

EVALUATION OF POTENTIAL PRE-MATING REPRODUCTIVE ISOLATION IN
TIGER BEETLES (CARABIDAE: CICINDELINAE)

BY

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DEDICATION

To Bill Conner. You have given me the tools to pursue a research life of bats, bugs, and breakthroughs.

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There really aren't words to express the gratitude I feel for all those who have guided me to the completion of this work. I have been extremely fortunate in finding mentors who listened to me, pushed my limits, and helped me in becoming a better researcher. So, I offer my sincerest appreciation to Traci Porter, Nickolay Hristov, and William "Bill" Conner. I am also thankful for my academic committee members, Miriam Ashley-Ross and T. Michael Anderson for their contributions in shaping my entire experience as a student. Last but not least, I am forever in debt to my lab mates, Nicolas Dowdy and Yohami Fernández-Delgado for putting up with me and sharing their knowledge and time.

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LIST OF ABBREVIATIONS

AZ	Arizona
NC	North Carolina
LMM	Linear mixed-effects model
MANOVA	Multivariate analysis of variance
MTB	Mandible-to-body-ratio
PCA	Principal Component Analysis

ABSTRACT

The biological species concept depends on mechanisms isolating reproduction to taxonomically define a species and to explain the disadvantages, and subsequent lack, of hybridization. In co-occurring tiger beetles (Carabidae: Cicindelinae), these between-species barriers are not readily apparent. To understand the characteristics potentially responsible for this isolation, we conducted behavioral and morphological experiments with two pairs of tiger beetle species. Choice trials showed males use visual cues to trigger the pursuit of potential mates and prefer females who appear similar in color. Geometric morphometric techniques allowed us to quantify shape differences in the mandibles of all four species. Previously, it was proposed that the mandible-coupling sulcus pairing acted as a lock-and-key mechanism to provide feedback necessary for species recognition. Our results, however, suggest that although there are significant differences in shape, these differences do not provide any feedback to the male or enhance his ability to retain his grasp on the female. Taken together, these findings suggest that vision, rather than the traditional mandibular lock-and-key hypothesis, may play a key role in reproductive isolation for co-occurring tiger beetle species.

OVERVIEW

Insects are terrific models for ecology, evolution, and physiology because of their sheer diversity. The tiger beetles are a species-rich sub-family of the ground beetles (Carabidae: Cicindelinae) and are so named for their fierce appearance and rapacious appetites, both as larvae and adults. They are predatory animals and diurnal species are thought to rely primarily on vision for identifying and capturing prey (Gilbert, 1997) and predator detection. Tiger beetles, though mostly capable of flight, spend much of the time on the ground running down their prey and mates. Relative to their size, tiger beetles are some of the fastest terrestrial animals, capable of sprinting more than 170 body lengths/second (Kamoun and Hogenhout, 1996). Moving at these speeds, the visual system of tiger beetles cannot keep up (Zurek and Gilbert, 2014), resulting in the jerky start/stop motion observed during prey pursuit as individuals reorient themselves relative to the position of their target.

TIGER BEETLE DISTRIBUTION AND THE PROBLEM OF SPECIES OVERLAP

Currently, more than 2,700 individual species have been documented globally (Pearson and Vogler, 2001). Tiger beetles are found nearly everywhere in the world (Fig. 1), with the exception of the polar regions (Cassola and Pearson, 2000). They are often used to assess ecosystem health (Pearson, 1988) and these insects are valuable indicators of ecosystem health because they spend their multi-year life cycles (Willis, 1967) in the same area in which they hatched.

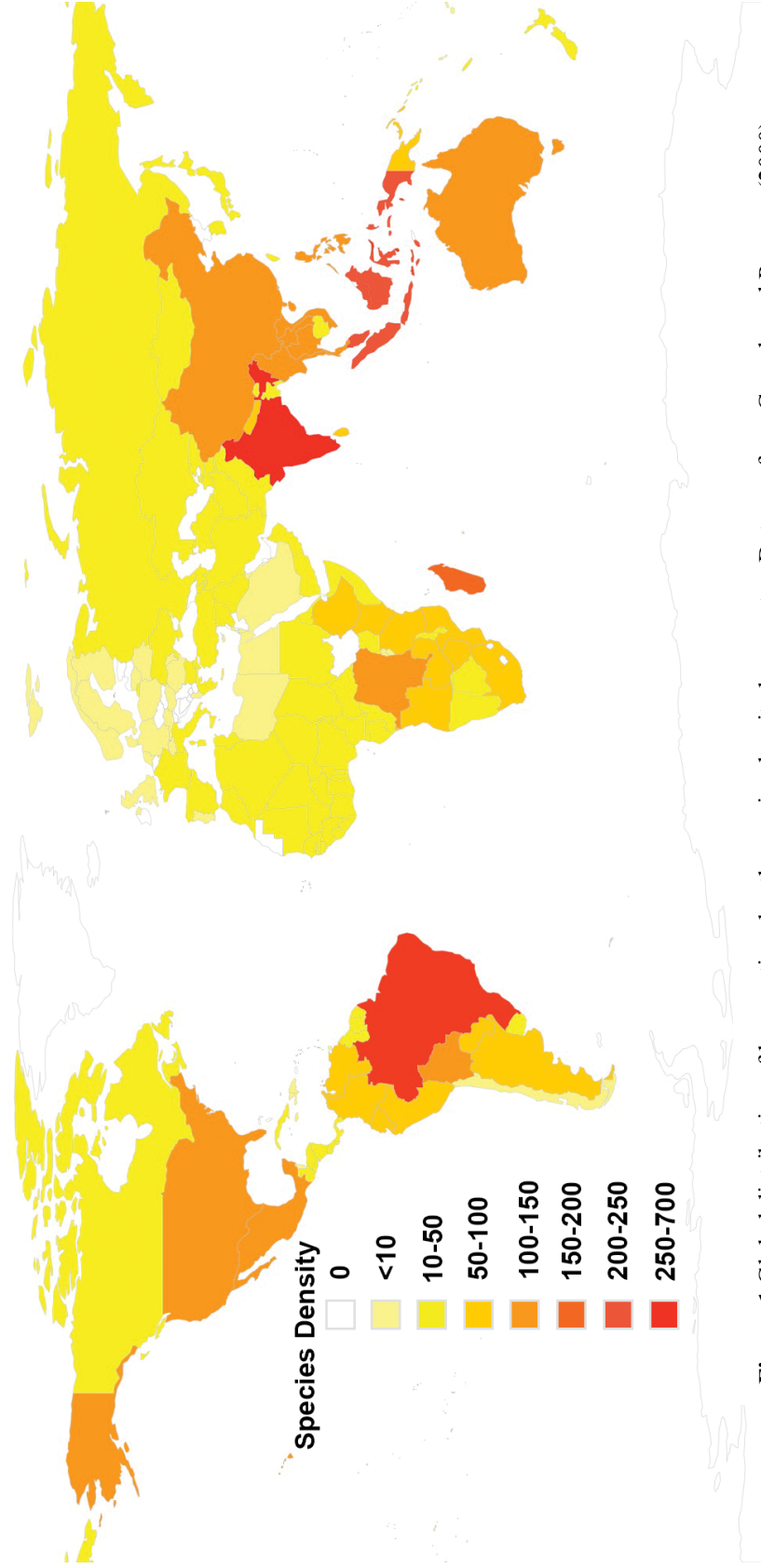


Figure 1 Global distribution of known tiger beetle species density by country. Data are from Cassola and Pearson (2000).

Despite extensive cataloguing of species, there is a dearth of studies utilizing controlled behavioral experiments to understand more about this subfamily. The goal of this thesis is to explore the essential cues of mate selection leading to pre-zygotic reproductive isolation in co-occurring species. I do so by examining the mating behavior of two sets of co-occurring North American species, *Cicindela sexguttata* and *C. repanda* in North Carolina and *C. sedecimpunctata* and *C. ocellata* in Arizona (Fig. 2), which overlap spatiotemporally in reproduction (Table 1).

