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Male Color Badges Advertise Multiple Messages: Tradeoffs Among Color Badge Intensity, Size, and Ectoparasite Load

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MALE COLOR BADGES ADVERTISE MULTIPLE MESSAGES: TRADEOFFS AMONG COLOR BADGE INTENSITY, SIZE, AND ECTOPARASITE LOAD

by

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(Under the Direction of Lance McBrayer)

ABSTRACT

Condition-dependent signals can be used by conspecifics to obtain information on an individuals' quality such as health, fighting ability, or immunocompetence. Variation in the severity of parasitic infections could mediate the differential expression of sexual signals in distinct populations of the same species. This could create diverse condition-dependent relationships between signals and quality. In this study, I examine the relationship between male signal expression and quality of *Sceloporus woodi* that inhabit areas with distinct ectoparasitic pressures. First, I examined if the brightness or size of male signals is indicative of body size or body condition in males without ectoparasites. Second, I used *S. woodi* from habitats with heightened parasite pressure to evaluate if brightness or size of male signals is indicative of the male qualities of body size or body condition. Third, I tested whether ectoparasite load is a predictor of signal brightness or size as predicted by the good genes and immunocompetence handicap hypotheses. Males without mites were caught only in the early breeding season and exhibited a negative correlation between the brightness of the black throat border and body size, suggesting that there may be seasonal trends and tradeoffs mediating this relationship. Males with mites were captured during the middle to late breeding season and displayed no correlations between size or brightness of badges and quality (body size or body condition). Thus, body size and/or body condition may not be ecologically important for male-male competition or female mate choice during the later breeding season. The brightness of the blue throat badge was not related to body size, body condition, or mite load, and likely serves as a signal for sex identity. Males with moderate mite loads had the darkest, most fully expressed abdomen badges indicating that the abdomen badge may be a signal of immunocompetence and ability to tolerate heightened parasitic pressure, providing partial support towards the good genes and immunocompetence handicap hypotheses. My findings reveal that each signal in male *S. woodi*

likely provide information on ecologically and seasonally relevant traits, supporting the multiple messages hypothesis.

INDEX WORDS: Signals, Communication, Sexual selection, Color, Ectoparasites, Immunocompetence

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CHAPTER I

LITERATURE REVIEW

The communication of information via colorful morphological ornamentation is shaped by natural selection and sexual selection (Laidre & Johnstone, 2013). Signals are features that have evolved to intentionally convey information between a signaler and receiver and often take the form of large or boldly colored ornaments. The communication of information through signals relies upon (1) a signaler's ability to convey information efficiently and accurately, (2) a receiver to understand the signal, and (3) to change their behavior based on its content. Both the receiver and the signaler benefit from this exchange (Bradbury & Vehrencamp, 1998; Smith & Harper, 1995). However, expression and maintenance of elaborate signals increase risk associated with predation and parasitism (Endler, 1978; Johnstone, 1997; Stuart-Fox, Moussalli, Marshall & Owens, 2003). Furthermore, elaborate signals can be energetically costly (Hill, 1996). Selective pressures are exerted on effective signalers and on receivers who can interpret the signaler's intention and respond accordingly (Johnstone, 1997).

Signals take many forms, including visual (morphological), olfactory, tactile, and behavioral traits. Visual signals function well with diurnal species that associate in close-range interactions with unobstructed views between individuals (Duellman & Trueb, 1994; Endler, 1992). Examples of visual signals include the elaborate train of a peacock, vivid coloration in birds or lizards, or aggressive gestures by chimpanzees. Effective signals rely on a receiver extracting information based on the signal's color, size, or movement. Males, in particular, display various visual signals to communicate information to competitive rivals and potential mates. Females rely on males to advertise their quality as potential mates through honest, condition-dependent sexual signals.

Male visual sexual signals often advertise condition-dependent information to female and male conspecifics about a male's quality (Andersson, 1994; Kelly, Pfennig & Pfennig, 2021; Keyser & Hill, 1999; Uetz, Papke & Kilinc, 2002; Zúñiga-Vega, Pruett, Ossip-Drahos, Campos, Seddon, Price, Romero-Diaz, Rivera, Vital-García & Hews, 2021). Display of an honest visual signal benefits males by preventing agonistic interactions and increasing reproductive success (Clutton-Brock & Albon, 1979; Reby, McComb, Cargnelutti, Darwin, Fitch & Clutton-Brock, 2005). Condition-dependent signals benefit females by providing an opportunity to appraise a male before mating. Hamilton and Zuk (Hamilton & Zuk, 1982) hypothesized that sexual selection directs male signal evolution if signals honestly represent a male's genetic quality. If so, females would use signals to obtain genetic benefits for her offspring (Andersson, 1994; Fisher, 1915), thus gaining indirect reproductive fitness and passing on the preference for that signal. For male signals to be informative to conspecifics and direct morphological evolution, males must differ in signal expression based on their health and fitness (Cotton, Fowler & Pomiankowski, 2004; David, Bjorksten, Fowler & Pomiankowski, 2000; Vergara, Mougeot, Martínez-Padilla, Leckie & Redpath, 2012). Multiple signals, such as badge size and color, may increase the informational content or its specificity from signaler to receiver.

Multiple signals enforce the signal's honesty and conspicuousness towards conspecifics (Doorn & Weissing, 2006; Lehtonen, Rintakoski & Lindström, 2007; McGlothlin, Parker, Nolan & Ketterson, 2005; Møller, Saino, Taramino, Ferrario & Galeotti, 1998). Multiple signals could reflect different genetic or health components that a female benefits from passing to her offspring, such as immune response and endurance (Coleman, Patricelli & Borgia, 2004; Johnstone, 1997; McGlothlin et al., 2005). Multiple signals may give rival males information simultaneously on an individual's bite force (Pérez I De Lanuza, Carazo & Font, 2014) or

dominance (Marty, Higham, Gadsby & Ross, 2009). Male Schreiber's green lizards, *Lacerta schreiberi*, display different colored badges on the throat, sides, and chest (Martín & López, 2009). The different chromatic combinations of these badges correlate with variation in the individual's health, body size, dominance, and pairing status (Martín & López, 2009). However, male signals may also advertise redundant information and act only to increase the detection and visibility of the signal to receivers (Rowe, 1999). The additive and interactive effects of multiple signals may be more important and reveal stronger relationships with individual quality than single traits (Day, 2000; Hamilton, Gaalema, Laage & Sullivan, 2005).

Identifying the relationship between multiple male ornaments and male health and quality is necessary to determine how signals are interpreted and used within a species for sexual selection. Many visual signals have been indirectly linked to male qualities that increase fitness, such as body size, body condition, and immunocompetence. A larger body size indicates increased survivability in species that exhibit positive associations with age and size (Halliday & Verrell, 1988; Halliday, 1992). A larger male may also be able to forage more effectively, grow faster, and have higher tolerance for stressful events (Ruby, 1984). In territorial and non-territorial species, larger males are more successful in aggressive encounters and copulate more frequently (Anderson & Vitt, 1990; Cooper Jr & Vitt, 1993). Color badges correlated with body size would function as multiple signals, with body size itself being a signal, increasing the attention received or ensuring the information is appropriately communicated to the receiver. While body condition is harder to assess by conspecifics (Hasson, 1991), badges that show correlations with body condition could provide information to females on general health or foraging efficiency of a male (Donnelly & Sullivan, 1998; Giles, Harris, Rands & Nicol, 2020).

Strong correlations between signals and fitness components would support male and female conspecific use of these signals for sexual selection.

Colorful badges are used for intra- and inter-sexual interactions, allowing conspecifics to judge a male's quality based on the badge's chromatic qualities or size (Orton, Kinsey & McBrayer, 2020; Swierk, Ridgway & Langkilde, 2012). The location of these badges allows males to display when interacting with conspecifics yet remain cryptic when necessary to avoid predation. Structural-based coloration (e.g., blue and black) has been regarded as an honest signal of quality in several species, including blue grosbeaks (*Guiraca caerulea*) (Keyser, 2000), Tenerife lizard (*Gallotia galloti*) (Megía-Palma, Martínez & Merino, 2016), and wall lizard (*Podarcis muralis*) (Pérez I De Lanuza et al., 2014). Black coloration only requires melanin in underlying melanophores to absorb all wavelengths, while blue coloration also requires iridophores to reflect short (blue) wavelengths (Cooper, Greenberg & Gans, 1992; Morrison, Rand & Frost-Mason, 1995; Quinn & Hews, 2003). Often, a black border surrounds the throat badge in lizards, resulting from the melanophore layer extending beyond the iridophore layer, potentially increasing the contrast and conspicuousness of blue badges to receivers (Bókony, Liker, Székely & Kis, 2003)..

Androgens mediate the enforcement of honesty and the relationship between the expression of colorful badges and male quality Testosterone, a key androgen mediating sexual activity, particularly during the reproductive season, primarily affects body size (Abell, 1998; Borski, Tsai, DeMott-Friberg & Barkan, 1996; Cox & John-Alder, 2005; Fennell & Scanes, 1992; Ford & Klindt, 1989; Gatford, Egan, Clarke & Owens, 1998; Holloway & Leatherland, 1998; Huggard, Khakoo, Kassam & Habibi, 1996; Kuwaye, Okimoto, Shimoda, Howerton, Lin, Pang & Grau, 1993; Lerner & Mason, 2001; Wehrenberg & Giustina, 1992), but can also affect

color signals (Ligon, Thornhill, Zuk & Johnson, 1990; Rand, 1992). Testosterone promotes color in fish (Shahidian, Jamili, Rezaiee & Amri, 2014), reptiles (Seddon & Klukowski, 2013), birds (Lindsay, Webster & Schwabl, 2011), and mammals (Rhodes, Argersinger, Gantert, Friscino, Hom, Pikounis, Hess & Rhodes, 1997). Testosterone is implicated in the physical development and maintenance of color badges. In *Sceloporus* lizards, androgens may permanently organize iridophore structure at maturation to reflect blue light (Morrison et al., 1995; Quinn & Hews, 2003), but circulating testosterone is then required to maintain melanophore quantity and the vivid expression of both black and blue coloration (Kimball & Erpino, 1971; Morrison et al., 1995). Testosterone can cause melanophore expansion resulting in increased badge size (Kimball & Erpino, 1971; Morrison et al., 1995). For example, male house sparrows display a black chest badge whose size positively correlates with testosterone level (Buchanan, Evans, Goldsmith, Bryant & Rowe, 2001; Evans, Goldsmith & Norris, 2000; Gonzalez, Sorci, Smith & Lope, 2001; Laucht, Kempenaers & Dale, 2010). Interestingly, castration of male *Sceloporus undulatus* during the breeding season inhibits the development of male-specific coloration, and exogenous replacement of testosterone restores normal male coloration (Cox, Skelly, Leo & John-Alder, 2005b).

A negative correlation between testosterone and immunocompetence provides a potential mechanistic link between signal expression and parasite load (Boonekamp, Ros & Verhulst, 2008; Duckworth, Mendonça & Hill, 2001; Oppliger, Giorgi, Conelli, Nembrini & John-Alder, 2004). High testosterone is positively correlated with parasite load in many studies, but relationships vary by parasite type (e.g., ectoparasites vs endoparasites) and by species (Argaez, Pruett, Seddon, Solano-Zavaleta, Hews & Zuniga-Vega, 2021; Cox & John-Alder, 2007; Grear, Perkins & Hudson, 2009; Halliday, Paterson, Patterson, Cooke & Blouin-Demers, 2014;

Mougeot, Irvine, Seivwright, Redpath & Piertney, 2004; Mougeot, Redpath & Piertney, 2006). Testosterone can be linked to parasite load in two ways, though not mutually exclusive. First, testosterone is correlated with increased activity, behavioral displays, and home range size in lizards (Beach & Inman, 1965; Cox, Skelly, Leo & John-Alder, 2005; Denardo & Sinervo, 1994; John-Alder, McMann, Katz, Gross & Barton, 1996; Klukowski, Ackerson & Nelson, 2004; Klukowski, Nelson & Jenkinson, 1998; Marler & Moore, 1988; Moore, 1988; Moore & Marler, 1987; Valenstein & Young, 1955). Increased activity can increase exposure to ectoparasites in males (Cox, Skelly & John-Alder, 2005), but evidence of this in free-ranging populations is lacking (Lane, Kleinjan & Schoeler, 1995; Schall, Prendeville & Hanley, 2000). Second, circulating testosterone levels can be suppressed by activation of the immune system (Boonekamp et al., 2008). Thus, increases in immune activity resulting from high parasite load, may block the release of testosterone (Belliure, Smith & Sorci, 2004; Folstad & Karter, 1992; Foo, Nakagawa, Rhodes & Simmons, 2017; Mills, Grapputo, Jokinen, Koskela, Mappes, Oksanen & Poikonen, 2009). The negative relationship between immunocompetence and testosterone level appears to create a physiological tradeoff that can maintain the honest advertisement of male sexual signals. Honest signaling of male condition occurs when sexual signals are intimately linked to physiological and cellular processes (Biernaskie, Grafen & Perry, 2014; Hill, 2011; Smith & Harper, 1995). Thus, testosterone may be critical in regulating the relationship between the development and honesty of sexual signals, parasite load, and immunocompetence. The trade-off between immunocompetence and sexual signal production creates an opportunity for females to judge male quality accurately. Only high-quality individuals can resist parasites while maintaining high testosterone levels for increased signal

expression and associated reproductive benefits (Immunocompetence Handicap Hypothesis (ICHH); Folstad & Karter, 1992).

Fragmented populations of a species that differ in environmental parasite infection rates provide an interesting opportunity to examine the effect of parasites on links between sexual signals and male quality. Environments with higher parasite prevalence exhibit stronger correlations between individual quality and sexual signals (Giery & Layman, 2015; Vergara, Martínez-Padilla, Redpath & Mougeot, 2011; Vergara et al., 2012). This indicates tight associations between individual condition and signal expression in environments with high parasite infection and weaker relationships between condition and signal in environments with lower parasite infection. Those individuals facing increased ecological pressure, in the form of increased parasitism, should allocate more energetic resources towards immunocompetence, increasing survival, instead of sexual ornament expression, particularly if they are of poor genetic quality or health. Therefore, individuals in prime condition, i.e., that are better able to cope with stressors, are capable of producing the strongest sexual signals. When ecological stress is low, less restraint is placed on the allocation of energy resources allowing for a broader range of individual conditions to display strong signals. The strength of environmental stressors in a habitat can constrain or enhance the relative degree of difference between low- and high-quality individuals (Vergara et al., 2011). Thus, in examining fragmented populations of a species, we expect stronger relationships between male quality and sexual signal expression in habitats with high parasite abundance, maintaining increased honesty of the signal to conspecifics.

The Florida scrub lizard, *Sceloporus woodi*, produces multiple male-biased visual signals, including blue badges on the throat and abdomen that vary in size and brightness and are potentially correlated with male fitness qualities. *S. woodi* inhabit two diverse habitat types in

central Florida's Ocala National Forest: longleaf pine (LLP) and sand pine scrub (SPS). Different vegetation structures characterize these habitats, consequently varying parasite abundances (Campbell & Christman, 1982; Greenberg, Neary & Harris, 1994; Hokit, Stith & Branch, 1999; Orton et al., 2020). Lizards in SPS have significantly more ectoparasites (*Eutrombiculis cinnibarensis,* chigger mites) than lizards in LLP (Orton et al., 2020), providing an opportunity to compare the effect of divergent parasite abundance on sexual signal expression.

