographic designation of the Coastal Plain) within the tortoise's natural range in southern Alabama. Many of Fort Rucker's training areas have ideal habitat characteristics required by the tortoise, including spatially open understories, sandy uphill landscapes, longleaf pine-tree assemblages, and preferred grasslands.

We trapped adult tortoises from May-June, within the active tortoise nesting season (Ott et al., 2000), in both 2017 and 2018 using live-animal Tomahawk (Wisconsin, USA) traps placed at the mouths of active tortoise burrows (n = 40; 20 tortoises/year). Upon capture, individuals were assessed for health and size. Individuals >180 mm carapace length, indicating sexual maturity (McRae et al., 1981), and not exhibiting active respiratory disease symptoms were transported to Auburn University's outdoor animal handling facility and housed in artificial enclosures (1.52 x 3.048 m^2) where all behavioral trials took place. Each enclosure was surrounded by chicken wire and black construction-grade silt fencing, both of which were dug into the ground to prevent burrowing outside the enclosure. All enclosures included vegetation (e.g. food *ad libitum*), shelter (in the form of artificial burrows), and water. All tortoises were provided with at least >1 month of acclimation prior to commencing behavioral choice trials.

Experimental Design

Experimental arenas (1.3 x 1.3 m^2) were created within a single unused enclosure (described above) with the same materials as the enclosures (chicken wire and black silt fence). Before each experimental trial, mental gland secretions were collected fresh (i.e., on the day of the trial) with cotton swabs by gentle palpation of the gland. MG secretions from 3-4 actively-secreting, sexually active males were pooled and used in trials. MG secretions were pooled to keep volume and concentration of the signal consistent between presentations in the pen (see also Kelley and Mendonça, 2020). Secretions were collected from males that were not used in behavioral analyses to avoid self-recognition confounding of signals.

We performed two separate experiments to investigate behavioral responses to male MG secretions. In the first experiment, 20 adult gopher tortoises (n=10 males; n=10 females) were exposed to paired swab presentation trials consisting of male MG secretions or distilled water (a neutral odorant control; hereafter NC) in 2017 (August-September). Because distilled water is a neutral scent and was not thought to illicit social behaviors in tortoises, it was used as the NC in the first experiment (similar to what has been used as a negative control in squamates: Cooper and Burghardt, 1990; Cooper, 1998; also used in tortoises: Kelley and Mendonça, 2020). In the second experiment, another 20 adult tortoises (n=10 males; n=10 females) were exposed to paired swab presentation trials consisting of MG secretions or acetone (pungent odorant control; hereafter PC) in June 2018. Acetone was chosen as the PC in the second experiment because its vapours maintained pungency on a saturated swab after a 10-minute duration (e.g., the period of a single trial), and previous electrophysiological experiments have shown responsiveness of tortoise olfactory neurons elicited by acetone presentation (Matthews, 1972). Furthermore, because acetone is not thought to be biologically relevant in the natural environment, acetone was not expected to affect tortoise social behaviors. For both experiments, all 20 behavioral trials were performed within the same week, to minimize possible seasonal influences on behavior. All trials were performed within the same block of hours, when tortoises are most active (Ott et al. 2000; Johnson et al. 2007), and at temperatures >21°C.

For each trial, chemical treatments (e.g., acetone/distilled water or MG) were placed onto cotton swabs *de novo*. Treated swabs were then placed at opposing corners in the behavioral arena to enable focal behavioral observations towards a particular treatment. Tortoises were then placed directly into the middle of the arena, facing away from both swab treatments, and monitored for 10 minutes with a timer to investigate immediate behavioral responses to chemical cues.

Eight quantifiable behaviors were defined *a priori* following previous observations of tortoise-to-tortoise interactions (data not included). Quantifiable behaviors included sniffing, head bobbing, head extension, doubleback (walking past a swab treatment and immediately turning back to the same swab treatment), searching, biting at a swab, or resting or eating directly near a swab. Each behavior was quantified in duration (seconds) of performance (towards a particular swab treatment) and were only quantified if a tortoise performed the behavior within 10 cm of the swab treatment.

For both experiments, all trials were performed within 2-3 days across the span of the same week, with an average range of 8-14 hours at most between any given trial. Between behavior trials, efforts were taken to eliminate prior tortoise smells from arenas. Briefly, the placement of a swab treatment within the arena was randomized for each trial, in such a manner that the same treatment swab was not placed in the same arena corner for more than two trials in a row to avoid bias by tortoises for certain corners of the arena. Also, in the event of focal tortoise defecation, faeces were removed, as well as any substrate that touched it, and tortoises were rotated between two similarly sized behavioral arenas next to each other to eliminate any obvious bias of odours. Human scent in behavioral arenas was also minimized (i.e., nitrile gloves worn at all times and observer did not enter behavioral arena except for waste removal/suspending of trials for the day) to prevent tortoise avoidance of certain arena areas.

Statistical Analyses

Linear mixed effect models (R package: lme4; Bates et al., 2015) were used to analyse total time spent with a treatment (in seconds) for summed behavior durations (Total Time) and the durations of individual behaviors. Fixed effects included Treatment (Experiment 1: NC vs. MG; Experiment 2: PC vs. MG), Sex (Male vs. Female), and the interaction of Treatment×Sex. LME statistics are presented as F-statistics. To further investigate Treatment×Sex interactions, multiple comparisons were performed (when significant) with Tukey's adjustment. Trial date and time were also used as covariates to account for diurnal and daily variation between trials. However, because neither date nor time affected treatment differences or patterns observed, both were removed in final models. To link individual identification with repeated measures of differing behaviors within the same 10-minute observation period, ID was included as a random effect (R Core Team 2019). Univariate descriptive statistics of spread are indicated in the results section as mean ± standard error.

Using a generalized linear mixed model (R package: GLMMRR; Fox et al., 2016) with Poisson distribution (family = poisson) for count data, the numbers of individual behaviors were counted per trial and quantified in analysis by treatment and sex, similar to previously described univariate analyses. These count data were used as a general behavioral diversity index to assess if tortoises were behaviorally stimulated to perform more total numbers of behaviors in any given trial, as would be hypothesized if mental glands produced social cues. Therefore, behavioral diversity was assessed for each experiment through the performing of a Poisson distribution-generalized linear model (GLMs) of raw total numbers of behaviors performed at each treatment. Again, treatment and sex were fixed effects, including the interaction term, if any significant effects occurred. GLM statistics are presented as z values to account for the count data in the distribution. All univariate mixed models were analysed in R i386 3.4.2 (R Core Team 2019).

Multivariate principal components analyses (PCAs) were also performed to assess relationships among behavior patterns overall to construct motifs of correlation, if any occurred (similar to Finger et al., 2019). Component scores were reported if eigenvalues were >1, and individual behaviors were included in a "defining" component if their loading value exceeded 40%. Factor loading scores for the significant components were then analysed for the effects of sex and swab treatment in a two-way ANOVA. Multivariate analyses were performed in JMP Pro 14, and ANOVA analyses were also performed in R i386 3.4.2.

Results

General Univariate Linear Mixed Model for Total Time

In experiment 1, Treatment had no effect on the amount of total time tortoises spent with the MG swab or the NC swab (Sample size (N)= 20; $F_{1,38}$=1.69; p = 0.27). Treatment×Sex also had no effect on the amount of total time tortoises spent with either swab (N=20, $F_{3,36}$=1.60, p=0.12; see Table 1).

In experiment 2, Treatment significantly affected the total time tortoises spent with a swab (N=20, $F_{1,38}$= 9.35, p = 0.004); tortoises spent significantly more time (22.72 ± 5.83 more seconds on average ± standard error) with the MG swab compared to the PC swab. Treatment×Sex also had a significant effect on total time spent with a swab (N=20, $F_{3,36}$=8.12, p = 0.0061). Female tortoises spent significantly more time with the MG swab than the PC swab (22.73 ± 5.88 seconds; *t*=-3.86, p = 0.0001). Female tortoises also spent significantly more time with the MG swab than males did (11.49 ± 5.40; *t*=-2.12, p = 0.0361). However, there was no difference in the amount of time male tortoises spent with the MG swab than the PC swab (p = 0.9678; see Table 2).

Individual behaviors below can be visualized in Figure 1. There were no fixed effects of sex for any individual behaviors throughout this study, so results are described by treatment effects and Treatment×Sex interactions (see Tables 1 & 2).

Month effects of the two experiments were taken into account, but differences for effect of month were only found for the head extension behavior ($F_{1,38}$=5.095, p=0.03) among the MG treatment durations across both experiments. All other individual behaviors yielded non-significant fixed differences by month.

Sniffing

In experiment 1, Treatment significantly affected duration of sniffing: tortoises spent more time sniffing (6.40 ± 1.80 more seconds; $F_{1,38}$=11.83, p = 0.001) the MG swab than the NC swab. Treatment also significantly affected tortoise duration of sniffing in Experiment 2, as tortoises spent more time sniffing (9.00 ± 2.25 more seconds; $F_{1,38}$= 16.33, p = 0.0003) the MG swab than the PC swab. Treatment×Sex did not affect sniffing behavior in experiment 1 or experiment 2 (NC study: $F_{3,36}$=4.37, p=0.66; PC study: $F_{3,36}$= 5.36, p=0.61).

Searching

Searching behavior duration was significantly affected by Treatment in Experiment 2 ($F_{1,38}$=0.84, p = 0.02), but not in Experiment 1 ($F_{1,38}$=0.53, p = 0.23). Tortoises spent more time searching (44.50 ± 17.89 seconds) near the MG swab than the PC swab. Treatment×Sex had a significant effect on searching duration in experiment 1 ($F_{3,36}$=2.28, p = 0.04, biasing males spending more time searching near MG swab) and experiment 2 ($F_{3,36}$=1.14, p = 0.04, biasing females searching more near the MG swab).

To further account for this interaction difference between NC and PC studies, the MG treatment was subset to compare Month (June or September), Sex, and Month×Sex interaction for the duration of search behavior and found that of the three analyses, there was a significant effect of Month×Sex ($F_{3,36}$=2.14, p=0.02), in which males were more likely to search in September than June, suggesting some seasonality for this behavior.

Head Extension

Duration of head extension was also affected by Treatment suggestively during Experiment 1 ($F_{1,38}$=3.98, p = 0.05), as tortoises spent more time performing head extensions towards the MG swab than the NC swab (1.15 ± 0.58 more seconds). However, head extension duration was not affected by Treatment in Experiment 2 ($F_{1,38}$=0.07, p=0.79). Treatment×Sex had no effect on head extension duration in both experiments (NC study: $F_{3,36}$=1.68, p=0.76; PC study: $F_{3,36}$= 0.1, p=0.64).

Resting

Neither Treatment ($F_{1,38}$=0.73, p = 0.40) nor Treatment×Sex ($F_{3,36}$=0.78, p=0.18) affected resting behavior duration in Experiment 1. Treatment had a significant effect on resting duration in Experiment 2 ($F_{1,38}$=4.73, p = 0.033), with tortoises resting more near the MG swab than the PC swab (43.85 ± 19.04 seconds). Treatment×Sex also significantly affected resting duration in Experiment 2 ($F_{3,36}$=3.89, p = 0.0217). Female tortoises spent significantly more time resting near the MG swab than the PC swab (*t*=-3.40, p = 0.0152; 91.70 ± 26.93 seconds).

Eating

Eating duration was not affected by Treatment ($F_{1,38}$= 0.04, p = 0.85) or Treatment×Sex ($F_{3,36}$=0.92, p =0.13) in Experiment 1. Eating had a significant effect on Treatment ($F_{1,38}$=5.18, p = 0.0068) in Experiment 2, as tortoises spent more time eating near the MG swab than the PC swab (31.40 ± 8.31 seconds). Treatment×Sex also significantly affected eating duration ($F_{3,36}$=3.1, p = 0.0343) in Experiment 2, with female tortoises spending more time eating at the MG swab than the PC swab (*t*=-3.78, p = 0.0068; 31.40 ± 8.31 seconds).

Doubleback

Neither Treatment ($F_{1,38}$=1.12, p = 0.29) nor Treatment×Sex ($F_{3,36}$=1.94, p =0.23) affected doubleback behavior duration in Experiment 1. Doubleback behavior did not occur in Experiment 2.

Head Bobbing

Head bobbing did not occur in Experiment 1. While head bobbing occurred in Experiment 2, neither Treatment ($F_{1,38}$=3.46, p = 0.07) nor Treatment×Sex affected its duration ($F_{3,36}$=2.44, p =0.19).

Biting

Biting behavior was not observed in Experiment 1, indicating that tortoises did not try to extract water or eat cotton of the NC treatment. While biting was observed in Experiment 2, it was not affected by Treatment ($F_{1,38}$= 3.26, p = 0.07) or Treatment×Sex ($F_{3,36}$=3.73, p=0.07).

Behavior Diversity

We used GLMs to examine the number of behaviors occurring in each experiment, and test statistics for Poisson distribution-GLMs are presented as z values. In Experiment 1, mean count data of all behaviors did not differ significantly between treatments (z=0.96, p=0.33) nor was there any effect of sex (z=-0.46, p=0.64). In the PC (acetone) study, there was a treatment effect on total number of counted behaviors. The mean total number of all behaviors was significantly higher for the MG swab (z=2.98, p=0.003), and sex of the tortoise subject did not affect this finding (z=1.11, p=0.27; see Table 2).

Principal Component Analyses

For each study, a principal component analysis (PCA) was performed on the duration of each behavior observed. Our goal was to identify possible groupings of behaviors performed commonly together. For the NC study, two components had eigenvalues greater than 1.0, and they accounted for 46.24% of the total model variation. Component 1 included behaviors of sniffing, head extension, eating, and was inverse to resting near a treatment, accounting for 24.8% of model variation, and Component 2 included doubleback and resting behaviors, which were inverse to searching behaviors, accounting for 21.4% variation (Figure 2A; Table 3). In two-way ANOVAs on both component scores for effects of sex and treatment, there were no differences in the model seen for either independent variable.

For the PC study, three components with eigenvalues greater than 1.0 were observed, accounting for 62.63% of the variation. Component 1 included sniffing, eating near, head bobbing, and biting at a swab (26.5% variation); component 2 included resting and eating near a swab, head extension, and inversely related to biting (20% variation); and component 3 included search behavior and resting near a swab (16.13% variation; Figure 2B; Table 4). There was no difference between the sexes on factor loading scores on any component. For component 1, there was a significant effect of treatment, favoring the MG treatment versus acetone ($F_{1,38}$=20.09, p=0.0003). No other components exhibited significant treatment effects.

Discussion

Mental glands, which are a distinguishing sexually dimorphic characteristic of *Gopherus spp*. (mental glands are male-biased in size), have been considered secondary sexual glands capable of secreting volatile chemical substances with olfactory species-specific information and sex- or individual-specific information (Rose et al., 1969; Rose, 1970; Winokur and Legler 1975). Yet, the behavioral support for these anecdotal allegations of pheromone use in gopher tortoises (*Gopherus polyphemus*) have remained largely unexamined prior to current studies (see also Kelley and Mendonça, 2020). In other *Gopherus spp.* tortoises, evidence suggests that MG secretions may be used as pheromones (see Bulova, 1997; Alberts et al., 1994; Tuberville et al., 2011; Rose, 1970). However, in addition to the paucity of chemical ecology work with gopher tortoises, few studies have investigated MG secretions in general or the extent to which any *Gopherus spp.* tortoise may use scent cues in MG secretions to locate other conspecifics. There has also been little work in general on specific types of behaviors (possibly social or courtship behaviors) that could be stimulated alone by MG chemical cues in secretions and/or any other derived possible origin of social cues for tortoises, especially without a simultaneous visual cue present (Kelley and Mendonça, 2020, Rose, 1970, Weaver, 1970). Therefore, to our knowledge, this study represents the first true chemical to behavioral bioassay of MG secretions in gopher tortoises.

Overall, tortoises performed behaviors for longer durations toward the MG swab than either the PC or NC control swabs in both experiments. A total of 6 behaviors were observed in the NC experiment and 8 behaviors were observed in the PC experiment, in which all statistically significant comparisons illustrated that tortoises spent more time with the MG swab. These findings support our primary hypothesis of increased behavioral awareness towards the MG treatment. In particular, our hypothesis that olfactory behaviors would be performed for longer periods of time towards the MG-swab in both experiments was supported. In the NC experiment, the two identified olfactory behaviors of sniffing and head extension occurred for longer periods of time with the MG swab. Likewise, in the PC experiment, both females and males spent significantly more time sniffing near the MG swab. It seems likely that MG secretions from the signaler are acting to initiate olfactory investigation by the receiver, which supports a putative role in chemical signaling. Olfactory investigation, including sniffing behaviors and head extension, have been shown to be possible preliminary behaviors necessary to initiate close-encounter social interactions between conspecifics for courtship in both tortoises and freshwater turtles (Bels and Crama, 1994; Bulova, 1997; McLeod, 2012; Sacchi et al., 2003). In freshwater turtles, there are three commonly accepted phases of courtship including the "tactile phase" (when first touch occurs between conspecifics after sensory stimulation), the "mounting or copulatory phase," and the "biting or rubbing post-copulatory phase" (Barbour and Ernst, 1972; Bels and Crama, 1994; McLeod, 2012). Therefore, the importance of "sniffing" or "head extension" observed in this study towards the MG cotton swab may indicate a "sensory stimulation phase" of tortoise social interactions that precedes the first sight or tactile touch of another conspecific. In other words, male-pooled MG secretions induced olfactory awareness in both female and male adult gopher tortoises without the visual sighting of a conspecific.

In the PC study, both male and female tortoises spent more total time, performed a higher average number of behaviors, and spent more time sniffing and head bobbing, towards the MG swab. Although all comparable parameters support our primary hypothesis of social awareness of mental gland secretions in both experiments, combined with the PCA analysis, our findings indicate a greater preference for the MG swab in the PC study than in the NC study, contrary to our original prediction. We originally expected that tortoises would display avoidance or demonstrate fear-based behaviors (e.g., continuous immobility or withdraw into carapace) towards the PC treatment since acetone is a volatile, non-biologically relevant scent (but still stimulates up to 19 olfactory neurons in gopher tortoises; Matthews, 1972). Nevertheless, neither females nor males displayed fear behaviors but instead performed greater numbers of total behaviors in the PC experiment, indicating tortoises did not avoid the PC treatment. One possible explanation for the observed increased activity in the PC study could be that tortoises of both sexes were more easily able to distinguish between the PC and the MG treatment (i.e., due to higher volatility or a more novel scent of acetone relative to the NC treatment of distilled water), thus stimulating a greater number and higher frequencies of behaviors, including aggressive social displays (e.g., head bobbing and biting) which have previously been documented in Texas Tortoises (Auffenberg, 1966; Rose 1970; Auffenberg, 1977).

Seasonality could be another possible explanation for the increased behavioral responses observed in the PC experiment. The PC experiment was performed in June, while the NC experiment was performed in late August-early September. These two time periods were chosen because June and August-September coincide with elevated plasma testosterone in both male and female tortoises (Ott et al., 2000; Lance and Rostal 2002). However, because peak testosterone, especially in males, actually occurs in August-September (when the NC study was performed), it is possible that tortoises at the end of their mating season might have already mated earlier in the season and consequently be less likely to display behaviors associated with looking for conspecifics (Ott et al., 2000; Lance and Rostal 2002). The interaction of sex and treatment for searching behavior observed in both experiments appears to support this hypothesis. Males were more likely to actively search near the MG treatment than the control in the NC experiment, whereas females were more likely to actively search near the MG swab than the control in the PC experiment. The difference in searching behavior between males and females may be associated with tortoise endocrinology. Females have their first peak in testosterone earlier in the mating season before nesting in July and, consequently, may be more actively searching for males for mating opportunities at that time (Ott et al., 2000). In August-September, it is likely that many females may have already mated and thus, be less likely to search for males. On the other hand, coinciding with their peak testosterone, males may be more actively searching in August-September due to proximity with the end of mating season, suggesting behavioral synchronicity of peak plasma sex steroids, if this speculation can be confirmed (Itoh and Ishii 1990; Ott et al., 2000; Lance and Rostal 2002). It is important to note however that although potential seasonality may have been observed for search behavior, no other behaviors showed this seasonality significantly with sex effects to support hormonal roles of the behaviors observed in this study. More work is necessary to understand the interaction of sex steroids and behavior in gopher tortoises.

Surprisingly, we observed no fixed effects of sex in behavior frequencies in this study, even though there were sex by treatment interactions between some behaviors and treatment. In Hermann's tortoises (*Testudo hermanni*), previous work suggested females were less acute to specific olfactory cues than males (Galeotti et al. 2007), leading to the hypothesis that females would be less likely to discriminate scent cues and/or actively pursue males for social interactions. The results of this study, however, are in contrast to this, with males and females spending similar amounts of time (both total time and time spent individually performing behaviors) with the MG treatment. Nevertheless, our results also highlight that females were more likely to perform passive, maintenance behaviors (e.g., eating or resting near) near the MG swabs in the PC experiment, suggesting that once females were able to discriminate between treatments, they were more likely to passively stay with the treatment than males. In contrast, males were more likely to continue to sample both treatments for the duration of a trial, possibly indicating female tortoises may adapt more quickly to olfactory cues (e.g., Sorokowski et al., 2019).

Although the results of this study indicate MG secretions are used by gopher tortoises as chemical cues, the small sample sizes (*n*=10 for both sexes in both experiments) may have impacted our results. Gopher tortoises are a threatened species due to habitat destruction and degradation, which precluded the sampling for high numbers of individuals for long-term controlled studies. Because of being endangered in Alabama in the western part of their range along the Mobile and Tombigbee Rivers (U.S. Fish and Wildlife Service 1986; TESII 1995), the number of tortoises for behavioral observations were limited for this study overall, thus necessitating this study to better understand how conspecifics are sought for mating opportunities for management purposes. Nevertheless, the sample sizes used in this study are similar to those that were robust enough to investigate behavior in the desert tortoise (Alberts et al., 1994; Niblick et al., 1994).

Conclusion

This is one of the first studies to investigate chemical cues in a tortoise species using a chemical-only bioassay. Our results illustrate that both male and female tortoises respond behaviorally to secretions contained within mental glands, indicating that mental glands are used as a source of social cues. Our results provide a firm foundation for further investigation of social interactions in gopher tortoises and the possible management implications that can best preserve this species.