Table 1 General information regarding size, seasonality, and preferred habitat types of each species used in this study.

Species	Average Adult Length (mm)	Adult Active Season	Habitat
<i>Cicindela sexguttata</i>	10-14	April – June	Sandy and loamy soil types
<i>Cicindela repanda</i>	11-13	January – June; August – September	Edge of sandy beaches
<i>Cicindela sedecimpunctata</i>	9-11	June – September; April – May	Multiple soil types; water edges
<i>Cicindela ocellata</i>	9-13	June - October	Moist, open ground; water edges

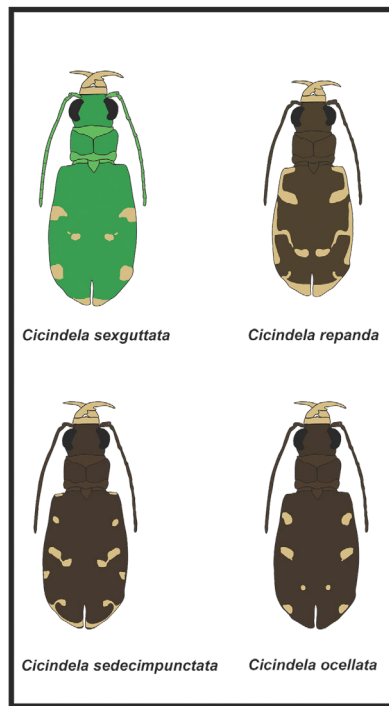


Figure 2 Illustration depicting the dorsal view of the four species of interest.

Generally, hybridization between two species is often maladaptive. The resulting hybrid offspring may be disadvantaged in comparison to non-hybrids or may be altogether inviable. Only a few instances of hybrids have been observed in tiger beetles (Brust et al., 2012a; Freitag, 1965), but many species overlap in their geographical distribution and inhabitation of similar habitats within that geographical range. In some cases, the species may be reproductively active at different times of the year, but this is not always the case. So, how do co-occurring species remain isolated?

Under sexual selection theory, the individual acts to increase its own reproductive success (Simmons, 2001) and males and females differentially invest in reproduction. Typically, males producing abundant, inexpensive sperm benefit by maximizing the number of mates they can obtain during the reproductive season. Females, on the other

hand, invest in the costly production of eggs (and sometimes extended parental care) and benefit from maximizing the quality/viability of offspring by choosing high quality males. This asymmetry in investment often leads to opportunistic males competing for access to females with a variety of behaviors including acoustic signals, gestural displays, and nuptial gifts (West, 2009). Thus, we would expect female tiger beetles to evaluate some aspect of male physiology or behavior and males to maximize their time investment by evaluating appropriate females (i.e. conspecifics) prior to copulation.

TIGER BEETLE MATING BEHAVIOR

A typical sequence of events in tiger beetle mating looks very similar to a predatory pursuit. Males approach females with the same start/stop trajectory of prey tracking and when close enough, mount the female. The male then grasps her thorax with his mandibles and immediately positions himself for copulation (Fig. 3A). Afterwards, males maintain physical contact with females from anywhere between a few seconds to more than 16 hours (Pratt, 1939; Rodriguez, 1998; Shivashankar and Pearson, 1994). Presumably this mate-guarding behavior is to prevent the promiscuous females from copulating with other males. This can help to ensure paternity and is an indicator that sperm-competition offers the last male an advantage (Alcock, 1994). Thus, for males, their time investment not only lies in finding and evaluating potential mates, but also in post-copulatory defense of his paternity.

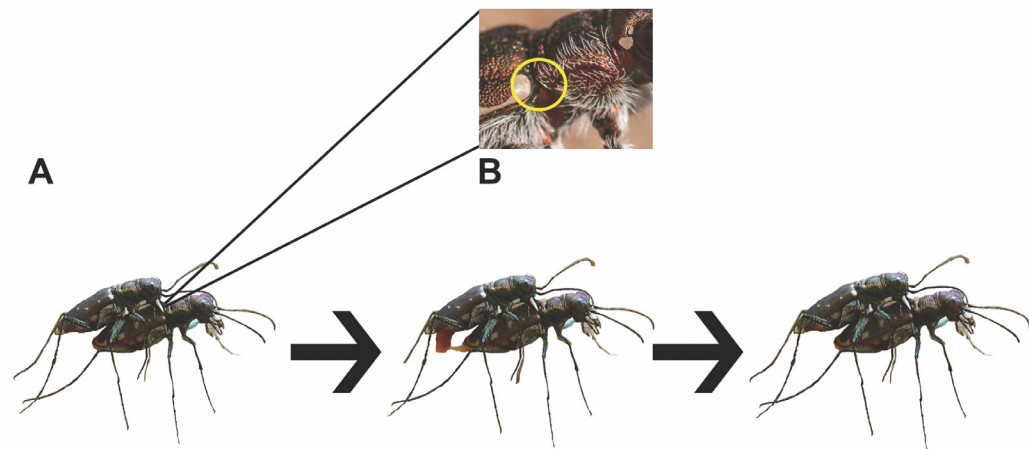


Figure 3 A) Mating sequence of two tiger beetles. Males mount females dorsally and immediately attempt to insert the aedeagus. During this time, females will vigorously try to dislodge the male. Copulation is in progress when intromission is observed. **B)** The male grasps the female with his mandibles which fit into the coupling sulcus located laterally between the thorax and abdomen.

To the observer, there appears to be no courtship behaviors prior to the initial rapid approach. Males are also noted to frequently mount heterospecifics and conspecific males, as if there is no external signal indicative of species, sex, or individual. Same-sex behaviors in mating are not uncommon in animals and generally result from social interactions like dominance displays (Lane et al., 2016), the intrinsic reward of sexual activity (Bagemihl, 1999), practice (McRobert and Tompkins, 1988), or simple misidentification (Scharf and Martin, 2013). These types of social interactions have not been documented in the tiger beetles nor have behaviors associated with mating been observed in the absence of a second individual. Because of this, I attribute the male-male mating behavior to misidentification, and male strategy seems to be to attempt to copulate with every tiger beetle it encounters. This mating paradigm poses two problems: i) it is unclear how male quality is evaluated and ii) there appear to be no species-specific cues to distinguish conspecifics.

In the experiments outlined in the next chapter, I sought to provide insight into the latter problem and evaluate the potential mechanisms allowing for pre-zygotic reproductive isolation. I chose to test whether tiger beetles made use of visual and/or mechanical feedback as pre-mating cues. First, I detail why I have excluded testing of signals in other sensory modalities.

TIGER BEETLE CHEMICAL CUES

To date, there is very little published data concerning the pheromones of tiger beetles. At the time of writing, documentation primarily exists for the production of benzaldehyde (Altaba, 1991; Moore and Brown, 1971), a defensive allomone produced from a cyanogenic precursor (Blum et al., 1981) within the pygidial glands. Given that there have been no pheromones described as having significance in reproduction, we do not attempt to test this.

TIGER BEETLE ACOUSTIC CUES

Tiger beetles possess functional hearing organs responsive to airborne sounds. They have been documented responding to ultrasonic bat cries and are capable of producing their own ultrasonic clicks while in flight, suggesting that hearing functions in predator evasion (Yager et al., 2000). Neural recordings illustrate that tiger beetles are capable of hearing sounds outside the typical range of echolocating bats (Yager and Spangler, 1995), suggesting that hearing may also function in intraspecific communication. Some tiger beetles also possess stridulatory structures, *pars stridens*, a

“file” on the flight wings located along the dorsal side of costal and subcostal veins. Sound is produced through the interaction of this flight wing file with the plectrum (ventral apical area of the elytra) (Freitag and Lee, 1972). The species of interest all possess this type of stridulatory structure but were not observed to produce sound in the context of the experiments. To date, there are no studies examining the function of these sounds in any behavioral context.