This thesis addresses two broad questions: (1) What do visual signals advertise about male quality in *S. woodi,* and (2) How could the relationships between these signals and individual male quality vary by parasite load?

CHAPTER II

INTRODUCTION

Male visual sexual signals are often condition-dependent and relay information about male quality to potential female mates and male rivals (Kelly et al., 2021; Keyser & Hill, 1999; Uetz et al., 2002; Zúñiga-Vega et al., 2021). Sexual selection directs the expression and evolution of condition-dependent male signals when those signals are honest representations of a male's genetic quality (Hamilton & Zuk, 1982). High-quality males that display vibrant, fully expressed signals benefit from avoiding costly aggressive encounters with competitors and increasing reproductive fitness by attracting females (Clutton-Brock & Albon, 1979; Reby et al., 2005). Condition-dependent signals allow females to appraise a male's quality before mating, thereby selecting specific genetic traits that can be inherited by her offspring and increasing her own indirect reproductive fitness (Andersson, 1994; Fisher, 1915). For male signals to be informative to conspecifics and direct the evolution of male morphology, males must differ in signal expression based on their health and genetic quality (Hund, Hubbard, Albrecht, Vortman, Munclinger, Krausova, Tomasek & Safran, 2020).

Male and female conspecifics may glean useful information from signals advertising males' body size, body condition, or parasite resistance. In species with positive allometry between size and age, body size is an important indicator of survivorship, a quality that directly increases offspring fitness (Halliday & Verrell, 1988; Halliday, 1992). Large body size may also confer benefits to offspring, such as increased copulation and energetic resources, resilience to stressful events, and more success in aggressive encounters (Anderson & Vitt, 1990; Cooper Jr & Vitt, 1993; Ruby, 1984). Body condition is an important indicator of health and has been positively associated with foraging capability and negatively associated with stress and disease

state (Donnelly & Sullivan, 1998; Giles et al., 2020). While body size and condition are themselves signals, additional signals such as colorful badges are often correlated with body size and body condition. Thus, colorful badges may also function as ancillary signals to increase the conspicuousness of the male or transmit additional information (Hasson, 1991). Multiple signals (e.g., throat, throat border, and abdomen badges) could advertise different information to different receivers (Multiple Messages Hypothesis, Johnstone, 1996). Therefore, each signal would independently display information dependent on the identity of the receiver (Moller & Pomiankowski, 1993).

Yet, non-adaptive hypotheses could also explain the evolution of color patterns and/or signals (Andersson, 1994). Non-adaptive hypotheses of the evolution of male color signals may include sensory bias, and other non-functional hypotheses (Andersson, 1994). In these scenarios we would not expect to find relationships between male qualities and badge traits. For example, foraging behavior of a species may lead to a high sensitivity to colors, patterns, or shapes. Organisms such as the peacock may then exploit this sensitivity whereby, they display colors that elicit the same level of attention and response (Ridley, 1981). In contrast, two adaptive hypotheses shown to lead to the evolution and maintenance of male signals include the good genes hypothesis (Hamilton & Zuk, 1982) and the immunocompetence handicap hypothesis (Folstad & Karter, 1992). In each, male color badges have evolved to advertise relevant condition-dependent information that is informative to conspecifics (David et al., 2000; Keyser & Hill, 1999; Martín, Moreira & López, 2007; Mougeot et al., 2004).

The ‘good genes’ hypothesis states that the full expression of sexual signals is dependent on a male’s genetic quality, specifically his genetic ability to resist parasitic infection (Hamilton & Zuk, 1982). Thus, male sexual signal expression mediates female mate choice based on male

genetics and immunocompetence. The immunocompetence handicap hypothesis (ICHH) proposed by Folstad and Karter (1992) expands on the 'good genes' hypothesis suggesting that the relationship between immunocompetence and signal expression is regulated via hormones such as testosterone. Testosterone levels have been negatively correlated with immunocompetence (Belliure et al., 2004; Boonekamp et al., 2008; Folstad & Karter, 1992; Foo et al., 2017; Mills et al., 2009) and positively associated with the production and maintenance of structural color badges (Alatalo, Höglund, Lundberg, Rintamäki & Silverin, 1996; Duncan, Cohick & John-Alder, 2020; Kimball & Erpino, 1971; Morrison et al., 1995). Thus, the negative correlation of testosterone with immunocompetence creates a tradeoff between costs for immune support and fitness benefits of enhanced signal expression. Thus, under the ICHH, sexual signal intensity should exhibit a negative relationship with parasite load, with intensely expressed sexual ornaments signaling male resistance to parasitic infection.

Signals that honestly advertise male qualities that are pertinent and beneficial to receivers are under strong selection (Andersson, 1994). For example, a male that honestly expresses his large size and enhanced fighting ability would benefit from avoiding agonistic encounters, and likewise, conspecific males would also benefit from preventing injury or death. A male that honestly expresses his prime body condition benefits from increased mating opportunities, and females indirectly benefit from obtaining strong genes for her offspring (Fisher 1915, Andersson 1994). This situation creates a cooperative signaling system, wherein the signaler benefits from eliciting a response that also benefits the receiver (Johnstone, 1997). For a cooperative signaling system to be evolutionarily stable, signals must be closely linked to physiological and cellular processes (Biernaskie et al., 2014; Hill, 2011; Smith & Harper, 1995). The 'good genes' hypothesis and the ICHH can be viewed as two ends of a continuum providing different

explanations for patterns of signal expression and a physiological link, immunocompetence. Evidence of males exhibiting intense, fully expressed sexual signals while simultaneously maintaining low parasite loads would support both hypotheses. However, this relationship may be affected by environmental context. Environmentally relevant pressures, such as high parasite abundance or phenotypic plasticity, may affect the relationship between signal expression and male genetic quality.

Sceloporus lizards are well-studied for relationships between male signals and individual male quality and are located in a broad range of environments that likely reflect different selective pressures (Sites, Archie, Cole & Flores-Villela, 1992). Most *Sceloporus* lizards exhibit male-biased blue color badges on the abdomen and throat, often outlined with a black border (Ossip-Drahos, Oyola Morales, Vital-García, Zúñiga-Vega, Hews & Martins, 2016). Males display badges towards male and female conspecifics, indicating that they are signals of some component of male quality (Zúñiga-Vega et al., 2021). The presence or absence of these badges is likely a signal of sex identity, wherein females display rudimentary badges or lack a badge all together (Cooper Jr & Burns, 1987). However, variability in brightness and size of badges may reveal other information relevant to male-male competition or female mate choice. Male abdomen color is related to success in male-male aggressive encounters in *Sceloporus virgatus* (Quinn & Hews, 2000) and badge size is correlated with body size, body condition, fighting ability and parasite load in *Sceloporus undulatus* and *Sceloporus jarrovi* (Halliday et al., 2014 Cooke, & Blouin-Demers, 2014; Langkilde & Boronow, 2010; Ossip-Drahos, Berry, King & Martins, 2018). Two populations of *Sceloporus undulatus* in similar but different habitats differ in their relationships between body size and color badge brightness (Langkilde & Boronow, 2010). Thus, subtle contrasts in habitat structure and environmental pressures may result in

variation in sexual signal expression. Male *Sceloporus woodi*, sister species to *Sceloporus undulatus*, display the characteristic blue throat and abdomen badge like *S. undulatus*. *Sceloporus woodi* occupies long leaf pine and sand pine scrub habitat types in central Florida that differ in canopy cover, ground cover, and open sand availability (Tiebout III & Anderson, 2001). This variation in habitat creates populations that face different ectoparasite loads (Orton et al., 2020), thereby providing the opportunity to test hypotheses between male sexual signal expression and signal quality.

This study will quantify the relationships between indicators of male quality (e.g., body size and condition), sexual signal expression (e.g., badge brightness and area), and ectoparasite load (*Trombiculariidae* mites) in *Sceloporus woodi*. First, I determine if signal expression is positively associated with quality (SVL and body condition) in males with zero ectoparasites as predicted by the 'good genes' hypothesis. Second, I focus on males that have variable, often high, ectoparasite abundance (Orton et al., 2020). Comparing the resulting patterns from males without ectoparasites to males with ectoparasites will provide context to how ectoparasites mediate tradeoffs between male quality, signal expression, and ectoparasite load. Signal expression is predicted to positively correlate with the quality indicators of SVL and body condition in *S. woodi* with and without ectoparasites. In contrast and as predicted by the ICHH, signal expression is predicted to be negatively correlated with increasing ectoparasite load, with the lowest ectoparasite load males expressing the most optimal signals (Folstad & Karter, 1992). Third, I demonstrate that very high ectoparasite loads negatively affect signal quality and depart from expectations of either the good genes or ICHH.

CHAPTER III

METHODS

Study Species and Area

Sceloporus woodi, the Florida Scrub Lizard, is a sexually dimorphic lizard that inhabits fragmented patches of Florida scrub and longleaf pine in central peninsular Florida (Enge, Tornwall & Bankovich, 2021; Jackson & Telford Jr., 1974). *S. woodi* prefers open, sandy environments near trees or shrubs that provide an escape from predation and a range of thermal microclimates. Individuals appear to be philopatric with limited dispersal (Heath, Schrey, Ashton, Mushinsky & McCoy, 2012), resulting in clumped populations in areas of suitable habitat (Greenberg et al., 1994). Adult males (snout-vent length, SVL = 45.5 ± 3.3 mm) are smaller than females (SVL = 50.7 ± 4.1 mm) and display male-specific blue badges on the throat and abdomen. The male throat badge is surrounded by a brown to black border (Figure 1). Male color badges are highly variable in both size and brightness, suggesting that size and brightness of male badges in *S. woodi* is related to male quality as shown in birds (Brawner III, Hill & Sundermann, 2000; Veiga, 1993), fish (Dijkstra, Hekman, Schulz & Groothuis, 2007; Houde, 1987; Kodric-Brown & Brown, 1984; van der Sluijs, van Alphen & Seehausen, 2008), amphibians (Grether, Kolluru & Nersissian, 2004) and reptiles (Lebas & Marshall, 2001), including other *Sceloporus* lizards (Langkilde & Boronow, 2010; Zúñiga-Vega et al., 2021). *S. woodi* are commonly parasitized by larval *Eutrombicula cinnabaris*, a terrestrial chigger mite (Johnston & Crossley, 1996), providing an opportunity to examine the relationship between male signals, quality, and an ectoparasite.

Populations of *S. woodi* inhabit longleaf pine (LLP) and sand pine scrub (SPS) habitats in Ocala National Forest. Longleaf pine stands are burned on an annual or biannual schedule and

are characterized by mature longleaf pines and turkey oaks with bunch grass and open sand groundcover (Campbell & Christman, 1982; Hokit et al., 1999 1999, Tiebout & Anderson, 2001). Sand pine scrub is managed with a combination of clear-cutting and roller chopping, resulting in a landscape of open sand and woody debris with a progressive increase in the vegetation density as stands regrow (Greenberg et al., 1994; Tiebout III & Anderson, 2001). *Sceloporus woodi* from SPS habitats have significantly higher mite loads of larval *Eutrombicula cinnabaris* (Orton et al., 2020). As such, males from LLP and SPS habitats provide an opportunity to examine sexual signal relationships related to variations in mite load.

Field Collection

I collected lizards from May to October 2021 and February to July 2022 so that males would be captured during the early breeding season (February to March), late breeding season (April to August), and non-breeding season (September to October). This was done to quantify any seasonal effects of signal expression. Lizards were stored in a cool environment and later weighed (+/- 0.1 g) and snout-vent length (SVL) measured (+/- 1mm). Larval chigger mites (*E. cinnabaris*) were counted on the entire body of every individual using a standard magnifying lens. The majority of mites were located in the nuchal pockets behind the tympanum and front of the shoulder. Individuals were returned to their exact location of capture within two weeks if taken to the lab at Georgia Southern University or within two hours if not.

Calibrated Photographs

Because temperature affects coloration in *Sceloporus* (Langkilde & Boronow, 2012; Sherbrooke, de Lauro Castrucci & Hadley, 1994), the internal body temperature of a subset of individuals (N = 63) was taken just before photographing. Body temperature ranged from 27 -

33.6 °C, and no color variation was observed. All photographs were taken with lizards in the range of body temperatures from 27 - 34 °C.

All calibrated photographs were taken in standardized lighting conditions for the individuals transported to the Georgia Southern laboratory. All photographs were taken in a windowless room lit from fluorescent ceiling lights and a small light attached to the camera tripod. Field photographs were ensured to be without the effects of shadows or glare. Orton and McBrayer (Orton & McBrayer, 2019) photographed lizards in the field and the lab using the same techniques and noted no significant differences in brightness by location of photographs. I took photos from a standard distance of approximately 0.3 meters. All photographs were taken and analyzed by the same individual (KMR).

I photographed lizards using an iPhone 11 with a 12MP camera, then used the app ProCamera (Cocologics GmbH, V15.1), which allows images to be processed as RAW and JPEG files. The ventral and dorsal surface of each lizard was photographed against eight color-aid basic gray scales (Color-aid), as well as one black standard and one white standard to allow for calibration and grayscale equalization in Adobe Photoshop (Adobe Systems, San Jose, CA) (Stevens, Párraga, Cuthill, Partridge & Troscianko, 2007) (see Figure 2).

Each component's brightness was estimated using the reflectance percentages for the red, blue, and green color channels as determined in Adobe Photoshop using the blur and average tools. This method of measuring red-green-blue (RGB) color was recently compared with spectrophotometric data. It showed strong positive correlations between results of hue, saturation, and brightness (Orton & McBrayer, 2019). The left and right badges were each sampled once per individual and averaged to obtain a single brightness value for the throat badge, throat border, and abdominal badge.

I measured brightness as the relative amount of white mixed with badge color (blue or black). Brightness is interpreted as a measure of lightness reported on a unitless scale where low values signify similarity to black and high values signify similarity to white (Cuervo, Belliure & Negro, 2016). High brightness in the context of the *S. woodi* badge would mean a color badge with less intense color or less blue (or black) pigment. Low brightness, indicating darker colors, would signify more intense blue (or black) coloration. The pigments required for expressing vivid (i.e., highly pigmented) color badges of lizards absorb light rather than reflect it (Megía-Palma, Barrientos, Gallardo, Martínez & Merino, 2021), indicating that those individuals with more pigments, and thus fully expressed badges, have decreased brightness values. Increased pigmentation is costly and at least partially mediated by testosterone (Fitzpatrick, 1998; Kimball & Erpino, 1971; Morrison et al., 1995), so it can be inferred that those males who produce more pigmented, fully expressed badges are in better condition (Ethier, Gasse, Lake, Jones, Evenden & Despland, 2015; Jawor & Breitwisch, 2003; Kimball & Erpino, 1971; Morrison et al., 1995). For this study, we refer to increased signal expression as darker badges or those with decreased brightness.

From photographs, I measured the area of the left and right throat, throat border, and abdominal badge using the measure tool in ImageJ (National Institutes of Health, Bethesda, MD). Measurements were standardized for the number of pixels against a known measurement indicated by a ruler in each photograph (1 mm). Left and right badge measurements were measured once per individual and averaged to obtain a single measure of each individual's throat, throat border, and abdominal badge area. In addition, the throat area and throat border area were added together to get a total measurement of the throat badge area, hereafter referred to as the combined throat and throat border badge.