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Table 1: Statistical analyses for negative control study (DI water vs. male mental gland secretions)

Negative Control Experiment	*Treatment*	*Sex*	*M:F Biased*	*Treatment*Sex*	*DF*	*Residual Error*	*F-Statistic*
Total Time	5.44	3.22	M	p>0.05	237	38.11	0.82
SE	4.92	5.02					
t-value	1.11	0.64					
p-value	0.27	0.52					
Searching	-31.88	-18.25	M	73.96	18	51.4	—
SE	25.7	23.46		33.18			
t-value	-1.24	-0.78		2.23			
p-value	0.23	0.45		0.04*			
Biting	NA	NA	NA	NA	NA	NA	NA
Doubleback	0.95	1.56	M	p>0.05	37	2.77	2.115
SE	0.88	0.89					
t-value	1.09	1.75					
p-value	0.29	0.09					
Eating	-2.5	-5.29	F	p>0.05	37	42.06	0.09
SE	13.3	13.58					
t-value	-0.19	-0.39					
p-value	0.85	0.7					
Head bobbing	NA	NA	NA	NA	NA	NA	NA
Head extension	1.15	-0.63	F	p>0.05	37	1.82	2.57
SE	0.58	0.59					
t-value	2	-1.07					
p-value	0.05*	0.29					
Resting near	14.15	7.1	M	p>0.05	37	53.06	0.44
SE	16.78	17.12					
t-value	0.84	0.42					
p-value	0.4	0.68					
Sniffing	6.4	-2.15	F	p>0.05	37	5.86	6.6
SE	1.85	1.89					
t-value	3.45	-1.13					
p-value	0.001*	0.26					

Table 2: Statistical analyses for positive control study (acetone vs. male mental gland secretions)

Positive Control Experiment	*Treatment*	*Sex*	*M:F Biased*	*Treatment*Sex*	*DF*	*Residual Error*	*F-Statistic*
Total Time	22.72	11.48	M	-22.96	298	36.88	—
SE	5.83	5.83		8.25			
t-value	3.9	1.97		-2.78			
p-value	0.004*	0.06		0.006*			
Doubleback	NA	NA	NA	NA	NA	NA	NA
Eating	31.4	20.4	F	-26.9	18	18.57	—
SE	8.31	10.89		11.75			
t-value	3.78	1.87		-2.29			
p-value	0.001*	0.08		0.03*			
Resting near	91.7	41.1	F	-95.7	18	60.22	—
SE	26.93	27		38.09			
t-value	3.4	1.52		-2.51			
p-value	0.003*	0.15		0.02*			
Searching	44.5	29	F	-56.5	18	40.01	—
SE	17.89	24.93		25.31			
t-value	2.49	1.16		-2.23			
p-value	0.02*	0.26		0.04*			
Head bobbing	1.05	-0.75	F	p>0.05	37	1.77	2.67
SE	0.56	0.56					
t-value	1.88	-1.34					
p-value	0.07	0.19					
Head extension	0.5	0.1	M	p>0.05	37	5.94	0.04
SE	1.88	1.88					
t-value	0.27	0.05					
p-value	0.79	0.96					
Sniffing	9	1	M	p>0.05	37	7.12	8.09
SE	2.25	2.25					
t-value	3.998	0.44					
p-value	0.0003*	0.66					
Biting	1.35	-1.35	F	p>0.05	37	2.29	3.47
SE	0.72	0.72					
t-value	1.863	-1.86					
p-value	0.07	0.07					

Table 3: Multivariate PCA Loadings and Final Communalities for the Negative Control Swab Experiment

Behaviors	***PC1 Loadings***	***PC2 Loadings***	***Final Communality Estimate***
Sniffing	0.471	0.26	0.29
Head Extended	0.785	-0.0745	0.622
Searching	-0.318	-0.532	0.383
Doubleback	0.013	0.688	0.473
Rest Near	-0.479	0.662	0.668
Eat Near	0.565	0.133	0.337

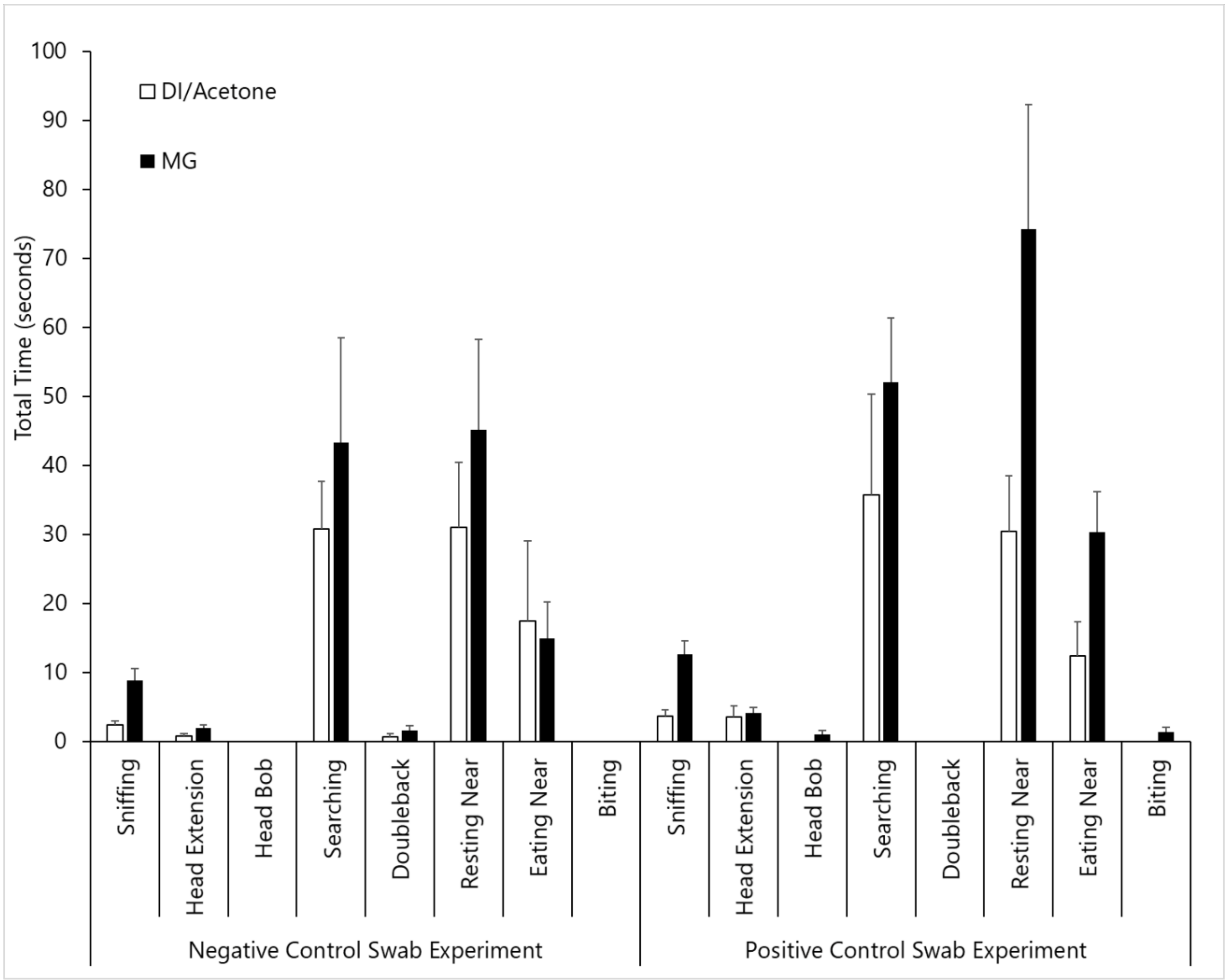

Figure 1: Behaviors of Negative and Positive Control Swab Experiments. Univariate comparison between duration time of individual behaviors performed by both sexes of tortoises and effect of treatment. Mean (± standard error) time spent (in seconds) performing behaviors are indicated "NA" indicates behaviors that were not performed or observed during the experiment. "DI" represents distilled water in the negative control swab experiment (performed in August-September 2017), and "Acetone" represents the positive control swab experiment (performed in June 2018; both pictured in gray depending on experiment as indicated).

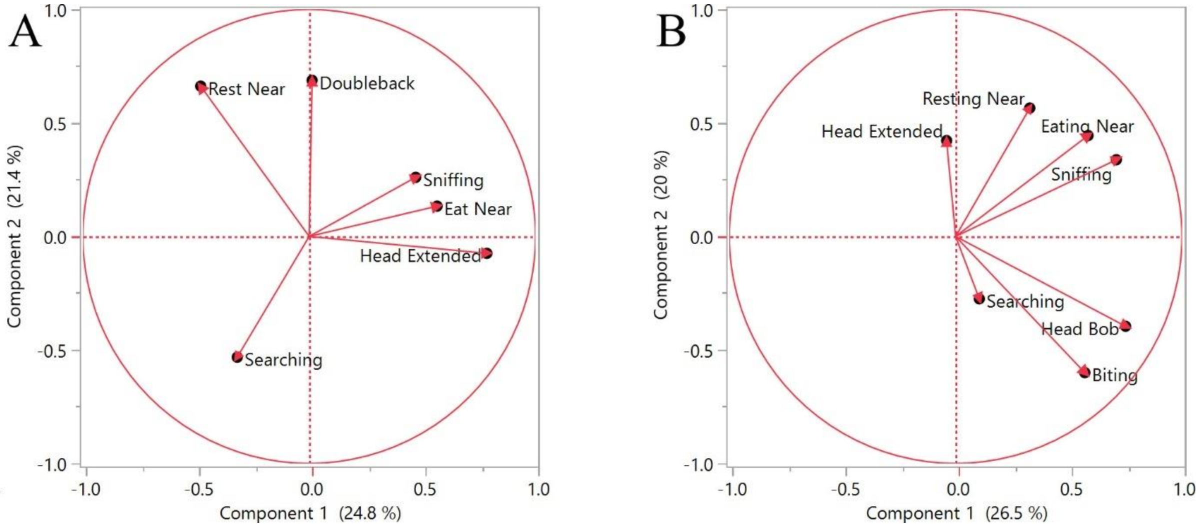

Figure 2: PCA for Both Swab Experiments. Principal components analyses (PCA) for durations of behaviors. **A)** PCA of the neutral control study (NC study: male chin gland secretions vs. distilled water). **B)** the pungent control study (PC study: male chin gland secretions vs. acetone).

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CHAPTER TWO

MENTAL GLAND SECRETIONS AS A SOCIAL CUE IN GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*): TORTOISE PRESENCE STIMULATES SOCIAL BEHAVIOR WITH CHEMICAL CUES[2]

Introduction

Multimodal signaling is common in social interactions, in which repetitive or novel information may be shared between conspecifics through multiple senses (Johnstone 1996; Candolin 2003; Partan 2004). One such combination of multimodal presentation could include sensory cues such as visual and/or olfactory presentation of a single characteristic (i.e., the color *or* scent of floral blooms advertising a nectar reward; Faegri & Van Der Pijl 1971; Kelley et al. 2017) or a complex whole conspecific presentation (i.e., presence of a female red-sided garter snake, leopard gecko, or Spanish terrapin, respectively, with high levels of integumental semiochemicals that serve as pheromones; Mason et al. 1989; 1990; Mason & Gutzke 1990; Ibáñez et al. 2012), in which the visual sighting of a conspecific and/or the olfactory recognition of volatized chemical exudates from a conspecific could alert others to social or mating opportunities. While it is difficult to ascertain if visual or olfactory cues are more important in stimulating social interactions in most species, namely if conspecifics prioritize sensory cues for maintaining those social interactions, a simpler question would be to ask about the role or importance of one type of information when other information is already present. For instance, when animals first detect conspecifics via visual cues, we can ask what is the role that remains for chemical cues regarding the initiation and maintenance of social interactions? Olfaction is the

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[2] Kelley M.D. and Mendonça M.T. 2020. Mental gland secretions as a social cue in gopher tortoises (*Gopherus polyphemus*): tortoise presence stimulates and maintains social behavior with chemical cues. *Acta ethologica* 23: 1-8.

oldest of the physical senses (Taniguchi and Taniguchi 2014), and as such, it is likely that even in the presence of visual information animals will still respond to chemical cues. Moreover, because chemo-olfactory receptors are highly conserved across taxa and because of parallel olfactory and behavioral neurological pathways (Nelson & Kriegsfeld 2017), it may not be surprising that we observe courtship and mating behaviors that could be similar, or at least analogous, throughout species, such as pheromone-stimulated fixed action patterns of ordered courtship behaviors (i.e., hamsters; Darby et al. 1975; rabbits; Hudson et al. 1990) and other stereotypies, such as head bobbing displays observed in fish (Donaldson 1995), birds (Ota et al. 2015), lizards (Noble and Bradley 1933; Orrell & Jensson 2002; Martins et al. 2015; Pruett et al. 2016), and tortoises (Auffenberg 1977).

Reptiles may be key model species for understanding the interplay between visual and chemo-olfactory cues because chemo-olfaction is present in every aspect of their life and evolutionary histories (Brattstrom 1974; Apps et al. 2015). Reptiles have adept olfactory receptor systems and diversity of integumental volatized compounds that could serve as pheromones (Apps et al. 2015). However, the visual presence of a conspecific can also be required for some reptile species to initiate social interactions. For instance, in red-sided garter snakes, visual presence of a female stimulates males to begin attempting courtship behaviors, even when the female is recently deceased (e.g., not requiring female behaviors for males to initiate courtship; Garstka et al. 1982). Conversely, relevant olfactory cues may also be sufficient to stimulate social behaviors in other species, including competition and courtship in some tortoises (Texas tortoises: Rose 1970; Desert tortoises: Alberts et al. 1994). Even though visual and olfactory cues may both stimulate the initiation of social behaviors in reptiles, studies so far do not clarify whether both sensory cues (visual and olfactory cues) are simultaneously necessary to maintain those social interactions that may lead to courtship advances or reproduction.

In this study, we investigated if, in the presence of visual cues, olfactory cues of the chin gland (hereafter, referred to as mental gland) are still necessary for the maintenance of social behaviors in gopher tortoises (*Gopherus polyphemus*). We studied mental gland olfactory cues in conjunction with visual cues of resin tortoise models as a visual representative of a tortoise marked with mental gland (MG) secretions vs. distilled water (DI; as a control) in a paired-choice design, measuring first choice, time spent performing behaviors, and total numbers of behaviors performed to either treatment. Because tortoises do not have one known rapid response behavior that occurs consistently to social cues (i.e., tongue-flicking as in lizards or snakes; Cooper 1994; Bryant et al. 2011) and also because there are many courtship or competition behaviors within the tortoise behavioral repertoire that could be considered a social acknowledgement, we decided on this paired-choice presentation to examine any behaviors observed from focal tortoise individuals that could be social and/or lead to courtship. Both visual and olfactory cues may stimulate social behaviors in tortoises (Auffenberg 1977), but a paired-design such as the one used in this study allows for investigation of tortoise individual engagement and maintenance of social behaviors over a set period of time, using a visual-only presentation in the form of a tortoise model, relative to a more complex multimodal presentation of visual + olfactory cues (e.g., MG secretions on a tortoise model).

Our primary hypothesis is that although visual cues are important to detect conspecifics, other stimuli, such as chemical cues, are also necessary to stimulate and maintain tortoise social behaviors by both sexes of gopher tortoises. We would predict that if a simple visual-only presentation is sufficient to engage and maintain social behaviors, tortoises of both sexes might be expected to spend equal amounts of time with either model, regardless of chemical treatment. However, if more sensory stimulation is required than only visual cues as we hypothesize, then more time or a higher number of behaviors should be performed to the MG treatment, indicating that olfactory cues (e.g., including MG secretions) are required by gopher tortoises to maintain social interactions. Our secondary hypothesis is that if multimodal stimulation of both visual and olfactory cues are necessary to both initiate and maintain social interactions, then more olfactory (e.g., sniffing, head extension) and social behaviors (e.g., head bobbing, scratching), in particular, will be performed by tortoises towards the MG treatment.

Methods and Materials

Study Species

Gopher tortoises are a social but long-lived species that live in fragmented populations in longleaf pine ecosystems (Diemer 1986; Aresco and Guyer 1999). They dig several burrows each year (which provide refuge for up to 330 other species as well; Jackson and Milstrey 1989) in sandy soils in which to dwell during seasonal temperature extremes when they are not foraging or looking for mates (Diemer 1986). Gopher tortoises are most socially active in the warmer months of the year (March to October; Ott et al., 2000), presumably due to optimum temperature, day length, and peak testosterone levels (Weaver 1970; Ott et al. 2000; Lance and Rostal 2002). At least, day length and testosterone during this time is also speculated as responsible for the sexually dimorphic enlargement of their mental glands (larger in males; Winokur and Legler 1975) that have been suggested as a source of pheromones in both male and female tortoises. Mental gland secretions have subtle chemical composition differences that may allow conspecific gopher tortoises to obtain information about conspecifics (e.g., sexual identity or other information, Rose et al. 1969; Rose 1970; Weaver 1970; Auffenberg 1977).

Additionally, given that related tortoise species (*Gopherus berlandieri;* Texas tortoises) have not been shown to be able to visually recognize sexual identity of individuals through external physical characteristics (*Gopherus spp.* tortoises lack visually sexually dimorphic characters seen in other chelonian species, such as colour differences, elongated foreclaws in males, dramatic size differences, etc.), mental glands also have the unique possibility of providing both visual or olfactory information about sex to tortoise conspecifics due to sexual dimorphism (Weaver 1970; Auffenberg 1977).

Gopher tortoises (*Gopherus polyphemus*) are a federally threatened species in most of the states of their range in the southeastern U.S. and have been suggested for endangered species status as they are restricted to Coastal Plain longleaf pine ecosystems.

Study Sites & Trapping

Tortoises were captured in the state of Alabama (U.S) at Solon Dixon Centre for Education and Forestry (Covington Co.) and Fort Rucker Army and Aviation Base (Coffee and Dale Cos.), during the active mating season of the gopher tortoise (June to August 2017; Ott et al. 2000). Solon Dixon is a wildlife and forestry research centre owned by Auburn University and is part of Conecuh National Forest. Fort Rucker Army and Aviation Base is a large helicopter training installation and has hundreds of acres of tortoise-suitable longleaf pine forests. Both sites are below the Coastal Plain fall-line, have longleaf pine forests that are regularly burned, and many active free-ranging adult tortoises. Only adult tortoises were trapped via live-animal Tomahawk traps (Wisconsin, USA) placed at the entrance of active tortoise burrows and temporarily transported to Auburn University for behavioral observations.

Experimental Design

At Auburn University, tortoises were housed in outdoor pens (5 X 10 m^2) with ample forage, water, and shelter in artificial burrows made with bisected corrugated pipe (0.4572 m diameter). Pens were created with chicken wire and construction-grade black silk fence; an army-grade vinyl tarp was placed over artificial burrows for thermoregulation. The behavioral arenas were small pens (1.3 X 1.3 m^2) located within one unused housing pen and were constructed with the same materials previously mentioned (Figure. 3a).

Adult male and female tortoises (males: N=28; females: N=17) were acclimated for 3-4 weeks prior to use in a trial. Mental gland (MG) secretions were collected from non-experimental male tortoises (i.e., tortoises that were not to be behaviorally observed the day of collection) by gently squeezing the glands and transferring pooled exudate onto cotton swabs. The secretions were collected fresh, directly before observations to ensure consistency in concentrations between trials. After gland secretions were collected, the pooled sample (3-4 male secretions) was applied onto one resin tortoise model (12” long X 7” wide X 4” tall; ©Collectible Badges; Figure 3b) painted with UV resistant acrylic paint to resemble a gopher tortoise. Pooled secretions were applied to a consistent surface area (e.g., on the chin and neck, gular projection of the plastron, and front of each forelimb) on the model during each application. Additionally, on a second resin tortoise model identical to the first model, distilled water (DI) was applied to the same central areas as the (MG)-treated model. Models were randomly placed diagonally across from each other in the behavioral arena with the focal tortoise placed centrally in the pen (Figure 3a).

Mental gland secretions were pooled in this experiment to negate any possible chance of individual male recognition as tortoises were communally housed with 1-2 other individuals of the same sex in pens. As other studies have shown, specific proportions of compounds are necessary in olfactory signals to extract information at the most specific-level of recognition, the individual; therefore, pooling 3-4 samples together likely did not affect the more general signals (i.e., species, sex, etc.) but should eliminate individual-bias (Apps et al. 2015), and no tortoises ever experienced their own secretions, even with pooling.

Behaviors (16) observed and timed included *a priori* behaviors identified from prior tortoise-tortoise interactions (similar to those found in other chelonian behavior studies: Bels and Crama 1994; Ruby and Niblick 1994; Kazmaier et al. 2001; Liu et al. 2013; Sacchi et al. 2013; Cutili et al. 2014), including: sniffing, head extension, doubleback to a treatment, eating or resting near a treatment, head bobbing, biting at model, nose-to-nose interactions, carapace alignment, charging or shoving model, mounting/climbing on model, tasting or biting at air near a model, scratching/rubbing, pulling into shell, and direct approach (e.g., sudden vigilance of model, leading to direct advance and confrontation of model from across the arena without other behaviors exhibited). Behavioral observations began immediately after the tortoise was placed in the centre of the behavioral arena, between, but facing away, from both treatments (Figure 3a). Once an initial treatment was approached by a focal tortoise, the first treatment choice was noted for binary analysis of choice. All behaviors and their durations were recorded if they occurred towards either treatment within 0.33 metres of the model and were quantified in seconds, in addition to total time (in seconds) spent with either treatment by a tortoise. Each tortoise was used in one trial only for a 10-minute duration of observations. Although tortoises are diverse in behavioral responses as indicated by *a priori* investigations, most tortoises also required on average a 1-2 minute acclimation to behavioral arenas before displaying any behaviors or showing distinction between treatments, when that occurred.

Scent applications were handled to minimize transfer of other scents (e.g., human scents) to the models and the arena itself (e.g., nitrile gloves worn at all times, observer never entered arena, and feces removed while rotating between two identical behavioral arenas side by side for the next focal tortoise). Models were cleaned with isopropyl alcohol before new secretions were applied for another trial (presentation; similar to methods used in Alberts et al. 1994). Treatment locations were also randomized, and no treatment was allowed to be in the same corner of a behavioral arena for more than two tortoise trials in a row.

Statistical Analyses

All statistical analyses were performed in R i386 3.4.2 (R Core Team 2019). First, we used linear mixed models (LMMs) to analyse total time spent engaging in all combined behaviors as a function of treatment and sex. We also used LMMs to analyse total time spent engaging in individual behaviors as a function of treatment, sex, and treatment×sex interaction. Tortoise ID was included as a random effect in all LMMs to account for individual variation in time spent engaging in behaviors and to account for potential pseudo-replication in testing for the effects of sex (e.g., two treatments of time durations for every individual behavior and total summed durations; Zuur et al., 2009). *F*-statistics are reported to account for model variation in behavior duration means (e.g., sample size between sexes were not equal; males N=28 & females N=17) where appropriate in univariate models with behavior durations [therefore, results are presented as sample size (N), *F*-statistic, *p*-value].

In addition, average counts of numbers of behaviors per tortoise were analysed using a Poisson distribution-generalized linear model (GLM) for the purpose of noting overall behavior diversity as it occurred towards either treatment with sex as a secondary fixed effect in each individual trial. A secondary GLM-binomial (logistic) distribution analysis (in which coding was used with “0” for the DI control model and “1” for the MG-treated model) was also performed to assess the effect of sex of focally-observed tortoises on their first immediate treatment choice in each presentation, immediately after the tortoise was placed in the pen. For all GLM models, z scores are presented with *p*-values, along with N and degrees of freedom (df) (i.e., N, df, *z* score, *p*-value). Statistical significance was set to $\alpha = 0.05$.

Results

LMM results for repeated measures indicated that both sexes showed more interest in models that were treated with conspecific MG secretions. Specifically, the total time individuals performed behaviors was 8.6 times greater towards the MG-treated model, relative to the DI-treated control (N=45; $F_{1,1528}=18.66$, $p<0.0001$). There was no sex difference in this total time (N=45; $F_{1,1528}=1.66$, $p=0.2$), nor was there an effect of treatment×sex on total time (N=45, $F_{3,1586}=6.96$, $p=0.46$).