In the case of both sexes, hybridization is not a good strategy for maximizing genetic payoff to the next generation. For females, permitting copulation with a heterospecific can potentially decrease the quality of her offspring. Even if females exhibit control of sperm usage, as many insects are capable of doing (Eberhard, 1996), even her temporary unavailability to conspecifics can have a negative impact. And although sperm is “cheap” for males, the time spent interacting with an inappropriate partner can negatively affect his overall reproductive success.

INTRODUCTION

Hybridization, often considered maladaptive, is not as rare as once thought. Though thought to exact high fitness costs, “adaptive” or “facultative” hybridization may occur in response to certain environmental pressures (Pfennig, 2007; Veen et al., 2001). Historically, studies have focused on parapatric species and their relatively small “hybrid zones” where the individuals of two species come into close contact (Barton and Hewitt, 1985). Studies on sympatric species are more rare, the assumption being that if hybridization were commonplace the species could not persist as distinct groups (Hochkirch and Lemke, 2011).

Tiger beetles (Carabidae: Cicindelinae) are a speciose subfamily of ground beetle that occur in nearly every part of the world (Cassola and Pearson, 2000; Pearson and Cassola, 1992). Many exhibit bright, iridescent elytral colors and maculations and many species overlap both spatially and temporally during respective periods of reproduction. Despite this overlap, there are very few studies that have experimentally explored the parameters that prevent or, at least, discourage hybrids. We proposed to examine both an existing and a novel hypothesis for understanding the factors that prevent tiger beetle hybridization by manipulating behaviorally relevant cues.

Often, mate selection is predicated on a signal to mediate species recognition and indicate mate quality. However, pre-copulatory courtship, where a male vies for initial contact with a female, is not common among the cicindelids. Rather, females remain unaffected by the presence of male conspecifics until mounted (Richardson, 2013). The result of this hasty interaction is no apparent pre-copulatory evaluation for either sex, making interspecific sexual interactions more likely.

The only hypothesis for how tiger beetles prevent hybridization is the mandibular lock-and-key hypothesis first proposed over four decades ago (Freitag, 1974). This hypothesis states that the mandibles of the male tiger beetle, when grasping a female, “lock” into grooves on either

side of the mesepisternum called the coupling sulci (Fig. 3B), only present in females of the species. These coupling sulci vary in groove depth and length between species and were hypothesized to be a form of rapid species recognition that arose from environmental pressures and a structure to provide additional “grip” during the females’ vigorous attempts to displace the male.

Diurnal tiger beetles are primarily active in open sand/soil patches and, while capable of flight, spend most of their time running in intermittent bursts on the ground. This type of activity reduces the risk of aerial predation but also makes targeting potential mates a challenge. Thus, a “lock-and-key” mechanism would permit males to quickly determine whether they had approached the correct partner and a heterospecific exhibiting a different coupling sulcus structure would not allow for a firm enough hold to permit successful insemination of the female. This logic would also apply to males who attempted to mount and subdue conspecific males, a frequent occurrence that would otherwise result in a loss of time and energy that could be spent pursuing additional females. There is little empirical evidence to support this hypothesis, although additional hypotheses concerning the strength and endurance to maintain mandibular hold as a proxy for measuring male quality and citing this as the basis of female choice are based on this assumption (Pearson 1988). It is not clear, either through behavior or morphological analysis whether the mandible-coupling sulcus pairing functions as a primary barrier to hybridization. Thus, we present an alternative explanation that could act concurrently or in place of the lock-and-key hypothesis.

Tiger beetles may make use of visual cues given that vision appears to be the primary sensory modality. Currently, coloration has only been implicated as functioning in predator avoidance via camouflage, dazzle interference, aposematism, and mimicry (Acorn, 1988; Pearson, 1985; Schultz, 1986) or possibly in thermoregulatory processes (Pearson, 1988). However, coloration has an effect on tiger beetle predatory behavior (Lovari et al., 1992) and has been shown to be an important component for mate choice in many other invertebrates

(Baldwin and Johnsen, 2009; Ellers et al., 2003; Kronforst et al., 2006; Morehouse and Rutowski, 2010) and these preferences should exclude heterospecifics that do not share similar appearance. Being able to recognize prospective mates at a distance could be an invaluable tool for preventing needless energy expenditure chasing incompatible females (or other males) and we propose that elytral coloration aids in identifying potential mates and ultimately reduce the chances of hybridization.

We attempt to provide the first rigorous experimental tests of both hypotheses by determining 1) if there are significant differences in the mandibular morphology between species of male tiger beetles and 2) if males use color cues to distinguish between attending a conspecific or heterospecific. If the mandible-coupling sulcus pairing is crucial to successful copulation, there should be significant differences in the mandible shape of males and this should lead to a reduced ability to remain mounted on heterospecifics. Should coloration act as a cue, we would predict that males would approach females sharing the same coloring more often and avoid females with dissimilar coloration. To explore these hypotheses, we used two co-occurring species in North Carolina and two co-occurring species in Arizona that exhibited different coupling sulcus structures and conducted mate choice experiments on both groups.

The North Carolina species, *Cicindela sexguttata* and *Cicindela repanda*, are green to blue-green and brown in color, respectively (Fig. 2) with coupling sulci categorized as “very broad” and “thin,” respectively. We expect that males will prefer to interact with conspecific females and show very dissimilar mandible morphology that reduces copulation success with heterospecifics. The Arizona species, *Cicindela sedecimpunctata* and *Cicindela ocellata*, are both brown in coloration with very similar maculation patterns (Fig. 2), meaning that they would regularly encounter similar-looking heterospecifics. *C. sedecimpunctata* and *C. ocellata* have coupling sulci categorized as “thin” and “elongate and deep,” respectively. Males are expected to attend equally to conspecifics and heterospecifics but still display dissimilar mandible morphology. These two groups test the behavioral response of male tiger beetles when choosing

females between a conspecific and a morphologically and visually dissimilar heterospecific and between a conspecific and visually similar, but still morphologically dissimilar heterospecific.

Coloration may be important to mate selection if in the experiments, *C. sexguttata* males do not approach heterospecific females but *C. sedecimpunctata* males do. If mandibular morphology is critical to species recognition or grip maintenance, the dissimilar morphology should result in both male's reduced ability to maintain his grip while attempting copulation with heterospecifics, if copulation is attempted at all after initial contact. This allows us to determine the relative importance of visual cues and morphology.

METHODS

NORTH CAROLINA: ANIMALS

Adult *Cicindela sexguttata* ($n = 24$ females/36 males) and *Cicindela repanda* ($n = 21$ females/6 males) were collected in May and June 2017 at several sites throughout Forsyth and Surry counties in North Carolina. Animals were transported to Wake Forest University where they were maintained in an incubator with a 12:12 light:dark cycle at 34°C. Beetles were housed individually in plastic insect cages (30 x 20 x 20 cm) and fed live boxelder (*Boisea trivittata*) nymphs, a common prey item in the field, and mealworms (*Tenebrio molitor* larvae) each day. The remains of the previous days' insects were removed at the time of feeding. Cages were lined with the soil/sand of the habitat in which the beetles were found and kept moist with distilled water. At the end of the behavioral experiments, specimens were euthanized with acetyl acetate vapor in a glass jar prior to collecting morphological data.

NORTH CAROLINA: BEHAVIORAL EXPERIMENTS

To determine if color, independent of size or other indicators, played a role in the females a male chose to attempt copulation with, the elytra of some females were switched with those of the opposite species (Fig. 4), denoted forthwith by placing an asterisk (*) by the species name. To do this, the elytra of deceased individuals were removed. Females receiving the switched elytra were anesthetized by being cooled at approximately 4°C for five minutes and then placed in a chilled dissection dish while the new elytra were glued in place. Females were then allowed to recover for 24 hours prior to being used in mating trials. Other than preventing flight, this procedure did not appear to affect other behaviors and the ability to fly was regained once the new elytra were removed using a small amount of acetone. Male *C. sexguttata* were then allowed to choose between the females of every two-female combination (Fig. 5).