Statistical Analysis

Males with SVL greater than or equal to 43 mm were considered adults (Jackson & Telford Jr., 1974) (N = 220 from SPS and LLP) and included in analyses. All variables were assessed for normality using the Shapiro-Wilk Goodness of Fit test. Mites and abdomen badge brightness were not normally distributed ($p < 0.05$). Mites were transformed using the square root, and abdominal brightness was transformed using log10 to achieve normal distributions. All other variables were normally distributed. The body condition index was calculated as the residuals of the regression of mass on SVL. The throat, throat border, abdomen, and combined throat and throat border badge areas were significantly correlated to mass and were thus sized corrected by calculating the residuals of the regression of badge area on mass.

To examine signal characteristics in males without mites (Mite # = 0, N = 21, pooled LLP and SPS populations), reduced major axis (RMA) regressions were performed using both SVL and body condition as independent variables, and the brightness and area of all badges (throat, throat border, and abdomen) as dependent variables to quantify signal quality in mite free males. Given their distributions, quadratic regressions were performed separately on males from SPS (N = 128) and LLP (N = 71) to A) determine if patterns differed for each habitat type, and B) quantify the relationships between mite load and the brightness of the abdomen, throat, and throat border badges. Additional quadratic regressions were also run separately for SPS (N = 128) and LLP (N = 71) to determine the relationship between mite load and the size of the abdomen, throat, throat border, and combined throat and throat border badges.

I corroborated that SPS males have higher mite loads than LLP males using an analysis of variance (ANOVA). Subsequently, I used only males from SPS to determine relationships between male quality and signal expression because they face the most intense parasite pressure.

Doing so allowed me to estimate how signal quality is affected by very high mites load (150+ mite) that occur at very low frequency in LLP yet are common in SPS.

To determine how signals are related to quality in males with mites (Mite # = 1+, N = 128), reduced major axis (RMA) regressions were performed using both SVL and body condition as independent variables, and the brightness and area of all badges (throat, throat border, and abdomen) as dependent variables. Next, I examined the distribution of mite load to identify similar bin sizes across the distribution. Subsequently, mite load was categorized into low (mite # $\leq$ 40), moderate (40 > mite # $\leq$ 100), and high (mite # $\geq$ 101) to make specific comparisons of signal expression based on the intensity of mite load that quadratic regression could not identify. An ANOVA with Tukey's post hoc tests was performed to test for differences in brightness and size of abdomen, throat, and throat border badges between mite load categories.

CHAPTER IV

RESULTS

Relationship between color, size, and body condition in mite-free males

Mites were common in both LLP and SPS habitats from April to October, with mite load ranging from 1 to 368. Males in LLP had mostly low (37.6%) and moderate (41.6%) mite loads with few males having high mite load (12.9%). In contrast, males in SPS had fewer males with low mite loads (20.2%), similar proportions of males with moderate mite load (37.1%), and roughly 2.5 times as many males with high mite loads (32.1%). Mite free males were rare (7.8% LLP, 10.5% SPS) and observed only in February and March (Table 1). Combining all mite-free males, there was no significant relationship between throat badge brightness and snout-vent length (SVL) or between throat badge brightness and body condition (Table 2). Likewise, there was no relationship between abdomen badge brightness and SVL, nor between abdomen badge brightness and body condition (Table 2). Black throat border brightness had a significant negative relationship to SVL, with larger males displaying darker throat borders (RMA; N = 21, $t_{1,20} = -2.97$, $p = 0.0079$, Table 1 and Figure 3). The area of the throat, throat border, abdomen, and combined throat and throat border badges did not have any relationship with SVL or body condition in mite free males (Table 2).

Relationship between color, size, body condition, and mite load in males with mites

In both LLP (N = 71; Quadratic Regression; $t_{2,68} = 4.43$, $p < 0.0001$) and SPS (N = 128; Quadratic Regression; $t_{2,125} = 3.73$, $p = 0.0003$) habitats, there was a significant relationship between abdomen badge brightness and mite load wherein those males with moderate mite loads had the darkest abdomen badges (Figure 4).

In SPS, mite load varied from 1 to 368. Mite load was higher in SPS than LLP (LLP N = 77, SPS N = 143; ANOVA; $F_{1,218} = 8.4$, $p = 0.0042$; Figure 5), thus, SPS males with one or more

mites were used in the analyses that follow (N = 128). There was no relationship between SVL and brightness of the throat, abdomen, or throat border badges, nor between SVL and area of the throat, abdomen, throat border, or combined throat and throat border badges (Table 2). Likewise, neither SVL nor body condition was related to mite load (Table 2). There was also no relationship between body condition and brightness of the throat, abdomen, or throat border badges nor between body condition and area of the throat, abdomen, throat border, or combined throat and throat border badges (Table 2).

Males with moderate mite loads (41-100 mites) have darker abdomen badges (mean = 88.85 ± 3.91 S.E.) and males with low mite load (1-40 mites) have whiter abdomen badges (ANOVA; $F_{2,110} = 6.4243$, $p = 0.0016$; mean = 115.21 ± 5.41 S.E.; Figure 4). Additionally, moderate mite load males have larger throat border area (ANOVA; $F_{2,108} = 3.2739$, $p = 0.0417$) than low mite load males (Figure 6). Males with moderate (41-100 mites) and high mite loads (101+ mites) have larger throat badge areas (ANOVA; $F_{2,110} = 4.9768$, $p = 0.0085$), larger abdomen badge area (ANOVA; $F_{2,109} = 3.7803$, $p = 0.0259$), and larger combined throat badge and throat border areas (ANOVA; $F_{2,111} = 5.1667$, $p = 0.0072$) than males with low (1-40) mite loads (Figure 6).

CHAPTER V

DISCUSSION

Our results suggest badges in *S. woodi* advertise different information for different receivers. The throat badge likely serves as a sex identifier to conspecifics because it displayed no relationships with body size, body condition or mite load. The abdomen badge likely signals mite tolerance, evidenced by males with the darkest and largest abdomen badges bearing moderate or high mite loads (Figure 4). The throat border may advertise an aspect of male body size (Figure 3), though this relationship could be dependent on growth and seasonal effects. These relationships are likely mediated by seasonal increases in mite abundance and tradeoffs with testosterone level. Collectively, my results suggest that elements of both the ICHH and good genes are supported. That is, signal expression is related to both male quality and tradeoffs with immunocompetence. However, as parasite load increases across the breeding season, selection likely acts on genes that enhance tolerance to infection rather than resistance to infection.

The brightness of the throat badge lacked any relationship with SVL or body condition in mite-free males. Additionally, the throat badge was not associated with SVL, body condition, or mite load for males with mites. Given this, I conclude that the throat badge serves as a sex identifier. Support for this conclusion is seen in Figure 1, with males displaying a darker throat badge than females (Cooper et al., 1992). Likewise, male *Sceloporus undulatus*, who display blue throat and abdomen badges, painted to resemble females with white throats and white abdomens, were treated as females by observer males, and females painted with blue throat and abdomen badges were treated as males (Cooper Jr & Burns, 1987). Male phrynosomatid lizards prominently display the throat badge in reproductive behaviors such as head-bobs to broadcast

information on sex and species identity (Martins, 1991; Thompson, Bissell & Martins, 2008). Therefore, variability in throat badge brightness may not reveal individual male qualities, functioning only to inform on sex identity and ensure appropriate sex-specific responses from conspecifics.

The immunocompetence handicap hypothesis (ICHH) predicts that parasite load is related to signal expression by inhibition of testosterone via activation of the immune system (Folstad & Karter, 1992). Males that do not possess high-quality genes for parasite resistance are thought to acquire more mites and mount an immune response, which would, in turn inhibit testosterone production and result in suboptimal sexual signals (Folstad & Karter, 1992; Geary, 2015; Zuk, Johnsen & MacLarty, 1995). I show that males with moderate mite loads have darker abdomen badges (Figure 4) males with low mite loads. All lizards captured after March had at least one mite. This result suggests that *S. woodi* abdomen badges advertise mite tolerance during the breeding season because 100% of males were infected and moderate mite load males have the darkest badge during this time. The high prevalence of mites suggests genes for parasite tolerance may be more beneficial to *S. woodi* than genes for parasite resistance (Megía-Palma et al., 2016; Miller, White & Boots, 2006; Råberg, Graham & Read, 2009).

Given that immune response is energetically costly, individuals that effectively tolerate parasites may not waste resources on mounting a strong immune response, thereby maintaining production of testosterone (Jacobs, Zuk, Demas & Nelson, 2012). Genes for tolerance to parasitic infection are defined as those producing a reduced fitness loss when infected. Tolerance genes are less costly than resistance genes and are thus more adaptive in habitats with high parasite prevalence (Olsson, Wapstra, Madsen, Ujvari & Rugfelt, 2005; Råberg, 2014; Råberg et al., 2009; Roy & Kirchner, 2000). Males with attractive signals, such as the darkest abdomen

badges, would therefore advertise their ability to tolerate moderate mite loads without detrimental effects on health or testosterone production. In wild sheep, individuals with high tolerance had greater lifetime reproductive success than those with low tolerance; however, it was indicated that there was little heritable genetic variation for tolerance (Hayward, Nussey, Wilson, Berenos, Pilkington, Watt, Pemberton & Graham, 2014). The potential for genetic tolerance of parasitic infections has important implications for parasite-host dynamics and sexual selection. My results demonstrate that populations experiencing heightened parasitic pressures may exhibit selection for tolerance instead of resistance, redefining the relationship between signal expression and mite load. Considering this, the ICHH and 'good genes' hypotheses partially support sexual signal expression in *S. woodi.* Those males with highest mite loads did not display the darkest abdomens, indicating a potential limit to a males' ability to tolerate mites without detrimental health costs. *Sceloporus woodi* that accumulate extremely high mite loads likely face increased fluid loss, evaporative water loss, decreased sprint speed and/or endurance, and decreased ability to recover from stress (Baldwin, 1998). Therefore, males with moderate mite loads exhibit tolerance for moderate mite loads while avoiding high mite loads by advertising dark abdomen badges to females. Future work should determine the plasticity of responses to mite infection to delineate tolerance vs. resistance for ectoparasites.

Males with moderate mite loads displayed larger throat borders than males with low mites loads (Figure 6). Additionally, males with moderate and high mite loads have the largest abdomen, throat, and combined throat and throat border badges (Figure 6). I found no relationship between body size and mite load thus it seems unlikely that larger or older males accumulate more mites. It is more probable that badge size, like abdomen badge brightness, is hormonally regulated via testosterone and is related to mite load via immunocompetence

tradeoffs (Laucht, Dale, Mutzel & Kempenaers, 2011). Interestingly, those males with the largest abdomen, throat, or combined throat and throat border badges had moderate or high mite loads, indicating that there may be a static nature to the size of these badges unlike the brightness of the abdomen which may decrease in expression due to high mite load costs (Baldwin, 1998). Osborne (2005) showed that badge size in the tawny dragon (*Ctenophorus decresii*) displays this static nature and did not vary by season or by condition. Stability of badge size could tentatively be explained by seasonal changes in the honesty of signals. For example, early in the breeding season when mite pressure is low, male badge size may honestly indicate mite tolerance, however, increased mite pressure later in the year may make this signal unreliable. Thus, males may display attractive signals later in the breeding season even though they are suffering costs of high mite load. Additional work on the seasonality of relationships between male condition, quality, and badge size is necessary to support this hypothesis.

Early in the breeding season (February to March), when mite prevalence is low or nonexistent, the throat border is indicative of male size (SVL). Larger males displayed darker throat borders (Figure 3). The black throat border may also be indicative of male aggression, dominance, or survivability (Carpenter, 1995; Halliday & Verrell, 1988; Halliday, 1992; Olsson, 1994; Thompson & Moore, 1991; Whiting, Stuart-Fox, O'Connor, Firth, Bennett & Blomberg, 2006). This relationship was not significant in males with mites captured after March suggesting that seasonal trends or tradeoffs between immunocompetence, hormones, and mite pressure may regulate the relationship between black border brightness and body size. Further research on seasonal differences in relationships of sexual signals and male qualities is required to quantify these relationships.

While we were unable to directly assess differences in sexual signal expression by parasite load in SPS vs. LLP populations, due to low samples of high mite load males from LLP, we show that both LLP and SPS populations show similar trends in the relationship between abdomen badge brightness and mite load (Figure 4). Future work should compare the relationships between badge traits, male qualities, and additional habitat variables such as changes in the sensory environment. Communication is affected by the sensory environment in which the communication occurs, and signals may evolve to be more conspicuousness or cryptic in certain environments dependent on the vegetation structure and light environment (Maan & Seehausen, 2011). Longleaf pine habitats provide diffuse canopy cover while clear cut SPS habitats provide little to none (pers. observ.). This provides an interesting opportunity to measure habitat variables related to the light environment and male signals.

Overall, my results demonstrate that *S. woodi* badges advertise different information and do so in sites that vary in ectoparasite load. The throat badge is likely a sex identifier allowing conspecifics to assess the sex of the signaler quickly. The abdomen badge relates information on male ability to tolerate moderate to high mite loads while allocating energetic resources toward signal expression. Male mating success likely relies on a combination of intrasexual success in obtaining territories and the display of attractive signals that correlate with habitat-relevant information such as parasite tolerance. Further experimental work that quantifies seasonal testosterone levels, male reproductive success, and female mate choice in conjunction with the manipulation of signals is needed to verify these hypotheses. Studies involving these relationships with other parasite types, such as haemoparasites and endoparasites, are also warranted.

TABLES

Table 1. Sample sizes of adult males by mite load and month from longleaf pine (LLP) and sand pine scrub (SPS). A total of 220 males were captured with 77 males from LLP and 143 males from SPS. Mite-free males (N = 21) were captured in February (N=2), March (N=3), and October (N=1) in LLP, but only February and March in SPS.

	Mite Load				
	Zero	Low	Moderate	High	Total:
Habitat					
LLP	6	29	32	10	77
SPS	15	29	53	46	143
Month (SPS only)					
February	14	0	0	0	14
March	1	8	0	0	9
April	0	1	7	13	21
May	0	6	10	1	17
June	0	6	22	24	52
July	0	4	14	8	26
October	0	4	0	0	4

Table 2. Regression statistics of the relationship between color, size, and body condition. Larger mite free males had significantly darker (more intense) throat border badges, while males with mites showed no relationships color, size, and body condition. Badge area was size corrected by obtaining the residuals of the relationship between badge area and mass. SVL = snout – vent length.