In simple univariate comparisons, individual behaviors that differed by treatment were always higher in duration towards the MG-treatment, regardless of sex (sex effect, p=0.72; Figure 4). Specifically, behaviors of doubleback (N=45, $F_{1,88}=5.08$, $p=0.03$), eating near (N=45, $F_{1,88}=4.05$, $p=0.05$), head bobbing (N=45, $F_{1,88}=6.05$, $p=0.02$), head extension (N=45, $F_{1,88}=3.54$, $p=0.02$), scratching at a model (N=45, $F_{1,88}= 5.29$, $p=0.03$), and sniffing (N=45, $F_{1,88}=11.83$, $p=0.0009$) significantly favored the MG-treated model. Similarly, direct approach was also higher towards the MG treatment, but this finding was not significant (N=45, $F_{1,88}=3.03$, $p=0.08$). While there were also no fixed effects of sex for any behavior, there were two behaviors that yielded trends of a possible impact of treatment×sex interaction, in which males were more likely to perform doubleback behavior (N=45; treatment×sex: $F_{3,86}$=4.61, p=0.06) and were less likely to scratch at the MG- treated models (N=45; treatment×sex: $F_{3,86}$=3.40, p=0.08), but neither of these findings were significant.

For behavior diversity analyses (count data of behaviors), tortoises of both sexes performed an average of 4.49 behaviors specifically towards the MG-treated model vs. 2.82 behaviors to the DI-treated model. Thus, when using a Poisson distribution-GLM for count data of behaviors performed, the MG-treated model received significantly more total behaviors (N=45, df=89, z=4.1, p<0.001), regardless of sex (N=45, df=89, z=0.19, p=0.19). However, the binomial distribution-GLM indicated a significant effect of sex on first immediate treatment choice when entering the pen, in which females were about 2.01 times more likely to visit the MG-treated models first than males (N=45, df=42, z=-2.37, p=0.02); however, males were just as likely (50%) to approach the MG-treated model as the DI-treated model first.

Discussion

To our knowledge, this is the first study to show gopher tortoises of both sexes performing behaviorally and socially towards MG secretions in a complex display, indicating that visual cues alone are not sufficient to maintain social interactions for this species. Specifically, we found that gopher tortoises of both sexes directed more total numbers of behaviors and spent more total time with the MG-treated model, relative to the DI-treated control model, supporting our primary hypothesis of social relevance for MG secretions. These findings indicate that with a multimodal presentation of both visual presence of a tortoise and olfactory cues in the form of MG secretions, tortoises of both sexes are likely to utilize both visual and olfactory information to initiate social interactions, but the chemical presence of relevant olfactory cues (such as from MG secretions) increases total engagement and duration of the interaction, as shown by both total time spent with the MG-treatment and behavioral diversity. Because the MG-treated model was preferred by adult tortoises of both sexes, it seems likely that olfactory MG scent cues seem to be driving choice between models in this experiment. Nevertheless, because both chemical and visual cues were presented simultaneously (in a paired-choice presentation), future studies should further investigate individual sensory cues and modalities (in a no choice design) involved in locating conspecifics and social clique formation that could lead to mating opportunities (Guyer et al., 2016). Individual behaviors that were performed for more time towards the MG-treated model, included doubleback, eating near, head bobbing, head extension, scratching at a model, and sniffing, thus encompassing both social and olfactory type behaviors by the recipient tortoise (see Figure 4). These individual behaviors favoring the MG-treated model supports our secondary hypothesis that mental gland secretions may also be used to gain more information about conspecifics, thus stimulating not only awareness but also, both olfactory (i.e., sniffing and head extension) and social (i.e., head bobbing and scratching) behaviors that are also commonly observed in tortoise competition assessment and courtship interactions (Auffenberg 1966; 1977).

No current study has observed competition or courtship behaviors towards mental gland secretions in the gopher tortoise for either sex, making our study the first behavioral bioassay of mental gland secretions in a complex social choice presentation for this species. Previous studies have shown that mental glands are seasonally enlarged glands, particularly in males but also in females, due to seasonal peaks in testosterone (Weaver 1970; Ott et al. 2000), and that secretions contain chemical information that caused male-male combat in one *Gopherus spp.* tortoise, increased olfactory investigation, and possibly allowed for individual recognition (Rose 1970;

Bulova 1997; Tuberville et al. 2011). We observed a total of 16 behaviors towards both the MG-treated model and the DI-treated model, indicating that visual presence did also stimulate initiation of some social behaviors. However, only the MG-treated model yielded significant comparisons of total time or time of individual behaviors performed by observed tortoises, suggesting at least environmental awareness of the presence of mental gland secretions that could be used to approach and investigate conspecifics more closely. Signaling theory indicates that signals must be energetically efficient, must differ from other signals in a noisy environment, and must be able to reach and stimulate an intended receiver, which classifies MG secretions officially as social signals from the support observed in this study (Enquist et al. 2010). All tortoises in this experiment displayed some behaviors correlated with normal tortoise social displays and/or courtship (i.e., investigation, head bobbing, head extension dominance displays, biting, scratching/rubbing, carapace alignment, etc.; Auffenberg 1977), with preference to the MG-treated model. This indicates that tortoise mental gland secretions are consequential in social tortoise interactions because they stimulated social behaviors.

Of the behaviors that favored the MG-treated model, head bobbing, head extension, and scratching at the model could be interpreted as active social behaviors observed in dominance and courtship displays (Sacchi et al. 2013; Cutili et al. 2014). Other behaviors including sniffing, doubleback, or eating near the MG-treated model may have chemical implications through either olfaction or gustation. However, one key behavior not observed in this study was the mounting of either model treatment. Mounting is generally observed between competing males or in copulation attempts of females. Because the models lacked visual expression of enlarged mental glands (e.g., mental gland presence and size would be the primary visual sexually dimorphic trait in gopher tortoises; Winokur and Legler 1975; McRae et al., 1981), one unintended consequence of this study is that models may have appeared more female, in which case the male scent of MG secretions would be the only distinguishing character of possible sexual identity of the models. Yet, male tortoises did not attempt mounting of either chemically-treated model. A lack of mounting behavior could suggest that even more sensory stimulation may be necessary to observe the full suite of gopher tortoise social behaviors. For example, Auffenberg (1977) outlines the relative importance of other possible sensory stimulations including tactile presence (i.e., carapace alignment), receptivity displays, or other important olfactory cues (e.g., cloaca secretions or urination/defecation) that of course, were not displayed by the models in this experiment. If male tortoises identified MG secretions as male, especially considering that pooled secretions (secretions from 3-4 male tortoises) could be more pungent in olfactory cues than a singular male tortoise would produce, it is surprising that we did not also observe aggressive combat displays (e.g., charging or shoving models), considering aggressive behavior was previously observed in Texas tortoises (*Gopherus berlandieri*) towards models in a no-choice designed study (Rose 1970). Consequently, our results suggest potentially different uses and/or behavioral reactions to mental gland secretions between *Gopherus spp*. tortoises, or simply differences due to experimental design. Although tortoises behaviorally responded to our resin models, male tortoises especially did not seem to be maximally stimulated to social responses, even if some potential dominance and/or courtship behaviors were identified. While the use of resin models in this study allowed for better observation and subsequent characterization of focal tortoise behaviors, the use of inanimate models may have prevented some social behaviors from being displayed, requiring future studies of both natural observations of tortoise-tortoise interactions in addition to more controlled model studies with differing sensory cues.

One possible confounder in this study was the use of only male mental gland secretions vs. DI water in the paired-choice presentation. While both sexes may benefit from seeking scent cues from any conspecific regardless of sex (e.g., finding social groups could lead to mating opportunities), our design may have also biased females to be more interested in interacting with the MG-treated model than males, which may have actually been more likely to avoid an overly pungent, competing male scent. On the other hand, by using a strong tortoise-derived pooled sample, this design may have biased both sexes towards the MG-treated model as well in individual behavior durations (e.g., increased olfactory investigation). The use of male-only MG scent may also explain why male tortoises were not fully stimulated to display more active courtship behaviors to either model, despite olfactory and social interest. Nevertheless, in general, male social behaviors have already been broadly classified for a number of differing tortoise species, noting aggressive social behaviors (e.g., head bobbing, scratching) that led to successful reproduction, but female tortoise social behaviors are less well-known (Niblick et al. 1994; Alberts et al. 1994; Sacchi et al. 2013). Therefore, our study is one of the few tortoise studies to have thoroughly examined behaviors of both males and females, despite less obvious motivations for the behaviors of males in this study other than being aware of conspecifics. Often, female tortoises have only been observed in studies as a way to establish female choice for reproductively successful males, but female social behaviors for courtship and mating have rarely been discussed or examined and never in a controlled setting (Niblick et al. 1994; Alberts et al. 1994; Tuberville et al. 2011). Field observational studies of gopher tortoise courtship have suggested that females are only receptive to courtship advances of males, rather than being initiators of social behaviors themselves, contrary to what was observed in this study by using male-only scent cues (Auffenberg 1966; Johnson et al. 2009; Guyer et al. 2016). Specifically, past observations of females have traditionally been male biased by only quantifying behaviors that occur at female burrows, thus not allowing for observations of females seeking male burrows during the mating season (Landers et al. 1980; Boglioli et al. 2003; Moon et al. 2006).

In this study, females did significantly initiate more interactions with the MG-treated model than males on first presentation and performed all behaviors, including aggressive social displays such as head bobbing, similarly to males with no significant differences in the fixed effect of sex. One possible explanation is that the artificial nature of this presentation with a resin tortoise model may not have produced normal behavioral frequencies, especially by males. Conversely, because male mental gland secretions were used (e.g., not female scent cues), it could also mean that female tortoises may be more active in seeking out males or initiating social interactions (possibly courtship) than previously thought. More female observations are necessary to further elucidate the motivations of female tortoises in mating behaviors. Thus, our results illustrate that mental gland secretions are used as social cues that may be sex specific, but further work is required to better understand their role in social displays and mate choice, especially in relation to other sensory cues, including tactile cues. It is also not yet known the full scope of information (e.g., individual identity, health status, hierarchy, etc.) that may be transmitted by these gland secretions, requiring further work in both male and female gopher tortoises to better characterize reproduction behaviors and cues used for mate-choice for implementation in future management to help augment tortoise reproductive success in struggling populations.

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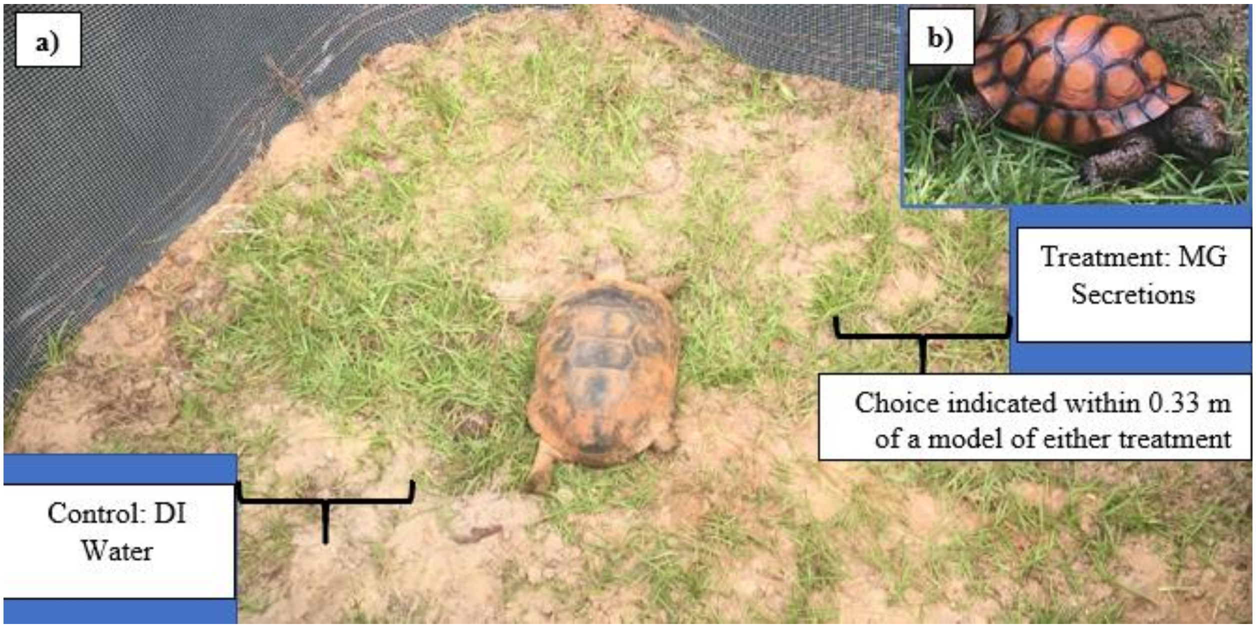

Figure 3: Experimental design for paired-model set-up. a) Experimental set-up for tortoise paired-choice experiment with resin models, in which blue squares represent actual model placement within the behavioral arena, about 1 meter on either side of a centrally place tortoise individual to observe and time behaviors. Choice indicated within 0.33 m of a model of either choice for behaviors to be observed, identified, and timed as directed towards either treatment. b) Painted resin tortoise model, like that used in this study.

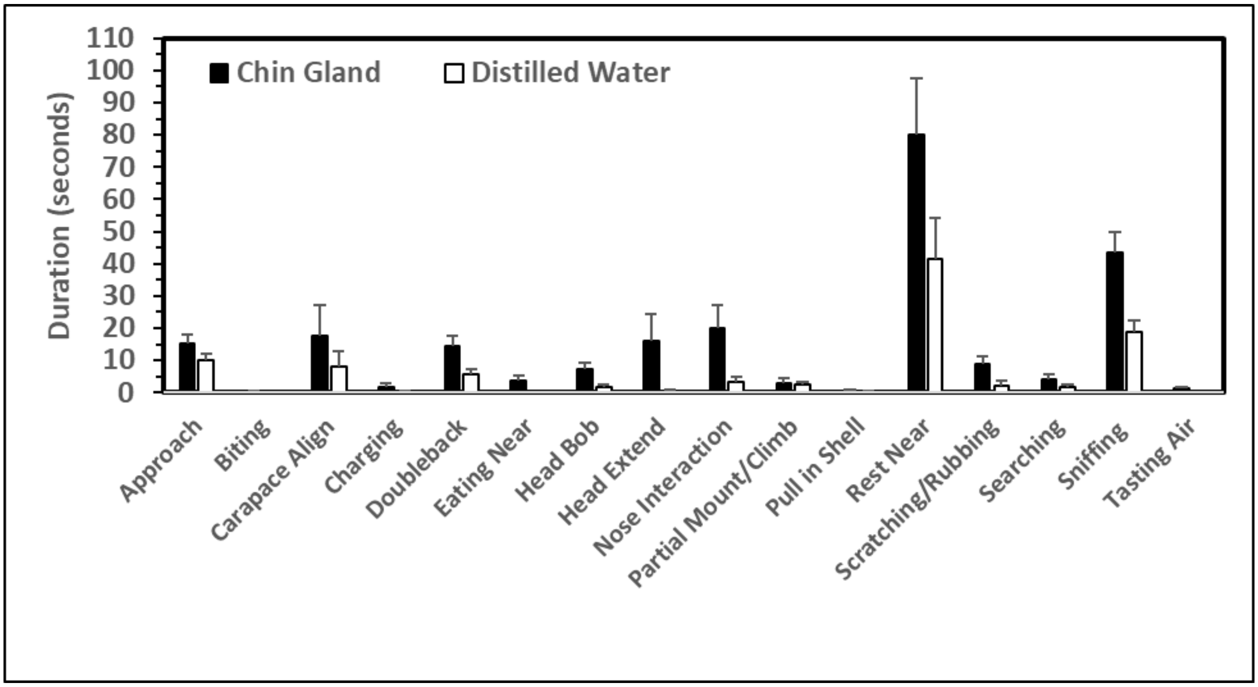

Figure 4: Behaviors of model control experiment. Mean duration (± 1 SE) of behaviors seconds, with both sexes combined, of gopher tortoises during the model experiment (black bars: mental or chin gland secretion treatment; white bars: distilled water control).

CHAPTER THREE

MALE GOPHER TORTOISE (*GOPHERUS POLYPHEMUS*) SOCIAL RESPONSES TO SERIALLY DILUTED MENTAL GLAND PHEROMONES

Introduction

Chemical communication is the oldest and most widely used form of communication in the animal world, but more specifically, pheromones evolved as chemical signals between conspecifics for social interactions within a species (Wyatt, 2014; Taniguchi and Taniguchi, 2014). The oldest and most general definition of a pheromone is a volatized chemical emitted from a conspecific that causes change in the receiver's physiology or behavior (Karlson and Luscher, 1959; Mason, 1992). As such, reptiles have been used as model species of study for the functional use of pheromones within their environments (e.g., scent-marking, trailing conspecifics, mate guarding, etc.), given that it has long been accepted that any exudate produced by the integument or gland of a reptile could be used in chemical communication and recognition among conspecifics, especially in male-male competition and mate-choice (Mason, 1992; Weldon et al., 2008). For example, in some of the oldest studies on pheromone use in rattlesnakes, male snakes were observed attempting mating behaviors towards a freshly shed female skin (Klauber, 1956; Mason, 1992), and since this classic work, the integument of red-sided garter snakes (*Thamnophis sirtalis parietalis*) has been shown to produce holocrine lipid secretions and volatized methylated ketones used in snake courtship and mating (Mason et al., 1989). More recently, representative species of snakes, lizards, and chelonians have also been shown to scent-mark with chemical secretions (snakes: LeMaster and Mason, 2001; lizards: Hews et al., 2011; and turtles/tortoises: Ibáñez et al., 2012; Alberts et al., 1994) with the potential of recognizing individual signature mixtures (i.e., recognition of a chemical sequence unique to an individual within a population; Wyatt, 2014; *Egernia striolata*, Bull et al., 1999; *Iberolacerta cyreni*, Martín and López, 2010).

In a complex terrestrial environment, especially in a fragmented habitat, chemical cues can become increasingly important as a primary mechanism for conspecifics within a population to locate one another for social or mating opportunities. For example, male timber rattlesnakes (*Crotalus horridus*) living in very fragmented habitats use mainly olfactory chemical trails of females for navigation across long distances in order to successfully encounter both mating (Coupe, 2002) and hunting opportunities (Clark, 2007). Similarly, olfactory preferences are also key in social interactions of far-reaching spatially distributed tortoise species as well, in which individuals that prefer conspecific signature mixtures for familiarity may also have to search relatively long distances to find females (*Testudo hermanni*: Galeotti et al., 2007; *Gopherus agassizzi*: Alberts et al., 1994; Tuberville et al., 2011). Therefore, one benefit to far-reaching olfactory signals is that chemical depositions that serve as social or mating cues can persist in an environment after the signaling conspecific has left the immediate area. However, temporal and spatial placement of cues is also an integral part of chemical depositing because chemical cues are subject to breakdown or dilution over time from environmental conditions (e.g., rain, wind, physical damage, etc.; Alberts, 1992; Bossert and Wilson, 1963). Because chemical cues in the form of pheromones can possess complex chemical information about conspecifics and breakdown of cues over time is eminent, pheromones are only as useful as signals, as receiving conspecifics are adept at recognizing even degraded variations of scent cues. Thus, investigations for how pheromones are affected by habitat fragmentation are necessary.

Interestingly though, another benefit to chemical cues as pheromones is that olfactory-chemical acquisition can provide a direct mechanism for physiological priming (e.g., hormonal changes relative to receptivity) or behavioral releasing effects (e.g., rapid behavioral reaction to scent cues) in receiver conspecifics, especially in potential mates (Wyatt, 2014). While priming effects can be physiologically complex (Brennan, 2009; Wyatt, 2014), behavioral releasing effects have been studied through attraction and aversion behaviors between conspecifics that can be seen immediately at the time of scent presentation. Behavioral releasing effects have been particularly observed in the formation of monogamous male-female interactions (e.g., the monogamous lizard the alpine skink *Niveoscincus microlepidotus* recognizes and forms bonds to conspecific signature mixtures the longer they spend following a scent-trail; Olsson and Shine, 1998) or in the avoidance of marked territories (e.g., male *Acanthodactylus boskianus* avoid scent-marks of other males; Khannoon et al., 2011). Both priming or releasing effects could play a role in determining if a conspecific is attracted to or averse to the information within the chemical cue. Thus, assessing the behavior of conspecific receivers to a cue presentation gives insight into the physiological effect of the chemical cues or pheromone signals (Campos et al., 2016; Kelley et al., 2021).

Here, we use behavioral observations of male gopher tortoise reactions to chemical cues in mental gland secretions by other male conspecifics. We presented diluted mental gland pheromones to determine the ability for male gopher tortoises to detect low concentrations of mental gland secretions of other males that might be present in a complex, fragmented habitat. For example, gopher tortoises reside exclusively in the fragmented habitat of the longleaf pine forest of the southeastern United States. Interacting male gopher tortoises may have the need to detect olfactory cues of other males because they are competitive with one another over available females for mating opportunities, which can result in male-male combats. Despite the mortal risks of male-male combats (flipping onto back and desiccating in the sun) that could be avoided and the findings that larger males are most likely to be successful in winning direct conflicts over smaller males for mating opportunities (Douglass, 1986; Moon et al., 2006; Tuberville et al., 2011; White et al., 2018), smaller males still seem to seek out other gopher tortoises (both males and females) and can gain partial paternity in clutches, suggesting that direct male-male conflicts do not entirely inhibit reproductive success for smaller male tortoises in this species (White et al., 2018). Therefore, partial paternity in clutches and the ability for female tortoises to also store sperm across reproductive years (Moon et al., 2006; White et al., 2018) indicates utility of following olfactory cues to find mating opportunities in a spatially segregated habitat, in spite of possible male conflicts. One such pheromone source in tortoises that could be important in environmental trails or eliciting behavioral reactions are mental gland secretions (or chin gland secretions) that have been observed to be wiped on forelimbs, the carapace of a female in courtship, or grass/herbivory (Auffenberg, 1966; Auffenberg, 1977). Mental gland secretions have been recently shown to be recognizable to tortoises of both sexes and do elicit social behaviors, both with and without a visual stimulus present (Kelley and Mendonça, 2020; Kelley et al., 2021). As a social reptile that forms “cliques” of repeated interactions with the same individuals, it has long been hypothesized that far-reaching volatized chemical cues, such as those from mental gland secretions, could be driving these repeated encounters (Boglioli et al., 2003; Johnson et al., 2009; Guyer et al., 2016).

In this study, our focus was to examine the behavioral reactions to ecologically relevant ranges of mental gland secretions in a series of paired-choice experimental dilutions. In a previous study, pooled samples of mental gland secretions elicited greater total attention in time and individual behavioral reactions, relative to a negative distilled water control (Kelley and Mendonça, 2020). However, durations of time for social behaviors by either sex of gopher tortoise were low overall in this previous study, suggesting potential aversions to strong pooled scent cues (Kelley and Mendonça, 2020). Therefore, we aimed in this study to serially dilute mental gland secretions to determine at what minimal concentration mental gland secretions might elicit pheromone-stimulated social behaviors. We did this by presenting variations of diluted mental gland (MG) secretions vs. distilled water (DI) on resin tortoise models, in order to parse out chemically stimulated and maintained behavioral reactions (e.g., nonrandom behavioral interactions with chemical presentations), similar to those seen in Kelley and Mendonça (2020), from visual-only (e.g., random or model-driven) behavioral reactions. We hypothesized that stronger concentrations of MG secretions might cause more pronounced treatment differences but could decrease social behavioral diversity, if male tortoises avoided stronger scent cues of other males. We also hypothesized that once a signal was too diluted to discriminate between treatments, tortoises would behaviorally respond only to the models without a clear choice for treatment effects.