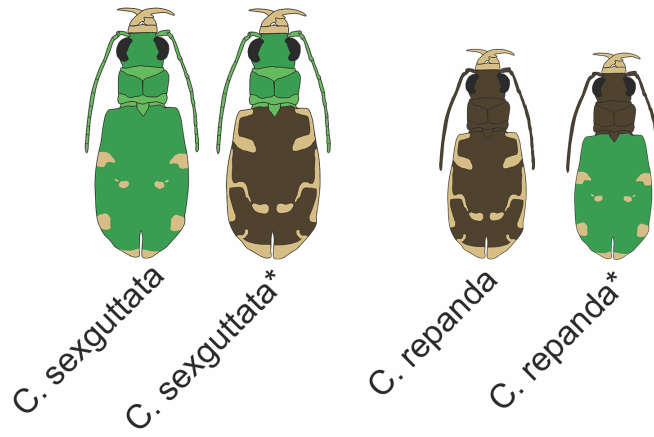


Figure 4 Illustration of North Carolina tiger beetles with and without the switched elytra.

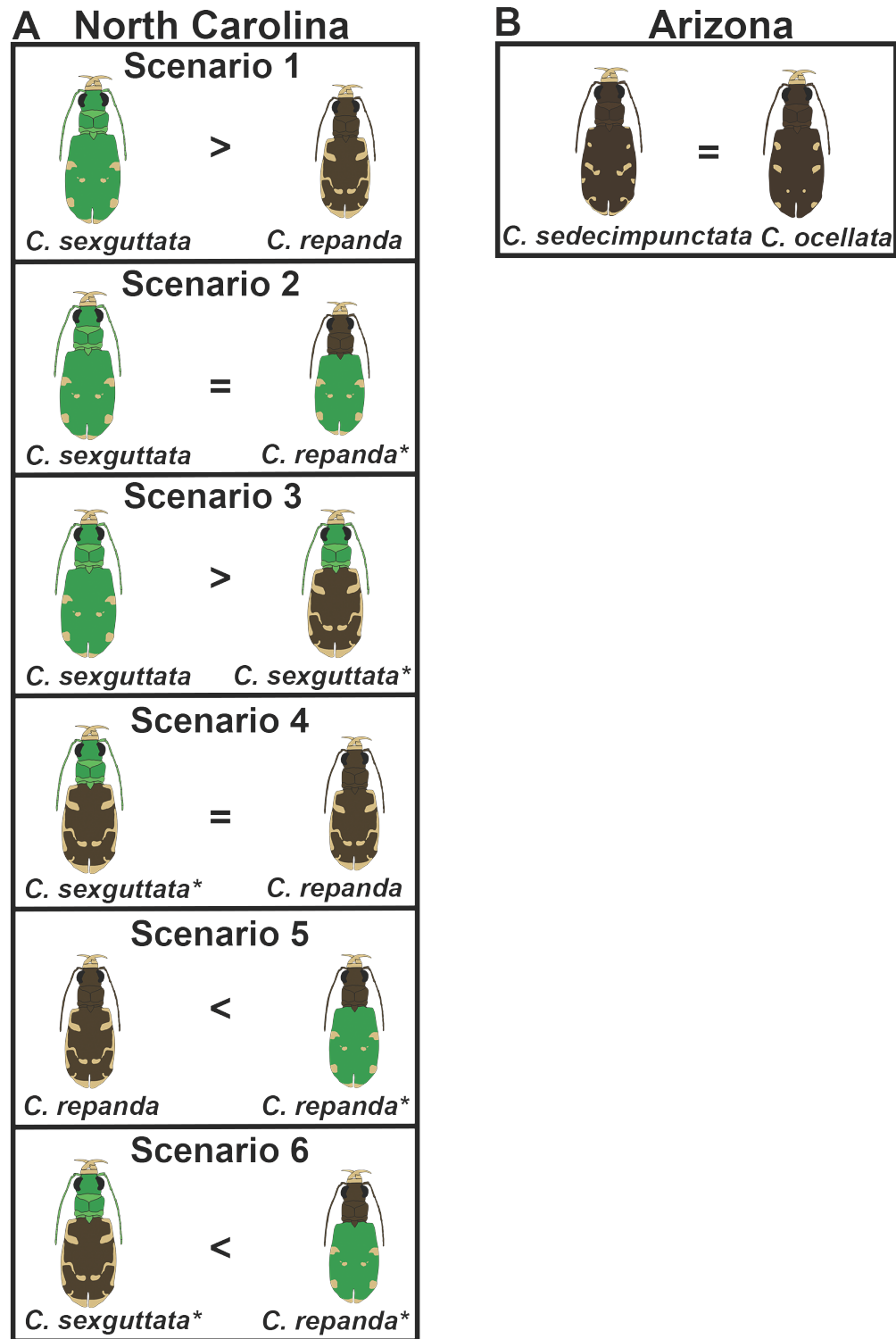


Figure 5 Every two-female combination tested for male preference in **A)** North Carolina and **B)** Arizona. Comparison symbols are used to indicate which female is expected to be approached more frequently if vision plays a role in mate selection.

Male beetles ($n = 21$) were placed into small groups consisting of no more than two females ($n = 14$ *C. sexguttata*; $n = 10$ *C. repanda*) and no more than one other male ($n = 48$ trials). These groups could move freely about an arena (51x25.5x30.5 cm; Fig. 6) and males frequently had multiple interactions with other individuals in a single trial ($n = 79$ interactions); these interactions were often brief mounting attempts and did not necessarily lead to copulation. The arena was placed outside in natural sunlight on days when the temperature was between 21°C - 28°C and covered with a mesh top to prevent escape. We recorded number of copulation attempts and the amount of time spent in pre-copulatory amplexus (prior to aedeagal insertion), copulation (during which aedeagus is inserted, if copulation is attempted), and post-copulatory amplexus (mate guarding). The trial was terminated after a male/female pair physically separated, a male chose to attempt to mate with another male, or after twenty minutes if no choice made. Instances where males interacted with other males were excluded from the analysis ($n = 8$ trials/13 interactions). Individual males were used in an average of 4 trials each. Male *C. repanda* were also used in some trials ($n = 15$), and although mating pairs were observed in the field, males never attempted interact with other individuals of either species and their data are excluded from the analysis.



Figure 6 Arena where behavioral trials were conducted. Beetles had access to shelter, a branch under which they could burrow, and a dish containing sand saturated with distilled water.

ARIZONA: ANIMALS

During the month of July 2017, adult *Cicindela sedecimpunctata* ($n = 12$ females/28 males) and *Cicindela ocellata* ($n = 11$ females/8 males) were collected within a five-mile radius of the Southwestern Research Station in Cochise County, Arizona. The same care and euthanasia protocols described above were followed.

ARIZONA: BEHAVIORAL EXPERIMENTS

Beetles were placed in groups of two females ($n = 4$ *C. sedecimpunctata*; $n = 4$ *C. ocellata*), one of each species, and one male *C. sedecimpunctata* ($n = 5$) and allowed to interact in the same type of arena described previously ($n = 11$ trials/15 interactions). Because of their similar appearance, no elytra switching was conducted between the females (Fig. 2). The same data were recorded as for the North Carolina experiments and trials were terminated after a male/female pair physically separated or after 20 minutes if no choice was made ($n = 2$ trials). Male *C. ocellata* were also tested ($n = 4$ trials), but only attempted to mate with females in two of those trials. Their data were not analyzed.

BEHAVIORAL ANALYSIS

We refer to pre-copulatory amplexus as time spent with the male mounted on the back of the female, without genital contact, prior to intromission. Copulation refers to intromission, regardless of the state of sperm or spermatophore transfer. In the tiger beetles, spermatophores have been documented in only a few species (Freitag, 1966; Freitag et al., 1980; Schincariol and Freitag, 1986; W. Paarmann et al., 1982) but it's possible that direct sperm transfer may also occur (Pearson and Vogler, 2001). Because we use males multiple times and it is not known whether spermatophores are used or the length of regeneration time, males were not used more than once within a 24-hour period.