	SVL			Body Condition		
	Slope	Intercept	R^2	Slope	Intercept	R^2
Mite # = 0						
Brightness						
Throat Badge	-7.45*	464.01	0.19	-1.90	122.13	<0.001
Throat Border Badge	-4.79***	273.43	0.37	-2.07	54.10	1.4e-3
Abdomen Badge	-0.01	2.52	0.03	-0.06	2.12	0.04
Area						
Throat Badge	-0.24	9.86	0.07	-1.53	-0.66	0.08
Throat Border Badge	0.39	-18.53	0.02	2.08	-1.39	0.02
Abdomen Badge	-0.13	2.77	1.5e-3	0.73	-3.44	1.4e-3
Throat + Throat Border	-0.06	2.69	<0.001	0.24	-1.60	2.1e-4
Mite # = 1+						
Brightness						
Throat Badge	0.09	111.03	<0.001	4.89	115.30	3.3e-3
Throat Border Badge	1.41*	-17.06	0.03	-6.17	47.91	0.02
Abdomen Badge	0.01	1.93	<0.001	0.02	1.98	6.1e-3
Area						
Throat Badge	0.06	-2.88	3.3e-3	0.48	0.08	6.7e-3
Throat Border Badge	0.05	-2.44	<0.001	0.49	-0.14	1.2e-3
Abdomen Badge	0.05	-2.37	<0.001	2.44	-0.03	0.014
Throat + Throat Border	4.1e-3	0.30	1.6e-6	0.97	0.1	3.0e-3

***p<0.01, **p<0.05, *p<0.1

FIGURES

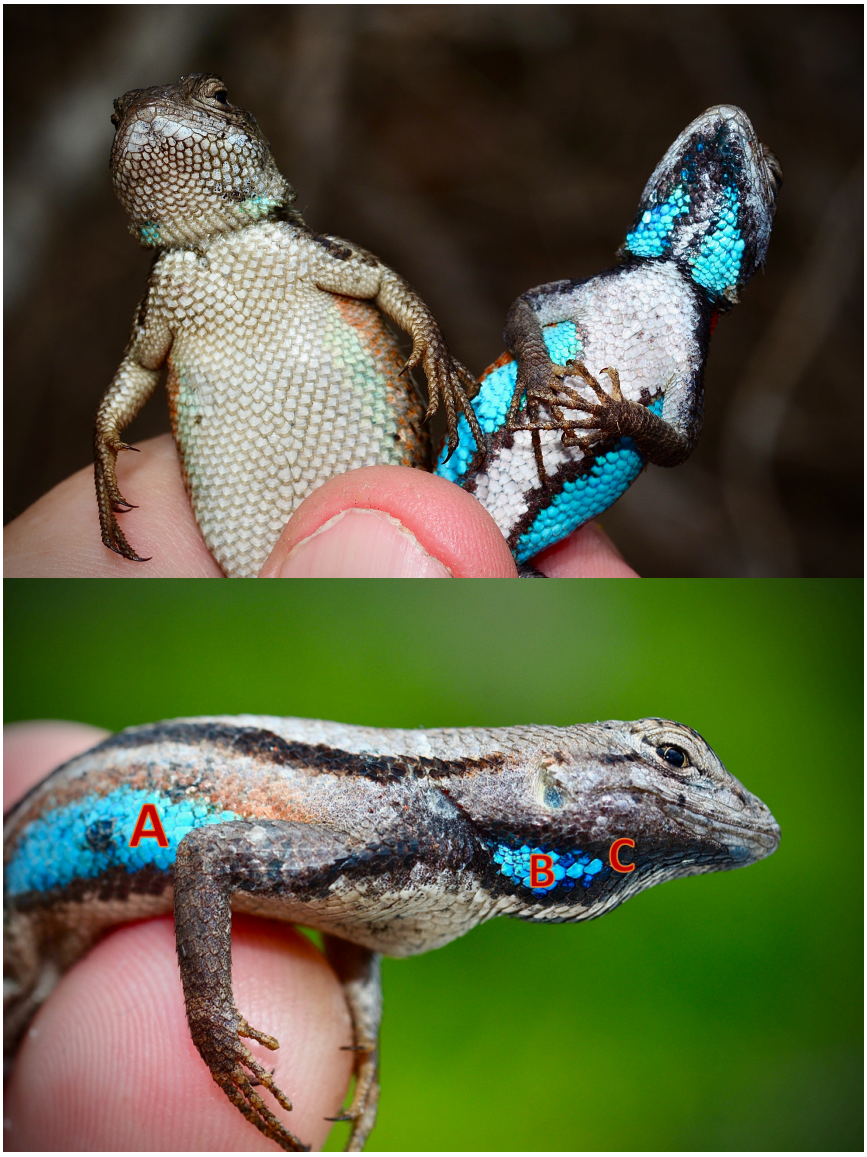

Figure 1. Coloration patterns of the Florida scrub lizard (*Sceloporus woodi*). Top photo shows a female (left) and a male with sexually dimorphic badges (right). Bottom photo shows the male-specific badges. Letters illustrate the different badges: the abdominal badge (A), the throat badge (B), and the throat border (C). Photograph credit to Janson Jones. Photographs were used with his permission.

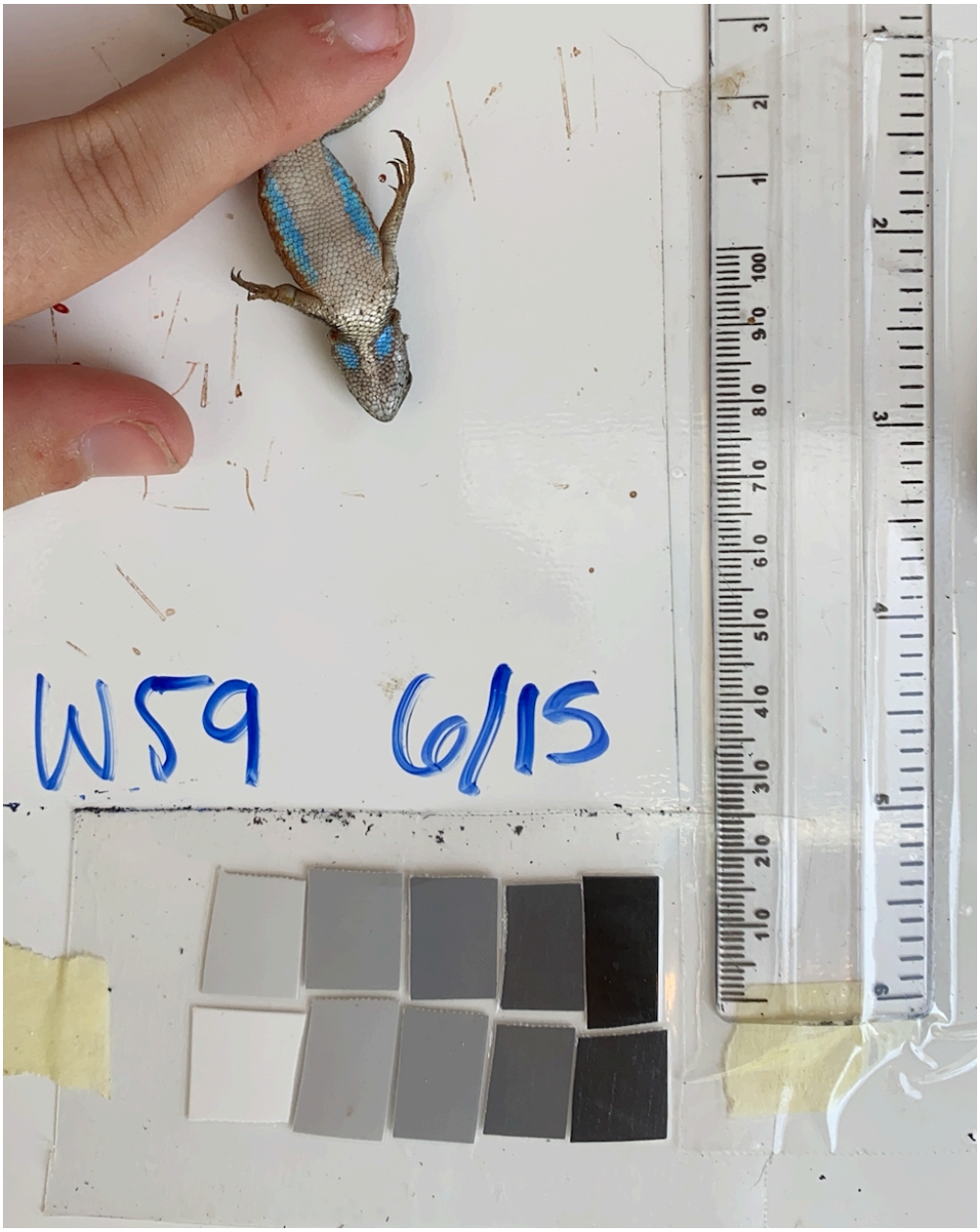

Figure 2. Example of calibrated photograph setup. Eight color-aid gray scales were used in addition to a black and white scale to calibrate brightness values across all photographs in Adobe Photoshop. A ruler was included in each photograph to allow for accurate patch area measurements in ImageJ. Note this male has minimal black border around its badges.

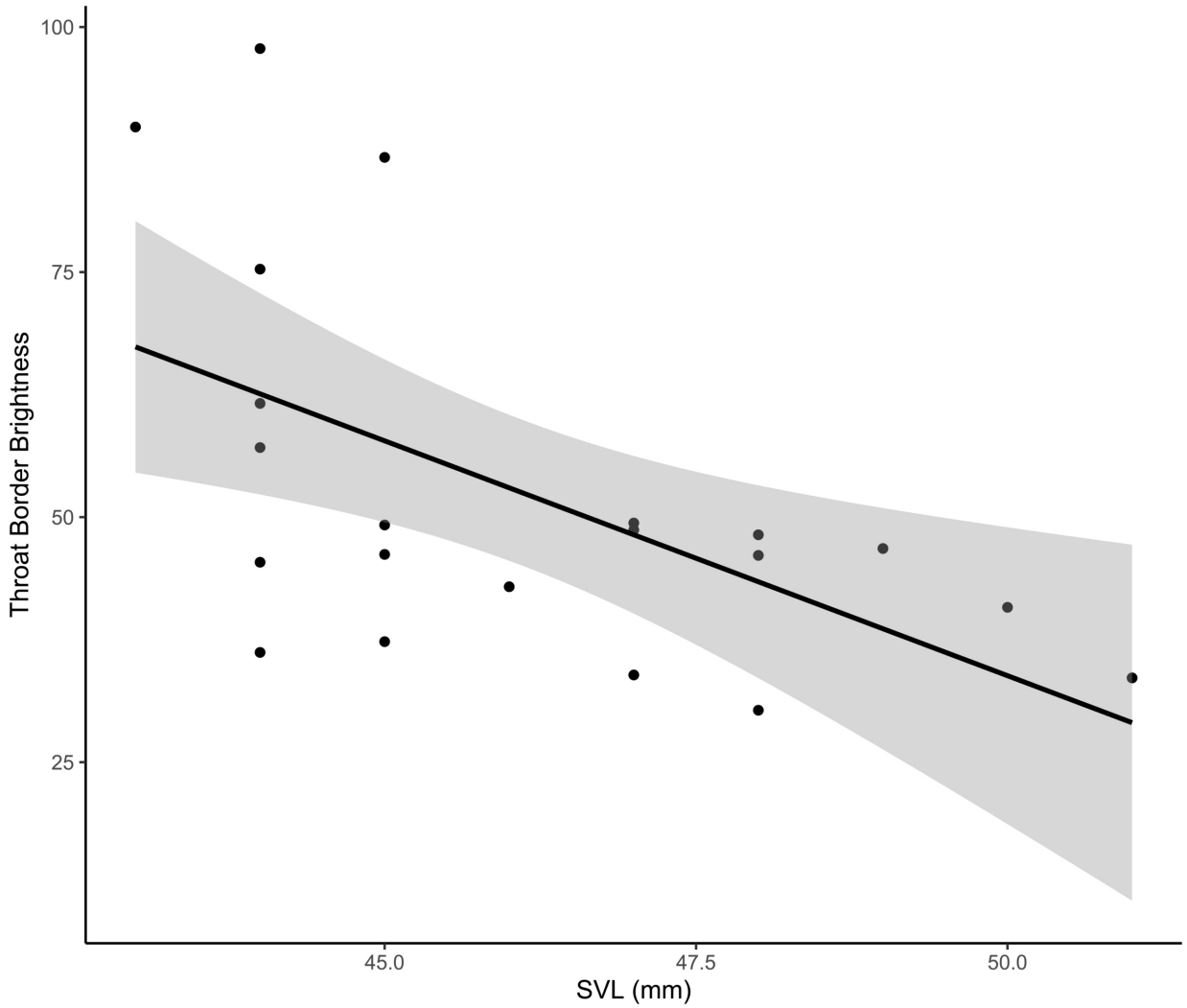

Figure 3. In mite-free males (N = 21), larger males have significantly darker throat borders. Small males have brighter (whiter) throat borders.

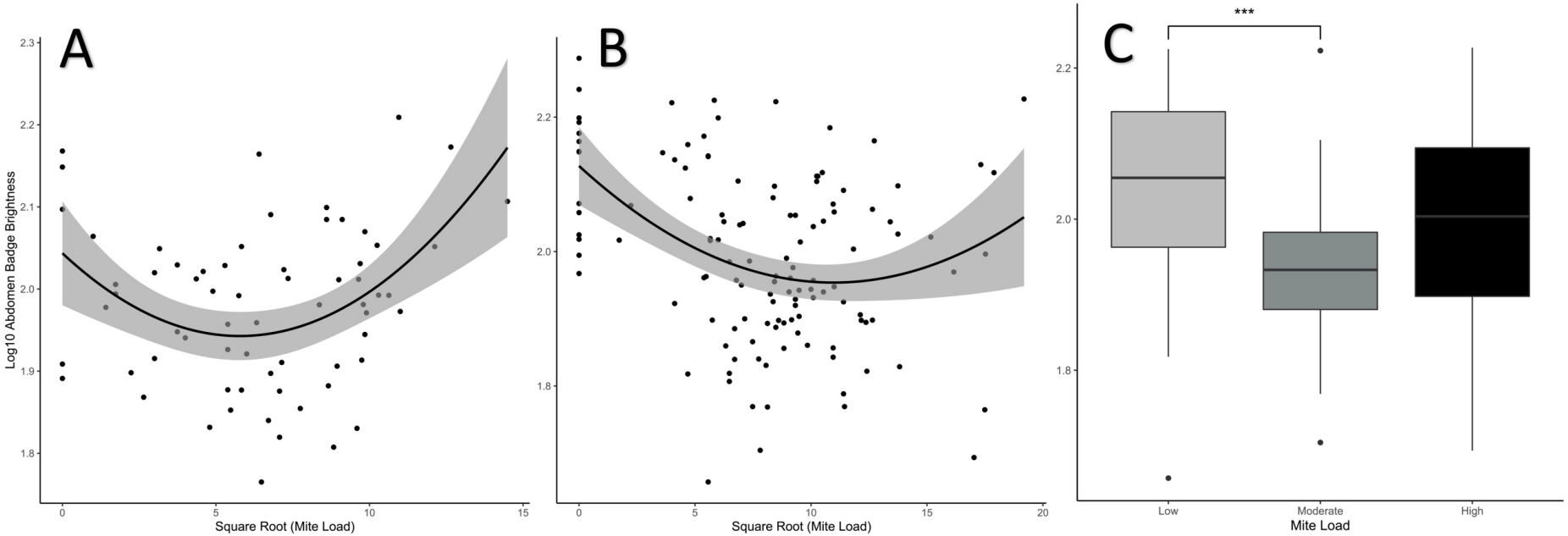

Figure 4. Males with moderate mite loads have the darkest abdomen badges in (A) sand pine scrub (SPS) and (B) longleaf pine (LLP). The same trend is seen with males with moderate mite loads having the darkest abdomen badge when males with zero mites are excluded (C). Abdomen badge darkness does not differ between moderate and high mite load males, nor between low and high mite load males. ***$p<0.01$