Materials and Methods

Study Species and Study Site

Gopher tortoises (*Gopherus polyphemus*) are a species of concern endemic to the southeastern U.S. longleaf pine forest and savanna, in which habitat has declined by more than 80% in the previous century due to deforestation (Diemer, 1986; Aresco and Guyer, 1999). This landscape-wide fragmentation caused by deforestation separates both tortoise populations and individuals, sometimes by great distances, creating a challenge of finding potential mates, but also putting the species in jeopardy through habitat loss and other potential threats to individual mortality (e.g., road mortality, disease, etc.). Because gopher tortoises depend on adult survival for population maintenance and species sustainability, it is increasingly more important to understand how adults locate one another for mating and reproduction.

All tortoises were captured from Fort Rucker Army & Aviation Base (Dale and Coffee Cos., Alabama (GPS: 31.342901N, 85.713983W). Fort Rucker encompasses thousands of acres of surrounding land and wildlife, including pristine longleaf pine forests with white soft sandy soils preferred by tortoises, in addition to other tree and plant assemblages indicative of the ecosystem, such as turkey oak, yucca and prickly pear succulents, and a variety of grasses and forbes consumed by tortoises. This study site is also below the fall-line (the geographic designation of tortoise preferred habitat) and close to the Coastal Plain in the southern part of the state.

Capture and Housing of Tortoises

Ten adult male gopher tortoises were captured via Tomahawk live-animal traps (LxWxH: 101.6 cm x 25.4 cm x 25.4 cm; Wisconsin, USA) placed unbaited on the aprons of active burrows between May-June 2018 (the early part of the tortoise socially active season; Spring-October: Ott et al., 2000; Lance and Rostal, 2002; McRae et al., 1981). Pre-selection criteria for tortoises used in this study included only individuals that had no prior carapace-markings (e.g., triangular file tick marks or round drill marks on marginal scutes of the carapace); had a carapace length exceeding 180 millimeters (one indication of adulthood; McRae et al., 1981); and did not present any active symptoms of respiratory disease (i.e., wheezing, mucous, etc.; Jacobsen et al., 1991). Adult tortoises that met the pre-selection criteria were transported back to Auburn University's animal handling facility temporarily for the performing of behavioral trials.

Artificial tortoise housing pens at Auburn were approximately 1.52 x 3.048 m^2 in area, made of chicken wire and black construction-grade silk fencing (see Kelley and Mendonça, 2020; Kelley et al., 2021). Each pen also included an artificial burrow, covered by a military grade tarp for shelter and thermoregulation, and water and food *ad libitum*, including a multitude of vegetation types for both forage and shade within the enclosure. Tortoises received daily checks for health and condition.

Chemical secretion collection & dilution preparation

Tortoises were allowed a one-month acclimation period (in housing pens) prior to commencing behavioral trials. Mental gland secretions were collected two days before the start of the experiment (all samples collected within 15 minutes of each other). Secretions from three adult excreting males were collected into a single glass capillary tube (measuring exactly 25 ul total volume in the manner described in Kelley and Mendonça, 2020) and stored on ice. Samples were processed by preparing a dilution series (1/4; 1/20, 1/100, and 1/500; Figure 5) using 4 labelled glass test tubes. Using a pressurized catheter tube, the MG secretions were diluted in the first glass test tube with 75 ul of deionized water (total 100 microliters) to create a 1/4 dilution (labelled “ORIG”). Then 20 ul of the 1/4 dilution was transferred to 80 ul of deionized water (1/20 dilution, or “DIL 1”), and then serially repeated to produce the 1/100 and 1/500 dilutions (“DIL 2” and “DIL 3”, respectively). These test tubes were thoroughly vortexed and each aliquoted into four sterile eppendorfs per dilution, labelled, and stored at -80º C until used as a chemical cue presentation for the behavioral trials.

Behavioral Presentations

The four differing chemical dilutions of mental gland (MG) secretions (ORIG, DIL 1, DIL 2, DIL 3) were presented individually in paired displays versus distilled water (DI) on the same polyresin tortoise models (30.48 cm long X 17.78 cm wide X 10.16 cm tall; ©Collectible Badges) for all experiments in a small arena (1.3 x 1.3 m^2). Outdoor arenas were similar to those described in Kelley and Mendonça (2020) and Kelley et al. (2021). All four chemical presentations were separate experiments, presenting one paired-presentation at a time (one diluted MG scent, such as DIL 3 vs. DI, on the same day for all 10 tortoises when possible or during the same daylight hours on a subsequent day) with repeated displays to the same 10 adult male tortoises to account for individual variation of behaviors and preferences while within the arena. No more than one 10-minute presentation was allowed each day per tortoise, and male tortoises were randomized for each experimental presentation to allow tortoises sufficient time between trials and to account for the possible influence of order on behavior. All four paired-presentations were performed within the same two-week interval to minimize the possible influence of season on behavioral responses.

Before the beginning of each chemical presentation, the polyresin tortoise models were treated with one of the dilution treatments or the DI control, using cotton swabs to apply chemical/water treatments along the same standardized area of the gular-head region: the gular projections from the plastron of the model, and the front of the forelimbs, similar to what might be expected from a live tortoise that wipes their mental glands on their limbs (Auffenberg, 1977; also similar to Kelley and Mendonça, 2020). Treated polyresin models were placed diagonally across the pen from each other. All treatment placements were randomized in the pen between tortoise presentations, in which a model of either treatment was not presented in the same corner of the pen for more than two sequential presentations to minimize possible corner bias. Behaviors were recorded immediately after the focal tortoise was placed directly in the center of the pen (Kelley and Mendonça, 2020), and the directionality to either treatment for behaviors was noted within 0.33 meters of the model and timed accordingly (in seconds) for analysis. Models were cleaned between every trial with swabs of isopropyl alcohol as previously described (Alberts et al., 1994; Kelley and Mendonça, 2020) and fresh MG dilution/DI treatment was applied while wearing nitrile gloves before each presentation. Measures were also taken to avoid human scent bias (e.g., never entered the pen, stood more than 1 meter away from pen for observations, etc.). All observations were performed by the same observer (MDK).

Approximately 16 behaviors were defined *a priori* and quantified in seconds during the 10-minute duration of each presentation (see Table 1 for behaviors).

Statistical Analyses

Total time with either treated model (e.g., timed collective period of any behaviors performed) and individual behaviors were quantified in terms of their duration (in seconds). Both dilution experiment concentration (ORIG, DIL1, DIL2, DIL3) and treatment (DI vs. MG dilution) of directed behaviors were indicated in the averaging of behavior times across analyses. Univariate analyses were performed to determine average time of each behavior performed per TRT (Treatment), DIL (dilution), or the TRT X DIL interaction. General behavioral diversity analyses were also performed to quantify differences in raw numbers of behaviors performed per TRT, DIL, or TRT X DIL interaction. Multivariate analyses were used to assess behavioral correlations with one another using a principal components analysis (PCA), and significant components from PCA were again used to indicate any effects of dilution on behavioral displays and also, interactions between dilution and treatment. For all tests, multiple post-hoc pairwise comparisons were used to determine differences between means of all relative groupings.

Univariate Analyses

All individual behavior durations were included in univariate linear mixed effect models (LMEs) for maximum likelihood fitted by REML for each behavior separately, using repeated measures in the statistical model to account for repeated sampling of the same individuals across the 4 dilution experiments. In one set of analyses, TRT was the only fixed effect. In the second set of analyses, DIL factor was used to determine differences in individual behavioral responses between dilution experiments. Finally, DIL factor X TRT interactions were also examined for each individual behavior, for the purpose of elucidating dilution concentration on the ability for individual tortoises to discern between mental gland concentration and control-treated models. When performing a simple linear regression of Time of Day relative to Ambient Temperature that occurred while experiments were performed, it was determined that temperature significantly fluctuated with period of time during experimental trials (p=0.003). Thus, the variable Time of Day encompasses Ambient Temperature changes and therefore was also initially included in all original models of individual behaviors, reported when significant, or removed from further models when not significantly affecting the durations of individual behaviors.

A measure of the general behavioral diversity observed in the 4 dilution experiments included the raw counted number of behaviors performed per TRT and DIL for each tortoise. Generalized linear models (GLMs) using Poisson distribution for count data were performed to account for behavioral diversity during different dilution experiments and between the two treatments. Again, in separate models first, TRT and DIL were the primary fixed effects, followed by a third model type including a TRT X DIL interaction term, if any occurred. All univariate measures of spread include: coefficient of variation (effect size between groups) ± standard error. All univariate comparisons were performed in R-3.5.3, and significance was established at $p \leq 0.05$.

Multivariate Analyses

A principal components analysis (PCA) was performed for all durations (in seconds as a continuous dependent variable) of observed behaviors, and components with eigenvalues greater than one were considered significant and used in further analyses. Loading matrix correlation indices for each significant component were evaluated at greater than or equal to 50% in order for individual behaviors to be considered included in particular components for further analyses with orthogonal computation values.

Using orthogonal values from the PCA for each component, LMEs were again performed for differences, if any, between TRT, DIL, or TRT X DIL interaction, blocked for repeated measures of individuals. All multivariate comparisons were performed in JMP Pro-14, and all LMEs were performed in R-3.5.3. Significance was again established at $p \leq 0.05$.

Results

Univariate effects of Treatment:

Of the 16 expected behaviors, only 15 behaviors were performed (Charging not observed). The total summed time of 15 observed behavior durations and the durations of individual behaviors (time in seconds) significantly differed by treatment throughout the overall dilution series (all 4 experiments combined). Individual tortoises spent significantly (p=0.0067; 48.1 ± 17.22 seconds) more time with the MG model than the DI model.

Individual behavior durations that differed by treatment (i.e., MG vs DI) overall throughout the four dose presentations included: carapace alignment (p=0.03), doubleback (p=0.0016), and sniffing (p=0.01), all favoring the MG secretion treatment relative to the DI control (Figure 6).

For individual MG dose presentations, the individual behaviors that differed significantly by treatment included sniffing (p=0.02) for the 1/4 MG presentation; carapace alignment (p=0.0003) and head bobbing (p=0.01) for the 1/20 MG presentation; and mounting behavior (p=0.006) for the 1/500 MG presentation but biting (p=0.049) also favored the DI control during the 1/500 MG presentation [Figure 7(a-d)]. Because no treatment discriminations occurred for the 1/100 MG presentation relative to the DI control and treatment preferences for social behaviors (e.g., mounting and biting) were inconclusive for a single preference during the 1/500 MG presentation, male tortoises did not appear to be able to discriminate between treatment for any presentation more dilute than the 1/20 (DIL 1) MG presentation.

Univariate effects of Dilution:

Durations of some individual behaviors yielded significantly different effects of time when comparing dose concentrations of MG secretions, indicating that dose may be important to the signaling power of MG secretions in the field (i.e., Figure 11 shows total time differences per dilution is concentration-dependent; Vasudevan and Vyas, 2013). For example, approach behavior did not significantly differ in duration of time between the 1/4 and 1/20 dose presentations (the two most concentrated dilutions), but did occur significantly longer for the 1/4 dose and the 1/20 dose, relative to both the 1/100 (p<0.01 & p=0.002, respectively) and the 1/500 dose presentations (both p-values<0.01). Searching behavior was similar to approach behavior among dilution experiments [13.5±5.79 more seconds for 1/4 vs. 1/100 (p=0.02); 18.1±5.79 more seconds searching in 1/20 vs. 1/100 (p=0.0027); and 14.9±5.79 more seconds in 1/20 vs. 1/500 (p=0.01)]. Mount behavior had a weak but significant effect on dose, in which male tortoises mounted models more and for a longer time during the 1/500 MG presentation vs. the 1/20 MG presentation (p=0.05).

There were also significant TRT X DIL experiment interactions. For carapace alignment, treatment significantly interacted with dilution when comparing all other dilutions to 1/20 (1/4 :Trt: p=0.03; 1/100:Trt: p=0.02; 1/500:Trt: p=0.003), in which 1/20 had much greater time spent in tortoises aligning carapaces with MG models than the DI control as seen in the other dilution presentations. For head bobbing, similar to carapace alignment, TRT X DIL interaction occurred more for 1/20 vs. 1/100:Trt (p=0.05) and vs. 1/500:Trt (p=0.05), in which tortoises head bobbed towards the MG model more than the DI control in the 1/20 MG presentation vs. the less concentrated presentations (1/100 & 1/500). Mounting was the only behavior that significantly occurred greater towards the MG model in the least concentrated presentation (1/500), relative to 1/20:TRT (p=0.047; Figure 7a-d).

Analyses for behaviors Time of Day and Ambient Temperature

Only approach (p=0.0094) and searching behaviors (p=0.03) displayed significant variation with Time of Day, and approach was also the only behavior significantly affected by Temperature (p=0.0036).

Generalized linear model for numbers of behaviors

For every increasing dilution of MG secretions, an additional behavior occurred. For example, for the 1/4 presentation, 12 behaviors occurred; for 1/20 presentation, 13 occurred; for 1/100 presentation, 14 occurred; and for 1/500 presentation (the most dilute concentration), the full suite of 15 behaviors occurred. However, generalized linear models for treatment nor dilution yielded any significant differences in average numbers of behaviors performed by individual males across dilution experiments (all p-values>0.05).

Multivariate Analyses

A principal components analysis (PCA; PC for principal components) was conducted, and a total of 6 components (comprised of a total of 12 behaviors out of the original 15 behaviors observed; Figure 8) were significantly designated with eigenvalues >1, accounting for 62.98% of the model variation. Considering loadings of $\geq$ 0.5 or 50% correlation, PC1 included behaviors approach, carapace alignment, head bobbing, tasting air, sniffing, and doubleback (accounting for 14.8% of model variation); PC2 included approach and searching (accounting for 11.98% of model); PC3 included pull in shell and resting near model (accounting for 11.69%); PC4 included head bobbing and mounting/climbing behavior (accounting for 8.89%); PC5 included scratching or rubbing model (accounting for 8.03%); and PC6 included head extension (accounting for 7.55%).

One-way and two-way LMEs using orthogonal PC values indicated significant effects of both treatment and dilution on components but no interaction terms, so only fixed effects are reported. Only PC1 had a treatment effect (p=0.0007; Figure 9) favoring the MG secretion-treated model overall, and Time of Day only significantly impacted PC2 (p=0.03). For dilution experiment comparisons, PC1 [1/20 vs. 1/500, p=0.04], PC2 [1/4 vs. 1/100 (p=0.002) & vs. 1/500 (p=0.02) and 1/20 vs. 1/100 (p=0.004) & vs. 1/500 (p=0.04)], PC4 [1/4 vs. 1/500, p=0.047], and PC5 [1/20 vs. 1/500, p=0.04] differed between dilutions (see Figure 10).

Discussion

To our knowledge, this is the first study to examine serial dilutions of a pheromone, simulating possible environmental degradation of known chemical signals present in male gopher tortoise MG secretions (e.g., Kelley and Mendonça, 2020; Kelley et al., 2021). Our results illustrate male gopher tortoises are aware of and behaviorally respond to diluted MG secretions from other male conspecifics. In an environmental context, chemical cues present in MG secretions could be prioritized for use in pheromone trails, which may be especially important for many reptilian species, including gopher tortoises, that live in a highly fragmented habitats and must maximize fitness through following the scent cues or trails of other rival conspecifics (also seen in male red-sided garter snakes that follow other males to locate discontinuous trails of females, *Thamnophis sirtalis parietalis*; Shine et al., 2005).

The primary finding of this study is that male gopher tortoises could behaviorally discriminate chemically diluted (1/4 and 1/20 MG dilution secretion) olfactory treatments (i.e., MG vs. DI) on visually identical models. However, behavioral discrimination was concentration-dependent, with male gopher tortoises unable to discriminate treatments at higher dilutions (i.e., less concentrated; 1/100 and 1/500 presentations). This indicates a minimum chemical threshold (i.e., concentrations equal to or greater than those present in the 1/20 dilution) may be necessary for stimulation of olfactory receptors and ultimate signal transduction to induce nonrandom behavioral reactions towards MG olfactory cues in gopher tortoises (Mathews, 1972; Gerlach, 2005; Labra et al., 2005; Vasudevan and Vyas, 2013). The requirement of an olfactory threshold also likely means that intraspecific gopher tortoise (e.g., male-male or male-female) social interactions stimulated by olfactory signals are concentration-dependent, in which an optimum concentration of recognizable parts of the olfactory signal is needed to stimulate specific behaviors or behavioral displays that might lead to mating opportunities (Vasudevan and Vyas, 2013). Previously, concentration-dependent pheromone usage has been shown to be important in stimulating nursing rabbit pups to nurse and in stimulating chemosensory investigation in *Sceloporus jarrovii* lizards (Coureaud et al., 2004; Romero-Diaz et al., 2021). However, this has not been shown in most other reptilian species, including chelonians. Because olfactory cue-derived exudates like MG secretions are likely emitting complex signals that could be transmitting chemical characteristics as broad as species and sexual status or as specific as individual signature mixtures (snakes: O'Donnell et al., 2004; Wyatt, 2014), concentration of a chemical cue is likely key in awareness of the cue, recognition of the individual components (when possible), and stimulating a minimum amount of olfactory receptors that might allow for behavioral releasing effects, or immediate behavioral reactions (see Figure 11).

In this study, we observed the highest amount of total time spent performing social behaviors during the 1/20 MG presentation, as well as the most significant behaviors by treatment (i.e., univariate head bobbing and carapace alignment & multivariate PC1, which included approach, carapace alignment, head bobbing, tasting air, sniffing, and doubleback) relative to other dilution presentations. Interestingly, male tortoises spent a greater amount of total time performing social behaviors (e.g., carapace alignment) during the 1/20 MG presentation relative to the 1/4 MG presentation, the most concentrated presentation of MG secretions. This finding suggests that not only is the 1/20 dilution exceeding a threshold for behavioral responses not seen in the more dilute presentations, but it was also viewed more optimally by male tortoises for behavioral responses when compared with the most concentrated presentation of MG secretions (ORIG-1/4). Therefore, it is possible that the concentration of MG secretions present in the 1/4 dilution may be stimulating aversive or avoidance responses towards the MG-treated model.

The primary hypothesis of this study was that male gopher tortoises might avoid highly concentrated MG secretions, if concentrated MG secretions are an indication of “maleness” or presence of a stronger competing male conspecific that might intimidate subordinate males. Although univariate analysis appeared to support our hypothesis, to further investigate this, we performed multivariate analyses. Interestingly, in addition to the dominant behaviors observed at the 1/4 dilution, tortoises spent a slightly greater amount of time being immobile and pulling into their shells (i.e., PC3), which may be behaviors associated with avoidance or fear-based responses, at the 1/4 dilution than at greater dilutions, although this was not significant. Considering that the 1/4 dilution might stimulate avoidance/aversive responses, it seems likely, however, that avoidance of a cue may be difficult to directly measure if tortoises only had a lack of behavioral display or generally avoided the source of the intimidating olfactory cue presented on models. When a negative experience, such as male-male combat, is associated with the presence of a sensory cue, tortoises may learn avoidance of that cue or increase their investigation time prior to direct interaction. For example, insect pollinators have been shown to avoid certain cues when associated with a negative experience, such as the possibility of predation (Kelley et al., 2017). Similarly, in Kelley and Mendonça (2020), another paired-presentation of pooled MG secretions vs. DI water on models, more investigatory (e.g., sniffing, doubleback, scratching) or regulatory (e.g., eating) behaviors were found to significantly favor the MG treatment, but average time spent performing most behaviors was 30 seconds or less, suggesting possible avoidance of the MG-treated model, which could be associated with combative past experiences with dominant male tortoises and more pungent MG secretions. In lizards (*Sceloporus jarrovii*), a delay in chemosensory investigation was also seen for the most concentrated pheromone chemical presentation relative to other femoral chemical presentations (Romero-Diaz et al., 2021). Other studies in lizards and garter snakes have shown that concentrations of chemicals, especially due to increasing body size indicating dominance of larger animals, can be more informative to conspecifics than proportions of differing compounds (LeMaster and Mason, 2003; López et al., 2006; Martín et al., 2007; Khannoon et al., 2011). Even in chelonians, it has been more generally shown that males avoid strongly concentrated scents of other males in order to avoid costly male-male combats or agonistic interactions (Ibáñez et al., 2012).

Despite awareness of the MG-treated models at both the 1/4 and 1/20 dilutions, aggressive or agonistic displays (e.g., charging or high levels of biting) by male tortoises were not observed at any dilution. This lack of agonistic behaviors is in contrast to a similar study in which presentation of models treated with MG components induced combative displays of charging and flipping of MG-treated models took place in male Texas tortoises (Rose, 1970). On the other hand, head bobbing, which can be an aggressive display if performed between competing males, and carapace alignment, thought to be a more passive display, occurred at high durations during this study, especially during the 1/20 MG presentation. Auffenberg (1977) describes “lateral shell bumping” (e.g., which could be carapace alignment) in tortoises as a precursor to epiplastral ramming, pushing, or hooking, which are all aggressive displays between conspecific males. Head-bobbing is thoroughly observed in both aggressive competition displays and courtship displays by sexually mature male tortoises (Auffenberg, 1977; Sacchi et al., 2003; Sacchi et al., 2013). Therefore, because carapace alignment and head bobbing occurred significantly more towards the MG treatment at the 1/20 dilution when compared with other dilution experiments, these behavioral reactions could show preliminary displays of male dominance (e.g., without further stimulation or a behavioral trigger by the model itself) or could also show avoidance of a potentially aggressive encounter (e.g., not finishing the charging, ramming, etc.), indicating that male tortoises may have associated the MG secretions with the presence of another male, thus avoiding more contact as it appears was done for the 1/4 presentation. Indeed, PC1 (including approach, carapace alignment, head bobbing, tasting air, sniffing, and doubleback) was the only component that had a treatment effect for the MG dilution, in which the 1/20 presentation had higher values than the 1/500 presentation. The behaviors of PC1 collectively are very investigatory in focus, so it is possible that passive carapace alignment and head bobbing are the first challenges to another male tortoise while olfactory investigation takes place. Further support that male tortoises may have perceived the 1/20 MG presentation as "male" is the lack of performance of social behaviors that would lead to courtship or reproduction (e.g., no mounting, which did occur during the 1/100 and 1/500 presentations, or gentle biting of the carapace or forelimbs, which did occur during the 1/500 presentation).

Unlike the dominant displays observed at the more concentrated dilutions, mounting of tortoise models by male tortoises was observed during MG presentations at least concentrated dilutions (i.e., 1/100 and 1/500). Interestingly, mounting was not observed previously using pooled MG secretions (Kelley and Mendonça (2020), which may have been a consequence of avoidance or association with maleness. Significantly more mounting of models and higher differences for PC4 (i.e., mounting and head bobbing) were observed during the 1/500 dilution MG presentation relative to 1/20 dilution. Although all chemical presentations of MG secretions were diluted samples of male MG secretions, it is important to note that similar to the tortoise models used in Kelley and Mendonça (2020), the tortoise models used in this study did not have any physical characteristics of "maleness" (e.g., no enlarged mental gland regions or visibility of plastral concavity; Winoker and Legler, 1975; McRae et al., 1981). Therefore, the male MG secretions presented on the models were the only indication of sex. Combined, these results suggest male tortoises may have perceived models treated with the most dilute MG treatment as female by focusing exclusively on visual cues, rather than olfactory presentations.