Post-copulatory amplexus, or mate guarding, is the time a male spends mounted on the female after copulation, without genital contact (Fig. 3A). For both datasets, we analyzed the number of mating attempts each male made towards each female. A mating attempt was characterized as a male mounting a female and grasping her with his mandibles, even if copulation did not ensue. Mixed effects logistic regressions were used to model the males' choice between each female pair and these models were analyzed using a Wald chi-square test in both North Carolina and Arizona experiments. For the data collected in North Carolina, trials were sorted according to the color preference being tested. Scenarios 1, 3, 5, and 6 were combined as they all tested for a preference between green and brown elytral colors. Scenarios 2 and 4 served as controls, forcing males to choose between two green females and two brown females, respectively. The data obtained were adjusted for multiple comparisons using the Bonferroni method; no adjustment was needed for the data collected in Arizona. In all analyses, individual male identity was used as a random factor. The duration of the pre-copulatory amplexus was analyzed to determine how much time a male spent attempting to copulate with each group of females, regardless of whether the aedeagus was ever successfully inserted. According to the mandibular lock-and-key hypothesis, a male would not be able to maintain an adequate grip on a heterospecific and this should result in significantly less time spent in pre-copulatory amplexus. Males should also spend less time in pre-copulatory amplexus with heterospecifics if the coupling sulci serve as a species recognition cue independent of whether they provide additional grip assistance. Time spent in pre-copulatory amplexus was placed into a linear mixed-model (LMM) with female group as the fixed effect and the individual beetle identity as a random effect. These models were analyzed using a Wald chi-square test. We also recorded the percentage of successful aedeagal insertions. Males were observed to still evert the aedeagus and attempt copulation, even if they were never able to engage in intromission. The percentage of successful aedeagal insertions for each species were analyzed with a chi-square test of independence to test for quality of proportions. For North Carolina data, p -values were adjusted using the Bonferroni method. All

statistical tests were performed using R (R Development Core Team, 2016) and mixed-effects models were created using the lme4 package (Bates et al., 2015).

MORPHOMETRIC ANALYSES

To compare the overall size of the beetles, we obtained information on body area by photographing the dorsal side of the beetles using a Nikon D3200 with 40mm macro lens. Body area excluded the head and mandibles and was measured using FIJI (Schindelin et al., 2012). We also measured the surface area of the right mandible and used this to determine the relative mandible-to-body (MTB) ratio. Linear models were used to determine if body area significantly differed between species in each locale and if there were intraspecific sex differences.

To analyze shape information, we used geometric morphometrics to compare the mandible shapes of each species. Mandibles are complex shapes and the advantages this technique offers over traditional measurements, such as length or width, is that it captures and preserves the geometry of the mandible throughout the entire analysis. This geometry can then be visualized in meaningful ways as well as analyzed statistically.

The mandibles were removed from the beetle and the ventral side of the right mandible imaged in a standard orientation under a Zeiss VR16 microscope (Oberkochen, Germany). Only intact mandibles without excessive wear were used ($n = 100$). The images were then digitized for landmarks and semilandmarks (Fig. 7) using tpsDig2 (Rohlf, 2012), reducing the shape of the specimen to a series of two-dimensional Cartesian coordinates. We used 11 landmarks to define the gross shape of the mandible by placing them at the tip and base of the apical and terebral teeth (those extending from the terebral margin, or “cutting” edge of the mandible), as well as the molar complex. An additional 10-20 semilandmarks along the lateral curve of the mandible were used. The curve was then resampled to 20 evenly spaced points and these points were free to slide along the curve during the subsequent superimposition. These landmarks and semilandmarks are consistent with those used in previous studies (Richardson, 2013) and represent the homologous

structures that occur across the species, a critical requirement when conducting morphometric analyses. Control lines were removed from the resulting data file using the *tpsUtil* (Rohlf, 2012) and a “sliders” file created to handle the positioning of the semilandmarks.

The resulting data files were then imported into R and all remaining manipulations and analyses were conducted using the *geomorph* package (Adams and Otárola-Castillo, 2013). The shape data were processed with a Generalized Procrustes Analysis, an iterative method of optimally superimposing a set of shape data. This is done by translating, rotating, and scaling each specimen by minimizing the Procrustes distance (Rohlf, 1999), a measure of shape difference, between the specimens being compared. The resulting data are then free of any non-shape characteristics, such as size and orientation and the aligned Procrustes coordinates represent the shape of each specimen.

Data were visualized using a principal component analysis (PCA) on the superimposed shape coordinates. This process is based on the decomposition of the data covariation matrix, with respect to the Procrustes distance and yields a series of independent variables describing the morphological variation across the dataset. The variation can be further visualized using thin-plate spline interpolation to create a wire-mesh deformation that represents the displacement of the landmarks between a reference (mean) shape and target (individual) shapes. In this instance, the reference shape is the mean shape, an artificial construct based on the average of all the specimens included in the data set and resides at the origin of the new space generated through PCA. The target shapes can be those of the individual specimens or any point moving along the principal components (PCs).

To determine if the observed morphological differences were significant, we performed a Goodall’s F-test (Goodall, 1991), a MANOVA analog adapted for morphological analysis that uses the Procrustes distances of the shape data. This approach was used to determine if heterospecific males exhibited statistically significant differences in mandible shape and if intraspecific sexual dimorphism existed.

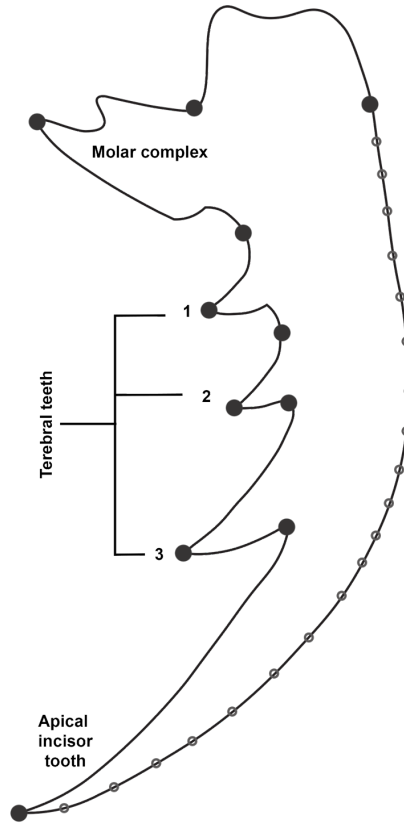


Figure 7 A generic sketch of a tiger beetle mandible with landmarks (large, filled circles) and the path on which semilandmarks (small, hollow circles) can slide between two landmarks.

RESULTS

NORTH CAROLINA: BEHAVIOR RESULTS

We observed males attempting to mount females in all categories as well as other males (Fig. 8), if they were present (data for multiple males not shown). But based on the number of mating attempts, male *C. sexguttata* showed a clear behavioral preference towards females with green elytra, regardless of their species (results in Table 2). In trials where beetles chose from between two females with green elytra, we observed a preference for conspecifics while trials in which both females appeared brown, males showed no preference.