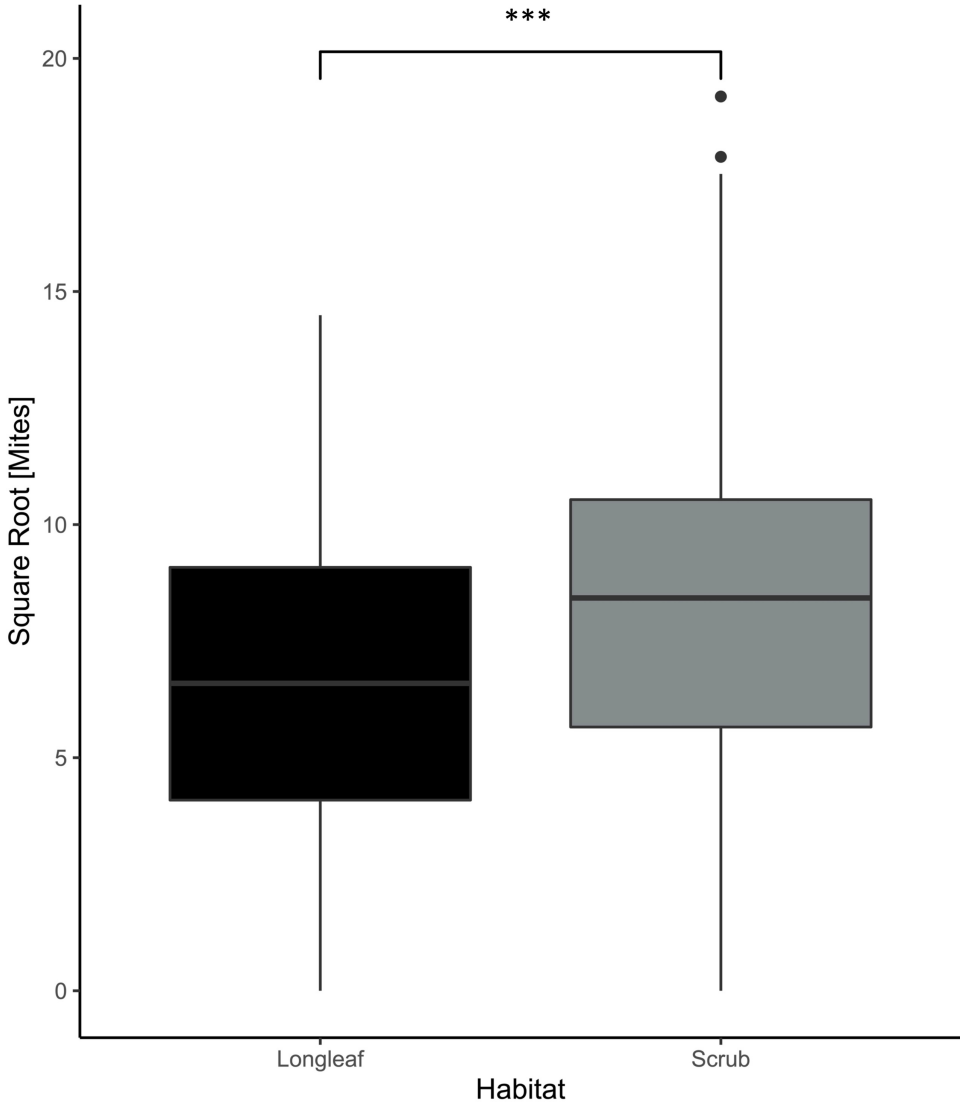

Figure 5. Males in sand pine scrub (SPS) have more mites than males in longleaf pine (LLP). *S. woodi* that inhabit scrub sites have significantly more mites than S. woodi in longleaf sites. The lowest mite load in both sites was 0 mites, while the highest mite load in longleaf was 210. The highest mite load in scrub was 368. ***$p<0.01$

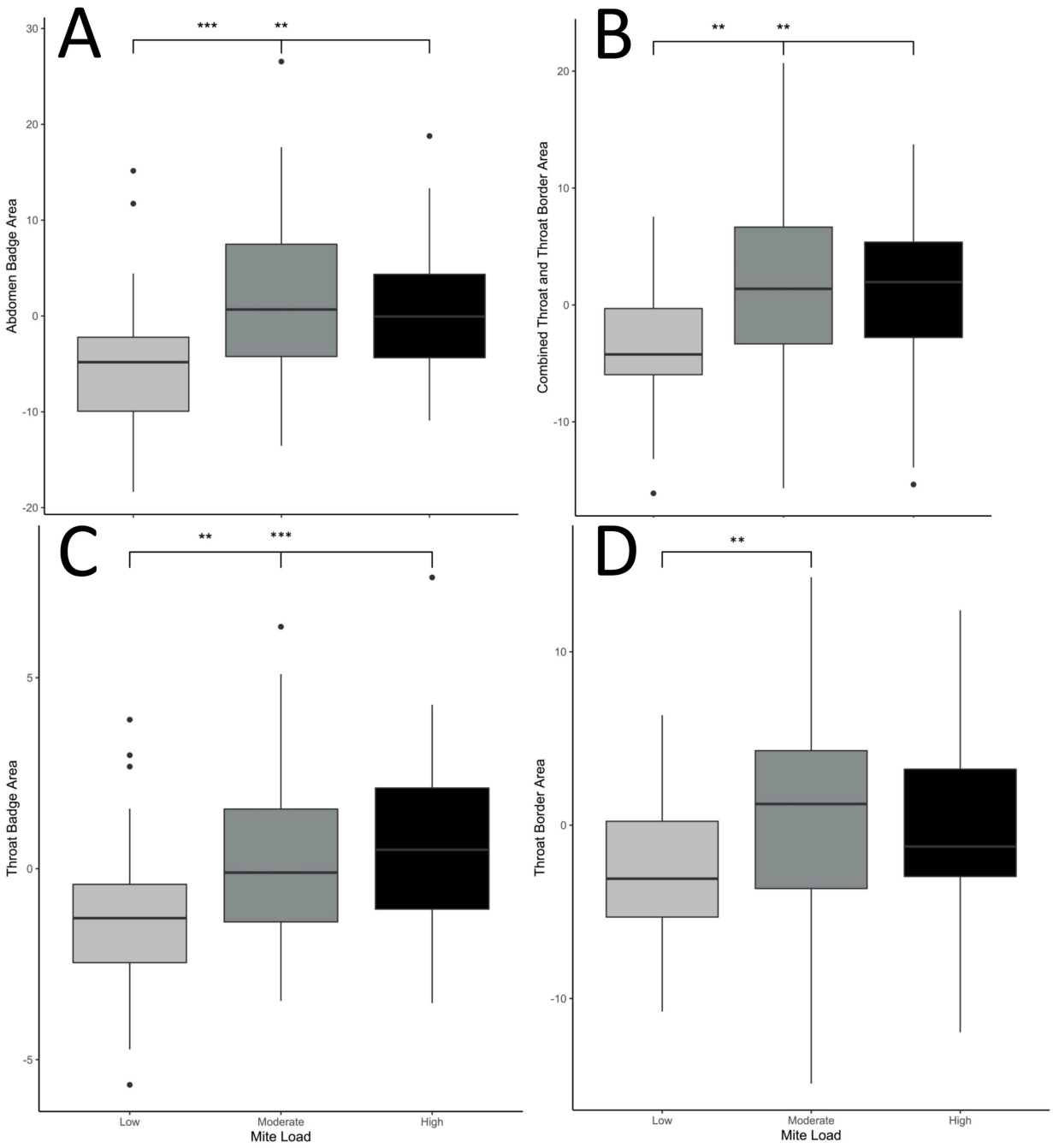

Figure 6. Moderate and high mite load males have larger (A) abdomen, (B) combined throat and throat border, and (C) throat badges than low mite load males. Moderate mite load males have larger (D) throat border badges than low mite load males. Badge area (mm^2) is corrected for body size. ***$p<0.01$, **$p<0.05$

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APPENDIX A

CALIBRATED PHOTOGRAPHY

INTRODUCTION

Research involving color in animals and plants is widespread. Spectrophotometry is a method of objectively analyzing color that measures the intensity of various wavelength reflected (Cuthill, Bennett, Partridge & Maier, 1999; Endler, 1990; Endler & Mielke, 2005; Gerald, Bernstein, Hinkson & Fosbury, 2001; Zuk & Decruyenaere, 1994). The use of spectrometry is often costly in terms of sampling time, as a spectrometer can only sample points, and can be more difficult for use in field situations (Cuthill et al., 1999; Endler & Mielke, 2005). An alternative to spectrophotometry is photography which is beneficial for its ease of use, availability, and power in analyzing entire color regions. See Stevens et al. (2007) and Johnsen (2016) for more information on the utility of calibrated photography.

Brightness is the term often associated with analysis of color. Brightness is measured as the relative amount of white mixed with pigment. Brightness is interpreted as a measure of lightness reported on a unitless scale where low values (0) signify similarity to black and high values (300) signify similarity to white (Cuervo, Belliure & Negro, 2016). It is important to note that high values of brightness do not necessarily mean ‘better’ or ‘more’ color. For example, high brightness in the context of sexually selected lizard badges (e.g., in *Sceloporus woodi)* would mean a color badge with less intense color, or less blue (or black) pigment. Low brightness, indicating darker colors, would therefore signify more vivid blue (or black) coloration. The pigments required for the expression of vivid (i.e. highly pigmented) color badges in lizards absorb light rather than reflect it (Megía-Palma, Barrientos, Gallardo, Martínez & Merino, 2021), thereby indicating that those individuals with more pigments have decreased

brightness values. Increased pigmentation is costly (Ethier, Gasse, Lake, Jones, Evenden & Despland, 2015; Jawor & Breitwisch, 2003; Kimball & Erpino, 1971; Morrison, Rand & Frost-Mason, 1995), thus those animals with more pigmentation (darker) are often higher quality individuals. For the lizard *S. woodi*, darker badges are likely 'better' badges, based on citation in other *Sceloporus* species. Be aware of the specific organism's biology before attempting to state whether darker or brighter is necessarily 'better', 'preferred', or 'more intense'.

Other color metrics commonly analyzed are saturation and hue. However, saturation and hue must be analyzed using spectrophotometry instead of photography (C. Bedore, pers. comm.), because a camera uses an RGB scale which cannot estimate saturation (how pure the color is). Photography is beneficial only for measures of brightness and only for comparison of brightness values among samples, not for absolute values. Absolute color values require use of a spectrophotometer (C. Bedore, pers. comm.).

The process of measuring brightness from a photograph starts with (1) deciding (via preliminary trials) how to consistently take the photographs, 2) taking calibrated photographs, (3) importing the photographs into Adobe Photoshop to analyze colors, and (4) creating a spreadsheet to calculate the brightness values from an RGB scale.

METHODS

Taking the Photographs

Specific color regions on organisms are used to adequately quantify brightness or color (Cuthill et al., 1999; Endler & Mielke, 2005). For example, in *S. woodi*, I analyzed multiple components on the body of the lizard (Figure 1). These same regions need to be measured on each specimen for accurate comparisons. Photographs should include color-aid basic gray scales

(e.g. Basic Gray Scale Coloraid TM), as well as one black standard and one white standard to allow for calibration and grayscale equalization in Adobe Photoshop (Adobe Systems, San Jose, CA) (Stevens, Párraga, Cuthill, Partridge & Troscianko, 2007). A ruler should also be included in every photograph to calculate the area of each region sampled (Figure 2). Every photograph should have the same view (or very close) of the color patch with as little shadow or glare as possible. All photographs should be taken from the same distance such as on a copy table or camera stand with a standardized setup of distance, lighting, and orientation. This can be achieved by taking all photographs in the same room under the same lighting conditions, or in the field with additional care. It is also important to note that some organisms may change color depending on temperature so do detailed background research, and/or color trials at different temperatures. It is best practice to also take the organism's body temperature before every photo.

A simple method of capturing photographs is using an app on a mobile phone. The app ProCamera (Cocologics GmbH, V15.1) allows images to be processed as RAW files and JPEG files which can be useful for further analysis. A digital camera can be used to take photographs if preferred. See Orton et al (2020) and other citations for more information on camera settings. The technical specifications and model of the equipment will need to be noted.

RGB Analysis in Photoshop

Photographs should be in JPEG format for use in Adobe Photoshop. Bring the photo to be analyzed into Adobe Photoshop. First, the photograph must be calibrated using the grayscale included in the photo. You will need to use the square selection tool to select the first gray scale. Next go to blur > average and then use the eyedropper tool to click inside this gray scale. This will give you the red-green-blue (RGB) values on the right-hand side. Write these down either in

your spreadsheet or elsewhere for future reference. Continue this same process for each of the remaining gray scale color units in the photograph separately. You will have 10 total measurements for the grey-scales if you have 10 grey-scales. Next, you will analyze the RGB values for each color patch. Draw a lasso around the entire color region on the organism you wish to sample. Next, go to blur > average. This will average the lasso-selected color region into one RGB value. Next use the eyedropper tool to select that color region. The RGB values will show up on the right side of the screen. These numbers should be recorded in your spreadsheet for future brightness analysis. Continue to follow the previous steps for each color patch in each photograph of each organism. This method of measuring RGB was recently compared with spectrophotometric data and showed strong positive correlations between brightness values (Orton & McBrayer, 2019). You will need to decide if you are only going to measure each patch once or multiple times. Multiple measurements are typically recommended. Once you have recorded all the RGB values for both the gray-scales and the color patches, you must move them to an excel spreadsheet with the required calculations (described below).

Brightness Analysis in Spreadsheet

An excel spreadsheet to compute brightness values from RGB measurements is available on the McBrayer server. In the top area of the sheet, you will input the RGB vales for each of the grayscales plus the black and white scale. This will automatically plot the % reflectance and give you the formula *y=me^nx.* You will need to copy the m and n value and input these into the formula for %R, %G, and %B below, where type the measured RGB values from the color badges. The formula should look like this: *=EXP(n value*I13)*m* value. In this example, I13 references either the R, G, or B value measured in Adobe Photoshop for each of the %R, %G, and %B measurements respectively. Make sure the formula for all these %RGB values match

those m and n values (use pull down + over entire %RGB formula area after doing one). Brightness is automatically calculated as the sum of the %R, %G, and %B rows. This value is your brightness value for that specific patch. The RGB values for each patch don't tell you much except that, in relative terms, this one has more "red" or "blue". Colors are hard to define using the photographic method because they aren't separated out into wavelengths. The real utility of these numbers is when you sum them and get the total brightness for the patch.

Area/length Measurements

Upload the photograph to the program ImageJ (National Institutes of Health, Bethesda, MD). Next, calibrate the number of pixels to a measurement on the ruler (each 1, 3, 5 mm, or 1cm) you included in the photograph. Do this by drawing a line on the ruler for the measurement you want (e.g., 1 mm), then select Analyze > Set Scale > input the correct measurement (e.g., 1mm) click done. Now you can either select a lasso for area measurements or the line tool for length measurements in your specified units.