Further supporting the hypothesis that male tortoises may have perceived dilute MG treatments as females, the highest number of behaviors were observed at 1/500 dilution. Behaviors observed at the 1/500 dilution included mounting, biting along the edge of forelimbs and carapace, and PC5 (including scratching/rubbing), all of which were significantly greater than for the 1/20 presentation. These behaviors are also commonly observed in tortoise courtship displays (Ruby and Niblick, 1994; Auffenberg, 1966; Auffenberg, 1977). Nevertheless, tortoises were unable to discriminate between treatments (i.e., MG vs. DI) at the 1/100 and 1/500 MG dilutions. If dilute MG presentations are perceived as more female and thus, eliciting more courtship behaviors (although not significantly), these random treatment effects in 1/100 and 1/500 may also indicate that MG secretions from females could be very weak in external pungency, relative to male MG secretions. Without strong olfactory determination between treatments in dilute presentations, male focal tortoises were likely more strongly relying on visual presence of the tortoise model rather than olfactory cues (if any), causing more courtship behaviors to be performed. Furthermore, a signaling tortoise may require tactile or receptivity cues that could not be presented by an inanimate model to stimulate full courtship and mating displays for this species (Auffenberg, 1977).

It is possible that the results of this study were confounded by the interaction of time of day and ambient temperature during respective experimental trials. All presentation trials were performed between 09:00 and 17:00 CDT and mean temperature for all trials was approximately 30°C. Both the time of day and the temperature intervals corresponded to field observations of peak tortoise activity during their active season (McRae et al., 1981; Boglioli et al., 2003; Johnson et al., 2007). Interestingly, time of day and temperature only significantly impacted PC2 (including both searching and approach behaviors; 1/4 & 1/20 presentations were significantly lower than 1/100 and 1/500 presentations for PC2), in which the most concentrated dilutions were less likely to be directly approached and less searching behavior occurred collectively. These findings of time of day and temperature affecting behaviors and potential social interactions highlights how behaviors might be variable or impacted due to impacts of potential climate change or increased global temperatures (Martín and López, 2013). In addition to potential aversion for more concentrated signals, time of day and temperature may also make tortoises less likely to interact with conspecifics, especially if elevated temperatures at certain times of day increase the degradation of the signal, thus making it more difficult to glean information in an ever-changing environment.

Conclusion

In summary, this study is the first study to determine a behavioral concentration-dependence reaction to social olfactory cues (in the form of mental gland secretion pheromones) between male gopher tortoise conspecifics. We determined that the 1/20 dilution was the optimum dilution for male gopher tortoises to spend the most time in treatment discrimination, especially for social behaviors such as carapace alignment and head bobbing, even more so than the most concentrated mental gland secretion presentation (the 1/4 dilution) that received mostly olfactory investigation only, suggesting some possible avoidance of stronger cues. In contrast, the most dilute mental gland secretion presentations (1/100 and 1/500) did not receive consistent treatment discrimination, in spite of an increased number of behaviors, which could mean that tortoises were relying more on visual cues and seeking tactile receptivity such as from a female tortoise that the model could not provide.

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Table 4: Review of chelonian behaviors in this study of Gopher tortoise males and other chelonian studies.

Behavior Name	**Definition**	**Other Names for Behavior (if any)**	**Other Species of Chelonian**	**Citation Where Observed**
Approach	Direct investigation of tortoise model	*Chase; Rapid linear movement*	*Sternotherus minor; Gopherus agassizzii; Gopherus berlandieri*	Bels and Crama, 1994; Ruby and Niblick, 1994; Kazmaier et al., 2001; Kelley and Mendonça, 2020
Carapace Align	Line carapaces in parallel with model; side by side	*Side display*	*Gopherus agassizzii*	Ruby and Niblick, 1994; Kelley and Mendonça, 2020
Head Bob	Vigorous vertical head shaking; some slow nodding	*Level, slow, or rapid head bobs*	*Gopherus agassizzii; Marginated, Greek, and Hermann's tortoises; etc.*	Ruby and Niblick, 1994; Liu et al., 2013; Sacchi et al., 2013; Kelley and Mendonça, 2020; Kelley et al., 2021
Head Extend	Head extended far out of carapace, usually over model's head or carapace	*Explore; High stand, head high; Neck extension*	*Gopherus agassizzii*	Ruby and Niblick, 1994; Kelley and Mendonça, 2020; Kelley et al., 2021
Nose Interaction	Nose-to-nose with model; direct facial contact	*Head to head; Sniff face to face*	*Sternotherus minor; Gopherus agassizzii*	Bels and Crama, 1994; Ruby and Niblick, 1994; Kelley and Mendonça, 2020
Mount/Climb	Interaction in which tortoise climbed on model; mount, when climbing occurred for several seconds and/or copulatory organs displayed	*Aggressive climb;*	*Sternotherus minor; Gopherus agassizzii; Marginated, Greek, and Hermann's tortoises*	Bels and Crama, 1994; Ruby and Niblick, 1994; Sacchi et al., 2013; Cutuli et al., 2014; Kelley and Mendonça, 2020
Doubleback	Walk passed model and immediately turn back	*Circling; Rotate; Circular moving*	*Gopherus agassizzii; Marginated, Greek, and Hermann's tortoises; etc.*	Ruby and Niblick, 1994; Sacchi et al., 2013; Kelley and Mendonça, 2020; Kelley et al., 2021
Searching	Hyper vigilance; looking all around pen	*Scanning; Looking around; Observing*	*Gopherus agassizzii; Marginated, Greek, and Hermann's tortoises; etc.*	Ruby and Niblick, 1994; Sacchi et al., 2013; Kelley and Mendonça, 2020; Kelley et al., 2021
Sniffing	Physical moving of the nares, either directed toward ground, model, or air	*Exporatory sniff; Nose explore*	*Sternotherus minor; Gopherus agassizzii; Marginated, Greek, and Hermann's tortoises*	Bels and Crama, 1994; Ruby and Niblick, 1994; Sacchi et al., 2013; Kelley and Mendonça, 2020; Kelley et al., 2021
Rest Near	Passively sitting in the vacinity of model without acknowledging it	*Sitting; Basking*	*Gopherus agassizzii; Gopherus berlandieri*	Ruby and Niblick, 1994; Kazmaier et al., 2001; Kelley and Mendonça, 2020; Kelley et al., 2021
Eat Near	Foraging grass near model without engaging with model	*Mashing; Food bite; Chewing; Rapid foraging movement*	*Gopherus agassizzii; Gopherus berlandieri*	Ruby and Niblick, 1994; Kazmaier et al., 2001; Kelley and Mendonça, 2020; Kelley et al., 2021
Tasting Air	Biting at the air near model	*Yawn*	*Gopherus agassizzii*	Ruby and Niblick, 1994; Kelley and Mendonça, 2020
Pull in Shell	Rapidly pulling into carapace; perceived fear behavior	*Head defensive; All defensive*	*Gopherus agassizzii*	Ruby and Niblick, 1994; Kelley and Mendonça, 2020
Biting	Physically biting forelimbs or carapace of model	*Head jerk in; Nip; Hold bite; Foot bite*	*Sternotherus minor; Gopherus agassizzii; Marginated, Greek, and Hermann's tortoises; etc.*	Bels and Crama, 1994; Ruby and Niblick, 1994; Liu et al., 2013; Sacchi et al., 2013; Cutuli et al., 2014; Kelley and Mendonça, 2020; Kelley et al., 2021
Charging	Running at model or attempt to flip it over	*Ramming; Flip; Fall over; Running after*	*Gopherus agassizzii; Marginated, Greek, and Hermann's tortoises; etc.*	Ruby and Niblick, 1994; Sacchi et al., 2013; Cutuli et al., 2014; Kelley and Mendonça, 2020
Scratch/Rub	Using forelimbs to scratch model or rubbing head on model carapace	*Chin rub; Stroke; Shell scratch; Foreclaw display*	*Gopherus agassizzii, etc.*	Ruby and Niblick, 1994; Liu et al., 2013; Kelley and Mendonça, 2020

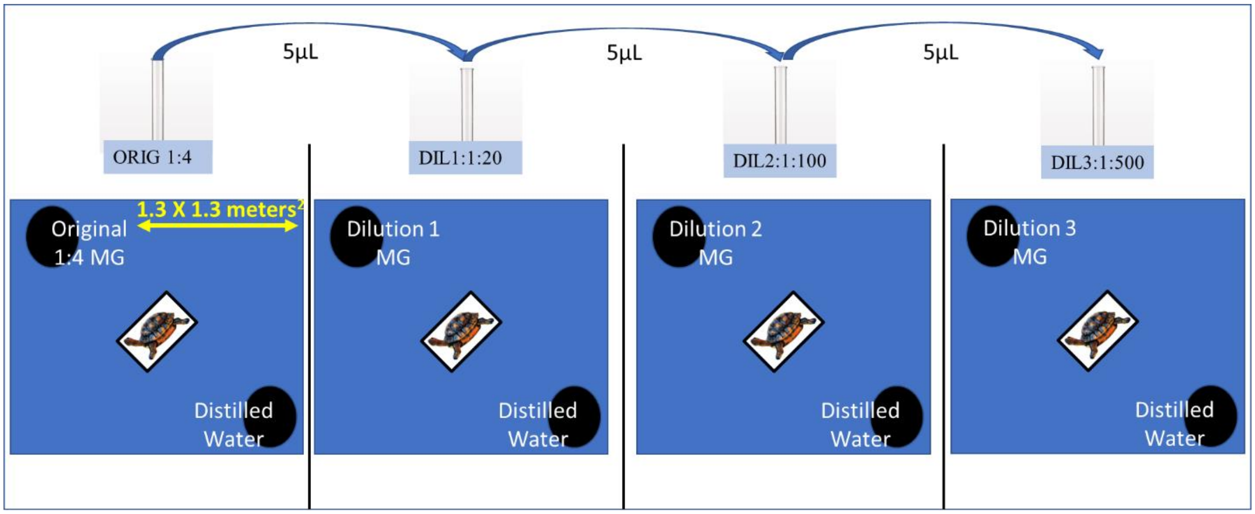


Figure 5: Serial dilution of male gopher tortoise mental gland (MG) secretions used in the four presentations and experimental design of presentations in the behavioral arena.

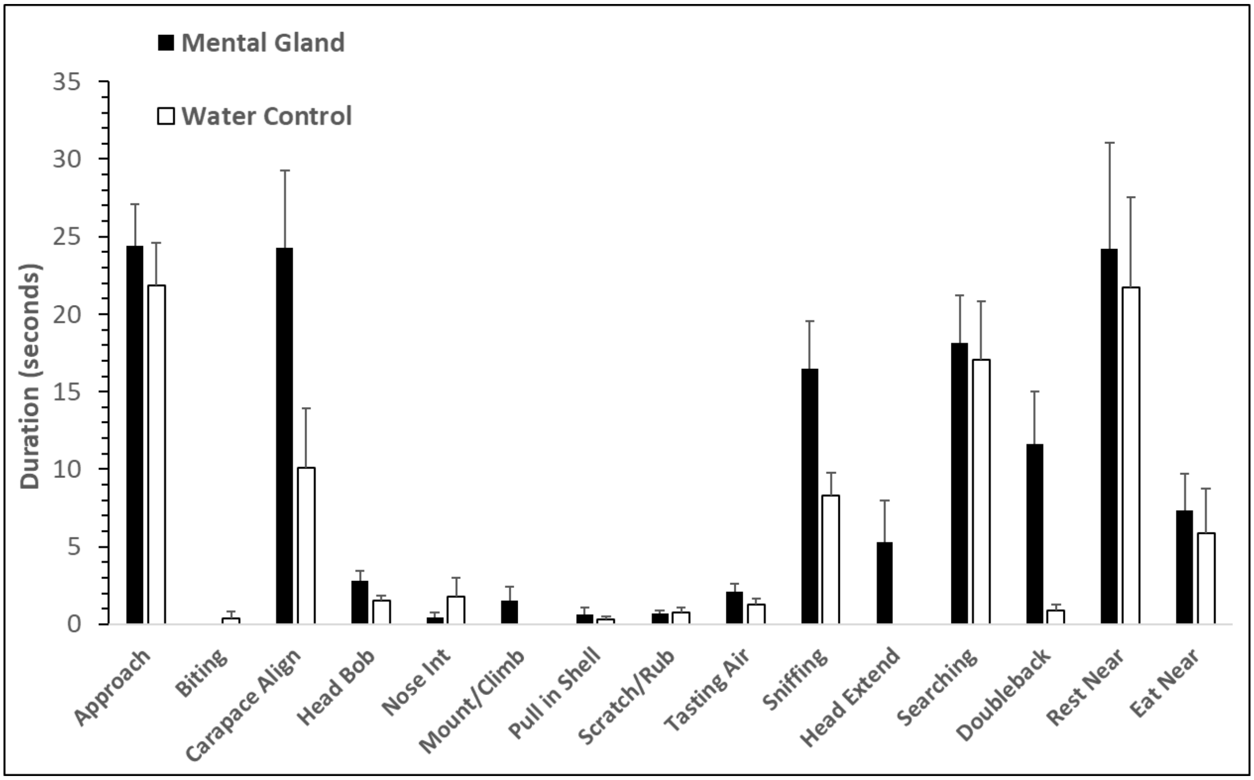


Figure 6: Average total time (in seconds) spent performing individual behaviors towards either treatment (MG experimental vs. DI control). Measures of spread indicated are Average Time ± Standard Error (SE).

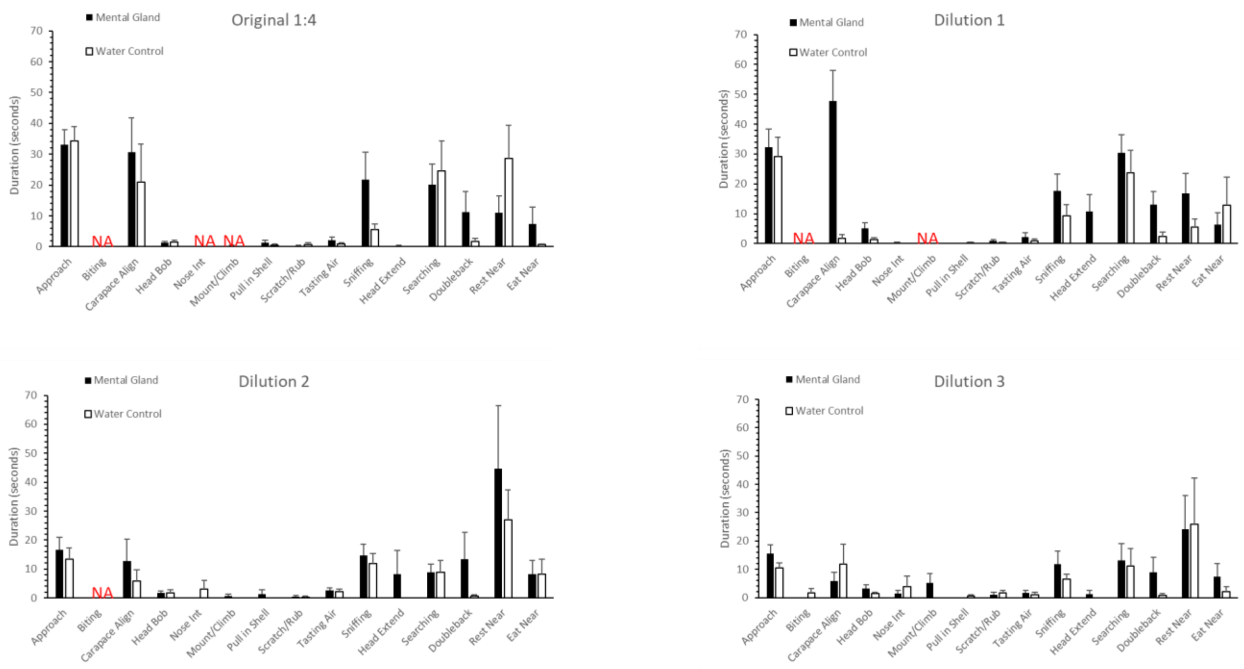
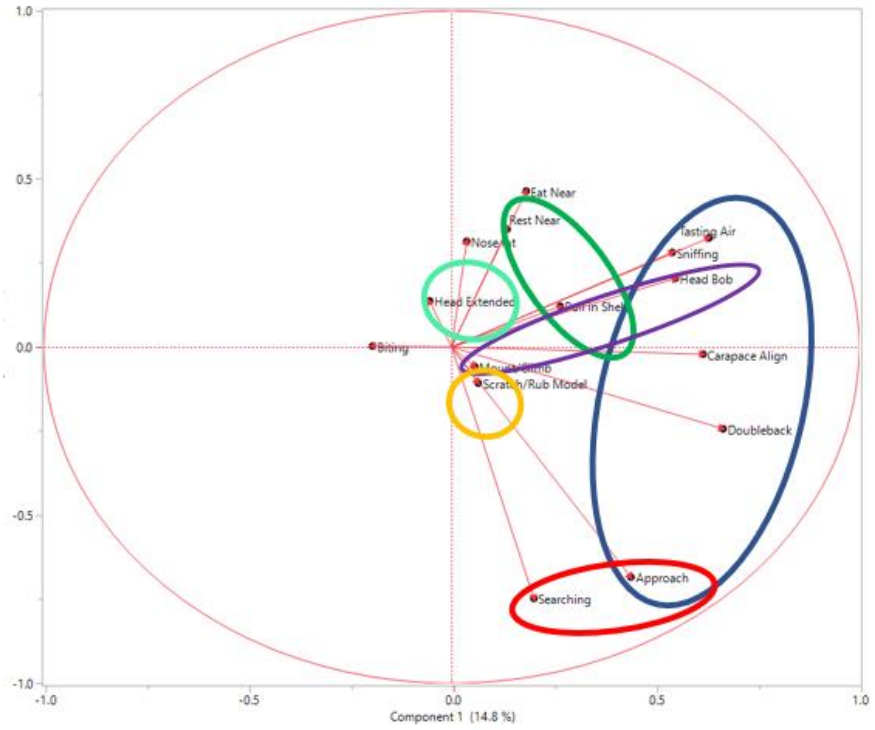


Figure 7 (a-d): Average time (in seconds) spent performing individual behaviors for each diluted MG presentation (1/4, 1/20, 1/100, and 1/500, respectively). Measure of spread indicated is Average Time ± Standard Error (SE).



PCA Correlated Behavior Groupings:

PC1: Approach; Carapace align; Head bob; Tasting Air; Sniffing; Doubleback
PC2: Approach; Searching
PC3: Pull in Shell; Resting Near
PC4: Head bob; Mount/climb
PC5: Scratch/Rub
PC6: Head Extension

=12 behaviors correlate in PCA

Figure 8: Multivariate principal components groupings of significant behaviors, using only loadings values that ≥ 0.5 or 50% correlated.

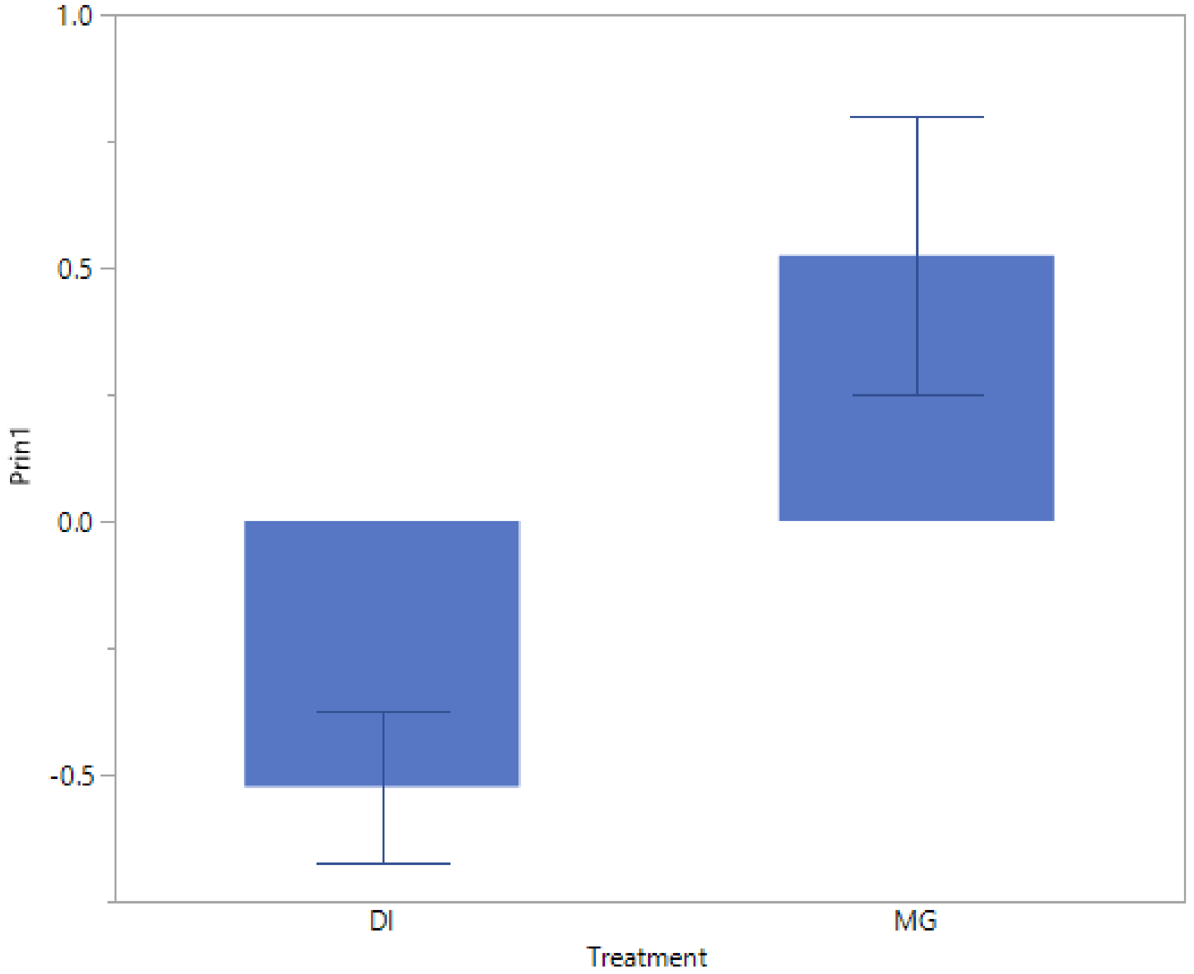


Figure 9: Principal component 1 as indicated by treatment differences, in which the mental gland (MG) treatment was significantly higher than the DI water control. Measures of spread are orthogonal values ± standard error (SE).