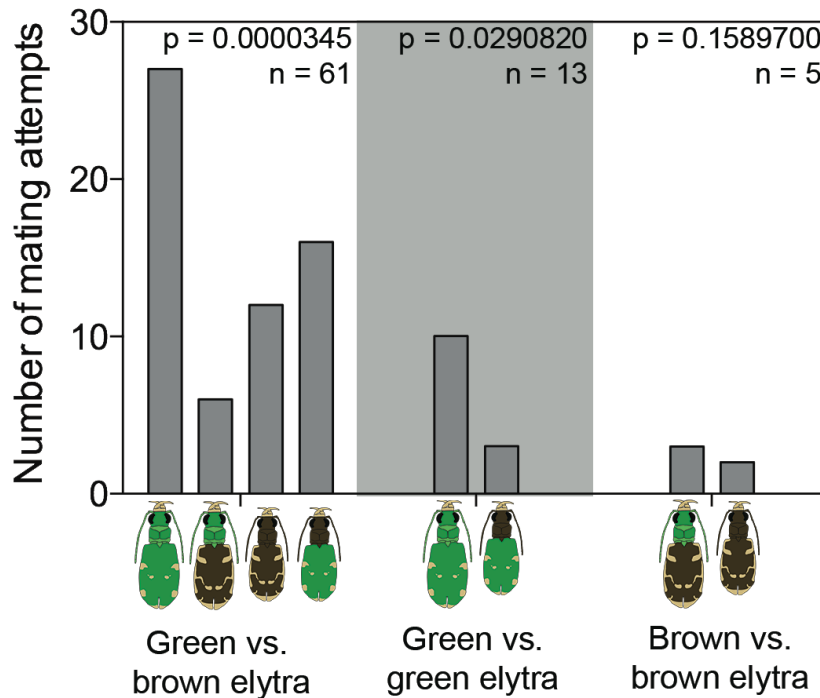


Figure 8 The number of mating attempts of *C. sexguttata* males. Trials testing for green vs. brown preference were collapsed (Scenarios 1, 3, 5, and 6) into a single analysis but each female type is displayed for reference. Colored diagrams of each female beetle are placed below their corresponding column. Sample size refers to number of interactions, not trials.

Table 2 Results of NC behavioral study documenting the number of mounting attempts of *C. sexguttata*.

Scenario	χ^2	Df	Bonferroni-adjusted <i>p</i>
Green vs. brown elytra	19.25	1	0.000035
Green conspecific vs. green heterospecific	6.69	1	0.029
Brown conspecific vs. brown heterospecific	0.39	1	0.16

There was no significant difference among groups in duration of pre-copulatory amplexus ($\chi^2 = 0.84$, $df = 3$, $p = 0.84$) and this time was quite variable (Fig. 9A). This result suggests that males did not experience difficulty maintaining their grip on females nor did it serve as a rapid species identifier, otherwise we would expect a significantly shorter period spent with heterospecifics. Males were, however, more successful inserting the aedeagus into conspecific females (Fig. 9B), even with switched elytra ($\chi^2 = 0.84$, $df = 3$, $p = 2.92 \times 10^{-7}$).

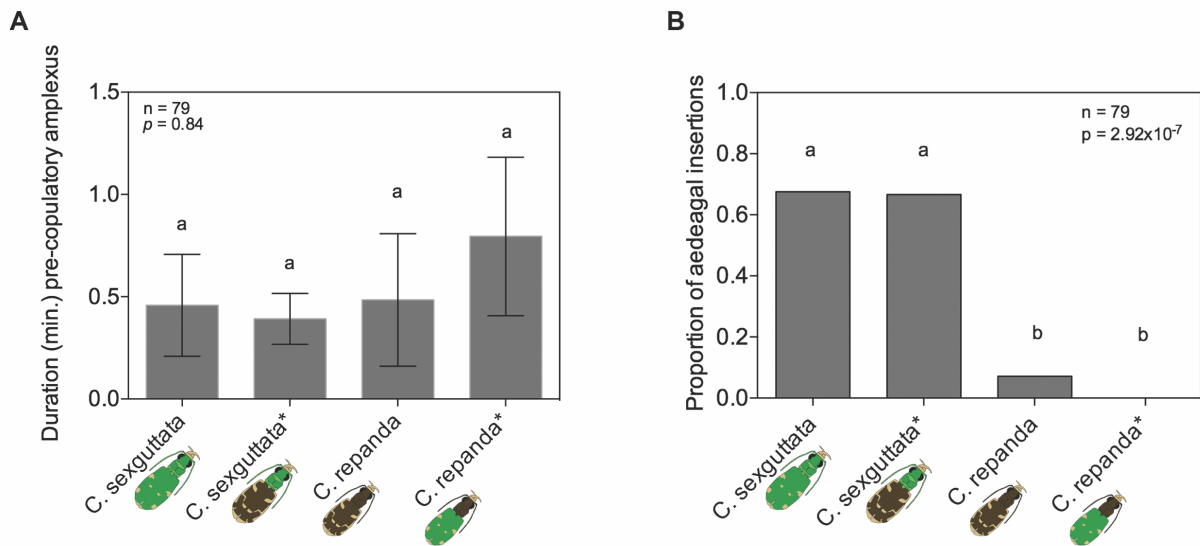


Figure 9 **A)** The average duration of pre-copulatory amplexus prior to aedeagal insertion of *C. sexguttata* males for each category of female. **B)** The number of successful aedeagal insertions for each category of female; males were more successful with conspecifics, even if the elytra had been switched. Sample size refers to number of interactions, not trials; error bars are mean \pm SEM.

ARIZONA: BEHAVIOR RESULTS

We observed no significant differences in the behavior of male *C. sedecimpunctata* when interacting with conspecific and *C. ocellata* females (Fig. 10). They attempted to copulate ($\chi^2 = 0.002$, $df = 1$, $p = 0.96$) and spent similar amounts of time in pre-copulatory amplexus ($\chi^2 = 0.88$, $df = 1$, $p = 0.35$) with both species. *C. sedecimpunctata* males were also able to successfully insert the aedeagus into females of both species ($\chi^2 = 0.31$, $df = 1$, $p = 0.58$).

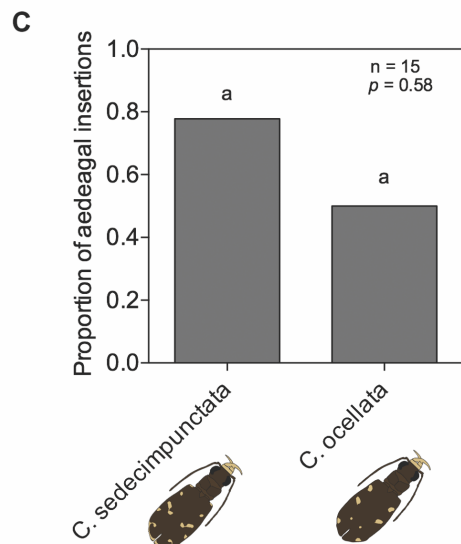
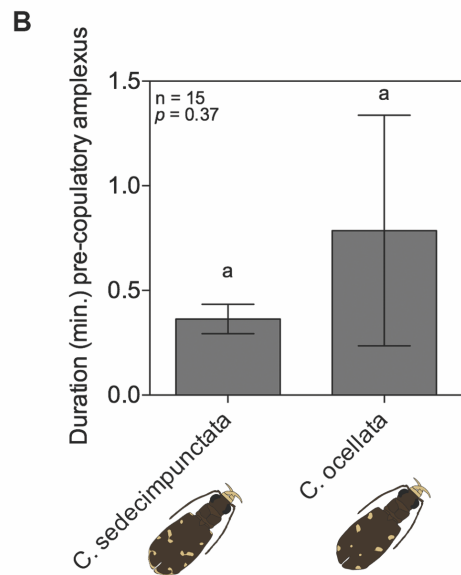
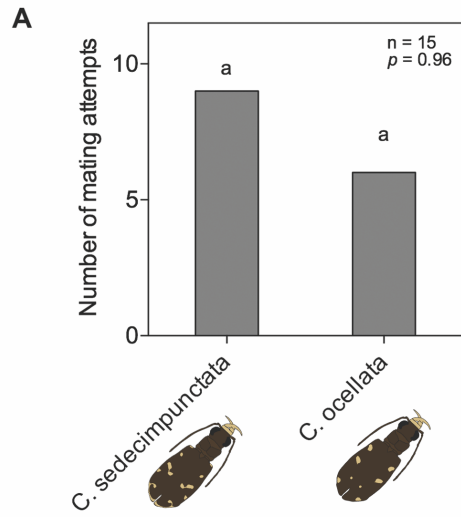


Figure 10 The results for the behavioral experiments for *C. sedecimpunctata* males in Arizona. There was no significant difference between conspecifics and heterospecifics for **A)** the number of mating attempts, **B)** the duration of pre-copulatory amplexus, and **C)** the number of aedeagal insertions. Error bars are mean ± SEM.