FIGURES

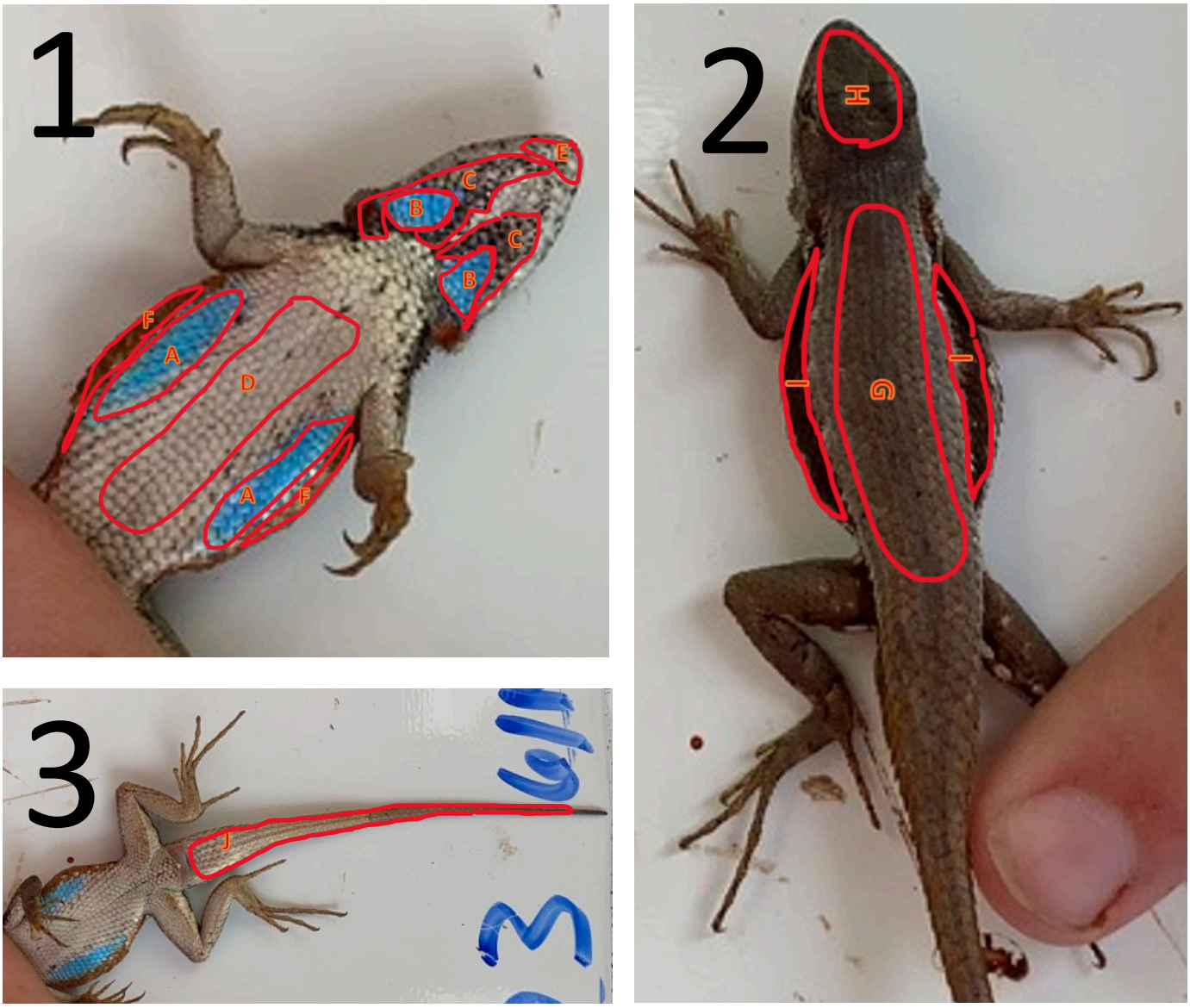

Figure 1. Badges examined in *S. woodi*. If a specimen did not display a color badge (e.g., no blue on abdomen or throat, or not black on throat border), then that badge was not measured at all and does not appear in results. Photograph 1 shows the ventral side of the lizard. Left and right badges are typically measured separately and then brightness values are average to obtain one value of brightness per badge per specimen. Letters illustrate the different color areas measured. (A) Abdomen, (B) Throat, (C) ThroatBlack, (D) Abdomen, (E) Midthroat, (F) Bellyside. Photograph 2 shows the dorsal side of the lizard. Letters illustrate the different color areas measured. (G) Back, (H) Head, (I) BlackStripe. Note: Some lizards display a dark stripe across the head this could be designated as Foreheadline. Photograph 3 shows the undertail side of the lizard, (J) Undertail.

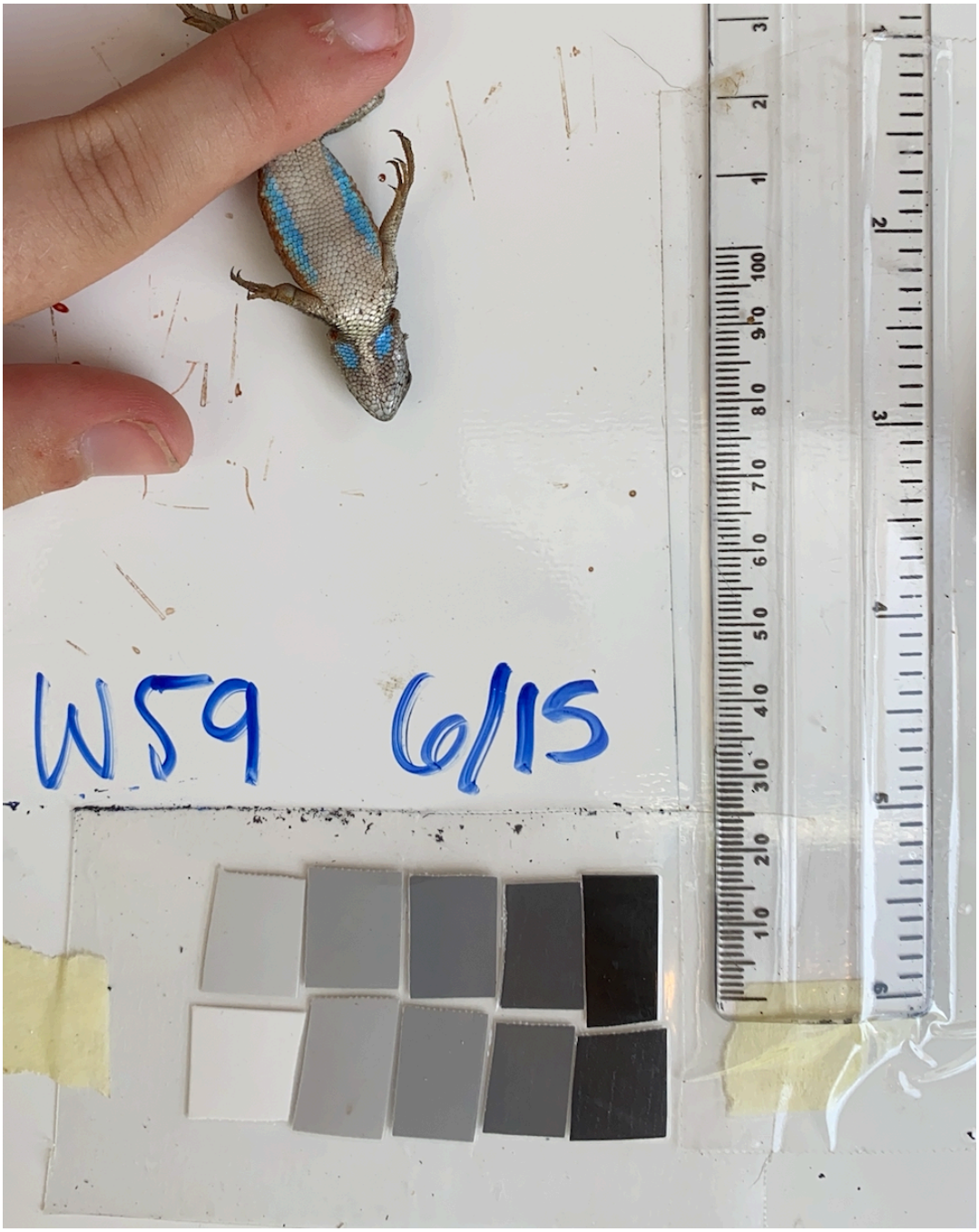

Figure 2. Calibrated photograph setup. Note that there is a ruler and 8 gray scale chips, plus 1 black and 1 white scale chip included (10 chips total). This or a very similar setup should be used for every photograph taken. It is also helpful to tape these to a small whiteboard and then use an expo marker to write the lizard ID and date and any other useful information so that when you upload your photos it is included.

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APPENDIX B

BLOOD SAMPLING FOR HORMONE ANALYSIS

INTRODUCTION

There are many ways to determine hormone concentration in animals such as via blood serum, feces, saliva, or urine. Common hormones of interest include androgens, such as testosterone and estrogen, and glucocorticoids, such as cortisol and corticosterone (Romero & Reed, 2005). Blood sampling is one of the most widely used methods of analyzing an organism's hormone levels to determine reproductive state or stress levels. In lizards, blood sampling is routine and there are many studies detailing methodology (Cox, Skelly, Leo & John-Alder, 2005; Moore, 1986; Moore, Thompson & Marler, 1991).

METHODS

Supplies

To obtain a blood sample you will need capillary tubes and heparin (or heparinized capillary tubes). The heparin prevents clotting of the blood so that the plasma and whole blood can be separated. If your capillary tubes are not heparinized, you will need to buy powdered heparin lithium salt (Example: https://www.sigmaaldrich.com/US/en/product/sigma/h0878) and dilute it with deionized water to make it liquid. This bottle of liquid heparin will need to be carried in the field or be available when you intend to draw the blood sample. Capillary tubes should be large enough to obtain enough blood but small enough to fit the region that you are drawing the blood from on the animal. For example, in lizards (e.g. *S. woodi*), blood is drawn from the orbital sinus behind the eye (Maclean, Lee & Wilson, 1973). Twenty microliter capillary tubes are needed to prevent injury to the eye. You will also need sealing clay for the

capillary tubes to prevent loss of blood. Depending on where you are when drawing blood (field vs laboratory), you may need a secure carrying case for your blood samples. It is possible to buy a waterproof match case (e.g., sporting goods store in the camping section) and cut two pieces of foam that fit horizontally inside so that you can place the filled and sealed capillary tubes snuggly between them and prevent any breakage of tubes. You will need a centrifuge designed for capillary tubes to separate the whole blood cells from the plasma. If spinning in the field, make sure you also have a car adapter to run the centrifuge from the car battery. There are various methods to mark capillary tubes for identification of individual samples once they have been spun. I used two methods. The first was placing a piece of tape on one end of the capillary tube and writing in permanent marker the specimen ID and date. I would place all the labeled capillary tubes from one date in a plastic bag for storage. Another method, which I found to be beneficial in case of tube breakage, is buying small plastic bags, commonly used for jewelry crafting, from a craft store and placing a single tube (or all tubes associated with one organism) in that bag. A piece of paper can be labeled with the specimen's name and placed in the bag as well. I had several tubes break and this allows you to still potentially obtain plasma from those broken vials while still knowing the identity of the blood. If you are obtaining blood samples in the field, you must have a cooler with ice or dry ice to keep spun samples cold until you can get them in a 0-degree F freezer. Make sure to put your baggies of samples in a hard case for transport where they will not be squished.

Obtaining Blood

Depending on the hormone that you wish to quantify, blood sampling may need to occur within seconds of capture. Glucocorticoids can rapidly rise when catching an organism so blood would need to be drawn within less than 3 minutes of spotting and/or capturing the organism

(Romero & Reed, 2005). Androgens are more stable over short time spans and thus blood can be sampled within several hours (Wickings & Nieschlag, 1976). One should note how long you handled the specimen and how long it took from sighting to capture to drawing the blood in order to ensure consistency of the timing of blood collection per individual.

In lizards the best place to obtain blood is in the orbital sinus behind the eye but different specimens may need blood drawn from different areas (Maclean et al., 1973). For testosterone analysis, 30 microliters minimum plasma is required (H. John-Alder pers comm). Because of this you would need to collect at least 50 microliters of blood from each organism. If you use 20 microliter capillary tubes, you will need to get several capillary tubes from each individual. To do so, obtain a capillary tube, dip it in the jar of liquid heparin (you only need a tiny amount per tube), and then poke the capillary tube into the orbital sinus. You need to be firm when puncturing the orbital sinus but not enough to injure the animal. You can often feel something like a little bubble pop when you have hit the sinus artery. You will want to very slowly pull the needle out a little without completely removing it from the eye area to collect the blood (Figure 1). Leave a small amount of the capillary tube empty to allow for closure with the sealing clay. Next, seal both ends of the capillary tube with the sealing clay and place in your protective carrier. If you are in the field capturing multiple specimens and collecting multiple blood samples, I recommend putting the blood samples in the carrying case in order from first to last, just as in your notebook so that you can validate the capillary tube label with the order in your field notes. Hormone levels in blood samples are generally stable for a moderate period of time, particularly testosterone (Wickings and Nieschlag 1976), but if the temperature is extremely hot or humid, try to get samples spun and put on ice faster.

Centrifuging samples

To separate the whole blood cells from the blood plasma for hormone analysis you will need to centrifuge the blood samples before they are frozen. Note that your samples must be sealed with the sealing clay before centrifuging. I strongly recommend resealing all capillary tubes immediately before putting them in the centrifuge as this prevents any spills. The centrifuge I used had four slots for capillary tubes that were numbered. I went from oldest blood sample to most recent and wrote down which specimen went in which centrifuge compartment (1-4) for each run. You will only need to spin the samples for 1-2 minutes at the most. I recommend initially spinning samples for only 1 minute and looking to see if they are appropriately separated or not before continuing. I did not have any problems with tubes breaking inside the centrifuge, but it is good practice to balance your tubes within the centrifuge compartment by placing empty tubes in empty slots. Once samples are separated into whole blood cells and plasma within the capillary tubes, they will need to be labeled (see above) and safely placed in a cold area before being put in a freezer. I recommend that you measure (estimate) the amount of plasma and whole blood in each tube and for each specimen and record this information for later. To measure blood or plasma volume in capillary tubes, I drew the length of the capillary tube on a piece of paper and broke the drawn line into segments dependent on their length. Then, I held the capillary tube against this measurement and was able to estimate the amount of blood vs plasma dependent on where each measured to relative to the size of the microcapillary tube (ex: if the plasma inside the capillary tube extended ½ down the tube, then the plasma volume was estimated to be 10 microliters).

Separating whole blood and plasma for analysis

The whole blood cells and the plasma do not need to be separated outside of the capillary tube until you are ready to analyze them. To do so you will need microcentrifuge tubes, a permanent marker, a small blade (made for cutting capillary tubes), a bulb dispenser and storage containers for the microcentrifuge tubes. If your project involves collecting the whole blood on filter paper then you will also need filter paper. Take capillary tubes out of the freezer and allow them to thaw to room temperature (~5 mins) before continuing. To separate the whole blood cells from the plasma, etch a short line with the blade in the capillary tube between the whole blood and plasma. The tube should break along the edging with a little pressure. Try to prevent getting any whole blood in the side with the plasma. Etch and make another small break with the blade right above the sealing clay. Next, pick up the piece containing the plasma and place the bulb dispenser tool over one end. Put the microcentrifuge tube below the other end and squeeze out the plasma into the tube. Close the tube and label it. If you are collecting the whole blood on filter paper, squeeze the whole blood on the paper and label it. These samples can go back in the freezer before being analyzed or shipped. If you are shipping the samples, make sure to pack them securely with dry ice.

Preliminary Analyses

Due to low plasma yields, I was only able to obtain testosterone analyses for 13 males from LLP and 13 males from SPS. Table 1 and Figure 2 show this preliminary data.