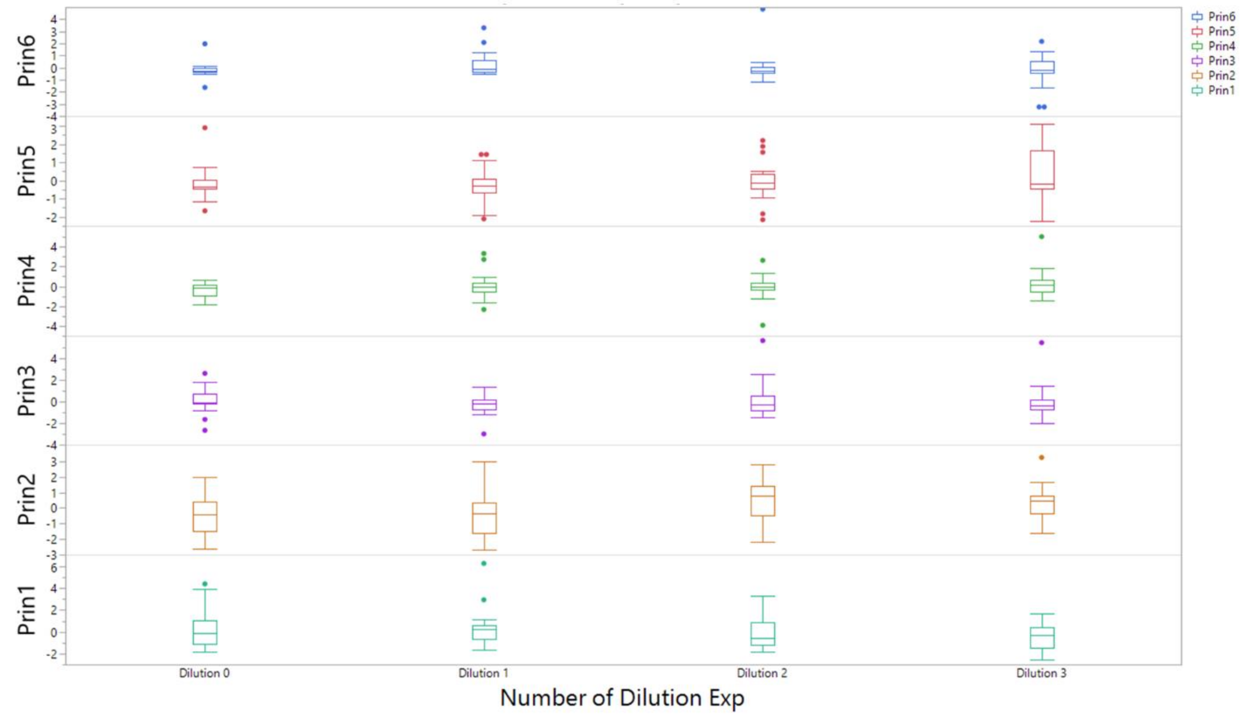


Figure 10: Six principal components for each dilution presentation box and whisker plots.

Measures of spread indicate orthogonal values of principal components ± standard error (SE).

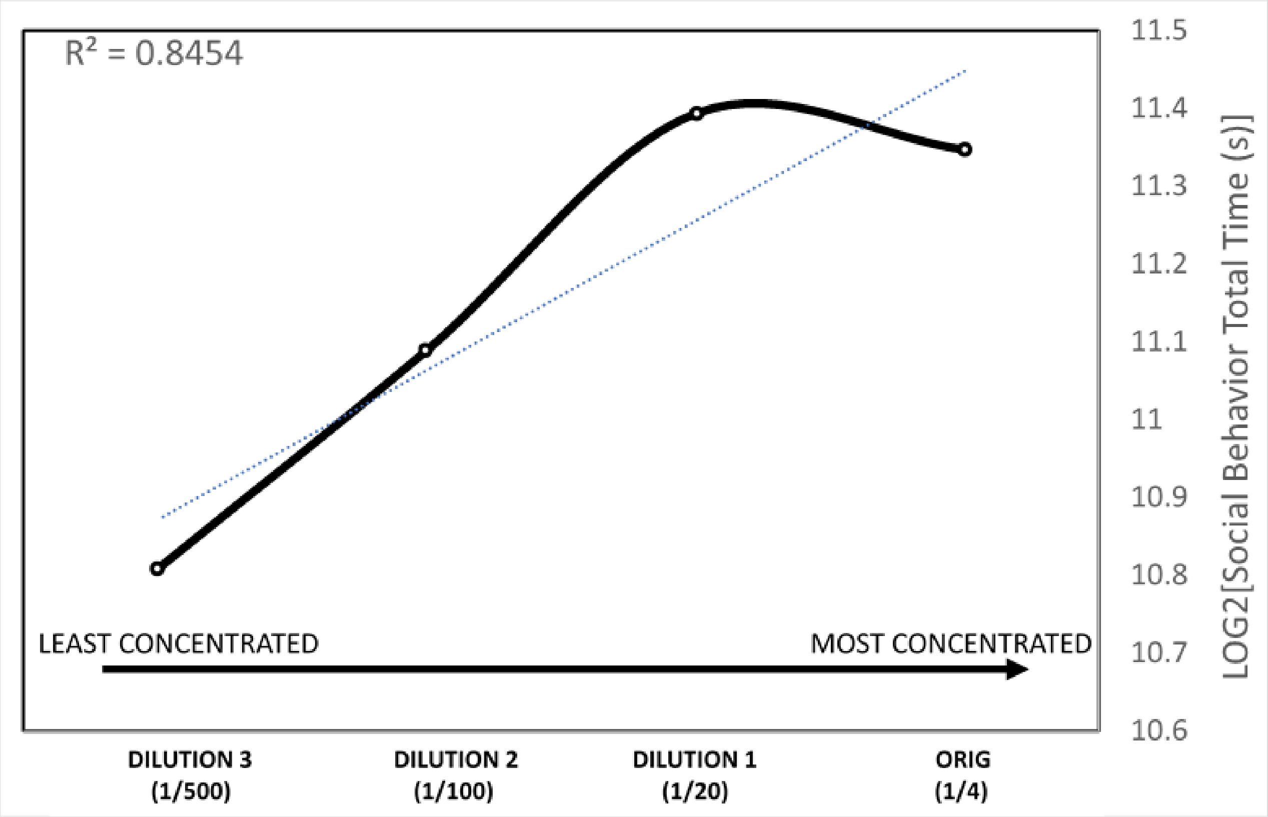


Figure 11: The total time (in seconds) spent performing social behaviors towards either treatment for each dilution of mental gland (MG) secretions indicates a concentration-dependent preferential response, favoring the 1/20-DIL 1 presentation overall. Data arranged from least to most concentrated and log2-transformed for linear comparison.

CHAPTER FOUR

CHEMICAL ANALYSIS OF MENTAL GLAND SECRETIONS OF BOTH SEXES OF THE GOPHER TORTOISE (*GOPHERUS POLYPHEMUS*) USING GAS CHROMATOGRAPHY-MASS SPECTROSCOPY

Introduction

Chemical signals shared between conspecifics, also known as pheromones, have been widely studied in a multitude of taxa, but have been predominantly identified and characterized for many insects, leaving vertebrate chemical ecology still largely unexplored (Eisner and Meinwald, 1995; LeMaster and Mason, 2003; López et al., 2006; Wyatt, 2014). In particular, characterized pheromones in insects have been able to provide insight into social and colonial behaviors that have yet to be examined in many vertebrate groups (e.g., >300 chemical structures of pheromones have been identified in over 1600 species, 90 families, and 9 orders of insects with an emphasis on Lepidopterans; Roelofs, 1995). However, similarities in species-specific signaling compounds can be found for many signaling animals, depending on the type of chemical signal being transmitted (e.g., attractant, alarm, etc.) and the environment in which the signal must be sustained in order to deliver its ‘message’ to a receiving conspecific (Alberts, 1992; Wyatt, 2010; Apps et al., 2015). For example, in many moth species’ pheromone-excreting glands and in the cuticle of other insects, biosynthetic pathways of fatty acid-derived molecules also can be formed into acetates, alcohols, aldehydes, and other potential chemical classes (e.g., esters and ketones), which can chemically provide species specificity and be used as attractant pheromones (Mason et al., 1989; Roelofs, 1995). In amniotes, lipids and free fatty acids in the skin play a large role in the keratin-epidermis complexes (that result in decreased integumentary water loss, allowing an increased terrestrial life). These lipids can also be secreted in volatile (e.g., airborne scents directly off the body) and nonvolatile (e.g., secreted markings in the environment) chemical signals (Menon et al., 1996; Weldon et al., 2008). Therefore, amniotes, reptiles in particular, have the ability to utilize lipids in biosynthetic production of identified pheromones (volatile fatty acids and aldehydes in lizards: Campos et al., 2020; fatty acid-methyl ketones in garter snakes: Mason et al., 1989; fatty acid-methyl esters in tortoises and possibly crocodilians: Rose, 1970; Weldon et al., 2008).

Pheromone usage has been broadly classified via behavioral responses in reptiles towards whole chemical secretions relative to controls (Price and LaPointe, 1981; Mason, 1992; Kelley and Mendonça, 2020; Kelley et al., 2021). However, few reptilian model systems have determined both the chemical components of specific secreting-exocrine glands that could be involved in pheromone production and associated chemical differences among ecological categories (e.g., sex, body size, location/populations differences, etc.). To date, the best studied reptilian model systems of chemical secretions used as pheromones include the integument or cloacal glands of garter snakes and the femoral glands of lizards. Red-sided garter snakes are capable of recognizing sex and body size via concentrations of long chained-saturated and unsaturated methyl ketones in the skin (Mason et al., 1989; 1990; Shine et al., 2003). Sex and body size have also been behaviorally indicated in chemical secretions of lizards by conspecifics, especially through the concentration of ergosterol, which indicates a more dominant male (López and Martín, 2012; Martín and López, 2012; López et al., 2006). Location differences of the same species have also been determined in chemical components of pheromones in differing populations of both snakes (LeMaster and Mason, 2003) and lizards (Gabirot et al., 2012). In contrast, little work has been done in chemically analyzing exocrine-secreting glands of turtles and tortoises (chelonians). For example, studies found chelonians able to distinguish conspecifics by sex (Muñoz, 2004; Poschadel et al., 2006; Ibáñez et al., 2012), body size (Ibáñez et al., 2012; 2014), and possibly location via recognition of familiar versus unfamiliar conspecifics (Alberts et al., 1994; Tuberville et al., 2011; Ibáñez et al., 2013), but it is unknown which chemical compounds might be causing these behavioral discriminations.

Chelonians have been widely studied for behavioral awareness of olfactory signals from conspecifics, but few studies have examined the chemical classes of the products produced by their exocrine-secreting glands. For example, one of the oldest studied scent glands unique to chelonians includes Rathke's glands, which are inguinal and axillary pores that occur along the plastron of most turtles except those from the family Testudinidae (tortoises; Ehrenfeld and Ehrenfeld, 1973). However, using scent cues from water of freshwater turtles (likely containing a mix of secretions from Rathke's, cloacal, or other integumentary glands), many studies have shown that some freshwater turtles orient themselves to the scent of conspecifics relative to controls (*Sternotherus odoratus*: Lewis et al., 2007; *Mauremys leprosa*: Muñoz, 2004; *Emys orbicularis*: Poschadel et al., 2006). It is still unclear, however, which chemical signals in these secretions are present in these studies to cause this orientation.

Another unique exocrine-secreting chelonian gland is the chin or mental gland. Tortoises (*Gopherus spp.*) have the largest subdentary mental glands of all chelonians (e.g. Type 1 mental glands are an identifying feature of the genus; Winoker and Legler, 1975). Studies have indicated mental gland secretions elicited both awareness of (e.g., increased olfactory investigation of secretions by conspecifics; Bulova, 1997) and discrimination by individuals in their environments (Weaver, 1970; Rose, 1970; Alberts et al., 1994; Tuberville et al., 2011; Kelley and Mendonça, 2020; Kelley et al., 2021). Rose et al. (1969) and Rose (1970) documented the general chemical classes (e.g., fatty acid-methyl esters and proteins), dominating *Gopherus spp.*

mental gland secretions with some sexually dimorphic variation, but no recent work has characterized the chemical classes further nor determined the ecological relevance of chemical signaling from these glands in this group.

In this study, we aimed to study the biochemistry of the mental glands of both sexes of the gopher tortoise (*Gopherus polyphemus*), which is a threatened species endemic to the southeast U.S. longleaf pine ecosystem (Diemer, 1986). The purpose of this work is twofold. First, characterization of general chemical components in gopher tortoise mental glands that might be involved in signaling ecology (i.e., cues to facilitate social interactions, potentially leading to finding mates in highly fragmented habitats or increasing courtship or mating behavior). Second, identification of individual mental gland chemical components that indicate morphometric or ecological characteristics about individual tortoises, such as sex, body size, or location.

Methods and Materials

Study Populations

Gopher tortoises were wild captured from four populations in Alabama, including Fort Rucker Army & Aviation Base [Coffee (GPS: 31.287347, 85.788226) and Dale Counties (GPS: 31.300007, 85.689266)], Solon Dixon Forestry Education Center (SDFEC; Covington County; GPS: 31.157119; 86.755901), Autauga Wildlife Management Area (AWMA; Autauga County; GPS: 32.612333, 86.548139), and private property in Macon County (GPS: 32.482778, 85.472778; Figure 12). Fort Rucker (FR) and Solon Dixon (DC) are located in southern Alabama below the fall-line and had the most dense tortoise populations we encountered, while Autauga and Macon tortoise populations were above the fall-line and much more sparsely populated. Since fewer tortoises were collected from these sites overall, we combined these individuals under the location “Northern Populations” (NP). Adult male and female tortoises were selectively captured via Tomahawk live animal traps at their burrows during the active mating season (June through September in 2016 and 2017; Ott et al., 2000) when tortoises would be most likely to utilize chemical signaling to seek each other out for mating opportunities. In order to be included in this study, tortoises of either sex had to be a total length of >180 mm (i.e., adulthood size; McRae et al., 1981), have enlarged and secreting mental glands, and not exhibiting active symptoms of respiratory disease (Jacobsen et al., 1991).

Mental Gland Examination and Secretion Collection for Chemical Analysis

Mental glands of all captured tortoises were examined externally and measured for volume [1/3π*(Length/2 x Width/2 x Depth); Alberts et al., 1994]. Males had consistently larger mental glands (MG) on average (captured in 2016: mean MG volume ± standard error: 48.24 ± 3.76 mm^3; 2017: 56.43 ± 4.1 mm^3) than females (captured in 2016: 13.07 ± 1.36 mm^3; 2017: 18.72 ± 1.89 mm^3), which is also consistent with the findings of other *Gopherus spp.* tortoise studies (Winoker and Legler, 1975). Briefly, secretions were collected by gently squeezing and palpating the mental gland secretions into a glass hematocrit capillary tube, which was stored in snap cap eppendorfs at -80˚C until samples could be processed further (similar to methods used in Kelley and Mendonça, 2020; Kelley et al., 2021). Tortoises kept in captivity were repeatedly sampled for MG secretions one time per month for all the months (June, July, August, September) that they were temporarily housed at Auburn University’s animal handling facility. All tortoises were released to their exact points of capture at the conclusion of the study, late in September of each collecting year.

Sample Preparation

MG secretions were removed from -80˚C storage and allowed to thaw; hematocrit tubes were weighed for secretion subtracted volume, and expelled into a 200µL glass vial insert with a total volume of 40 µL of 3 chloroform : 1 methanol. The hematocrit tube was weighed after drying for 10-15 min and the average weight was found to be less than 2 mg. Each evacuated sample was agitated via vortex for 30 seconds and placed in a water bath sonicator for a total of 15 minutes to ensure that the MG secretion residue were mixed thoroughly with the solvent. The dissolved liquid-only solution was collected into another glass vial insert and sealed into a crimp cap autosampler vial. Samples were not derivatized to focus the analysis on volatile (potentially pheromone) compounds, and the column is capable of analysis of fatty acids without the need for derivatization.

A total of 18 pure chemical standards (>98% Analytical grade standards; Sigma Aldrich) were purchased for verification in MG samples, including 13 fatty acids [stearic acid (catalog #: 85679), octanoic acid (catalog #: C2875), decanoic acid (catalog #: C1875), myristic acid (catalog #: M3128), oleic acid (catalog #: O1008), linoleic acid (catalog #: L1376), linolenic acid (catalog #: L2376), palmitic acid (catalog #: P0500), palmitoleic acid (catalog #: P9417), malonic acid (catalog #: M1296), propionic acid (catalog #: 402907), and benzoic acid (catalog #: 242381) and phenylacetic acid (catalog #: P16621; benzoic and phenylacetic acid are also aromatics)], three ketones [2-heptanone (catalog #: 537683), beta-ionone (catalog #: I12603), and 2-butanone (catalog #: 34861)], one methylated ester [methyl benzoate (catalog #: M29908)], and one steroid [cholesterol (catalog #: C8667)]. After preliminary examination of tortoise samples, a range of each verified standard was calculated and diluted into concentrations appropriate for measure. Standards were dissolved into 3:1 chloroform: methanol solutions for their appropriate concentration in a final volume of 5 milliliters each, and a series of 1:5 dilutions were created for each standard for the creation of a standard curve. Two master mix solutions of varying high vs. low concentrations of relative standards were also created to verify with MG samples as they were run on a gas chromatography-mass spectroscopy (GC-MS) machine.

Sample peaks were verified to be present in at least three samples with a similar for each retention time and mass spectra. The aforementioned master mix of standards were used to calculate the concentration of the compounds that had the same retention time and spectra in the samples.

GC-MS Analysis

A total of 80 individual samples (N=40 for males and females; body size and location sample sizes vary; see *Statistical Analysis* for details) were analyzed on an Agilent 6890 GC with a 5975 MS by injecting 1 µL in splitless mode onto a Stabilwax-DA column 30m x 250 µm with 0.25 µm film thickness (Restek part # 11023). The inlet temperature was 250 °C and was purged 1 minute after the sample had been injected. The initial oven temperature of 50 °C was held for 4 min, increased to 180°C at 50°C/min, then increased to 260 °C at 3°C/min where the temperature was held for 27 min. The MS transfer line was kept at 250 °C, the source was at 250 °C and the quad temperature was at 150 °C. Calibration curves and sample concentrations were determined with Enhanced Chemstation Software. Files were exported with the same software into the .CDF format for analysis with mzMine 2.29. If the chromatogram appeared similar to a blank solvent injection, the sample would be injected again. Due to the volatile nature of the solvent, some samples were also diluted to the original volume and re-injected. Due to the low amounts of secretion, 6 of the samples still appeared blank and were not included in the data processing.

Data Processing

The netCDF files were imported into MZmine software (Pluskal et al., 2010) and processed with the ADAP-GC software tool using the unit mass resolution parameters (Smirnov et al., 2018) with the following modifications. Mass detection was completed in centroid with a noise level of 0. Scans were filtered for the mass range of 46 to 550 and baseline was corrected with the RollingBall method (wm 200, ws 5) using m/z bins with a width of 1. The ADAP chromatogram builder was used with min group 5, group intensity threshold 2e2, min highest intensity 2e2, mz tolerance 0.05 m/z or 5000 ppm. Chromatograms were smoothed with a filter width of 11. Then chromatograms were deconvoluted with ADAP wavelets S/N threshold 10, min height 11, coefficient/area 200, peak duration range 0.2 to 10, RT wavelet range 0.1 to 1.0. The samples were aligned with the join aligner with m/z tolerance 0.2 or 5000 ppm, 5% relative retention time tolerance, and equal weight for m/z and RT. The ADAP and MZmine data processing workflow occasionally created different features with very similar retention times and spectra. There may actually be differences between these features and the differences may be apparent when a GCxGC instrument and/or higher resolution mass spectrometer is used for the sample analysis. Gap filling was performed and then the resulting aligned peak list was exported in the metaboanalyst format and subsequently imported into metaboanalyst (Chong et al., 2018) for statistical tests and graph generation. Compounds were identified with AMDIS and NIST 2.0, and with the similarity search in the mass bank of north America (MONA).

Statistical Analyses

The GC-MS peak spectra data matrix was exported into metaboanalyst 5.0, including a format of unpaired columns of individual samples and unpaired rows identified by features (i.e., number of peak inflection; retention time of peak; chemical charge, or m/z of peak). All data was log-transformed with every sequential analysis for each grouping. In the identifying row below sample names, categorical classifications were given for each sample for each analysis as allowed by metaboanalyst (e.g., by sex, by body size, and by location). For sex (N=40 for each sex), individual samples were given a “0” for female or a “1” for male. Following the sex analysis, for simplicity, tortoises were separated for *male only* and *female only* analyses of other parameters (body size and location). For body size (Sample sizes for Males: Large= “1”=13; Small=”2”=27; Females: “1”=26; “2”=14), individual tortoises that were greater than 250 millimeters in length were given a “1,” and individuals that were less than 250 millimeters in length were given a “2.” For location, in initial analyses, three locations were indicated, including Fort Rucker (FR=“1”), Solon Dixon (DC=“2”), and Autauga and Macon Counties called Northern Populations (NP=“3”). However, due to small sample sizes even with the pooling of the Northern Populations, an ANOVA found inconclusive results, causing the removal of the Northern Populations from the location analysis in favor of more stable sample sizes in the two more southern populations (Sample sizes for Males: FR=”1”=20; DC=”2”=16; Females: “1”=19; “2”=21). Therefore, all analyses reported in this study were for binary categorical groupings, using t-tests. All analyses were performed in metaboanalyst 5.0 for chemical spectral data, and all significance values were set to $p \leq 0.05$.

Results

General Chemical Analyses

We found a total of 1189 peaks with unique mass spectra within this dataset that corresponded to at least 69 identified chemicals (via database matching and chemical standards; see Table 5 and the corresponding numbered peaks in chromatograms in Figure 13A & 13B).

Predominant identified chemical classes found in the GC-MS analysis with greater than 70% matching in the NIST database, included primarily **amides** [AD; 12; Pyrolo[3,2-d]pyrimidin-2,4(1H,3H)-dione; 2-Amino-acetamide; 2-Aminocyanoacetamide; Ethyl urea; 1,3,5-Triazine-2,4,6(1H,3H,5H)-trione; N-Methoxymethyl-N-methylformamide; N-methyl-N-nitro-1-butanamine; N-Acetyl-dl-alanine methylamide; N-formamidoformamide; 2-Diethylamino-N-p-tolyl-acetamide; L-5-Propylthiomethylhydantoin; and Ethanediamide]; **esters** [ES; 10; 2-Nitroethyl propionate; Methyl carbamate; Methyl benzoate; 2-Chloroethyl dimethyl phosphate; 1,3-Dioxolan-2-one; DL-Norvaline ethyl ester; 4-methylpentyl 2-(methoxycarbonylamino)acetate; Methyl 2-methyl-2-(methoxy)amino-propanoate; Leucine methyl ester; and Ethyl 3,4-dimethyl-1H-pyrrole-2-carboxylate]; **fatty acids** [FA; 9; Propanoic acid; Isocrotonic acid; Butanoic acid; 2-Ethylbutanoic acid; Octanoic acid; Myristic acid; Palmitic acid; Stearic acid; and Oleic acid]; **aromatics** [AR; 8; 1,4-dihydroxy-2,3-dimethylanthracene-9,10-dione; 1-(6-Methoxy-4-methyl-3-quinolinyl)-3,4-dimethyl-1H-pyrazol-5-ol; 2-(4-Methoxy-phenoxy)-6-methyl-4-phenyl-quinazoline; Benzhydrazide; Benzoic acid; Phenylacetic acid; N-(2-benzoyl-3-benzofuryl)-2-diethylamino-acetamide; and 9-Acetylhydrazono-3,6-dichloro-2,7-bis-[2-(diethylamino)ethoxy]fluorene]; **amines** [AM; 8; 4H-1,2,4-Triazol-4-amine; N-Nitrosodimethylamine; (1-Ethyl-2-methylpropyl)methylamine; Thiazolidine; 1,1-Dimethyl-2-ethylhydrazine; Guanylurea; N,N-dimethyl-3-Octananime; and N-Methoxy-N-methylamino-N,N-diethylaminomethane]; **alcohols** [OH; 7; 4-methyl-1-hexanol; Diethanolamine; 4-methyl-1-pentanol; 1-(furan-2-yl)but-3-ene-1,2-diol; 3-(Diethylamino)-1,2-propanediol; dl-2-Amino-1-hexanol; and Methyldiethanolamine]; **fatty acid methylated esters** [FAME; 3; Methyl hexadecanoate; Methyl octadecenoate; and Unidentified compound #27] ; **steroids** [ST; 3; 3-Hydroxy-16,16-dimethoxyandrost-5-en-17-one; Cholesterol; and Unidentified compound #8]; **ketones** [KT; 2; 2,3,3-trimethyl-cyclobutanone and 2-Butanone]; ethers [ETH; 2; 1,2-Dimethoxy-ethene and 2-methyl-2-(4-methyl-3-methylenepentyl)-1,3-dioxolane]; and unidentified/unknown classes (UNK; 2). Some compounds were preferentially labelled aromatic because aromatic compounds could be preferentially found as pheromones, but multiple chemical classes could be indicated for more than one molecule found in this study. Future studies will characterize some of the compounds which resulted in significant behavioral modification.