GEOMETRIC MORPHOMETRIC RESULTS

To test if the tiger beetles used in this study exhibited sexual dimorphism, we analyzed both body area and relative mandible size. In three species, *C. sexguttata*, *C. sedecimpunctata*, and *C. ocellata*, males exhibited significantly smaller body sizes (Table 3; Fig. 11A, C) than conspecific females, but larger MTB ratios than females (Table 4; Fig. 11B, D). Males and females of each species were also compared to their heterospecific counterparts. Male *C. sexguttata* were significantly larger than male *C. repanda* and the same pattern holds true for females (Table 5). *C. sedecimpunctata* males and females were not different in body size than male and female *C. ocellata*. The MTB ratios were not different between same-sex heterospecifics (Table 6).

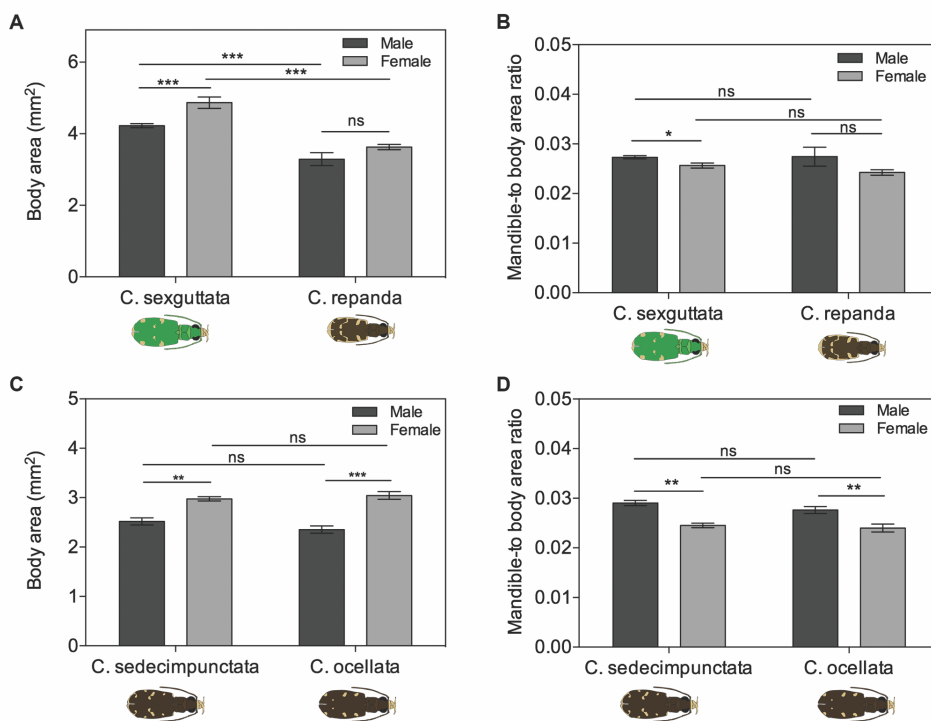


Figure 11 Comparisons of body area and relative mandible size between species and sexes in North Carolina (A, B) and Arizona (C, D). Significance codes: $p < 0.001$ ‘***’, $p < 0.01$ ‘**’, $p < 0.05$ ‘*’, not significant ‘ns’. Error bars are mean \pm SEM.

Table 3 Summary of ANOVAs comparing body areas between conspecific males and females.

Comparison	F_{df}	Bonferroni-adjusted p
Male <i>C. sexguttata</i> – female <i>C. sexguttata</i>	$F_{1,33} = 21.08$	$p = 2.45 \times 10^{-4}$
Male <i>C. repanda</i> – female <i>C. repanda</i>	$F_{1,13} = 3.90$	$p = 0.28$
Male <i>C. sedecimpunctata</i> – female <i>C. sedecimpunctata</i>	$F_{1,15} = 15.44$	$p = 0.0054$
Male <i>C. ocellata</i> – female <i>C. ocellata</i>	$F_{1,13} = 36.99$	$p = 0.017$

Table 4 Summary of ANOVAs comparing mandible-to-body ratios between conspecific males and females.

Comparison	F_{df}	Bonferroni-adjusted p
Male <i>C. sexguttata</i> – female <i>C. sexguttata</i>	$F_{1,33} = 8.15$	$p = 0.030$
Male <i>C. repanda</i> – female <i>C. repanda</i>	$F_{1,13} = 5.15$	$p = 0.16$
Male <i>C. sedecimpunctata</i> – female <i>C. sedecimpunctata</i>	$F_{1,15} = 19.46$	$p = 0.002$
Male <i>C. ocellata</i> – female <i>C. ocellata</i>	$F_{1,13} = 11.99$	$p = 0.017$

Table 5 Summary of ANOVAs comparing body areas between male heterospecifics and female heterospecifics.

Comparison	F_{df}	Bonferroni-adjusted p
Male <i>C. sexguttata</i> – male <i>C. repanda</i>	$F_{1,24} = 29.28$	$p = 5.88 \times 10^{-5}$
Female <i>C. sexguttata</i> – female <i>C. repanda</i>	$F_{1,22} = 49.31$	$p = 1.92 \times 10^{-6}$
Male <i>C. sedecimpunctata</i> – male <i>C. ocellata</i>	$F_{1,16} = 2.06$	$p = 0.68$
Female <i>C. sedecimpunctata</i> – female <i>C. ocellata</i>	$F_{1,12} = 0.3787$	$p = 1.00$

Table 6 Summary of ANOVAs comparing mandible-to-body ratios between male heterospecifics and female heterospecifics.

Comparison	F_{df}	Bonferroni-adjusted p
Male <i>C. sexguttata</i> – male <i>C. repanda</i>	$F_{1,24} = 0.012$	$p = 1$
Female <i>C. sexguttata</i> – female <i>C. repanda</i>	$F_{1,22} = 3.44$	$p = 0.31$
Male <i>C. sedecimpunctata</i> – male <i>C. ocellata</i>	$F_{1,16} = 1.20$	$p = 1.00$
Female <i>C. sedecimpunctata</i> – female <i>C. ocellata</i>	$F_{1,12} = 0.10$	$p = 1.00$

In addition to size, each species' mandibles were analyzed for shape differences. We first conducted PCAs on the aligned Procrustes coordinates. For both North Carolina (Fig. 12A) and Arizona (Fig. 12B) specimens, this yielded two orthogonal axes explaining a total of 70% and 78% of the overall variation in shape, respectively. The differences in mandible shape were further characterized using deformation grids to reveal the localized shape variation (Fig. 13).