TABLES

Habitat	N	SVL (mm) Mean ± SD	Testosterone (ng/ml) Mean ± SD	Mite Load Mean ± SD
LLP	13	45.53 ± 3.30	20.99 ± 11.70	36.53 ± 34.79
SPS	13	44.53 ± 2.30	28.93 ± 17.91	92.70 ± 89.98

Table 1. Preliminary summary statistic testosterone data from LLP and SPS males. SVL = Snout-Vent Length.

FIGURES

Figure 1. Blood sampling in the orbital sinus in *S. woodi*. The heparinized capillary tube is inserted into the orbital sinus and gently pressed until the sinus is ruptured.

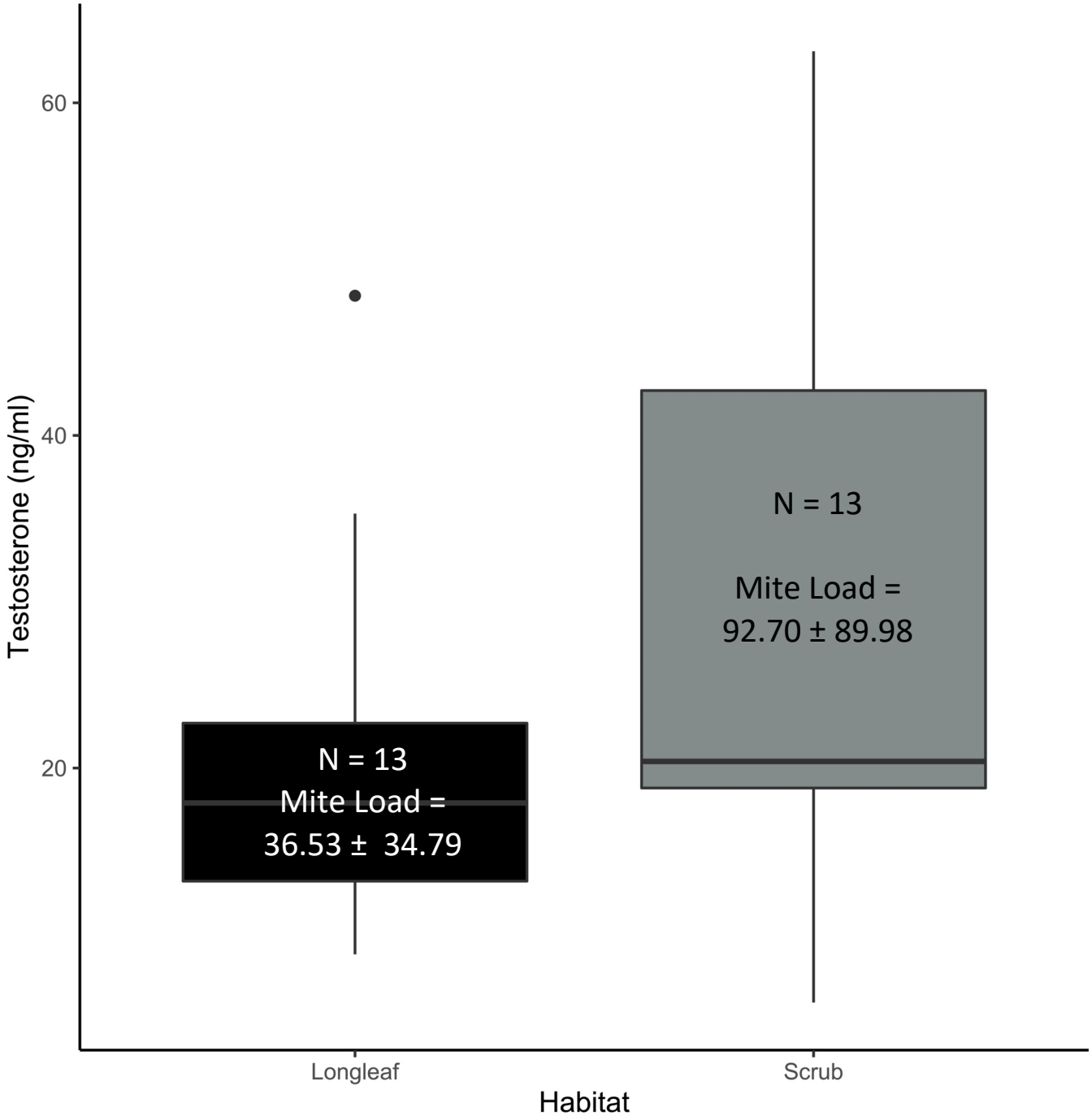

Figure 2. Preliminary testosterone results by habitat type. Testosterone did not differ between LLP and SPS. Mite load is given as mean ± std. dev.

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APPENDIX C

FEMORAL PORES AND GC-MS

INTRODUCTION

Knowledge of chemical communication in many vertebrates is limited. Squamate reptiles commonly exhibit a series of epidermal holocrine glands that produce a waxy substance implicated in chemical communication (Mayerl, Baeckens & Van Damme, 2015). These glands, referred to as femoral pores, are located on the ventral side of the hind limbs and can vary in number by species and individual (Baeckens, Edwards, Huyghe & Van Damme, 2015 Van Damme 2015). Femoral pore exudates are thought to be signals for intrasexual competition or mate choice (Mason, 1992). Exudates are composed primarily of lipids and proteins (García-Roa, Megía-Palma, Ortega, Jara, López & Martín, 2017 2017; Ibanez, Menke, Quezada, Jiménez-Uzcátegui, Schulz & Steinfartz, 2017; Khannoon, 2016; Mangiacotti, Fumagalli, Scali, Zuffi, Cagnone, Salvini & Sacchi, 2017). The quantitative variations of these compounds may produce different responses from receivers (Kopena, Martin, Lopez & Herczeg, 2011; Martin & Lopez, 2015), thus, it is important to study the composition of femoral pores in relation to individual traits such as color, size, and behavior. Gas-chromatography mass-spectrometry (GC-MS) is an analytical method that can identify (estimate) the composition and the abundances of chemicals within a sample.

METHODS

Obtaining the femoral pores

To collect the femoral pores you will need GC-MS vials with caps or microcentrifuge vials. You will also need small forceps and ethanol to sterilize the forceps after each femoral

pore. To collect the femoral pore from the lizard, dip the forceps in ethanol and ensure they are clean. Next gently press around the femoral pores until you can see a small waxy substance protrude. Collect the exudate with the forceps and place inside the vial. Collect as much of the femoral pores as possible and place in the same vial. Seal the vials, label, and place in a cool location, preferably on ice. I recommend weighing the vial before and after placement of the femoral pores to determine the mass of femoral pores for each animal. Femoral pores can be placed in a freezer before analysis.

Preparing for the GCMS

GC-MS vials with caps are necessary for running samples. These vials are recommended for small samples such as those from *S. woodi* femoral pores: Thermo Scientific™ SureSTART™ 0.3 mL Glass Screw Top Microvials for <2 mL Samples, Level 3 High Performance Applications Link: https://www.thermofisher.com/order/catalog/product/6PSV9-03FIVP. These caps will fit the vials and allow the plunger needle to go through them: Thermo Scientific™ SureSTART™ 9 mm Screw Caps, Level 3 High Performance Applications Link: https://www.thermofisher.com/order/catalog/product/6PSC9TST. You will also need a solvent to dissolve the pores in. We used n-hexane 97+/-% for HPLC Clear Liquid 1L from VWR International, LLC. Manufacturer: Acros Organicas. Part Number: AC44572-0010. It is usually recommended to also have an internal standard to put in with each sample, but we did not do this.

To prepare femoral pores for use in the GC-MS first take them out of the freezer and allow to thaw for ~1 hour. I initially added 250 microliters of hexane to each sample and thoroughly shook them before leaving them in the GC-MS machine for analysis. However, because lizard femoral pores are very small we need to minimize the amount of hexane we are

using to maintain a higher concentration of lipids from the pores. I then started using the method described in Garcia-Roa et al. (2018). In this paper, they outline a step-by-step procedure (Sample Preparation) wherein you add a calculated amount of hexane dependent on the mass of the pores.

Once all the GC-MS samples are prepared you will need to prepare the GC-MS machine. The plunger needle, which injects the sample into the GC-MS has to be rinsed with 3 different chemicals to prevent contamination. These three chemicals should be filled each time you use the GCMS and one of them should be the solvent you used (e.g., n-hexane). The other two can be chemicals such as pentane, acetone, benzene, or toluene. These three chemicals should be the same every time you run the machine.

You must set the specifications of the GC-MS for what samples you are using. We used the procedure from this paper (López, Amo & Martín, 2006) to setup the GC-MS. The batch table should also be set each time with the correct order of the IDs of the samples as they are put into the GC-MS machine. It is important to check the levels of the carrier gas (helium) as well as any errors that come up such as plunger errors (the plunger needle needs to be replaced) or generic errors. Once the machine is set up correctly and the samples are appropriately placed in order the machine can be started.

Troubleshooting

We were unable to determine any significant peaks from our samples. This may have been due to low concentration of the lipids, ineffective dissolution of the pores in the hexane, incorrect machine setup, or errors in the GC-MS machine. We were able to contact Dr. Jose Martin who wrote the following:

> “First, secretion is made of lipids and proteins, and these proteins are not soluble in hexane, so you always will have an amount of solid material in your samples. So, I think that dissolution is not your problem. Rather, I think you have a very low concentration of lipids in the solvent (in the vial), because the amount of sample is low, and you use too much hexane in each sample. We use now glass inserts or vials with a conic bottom, so we can reduce the amount of hexane and increase concentration of the sample. With low concentrations you can just inject in the GCMS the solvent, so you won't find peaks (but plastic, etc.). So, first try to reduce the hexane in the vial as much as possible (but allowing the GCMS injector to take sample). For this the inserts are very good.
>
> Then and this depends on the GCMS machine you use (so ask technicians), sometimes you have to change the amount of sample injected (one microliter?) and sometimes you need to change the MS settings such as the Detector Voltage, temperature, etc. to increase the "detection power" of the equipment. You will have to discuss details with technicians as they know what the better option for each equipment and sample is. Just tell them you have a low concentration of the lipids you want to analyze (detect). Sometimes changing these parameters have made the peaks to appear. Note that as you inject only a very small volume of sample, you can repeat injections with different settings to find an optimal one.”

I believe that my error lies in incorrect programming of the GC-MS machine for our specific samples and low concentrations of the sample in the hexane solvent. There are several more recent papers that have methods on GC-MS use for analysis of femoral pores (Campos, Pruett, Soini, Zuniga-Vega, Goldberg, Vital-Garcia, Hews, Novotny & Martins, 2020; Raya-García, Suazo-Ortuño, Campos García, Martín Rueda & Alvarado-Díaz, 2021; Romero-Diaz,

Campos, Herrmann, Soini, Novotny, Hews & Martins, 2021; Zozaya, Higgie, Moritz & Hoskin, 2019; Zozaya, Teasdale, Moritz, Higgie & Hoskin, 2022).

As of December 2022, Dr. Darrin Moore and Dr. Brent Feske in the Department of Chemistry and Biochemistry were assisting to isolate the problem(s). Additional GCMS trials will be conducted whereby a standard of n-heptadecane will be included with each femoral pore sample. Also, two to four different concentrations of hexane will be run to further identify the proper procedures.

Samples can be analyzed qualitatively and/or quantitatively dependent on what you are studying. Qualitative analysis is determining the identity of the peak (e.g., which chemical compound it is) while quantitative analysis is determining the relative percentage of that compound within the sample. Both are valuable to know for relating femoral pore composition to individual traits. Qualitative analyses are done by averaging the spectrum within a peak and doing a similarity search within the program. Quantitative measurements are done by integrating all groups by height or area and searching the resultant compound table which will produce the percentages of each peak.

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APPENDIX D

FEMALE MATE CHOICE TRIALS

INTRODUCTION

Signal coevolution provides a unique opportunity to study sexual selection and its impact on organisms. As theorized by West-Eberhard (1983), sexual selection drives signal diversity and may even result in speciation events (Seddon, Botero, Tobias, Dunn, MacGregor, Rubenstein, Uy, Weir, Whittingham & Safran, 2013). In turn, female mate choice is likely under selection for the derived benefits, direct or indirect, of both the signaler (male) and receiver (female). Direct benefits would stem from females' gaining resources or energy. Indirect benefits are heritable genes from the male that would promote survival in offspring, indirectly increasing female fitness. For a female to assess indirect benefits, males must provide an honest signal of their genetic quality and health.

Male signals must be valid indicators of quality for their evolution and use in mate choice. Structural coloration patterns have been found to honestly indicate individual properties, with increased coloration correlating with better health and fitness (Keyser, 2000; Megía-Palma, Martínez & Merino, 2016; Orton, Kinsey & McBrayer, 2020; Pérez I De Lanuza, Carazo & Font, 2014). Chemical exudates from external pores correlate with immune and metabolic function (López, Amo & Martín, 2006; Martín & López, 2006). Specific behaviors related to courtship may only be viable for healthy individuals with increased stamina (Kelso & Martins, 2007). These signals that correlate directly with male quality are likely under selection by females. Females exert preferences upon male traits when those traits are honestly linked to male quality and can provide the female with increased fitness.

Female choice is an ideal avenue to explore the mechanisms facilitating male signals and the processes through which these signals evolutionarily evolved to produce vivid colorations, chemical exudates, and behavioral displays. Females discriminate amongst males via characteristics such as size, behavior, coloration, and chemical signatures. Based on chemical signals alone, females have been shown to discriminate among males age (López, Aragón & Martín, 2003), size (Huyghe, Vanhooydonck, Herrel, Tadić & Van Damme, 2012; López et al., 2006), color (López, Moreira & Martín, 2009), glucocorticoid levels (Kavaliers & Ossenkopp, 2001), immune response (Martín & López, 2006), parasite load (Martín, Civantos, Amo & López, 2007; Penn & Potts, 1998; Zala, 2004), and heterozygosity (Milinski, 2006; Olsson, Madsen, Nordby, Wapstra, Ujvari & Wittsell, 2003; Penn, 2002). Thus, chemical signals potentially give a more accurate assessment of female preference and its use in natural populations than visual. Because chemicals can relay a large amount of information in a single signal, their use reduces the cost to females of mate choice by lessening the amount of time required and predation risk during the mate search process. Using multiple modalities, such as chemical and visual, for mate choice decisions decreases mate choice costs by increasing information gained and decreasing energy and time spent assessing multiple mates (Candolin, 2003).

I performed a series of female mate choice trials wherein I attempted to assess female preference for one of two males. Two trials were conducted: a combination trial where females could visually see and interact with the male and his femoral pore secretions and a chemical only trial where the female could only see and interact with the male's femoral pore secretions.

METHODS

Setup

Females were only used once for each trial type. For example, Female A will only have two total experimental trials, one combination and one chemical. Males were used more than once per trial type. Pairings of males were chosen randomly. The same female was exposed to the same pairing of males for all trials to see if she uses different sensory modalities to make the same mate choice. Combination trials were performed first for all females, and then chemical trials were run within 1-2 days. All trials were conducted in the same room at a controlled temperature of 31 +/- 3 degrees C. Trials were conducted during normal peak activity hours (800-1300 hours) and were video recorded from above (Figure 1); the human observer was not present after the start of the trial to prevent human interference.