Chemical Standard Verification

Following chemical identification with chemical databases (NIST and MONA), compounds were also identified via pure chemical standards from a standard curve calculated in the same GC-MS run as the samples. Of the 18 chemical standards used in master mixes, 10 standards were chemically verified in mental gland secretion samples, including 7 out of 13 fatty acid standards (Stearic acid, Myristic acid, Oleic acid, Benzoic acid, Octanoic acid, Palmitic acid, and Phenylacetic acid), 1 out of 3 ketone standards (2-Butanone), and the methyl ester (Methyl Benzoate) and steroid (Cholesterol).

Ecological Parameters

Several different metabolites were found to be significant factors that seem to correlate with tortoise sex, body size, and location of capture. Some of the metabolites were identified with commercially available standard compounds while others have been determined by comparison to databases. In analyzing separately for sex, body size, and location, an additional 9 compounds were identified, in addition to stearic acid, which was already identified via databases and through pure standard comparisons.

Sex

When comparing male to female, three chemical compounds were found to significantly differ by sex (Figure 14 and Table 6), including mucic acid (101/126.1952 mz/ 8.82 minutes retention time; p=0.01), 13-methyltetradecanoic acid methyl ester (8/143.1976 mz/13.45 minutes retention time; p=0.01), and methyl (Z)-10-pentadecenoate (6/264.3029 mz/ 13.76 minutes retention time; p=0.03). Compounds 13-methyltetradecanoic acid methyl ester and methyl (Z)-10-pentadecenoate were more abundant from female tortoise MG secretions, whereas mucic acid was more abundant in male tortoise MG secretions.

Body Size

When comparing large to smaller body sizes in separate male and female analyses (Figure 15 and Tables 7 & 8), one compound was identified in both males and females [methyl (Z)-10-pentadecenoate: females (11/98.1005 mz/ 13.74 minutes retention time; p=0.02) and males (68/123.2000 mz/ 13.68 minutes in retention time; p=0.04). The male tortoises also had a compound that could not be identified which also correlated with body size (80/292.5696 mz/17.21 minutes retention time; p=0.046). For both males and females, larger tortoises had significantly more methyl (Z)-10-pentadecenoate in their mental gland secretions.

Location

For location differences between Solon Dixon and Fort Rucker tortoises (Figure 16), males and females were again analyzed separately, and 6 compounds were noted to be statistically different (Figure 17 and Tables 9 & 10). In males, compounds that differed by location included: 10-methylundecanoic acid methyl ester (28/267.2001 mz/ 10.72 minutes retention time; p=0.049); 2-(trimethylsilyl)oxy-eicosanoic acid trimethylsilyl ester (4/355.3947 mz/ 10.72 minutes retention time; p=0.006); 1-hexadecanol (56/ 111.1856 mz/ 16.31 minutes retention time; p=0.01); lauric acid (57/97.1253 mz/ 22.77 minutes retention time; p=0.04); stearic acid (18/55.0193 mz/ 27.60 minutes retention time; p=0.04); and 7-hydroxyoctanoic acid (inflections 34/97.1566 mz/ 8.83 minutes retention time; p=0.01 and 66/55.0687 mz/ 8.86 minutes retention time; p=0.005). There were also two unidentified compound peaks (14/281.3952 mz/ 16.37 minutes retention time and 104/97.1566 mz/ 8.83 minutes retention time; both p-values < 0.04). Fort Rucker male tortoises produced larger amounts of lauric acid, stearic acid, 1-hexadecanol, and one unidentified compound. Solon Dixon male tortoises produced larger amounts of 7-hydroxyoctanoic acid, 10-methylundecanoic acid methyl ester, 2-(trimethylsilyl)oxy-eicosanoic acid trimethylsilyl ester, and one unidentified compound.

For females, only one compound was identified despite 5 inflection identifications of the same chemical (7-hydroxyoctanoic acid; all p-values < 0.03) and one unidentified compound (14/281.3952 mz/ 16.37 minutes retention time; p=0.02). All inflection points for 7-hydroxyoctanoic acid and the unidentified compound were all greater in Solon Dixon females, relative to Fort Rucker tortoises.

Discussion

In general, chemical signals and pheromones used to communicate chemically with conspecifics may exhibit relative patterns of overarching messages (e.g., indicators of sex and body size or condition) that help to provide the background framework for an individual's chemical profile (also called an individual's specific signature mixture), which can vary greatly depending on a number of factors (Wyatt, 2010; 2014). Some of these factors include the type of secretion, individual hormone and immune biochemistry, location effects caused by diet or climatic conditions, or bacterial symbionts and their metabolic byproducts (Wyatt, 2010). In this study, we found and verified approximately 78 total compounds comprising approximately 11 differing chemical classes in mental gland secretions of both male and female gopher tortoises (*Gopherus polyphemus*), showing relative chemical differences in not only sex and body size, but also effects of location for both sexes. To our knowledge, this is the first study to finely document the chemical profile of mental gland secretions in both male and female gopher tortoises (Rose et al., 1969; Rose, 1970; Winokur and Legler, 1975).

In this study, the predominant chemical classes found in gopher tortoise mental gland secretions were amides, esters, aromatics, fatty acids, amines, hydroxyls, and other lesser represented groups (e.g., fatty acid methyl esters, ketones, steroids, and ether molecules; see Table 5 and Figures 13A & 13B). One important point to note is that the hot and humid conditions of longleaf pine ecosystems in the southeastern U.S., where the gopher tortoise resides, in addition to the presumed sandy soil substrate, may differentially affect the transmission and diffusion rates of differing chemical compounds (Campos et al., 2020). However, our findings are similar to and consistent with that of other studies investigating tortoise mental gland secretions in other *Gopherus spp.*, with some new additional chemical classes also found in this study.

Lipids and fatty acids

In male Texas tortoises (*Gopherus berlandieri*), free fatty acids such as capric, caprylic, myristic, palmitic, palmitoleic, oleic, stearic, lauric, and linoleic acids were previously found in mental gland secretions (Rose, 1970). In this study, we found stearic, oleic, palmitic, lauric, and myristic acid in secretions. Rose et al. (1969) identified four classes of lipids (phospholipids, triglycerides, fatty acids, and cholesterol) and proteins in mental gland thin-layer chromatograms from all four species of *Gopherus*, similar to findings of this study (i.e., several types of lipids and cholesterol). The identification of prevalent fatty acids and other lipids is not surprising as fatty acids are extremely common in the integument and sebaceous secreting glands of amniotes (like mental glands) and are also thought to be stabilizing compounds in olfactory signals for many vertebrate groups (Alberts, 1992; Weldon et al., 2008; Apps et al., 2015; Campos et al.,2020). Because fatty acids may have a large diversity of chemical side-chains, their ability to vaporize in warmer environments can be varied and could carry redundant signals (Campos et al., 2020). However, it is also possible that not all of the fatty acids identified may play a strong role in chemical signaling and instead could be metabolic byproducts (Ibáñez et al., 2020).

Amides

One primary difference found in the current gopher tortoise study relative to other tortoise work is that amides were found to be the predominant chemical class in mental gland secretions, rather than fatty acids and methylated esters. In contrast to fatty acids, amides are one chemical class found in lizards to vary specifically with sex and individual age and have been found to be used in chemical signals (Khannoon et al., 2011; Khannoon, 2012). Amides have also been indicated in pre-anal glands as an important chemical class related to inter-populational differences of geckos in warm climates, which may provide insight into individual signature mixtures, or individual chemical profiles with varying concentrations of individual amide compounds (Khannoon, 2012).

Amines and amino acids

Amines and amino acids were found in secretions of male and female tortoises in this study, and these compounds are likely direct metabolites but could also be due to the degradation of proteins during collection, storage, and sample preparation. However, whole proteins were not observed, in contrast to Rose et al. (1969). Because of the high molecular weight of proteins, they are not candidates for vaporization like other chemical classes (including under atmospheric conditions and possibly when using GC-MS methodology, such as that used in this study) and are therefore, more likely to only be found in direct contact with an olfactory source, such as presence on an individual's body or in waste deposition (e.g., feces; Apps et al., 2015). Therefore, other techniques may be needed to determine presence and amounts of proteins found in gopher tortoise mental gland secretions than those used in this study. However, in contrast to the high molecular weight proteins, low molecular weight aldehydes, which were recently noted as important in *Sceloporus spp.* lizards (Campos et al., 2020), were also not observed in this study. This lack of aldehydes could be from rapid vaporization or degradation of this compound in the field before analysis, if they are present in mental gland secretions at all (i.e., Apps et al., 2015). It has also been hypothesized by other studies that low molecular weight compounds are less likely to be found in a terrestrial species, especially if they live in hot environments due to rapid degradation (Apps et al., 2015; Campos et al., 2020; Ibáñez et al., 2020).

One of the primary objectives of this study was to chemically analyze potential pheromone chemicals in the mental gland secretions of gopher tortoises. Thus, we were most interested in chemicals that could be vaporized and transmitted as long-distance signals from chemical emissions that could also indicate sex or other physical conditions (i.e., body size). We detected sex differences in three chemicals, but only one of those chemicals, mucic acid, was preferentially displayed in males. Mucic acid ($C_6H_{10}O_8$) is a short-chained carboxylic acid-carbohydrate based compound, in contrast to long-chained fatty acids (i.e., longer than 8 carbons in chain length, such as what was found in Texas tortoises; Rose, 1970), was identified in male gopher tortoise secretions (tentatively identified with MONA score 624, which is less than spectral overlap score of $\geq$700). Mucic acid is extremely insoluble in water and has been found previously only in fruit (e.g., sugar beets, peaches, and pears) and putrefied blood (Anet and Reynolds, 1954; Jeffrey and Wood, 1982). One benefit of insolubility in water is that mucic acid will likely persist more effectively in an arid environment, unlike other short-chained carboxylic acids (e.g., benzoic acid) which are common in freshwater turtles (Weldon et al., 2008; Ibáñez et al., 2020). Mucic acid, similar to lactic acid, which is a potential pheromone found in Rathke's secretions of some freshwater turtles, is formed from nitric acid oxidation (Weldon et al., 2008). We also identified phenylacetic acid in the mental gland secretions of the gopher tortoise. This compound also is present in Rathke's gland secretions of aquatic chelonians (Weldon et al., 2008), making our study the first study to identify it in a terrestrial chelonian. These findings indicate that short-chained compounds may be used as signals in arid environments if compounds are structurally stable and have low solubility.

Two other mental gland chemical compounds, 13-methyltetradecanoic acid methyl ester and methyl (Z)-10-pentadecenoate, which are sex pheromones in some insects (e.g., Charlton and Roelofs, 1991; Cross et al., 1977; Subbaraman et al., 1998; Singh et al., 1985), were higher in females than male tortoises. Both compounds are long-chained fatty acid methyl esters, which would be less likely to quickly degrade over time in a signal and are more of what might be expected in a xerically habituated terrestrial organism with the highest heat tolerance of all *Gopherus spp.* tortoises (thermal critical maximum: 43.9˚C; Hutchison et al., 1966; Spotila et al., 2016). In addition to being a primary chemical found predominantly in female tortoises relative to male tortoises, methyl (Z)-10-pentadecenoate also significantly differed in both males and females by body size, in which larger tortoises (in both sexes) were more likely to produce this compound in secretions. Because reproductively successful females are generally larger than males (e.g., females have larger carapace length, body width, and body thickness than males; McRae et al., 1981), it is likely that the sex difference for this compound was driven in part by body size. Additionally, another potential driver for the production of methyl (Z)-10-pentadecenoate could be age when considering that tortoises, like other chelonians, have various periods of growth throughout their lives, suggesting that larger tortoises could also be older tortoises (Aresco and Guyer, 1999).

Chemical composition differed by location, with location effects observed on six components in males and one component in females. Male and female tortoises from Solon Dixon both had significantly higher amounts of 7-hydroxyoctanoic acid than tortoises from Fort Rucker. Similar to methyl (Z)-10-pentedecanoate, it is possible that higher amounts of 7-hydroxyoctanoic acid may reveal an effect of age and/or status in a social group between populations in the two differing locations. Interestingly, 7-hydroxyoctanoic acid is also found in pheromones in higher amounts in queen honeybees as they age and are mated within a colony (Rhodes et al., 2007). Since it is difficult to determine age in tortoises through the external counting of scute annuli that often overlap with periods of rapid growth (Wilson et al., 2003; Mushinsky, 2016), we were unable to determine age between populations or overall life histories to support these findings. However, 7-hydroxyoctanoic acid, also like methyl (Z)-10-pentedecanoate, is antibacterial in nature, contributing to nutrition in growing queen bee larvae and also contributing to the antibacterial and antioxidant properties of honey (Isidorow et al., 2018). As a result of tortoises being herbivorous, consuming a large variety of grasses, forbes, and wildflowers (Diemer, 1986), the increased presence of 7-hydroxyoctanoic acid may indicate a higher plant diversity or amount of certain wildflowers at Solon Dixon, relative to Fort Rucker. One study in lizards did indicate that a larger herbivorous diet allowed for greater chemical secretion diversity, including alcohols, fatty acids, steroids, wax esters, terpenes, etc. (including some of the other chemicals that differed for males by location) relative to eating an insect diet (Baeckens et al., 2017). Because Solon Dixon and Fort Rucker are located at similar latitudes (Figure 12), major climatic patterns may not be influencing organisms living in these areas (Baeckens et al., 2017b; Campos et al., 2020), but microclimatic variables (e.g., humidity and temperature; Martín et al., 2015) and simulation of natural conditions (i.e., regular burning of longleaf pine habitats) may alter plant diversity, which could influence chemical secretions of gopher tortoises (anthropogenic habitat burning for land management increased plant species richness and diversity up to 2500% in Georgia longleaf pine site; Brockway and Lewis, 1997).

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Table 5: Chemical Compounds Found in Male and Female Gopher Tortoises Using GC-MS

#	Chemical Name	Cmpd Class^a	RI^b	Σ RT^c	Number Found In^d	NIST Probability % Match
1	1,4-dihydroxy-2,3-dimethylanthracene-9,10-dione	AR	<1300	4.92	4	70-74%
2	1-(6-Methoxy-4-methyl-3-quinolinyl)-3,4-dimethyl-1H-pyrazol-5-ol	AR	1601	6.64	3	74-75%
3	4-methyl-1-hexanol	OH	1638	6.75	6	77-93%
4	2-(4-Methoxy-phenoxy)-6-methyl-4-phenyl-quinazoline	AR	1651	6.79	3	70-77%
5	Propanoic acid	FA	1663	6.83	9	78-92%

6	2-Nitroethyl propionate	ES	1672	6.85	7	75-87%
7	Diethanolamine	OH	1676	6.87	3	74-86%
8	4H-1,2,4-Triazol-4-amine	AM	1700	6.94	3	74-80%
9	4-methyl-1-pentanol	OH	1747	7.09	3	72-76%
10	Methyl carbamate	ES	1756	7.12	4	70-90%
11	2,3,3-trimethyl-cyclobutanone	KT	1763	7.14	4	72-89%
12	Benzhydrazide	AR	1790	7.23	4	83-89%
13	Methyl benzoate[1]	ES	1812	7.31	4	13.5%
14	1-(furan-2-yl)but-3-ene-1,2-diol	OH	1834	7.39	5	70-77%
15	Isocrotonic acid	FA	1926	7.75	4	84-92%
16	Pyrolo[3,2-d]pyrimidin-2,4(1H,3H)-dione	AD	2059	8.36	6	76-86%
17	Butanoic acid	FA	2074	8.47	6	75-83%
18	2-Ethylbutanoic acid	FA	2084	8.54	3	70-78%
19	1,2-Dimethoxy-ethene	ETH	2091	8.59	4	73-87%
20	Unidentified compound #20	UNK	2123	8.82	83	NA
21	Octanoic acid[1]	FA	2201	9.37	3	19.6%
22	2-Chloroethyl dimethyl phosphate	ES	2219	9.50	11	75-82%
23	Methyl hexadecanoate	FAME	2343	10.72	81	63.1
24	N-Nitrosodimethylamine	AM	2355	10.85	14	70-89%
25	Methyl octadecanoate	FAME	2553	13.45	8	75.3
26	2-Amino-acetamide	AD	2554	13.46	3	70-83%
27	Unidentified compound #27	FAME	2574	13.76	8	NA
28	Benzoic acid[1]	AR	2589	13.99	4	31.4%
29	Phenylacetic acid[1]	AR	2703	15.88	3	34.7%
30	Unidentified compound #30	UNK	2726	16.31	83	NA
31	2-Aminocyanoacetamide	AD	2907	17.77	5	70-82%
32	Myristic acid[1]	FA	2938	18.37	3	33%
33	1,3-Dioxolan-2-one	ES	2930	20.17	4	70-81%
34	Ethyl urea	AD	2933	20.28	13	70-89%
35	1,3,5-Triazine-2,4,6(1H,3H,5H)-trione	AD	2943	20.44	7	70-79%
36	Palmitic acid	FA	3055	22.74	3	73.4
37	(1-Ethyl-2-methylpropyl)methylamine	AM	3139	24.42	4	71-77%
38	2-methyl-2-(4-methyl-3-methylenepentyl)-1,3-dioxolane	ETH	3198	25.61	3	82-89%
39	Thiazolidine	AM	3236	26.38	3	72-73%
40	3-Hydroxy-16,16-dimethoxyandrost-5-en-17-one	ST	3246	26.58	3	70-75%
41	Stearic acid	FA	3278	27.23	3	73.5
42	N-Methoxymethyl-N-methylformamide	AD	3288	27.43	19	70-79%
43	2-Butanone	KT	>3300	27.73	3	89-92%
44	DL-Norvaline ethyl ester	ES	>3300	27.85	3	77-81%
45	N-methyl-N-nitro-1-butanamine	AD	>3300	27.96	3	71-75%
46	Oleic acid[1]	FA	>3300	28.26	3	17.89%
47	N-Acetyl-dl-alanine methylamide	AD	>3300	28.29	4	77-81%

48	N-formamidoformamide	AD	>3300	28.14	14	70-93%
49	N-(2-benzoyl-3-benzofuryl)-2-diethylamino-acetamide	AR	>3300	28.17	4	76-77%
50	2-Diethylamino-N-p-tolyl-acetamide	AD	>3300	28.11	5	73-91%
51	1,1-Dimethyl-2-ethylhydrazine	AM	>3300	28.43	4	71-82%
52	L-5-Propylthiomethylhydantoin	AD	>3300	28.51	3	71-75%
53	1-piperidinyl dimethylcarbamodithioate		>3300	28.71	12	72-76%
54	Guanylurea	AM	>3300	28.83	13	70-85%
55	N,N-dimethyl-3-Octananime	AM	>3300	29.28	3	73-74%
56	N-Methoxy-N-methylamino-N,N-diethylaminomethane	AM	>3300	29.52	11	79-80%
57	2,2'-Bithiazolidine		>3300	29.83	9	70-77%
58	4-methylpentyl 2-(methoxycarbonylamino)acetate	ES	>3300	30.16	5	70%
59	Methyl 2-methyl-2-(methoxy)amino-propanoate	ES	>3300	30.56	35	70-84%
60	3-(Diethylamino)-1,2-propanediol	OH	>3300	30.72	25	73-78%
61	Allyl hyponitrite		>3300	30.84	3	70-71%
62	Ethanediamide	AD	>3300	31.62	4	72-82%
63	dl-2-Amino-1-hexanol	OH	>3300	31.98	5	71-77%
64	9-Acetylhydrazono-3,6-dichloro-2,7-bis-[2-(diethylamino)ethoxy]fluorene	AR	>3300	32.02	23	71-83%
65	Methyldiethanolamine	OH	>3300	33.27	27	70-76%
66	Leucine methyl ester	ES	>3300	33.65	27	70-86%
67	Ethyl 3,4-dimethyl-1H-pyrrole-2-carboxylate	ES	>3300	36.02	7	70-80%
68	Unidentified compound #8	ST	>3300	48.00		NA
69	Cholesterol[1]	ST	>3300	55.70	73	45.6%

[a] compound class including aromatic (AR), alcohol (OH), ester (ES), ether(ETH), fatty acid (FA), fatty acid methyl ester (FAME), ketone (KT), amide (AD), amine (AM), sterol (ST), unknown (UNK) a single class was chosen as several compounds contain several different functional groups/classes

[b] Retention index calculated via alkane standard

[c] Average retention time for samples that contain the compound

[d] number of times a compound was found in the samples with at least 70% probability via AMDIS [1]Indicates chemicals with low NIST matching %'s that were also verified with pure chemical standards.

Table 6: Male Vs. Female Differential Chemicals

Chemical Identified	Feature	FC	log2(FC)	MONA Score	P-Value
Mucic acid	101/126.1952mz/8.82min	0.37665	-1.4087	624	0.011055
Octadecanoic acid methyl ester	8/143.1976mz/13.45min	2.2051	1.1409	926	0.012978
Methyl (Z)-10-pentedecanoate	6/264.3029mz/13.76min	2.0035	1.0025	895	0.033115

Table 7: Male Only Body Size

Chemical Identified	Features	FC	log2(FC)	MONA Score	P-value
Unknown chemical 3	65/147.1470mz/6.54min	2.489	1.3156		0.004451
Methyl (Z)-10-pentedecanoate	6/264.3029mz/13.76min	3.4523	1.7876	895	0.081653
	87/96.2000mz/13.70min	3.2524	1.7015		0.09583
	68/123.2000mz/13.68min	2.694	1.4298		0.037802
Unknown chemical 34	80/292.5696mz/17.21min	3.5954	1.8462		0.046378

Table 8: Female Only Body Size

Chemical Identified	Features	FC	log2(FC)	MONA Score	P-value
Methyl (Z)-10-pentedecanoate	11/98.1005mz/13.74min	2.3744	1.2476	854	0.023825

Table 9: Male Only Location

Chemical Identified	Features	FC	log2(FC)	MONA Score	P-Value
7-Hydroxyoctanoic acid	66/55.0687mz/8.86min	0.38732	-1.3684	744	0.0046
	34/97.1566mz/8.83min	0.44862	-1.1564	746	0.011998
12-(Trimethylsilyl)oxy-eicosanoic acid trimethylsilyl ester	4/355.3947mz/10.72min	0.021771	-5.5214	622	0.0064
10-Methylundecanoic acid methyl ester	28/267.2001mz/10.72min	0.15877	-2.655	615	0.04902
1-Hexadecanol	56/111.1856mz/16.31min	4.8078	2.2654	803	0.0114
Unknown chemical	14/281.3952mz/16.37min	0.40643	-1.2989		0.0379
Lauric acid	57/97.1253mz/22.77min	2.5049	1.3248	751	0.0427
Unknown chemical	104/227.3639mz/27.07min	6.6776	2.7393		0.02132
Stearic acid	18/55.0193mz/27.60min	2.0588	1.0418	724	0.04258

Table 10: Female Only Location

Chemical Identified	Features	FC	log2(FC)	MONA Score	P-Value
Unknown chemical	14/281.3952mz/16.37min	0.30533	-1.7116		0.0202
7-Hydroxyoctanoic acid	112/98.2000mz/8.87min	0.33932	-1.5593	745	0.0007
	66/55.0687mz/8.86min	0.1427	-2.8089	744	0.0012
	75/69.1735mz/8.91min	0.13023	-2.9408	744	0.0017
	53/70.2000mz/8.81min	0.33809	-1.5645	744	0.0033
	34/97.1566mz/8.83min	0.18758	-2.4144	746	0.0386

Figure 12: County map of the state of Alabama as modified from: http://www.fma.alabama.gov/. Black stars indicate four populations of gopher tortoises where trapping and collections took place for this study. Two southern stars represent Solon Dixon (left star) and Fort Rucker (right star) populations that are used in the location analyses.