Two 1.5 meter by 1-meter choice trial arenas were constructed from plywood covered in white contact paper (Figure 2 and 3). This size was chosen as a compromise to provide adequate distance between the males and female to replicate viewing in natural populations, allow for various proximity areas for each male, and minimize size to allow for easier construction and cleaning. The arena base consisted of textured plastic placemats, and the arena was cleaned with no smell antibacterial wipes after every trial to remove scent. The arena was modifiable for each trial type (e.g., the two arenas can be used for both the combination and chemical trials). The arena was subdivided into 20 cm increments to determine the distance between males and females upon video playback.

Trials

Combination Trial (Chemical and Visual)

Males were divided from each other by an opaque barrier and tethered to the back of their compartment to ensure that they cannot access each other, display aggressive behaviors, or force interactions on the female. Males were tethered using fishing line and small clear hair bands. Tethering the males increases the likelihood that female behaviors directed towards males will express female preference for or rejection of those males (Bleay & Sinervo, 2007; Hamilton & Sullivan, 2005). A cotton swab was dipped in distilled water and rubbed on the femoral pore secretion from a specific male for 60 seconds. The cotton swab was then rubbed on a smooth clay tile for 60 seconds. The tile was placed at the front of the males' compartment. The female was able to contact both males physically and access their scent marked tiles during trials.

Video recording began once lizards were placed in the arena. Opaque dividers were temporarily placed between the males and female. The female was temporarily placed in the middle of the compartment in a clear plexiglass box. Males and females were given 15 minutes for acclimation before the plexiglass box and dividers were lifted. For the next 60 minutes, the female was allowed to visually assess males, interact with their scent marked tile, and physically contact them.

To control for preferring one side of the arena, females were placed in an empty arena (no males present) for 60 minutes, and location and behavior will be recorded (Baird, Fox & McCoy, 1997).

Chemical Choice Trial

An opaque plexiglass divider was placed horizontally across the arena, separating the arena into two large rectangular compartments (Figure 3). This area was also subdivided into 20

cm increments to determine distance between tiles and females upon video playback. Using the same method as discussed above, two scented tiles (one from each male) were placed on opposite ends of the arena. The female as then placed in the middle of the compartment in a clear plexiglass box for 15 minutes to acclimate. The female was then released and allowed to interact with the scent-marked tiles for 60 minutes.

To control for preferring one side of the arena, females were placed in an empty arena (no males present) for 60 minutes, and location and behavior will be recorded (Baird et al., 1997).

Behavior Scoring

Combination trial videos were watched 3x, with the observer (KMR) focusing on one lizard (2 males, 1 female) each time. Female and male behaviors were scored based on literature records of reproductive behavior in closely related species (all *Sceloporus*) (Table 1). The observer recorded the behaviors performed, the time point, and for the female, the male that a behavior is directed towards (male A or male B) (Bastiaans, Bastiaans, Morinaga, Castaneda Gaytan, Marshall, Bane, de la Cruz & Sinervo, 2014). A distance score between the female and each male was be recorded every 5 minutes. The chemical trial recording was watched once and assessed for the same female behaviors directed towards the tiles. A distance score between the female and each male's scented tile was recorded every 5 minutes.

Location in the arena was recorded for females as proximity to a male is a valid indicator of female preference. Female proximity and association to a male in natural populations commonly indicates paternity (Abell, 1997; Olsson et al., 2003). Proximity has been considered a reliable indicator of mate preferences in lizard studies (Bajer, Molnár, Török & Herczeg, 2010; Hamilton & Sullivan, 2005; Lailvaux & Irschick, 2006; Lebas & Marshall, 2001; Olsson &

Madsen, 1994; Olsson et al., 2003; Sigmund, 1983; Smith & Zucker, 1997; Walling, Royle, Lindström & Metcalfe, 2010; Weiss, 2002). However, others have found that proximity does not accurately predict paternity in *Sceloporines* (Haenel, Smith & John-Alder, 2003). Proximity will be used as a proxy for female preference during this study, but associations will be analyzed additionally with other behavioral data. Distance between males and females during the combination trial were assessed using the 20cm distance markings in the arena during video playback. Distance was scored as 1 if the male and female are in physical contact or if the female is in contact with the scented tile, as 2 if the lizards are less than one body length apart, as 3 if the lizards are in the same 20 cm area, as 4 if the lizards are in adjacent areas, and as 5 if lizards are further than 1 area apart (Kelso & Martins, 2007). For the independent chemical trial, distance was scored as 1 if the female is on the tile or contacts the tile, as 2 if the female is within one body length of the tile, as 3 if the female is in the same area as the tile, as 4 if the female is in an adjacent area to the tile, and as 5 if the female is further than 1 area apart. Distance scores were averaged for each trial for each male (e.g., one score for male A and one score for male B) (Kelso & Martins, 2007). Female activity was measured by counting the number of times a female crossed an area boundary.

To test the effect of male display behavior on female preference, male display type and frequency were quantified, though we were not able to observe any male-typical behaviors (Table 1). The most common displays performed by male *Sceloporus* include push-ups, shudder-bobs, and full shows (Bissell & Martins, 2006; Carpenter, 1962; Carpenter & Ferguson, 1977; Martins, 1993a; Vinegar, 1972). Push-ups involve raising and lowering the head and trunk, using either two legs or four legs (Martins, 1991; Martins, 1993b). Shudder-bobs are rapid motions of the head from side to side, usually performed after the body is raised in a motion similar to that

of a push-up (Carpenter, 1962). Since shudder-bobs are generally very rapid, a single bout will be recorded instead of individual nods (Smith & John-Alder, 1999). A full show resembles a raised push-up with the addition of an arched back and lateral compression of the sides (Carpenter & Ferguson, 1977).

Female behaviors were recorded based on literature detailing typical phrynosomatid lizard rejection and preference displays. Push-ups and shudder-bobs are commonly considered rejection behaviors (Cooper Jr & Vitt, 1993; Hews, Castellano & Hara, 2004), while approaches to the male are considered indications of female preference (Hamilton & Sullivan, 2005). Carpenter (1962) observed female *Sceloporus undulatus* performing a series of "sidlehops", in which the chest was extended, the back was arched, and rapid, jerky movements to the side were displayed (Ferguson, 1971). In some individuals, siddlehopping was accompanied by a tail raise, oftentimes directed in the male's direction (Carpenter, 1962). When siddlehopping was observed, copulation between the male and female rarely occurred indicating that this behavior is indicative of female rejection. However, these female display behaviors have not been studied in *S. woodi* specifically, and there may be species-specific disparities in different contexts (Hardwick, Robertson & Rosenblum, 2013).

Troubleshooting

I was unable to determine any relationships between female preference and male traits due to lack of behaviors from females and males. I believe lizards were stressed and that there was not appropriate acclimation time and settings similar to their natural environment (e.g., sand and high temperature).

TABLES

Behavior	Behavior Description
Copulation	Male grasps female's shoulder with teeth, male and female cloacal regions come into contact
Lateral Compression	Female compresses sides and gular region laterally. May be performed alone or in combination with push-ups.
Push-up	Entire body raised and lowered vertically, due to bending and straightening of either front legs alone or front and hind legs simultaneously
Shudder-bob	Rapid motion of the head side to side. May be performed alone or in combination with push-ups
Full-show	Raised posture of push-up with arched back and compression of sides
Siddlehopping	Female arches back, extends chest, and moves rapidly from side to side. May be performed alone or in combination with a tail raise
Bite	Female grasps some part of male's body with teeth
Lick	Female touches some part of male's body with tongue
Touch	Female touches some part of male's body with a part of her body other than the tongue
Tail Wave	Female raises entire tail and waves it vigorously back and forth
Tail Vibration	Female vibrates the tip of her tail but does not raise it
Tail Raise	Female lifts tail and maintains a stationary position
Approach	Female moves towards male, while looking at male
Retreat	Female moves rapidly away from male, usually after some interaction between them
Substrate Taste	Females touches snout to area male has been
Tile Lick	Female tongue-flicks or licks the tile with the male chemical substance

Table 1. Potential behaviors to be assessed during trials for females and males. All behaviors listed above have been observed in Sceloporus. Preliminary trials and combination trials in which copulation occurs will provide *S. woodi* specific behaviors. Female behaviors will be assessed for preference vs rejection. Table information obtained from Bastiaans et al. (2014).

FIGURES

Figure 1. An arena set up for the combination trial. The video camera was mounted to the black Velcro attached to the plywood bar and plugged into the wall outlet for all trials.

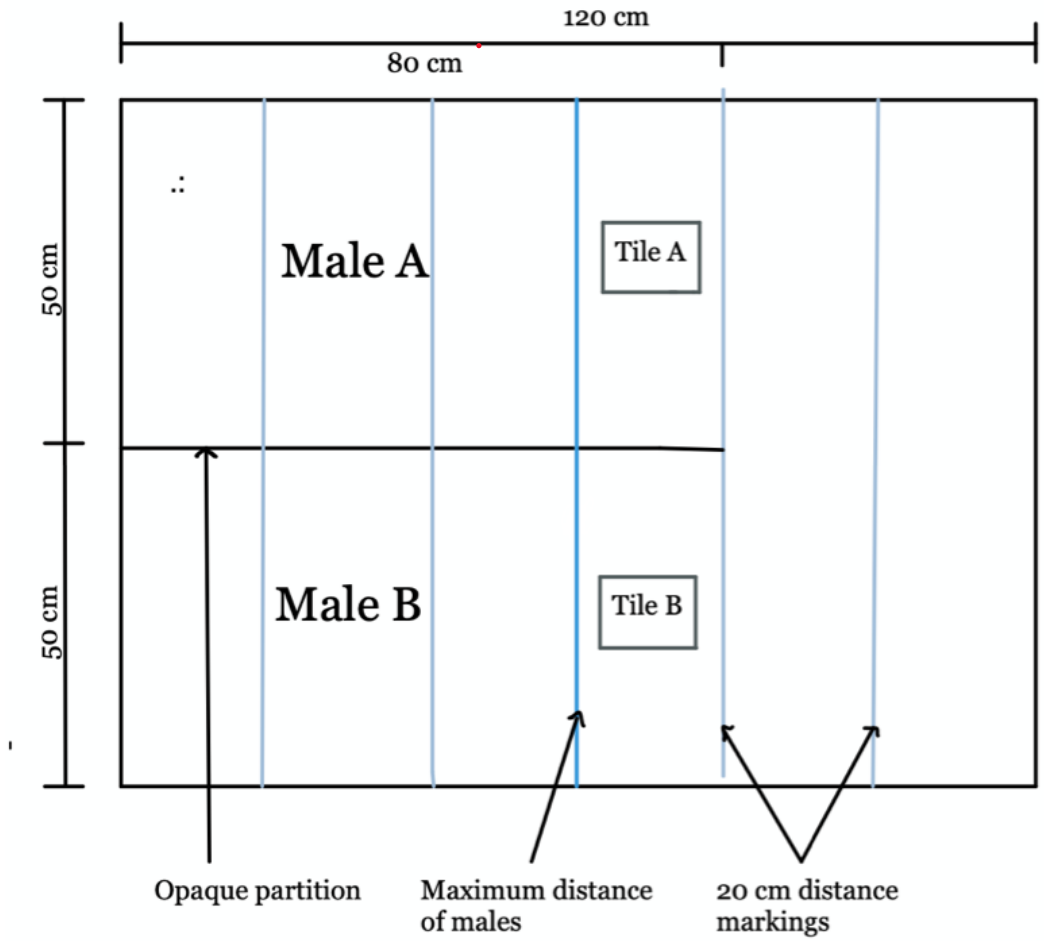

Figure 2. Combination mate choice arena design. For the combination mate choice trials, the female will be able to freely access all of the arena, including both males and their scent-marked tiles. Males will be tethered to the back of their area and separated from each other by an opaque barrier extending 2/3 of the way across the arena. Tiles from each male will be placed in front of that respective male's area. Tiles will also be separated via a barrier to prevent odor contamination. Males will not be able to access the tile.

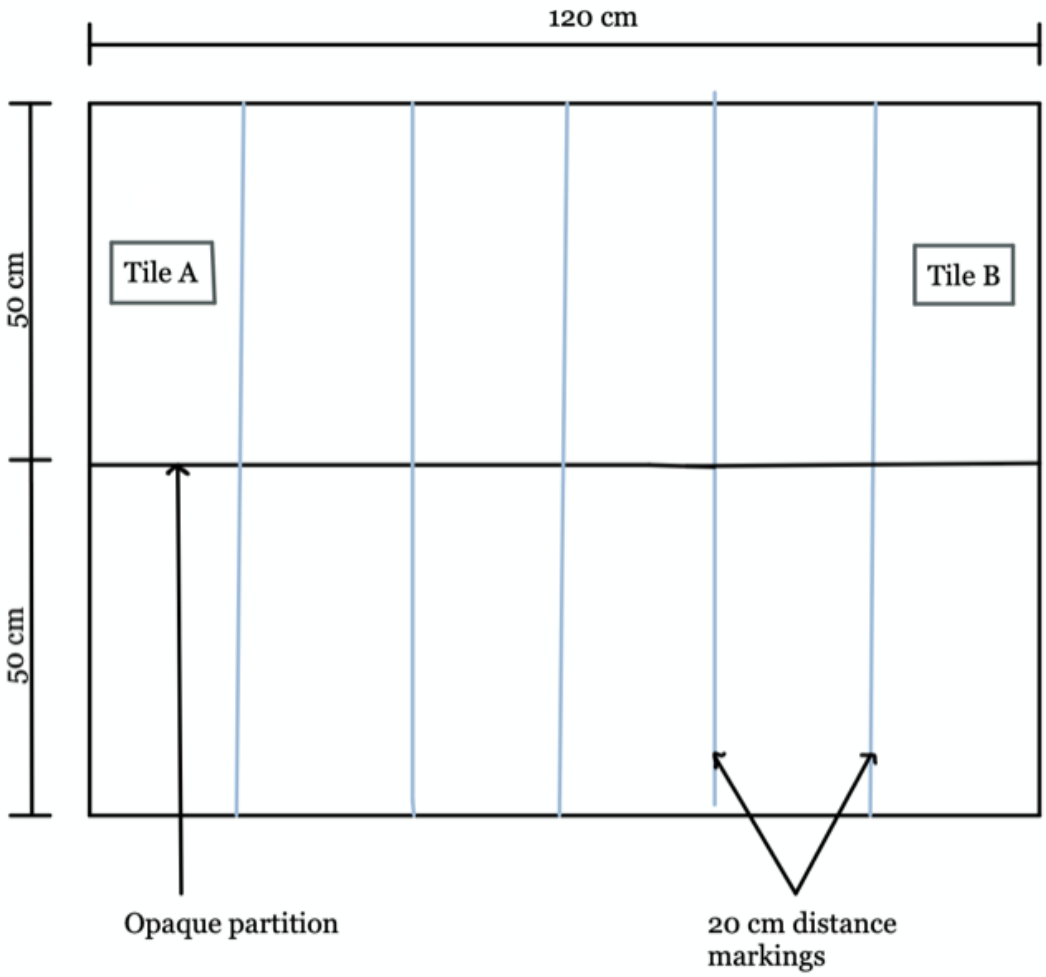

Figure 3. Chemical mate choice arena design. An opaque partition will be inserted into the arena, creating two rectangular compartments. Only one compartment will be used at a time to prevent odor contamination. Tiles marked with femoral pore secretions from male A and male B will be placed on opposite ends of the arena. Females will be able to access both tiles physically.

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