Figure 13A FR3

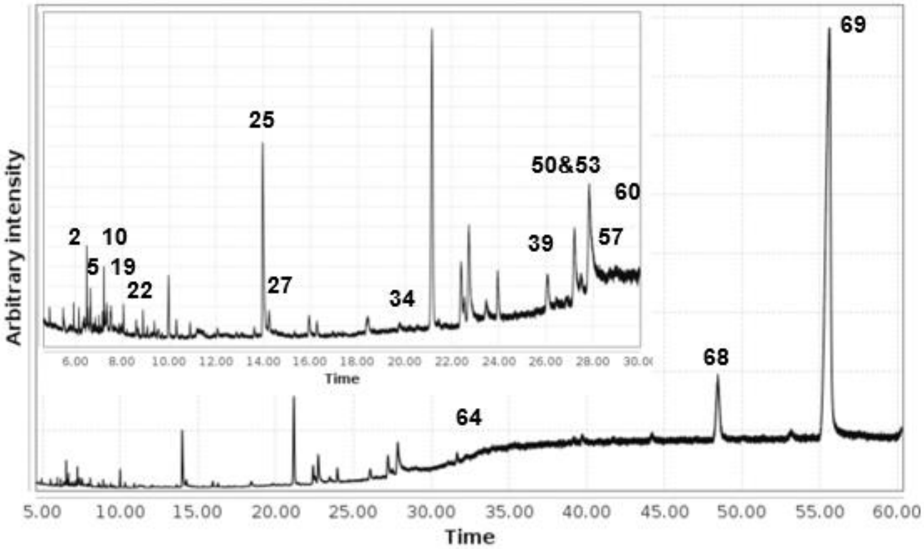

Figure 13B DC38

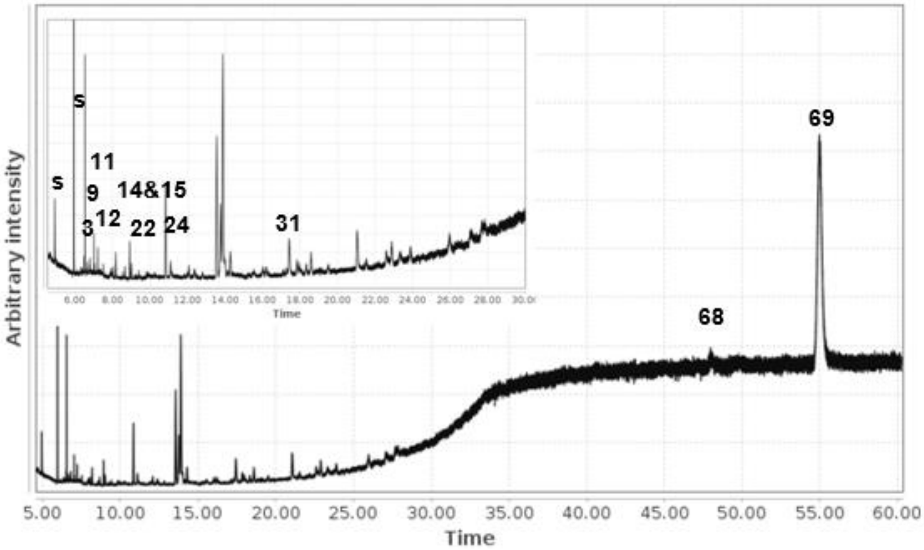

Figure 13A & 13B: Chromatograms of mental gland secretions of (A) male tortoise from Fort Rucker Army & Aviation Base collected in Sept (FR3) and (B) female tortoise from So collected in August (DC38) with an expanded inset of the first 30 min of the analysis in the upper left to show detail. Numbers indicate the compound described in Table 1. S = instrument or system peak not due to sample.

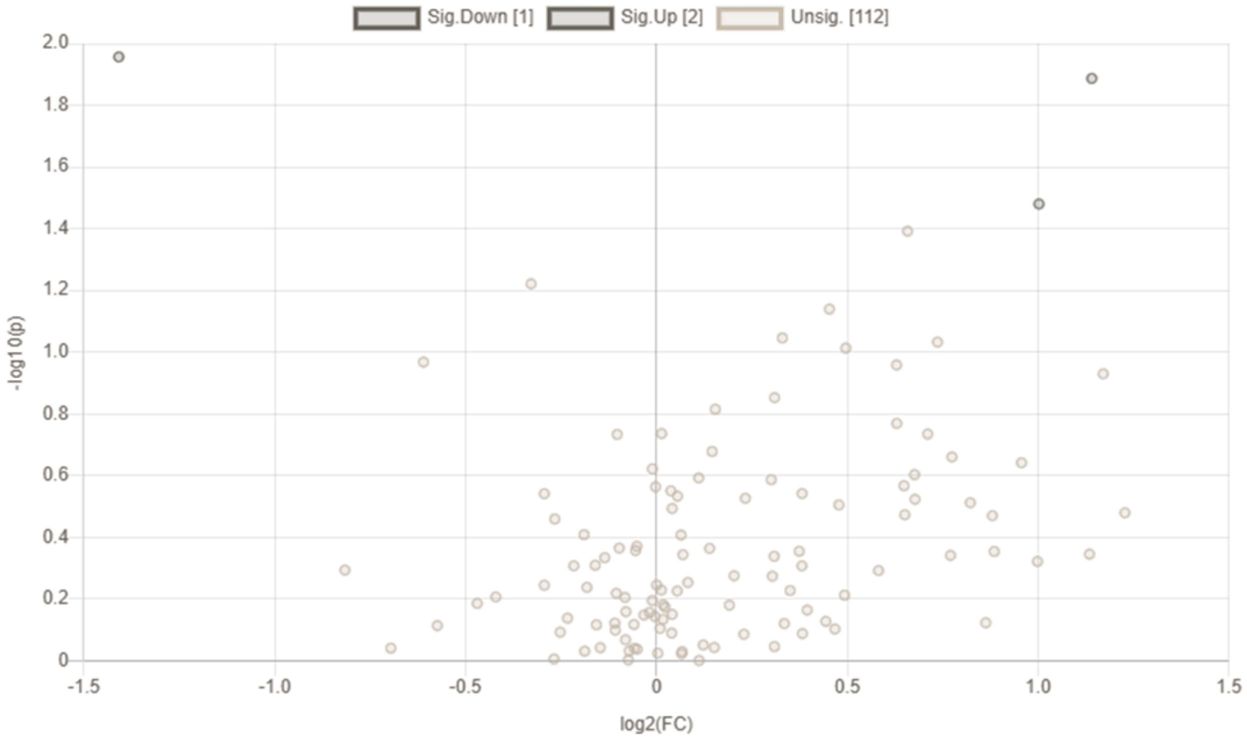

Figure 14: Male vs. female volcano plot for SEX chemical feature differences (see Table 6 for p-values of chemical constituents).

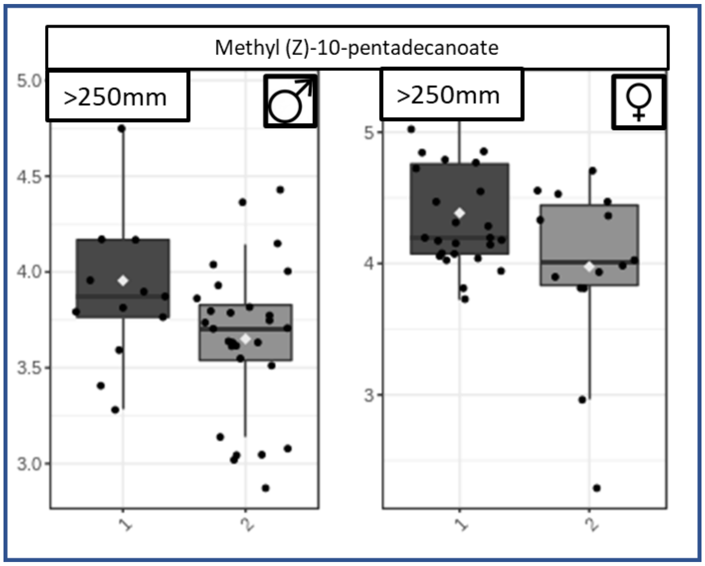

Figure 15: Representative inflections of BODY SIZE statistical chemical differences, shown as box and whisker plots. Sex and large body size indicated for both figures (see Tables 7 & 8 for p-values of all significant inflections). “1” represents >250 millimeters in length, and “2” represents <250 millimeters in length.

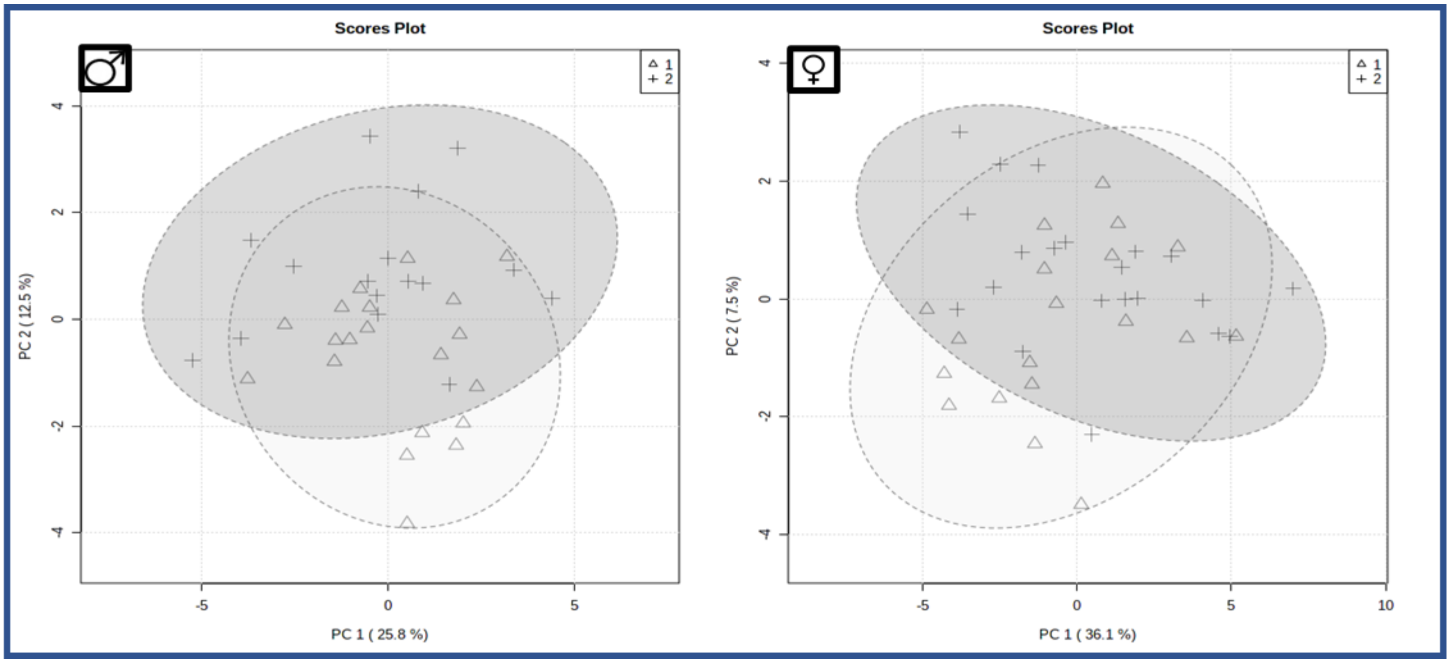

Figure 16: Principal components 2-dimensional analysis plots for effects of LOCATION for males (left) and female (right) tortoises. “1” represents Fort Rucker population of tortoises, and “2” represents Solon Dixon population of tortoises.

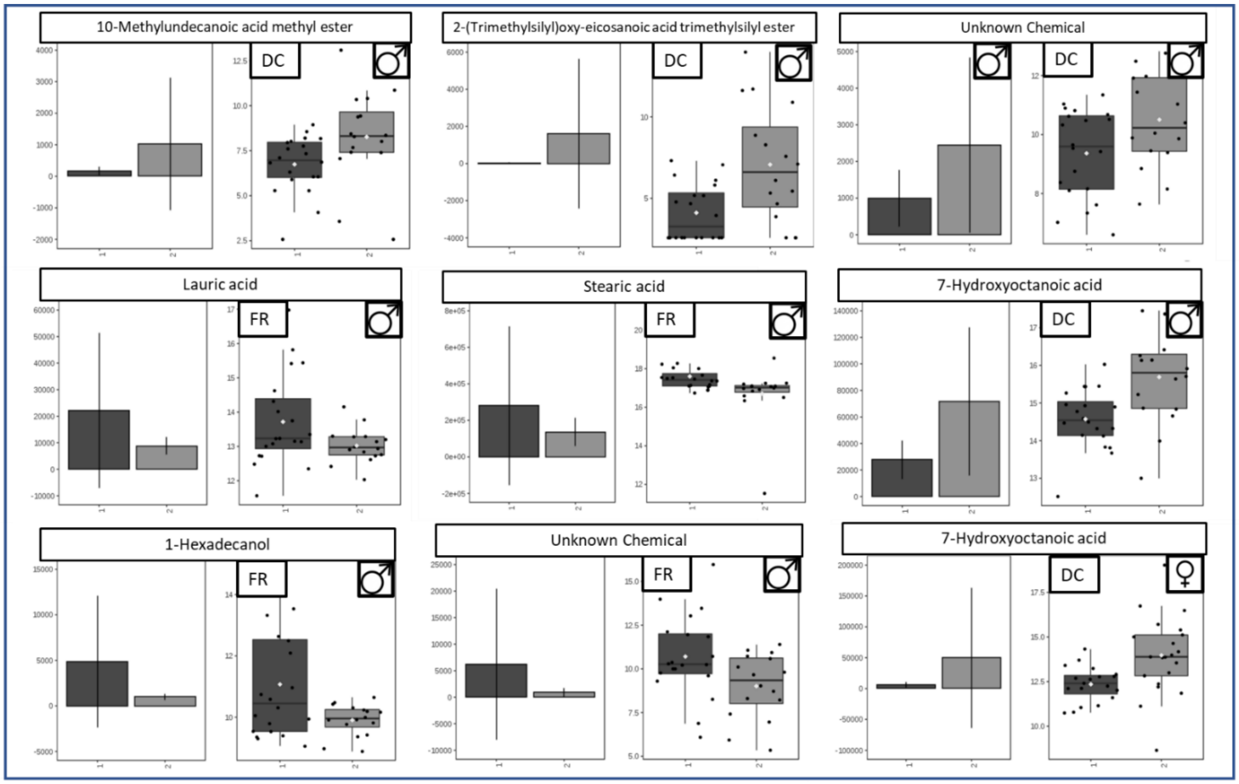

Figure 17: Major chemical components that differ by LOCATION. Sex and location (Fort Rucker=FR and Solon Dixon=DC) are indicated on each statistical box and whisker plot of significant chemical differences. For 7-hydroxyoctanoic acid that had multiple inflections significantly effected by location for each sex, representative inflections were chosen (see Tables 9 & 10 for all significant p-values).

CONCLUSION TO DISSERTATION

There are approximately 360 living species of turtles and tortoises on the planet today, and as a result of a number of mortality threats, most of which are anthropogenic threats, more than half of these chelonian species are threatened with extinction currently (Standford et al., 2020). Some of the major anthropogenic threats that threaten these species include deforestation and habitat decline, road mortalities, human consumption and illegal pet trade, invasive predators (e.g., domesticated animals, meso-predators such as raccoons, opossums, and foxes that live close to humans for food, etc.), and global climate change. Gopher tortoises, like other chelonians, also have the added risk of high nestling and juvenile life stage mortality that requires adults to live and to reproduce in order to be able to maintain already declining populations (Diemer, 1986; Burke et al., 2005; Rollinson and Brooks, 2007; Strickland et al., 2010; Smith et al., 2013). However, while much focus of past and present study has included gopher tortoise habitat management and fire ecology to maintain the also declining longleaf pine ecosystems across the southeastern United States, little work has been done with adult gopher tortoises to gain insight into their behavioral ecology and physiological needs to facilitate mating opportunities for reproduction on the individual level, which was a long-term goal of this work presented here. For example, the studies presented in this dissertation are the first studies to examine chemical signaling for this species, in both behavioral bioassay of the source of pheromone production (e.g., mental gland secretions) and the chemical analysis of candidate chemicals in the mental gland that differ by sex, body size (possibly body condition and health), and location.

Because longleaf pine has become so heavily fragmented over the last century due to logging and urbanization, gopher tortoise populations and individuals can often be separated by great distances (Aresco and Guyer, 1999), causing them potential difficulties in seeking out conspecifics for mating opportunities. We know from some of the social ecological work at female tortoise burrows that sometimes distances up to 30 meters do not affect female tortoise opportunities to mate (Boglioli et al., 2003), but we do not know how far tortoises will travel to find conspecifics, the strategies they use to locate conspecifics, or the sensory signals that are required for them to engage with one another in courtship and mating. With this work, we now know that mental gland secretions have sexually dimorphic chemical components with more than one chemical class represented, suggesting alternative potential chemical messages to be conveyed, and we also know that tortoises of both sexes can behaviorally discriminate these scent cues alone in their environment without a visual signal present. Importantly, I have also determined that mental gland secretions can be discriminated up to at least 20X diluted, allowing these secretions to be one effective chemical signal type that may be present in complex terrestrial habitats as potential trails, allowing especially male conspecifics (but also potentially females) to be able to follow mental gland secretions to find conspecifics for mating opportunities. Finally, using a multimodal paired-presentation with visual and olfactory cues, I also found that mental gland secretion cues are necessary to nonrandomly maintain social behaviors in social and potential courtship interactions between gopher tortoise conspecifics.

Gopher tortoises have been shown to be a socially aggregating species, that communally use burrows of other conspecifics, sometimes with multiple tortoises of both sexes found in a single burrow, and also in socially aggregating, studies have shown that tortoise individuals prefer repeated interactions with the same conspecifics in “cliques” (Douglass, 1986; Johnson et al., 2009; Guyer et al., 2016). If tortoises are in fact strategizing to find conspecifics using chemical cues or pheromones found in mental gland secretions as these studies indicate and support, they also likely have the ability to discern individual status, or a specific signature mixture (e.g., chemical signals specific to an individual; Wyatt, 2010; 2014) from the chemical profiles of mental gland secretions as well. Although I found chemical differences between sex and body size in mental gland secretions, these broader chemical messages are not the same thing as individual signature mixtures because any member of the sex or of a similar body size might be expected to share those same chemical emissions in various concentrations. However, if body size can be a metric of body condition, health status or immune profiles can be indicated as an “honest” chemical signal at the individual level (López and Martín, 2005; Ibáñez et al., 2012; Ibáñez et al., 2014). Immune profiling of chemical emissions of mental gland secretions could be a further avenue of future research to aid in the management of this species.

In contrast, while the “signaler” or the transmitter of pheromones from mental gland secretions may be indicating their health status in chemical emissions, it is simultaneously necessary for “receiving” conspecifics to be able to acutely detect chemical signals in these pheromones to locate conspecifics, which is made more challenging by habitat fragmentation and the threat of diseases such as Upper Respiratory Tract Disease syndrome (URTDs) and other respiratory illnesses. For example, studies have already shown that tortoises infected with predominant symptoms of URTDs (e.g., nasal discharge, nasal scarring and epithelial erosion, conjunctivitis, etc.; Jacobsen et al., 1991) are less likely to forage for optimum grasses and forbes because they are unable to smell (Germano et al., 2014). If tortoises are less likely to forage, they also are less likely overall to look for conspecifics for mating, given the trade-off between sex hormones and immune function in energetics. Furthermore, it has been shown in other tortoises that there is a complex relationship between self-secretion of mental glands and sex recognition of conspecifics (Weaver, 1970). If tortoises that are infected with URTDs still attempt mating, they may be unable to determine male vs. female and thus waste precious energy in pursuing non-reproductive or even incestuous interactions, which would reduce gene flow and cause genetic abnormalities to form in populations (i.e., zebra finches that were unable to smell were less likely to discriminate between siblings and other conspecifics to attempt mating; Caspers et al., 2015). The most detrimental consequence of URTDs (aside from mortality in terminal cases) that has yet to be fully explored is that if presence of this condition or other respiratory conditions (e.g., herpesvirus; etc.) erodes enough olfactory epithelium that tortoises become anosmic, this blocked olfactory condition could also block parallel brain pathways needed to produce sex hormones, which would prevent the ability to reproduce altogether (Wray et al., 1994; Yoshida et al., 1995; Forni and Wray, 2015). For management purposes, tortoises should not just be scanned for the presence of disease, but when possible, also the plasma-borne levels of sex hormones to assess their ability to reproduce in order to ensure that populations can at least be maintained through adult individuals being able to reproduce in the area.

Additional confounders observed in this work of challenges presented when using chemical emissions or pheromones from mental gland secretions for locating and interacting with tortoise conspecifics is the effect of climate and habitat on these emission signals. With the dilution behavioral experiments, we learned that ambient temperature and time of day may impact male tortoises from approaching or searching for conspecifics during daylight hours that had increased temperatures. While it is difficult to say if temperature is directly impacting male tortoise behavior in this case or hastening the breakdown of mental gland secretions that were presented, the potential effects of climate change on tortoise social behavior is worth noting; be it behavior or emission breakdown, we should be aware that gopher tortoises may not evolve fast enough or could be overly physiologically sensitive to socially coping with the strains of increased temperatures in the southeast. Furthermore, with the threat of potential temperatures on social behaviors, climate may also impact the ecosystem of longleaf pine herbivory that is both necessary for tortoises to forage, but also may allow for increased chemical diversity to be produced for various signals coming from the mental gland secretions (Baeckens et al., 2017). In the chemistry analysis of the secretions of the gland for both sexes of gopher tortoises, we found that there were location differences of chemicals in tortoise mental glands from two differing populations at about the same latitude in the state of Alabama. Since macroclimatic conditions are unlikely to cause these chemical differences (Baeckens et al., 2017b; Campos et al., 2020), microclimatic conditions (Martín et al., 2015) or improved fire maintenance (Brockway and Lewis, 1997) of a particular habitat may allow for increased plant diversity in longleaf pine ecosystems that could benefit the tortoise physically through eating and growth and also chemically through more diverse or stronger chemical emissions from mental gland secretions that could facilitate interactions of tortoises to find one another.

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