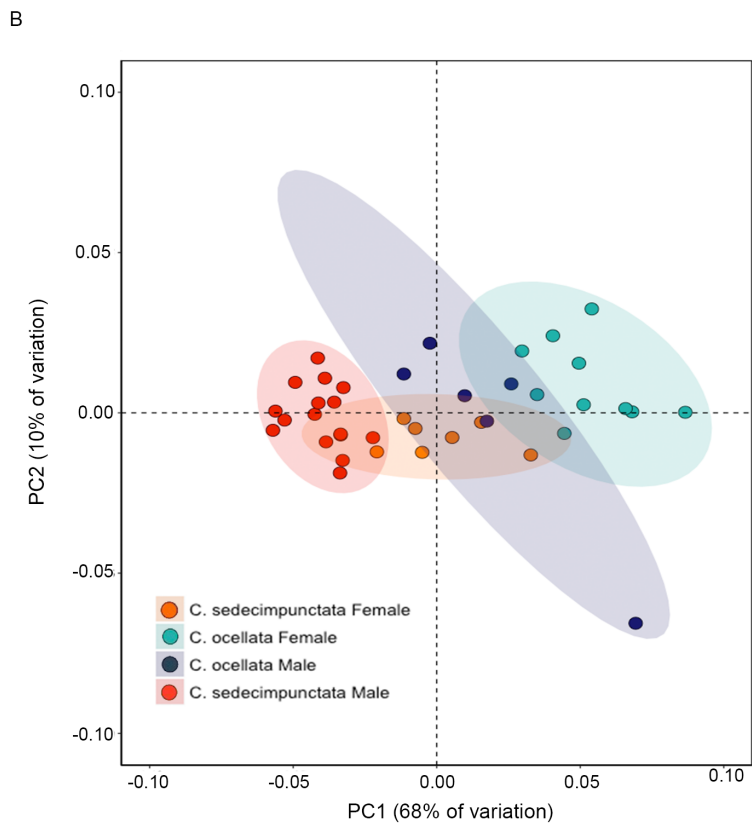
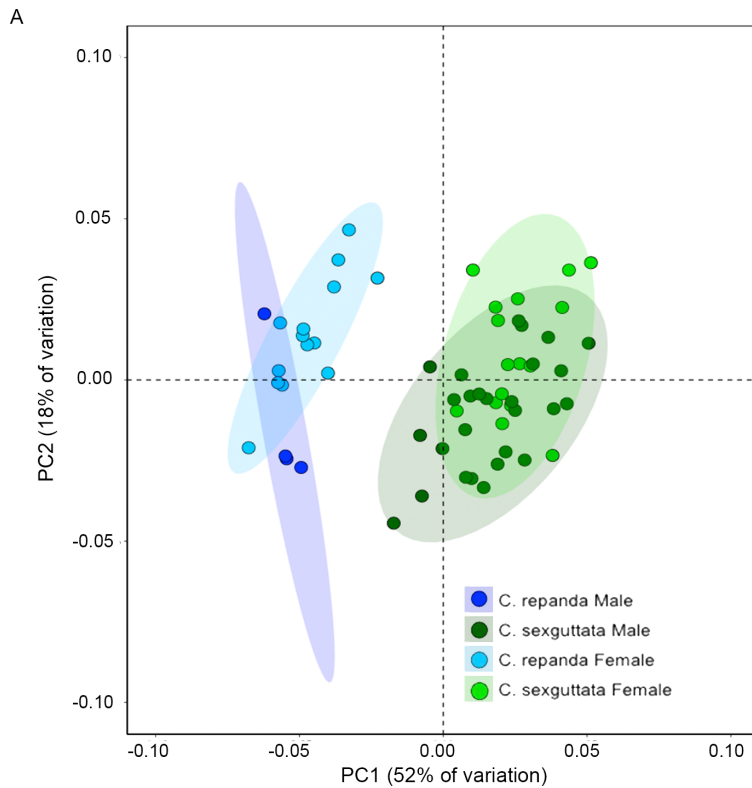
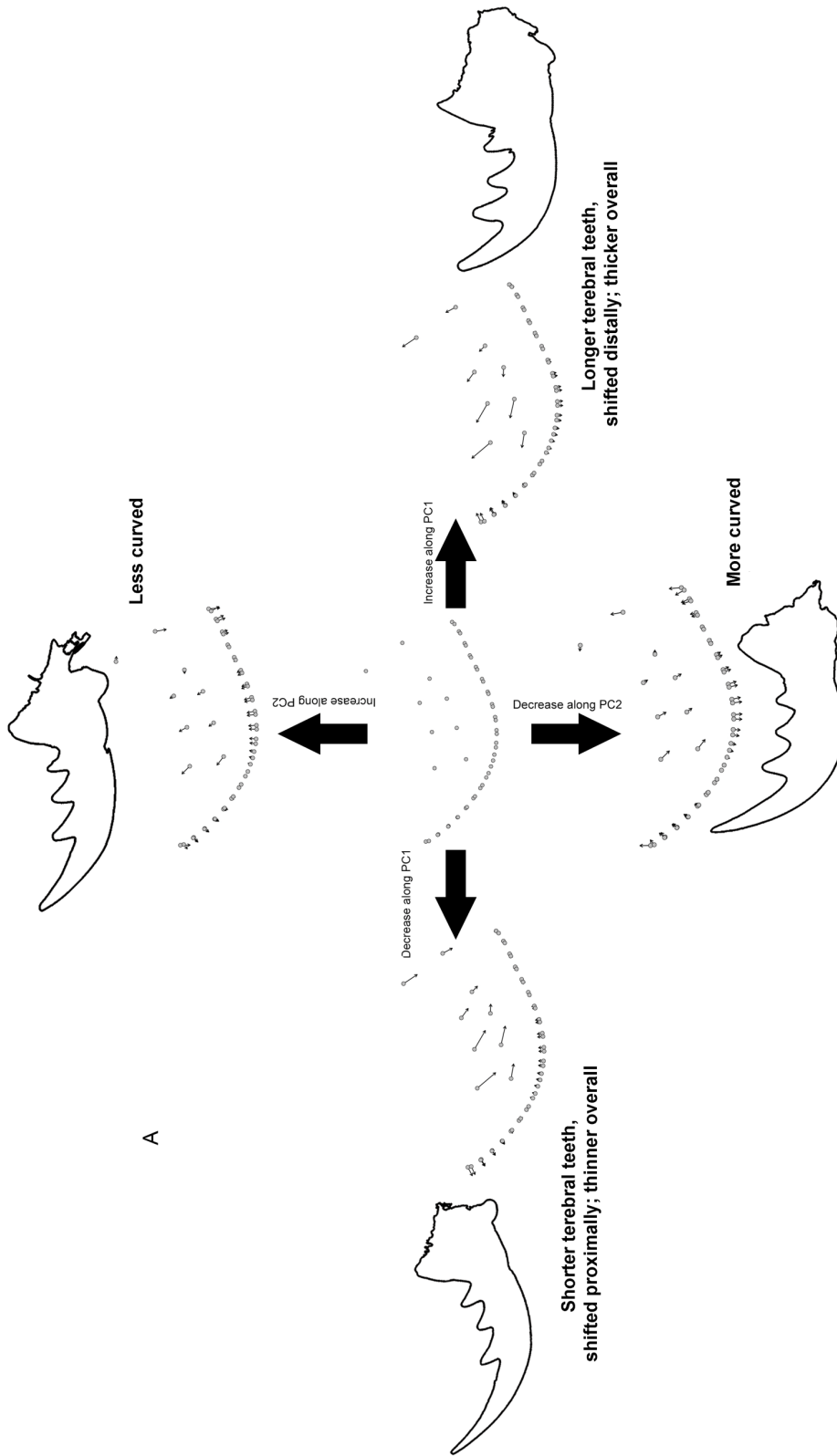


Figure 12 The results of the PCA for **A)** North Carolina species and **B)** Arizona species. These represent the two primary, uncorrelated components of mandible shape explaining the majority of shape variation present among groups. Dashed lines intersect at the origin where the mean shape, an artificially constructed “average” shape based on the data from all specimens, is calculated. Shaded areas represent the 95% confidence ellipses for each sex and species using our *a priori* classifications. These provide an estimation of the mean smaller ellipses indicate a better estimation.

To quantify these differences, we conducted a series of Goodall's F tests on the data. We chose to look at both intraspecific sexual dimorphism as well as interspecific differences for specimens in each location. In all comparisons, Bonferroni-adjusted p -values were < 0.05 , confirming that heterospecific males differ in shape, heterospecific females differ in shape, as well as conspecific males and females, though to a lesser degree. Increasing along PC1, mandibles appear to have longer terebral teeth shifted more distally from the mandible base and are thicker overall. Decreasing along PC2, they display more curved mandibles along the lateral edge (Fig. 13A). For, the Arizona PCA (Fig. 13B), moving rightward along PC1 yields a similar pattern of lengthening and shifting the terebral teeth and overall thickening while increasing along PC2 yields increasing curving of the apical incisor and lengthening of the of the mandibular complex.



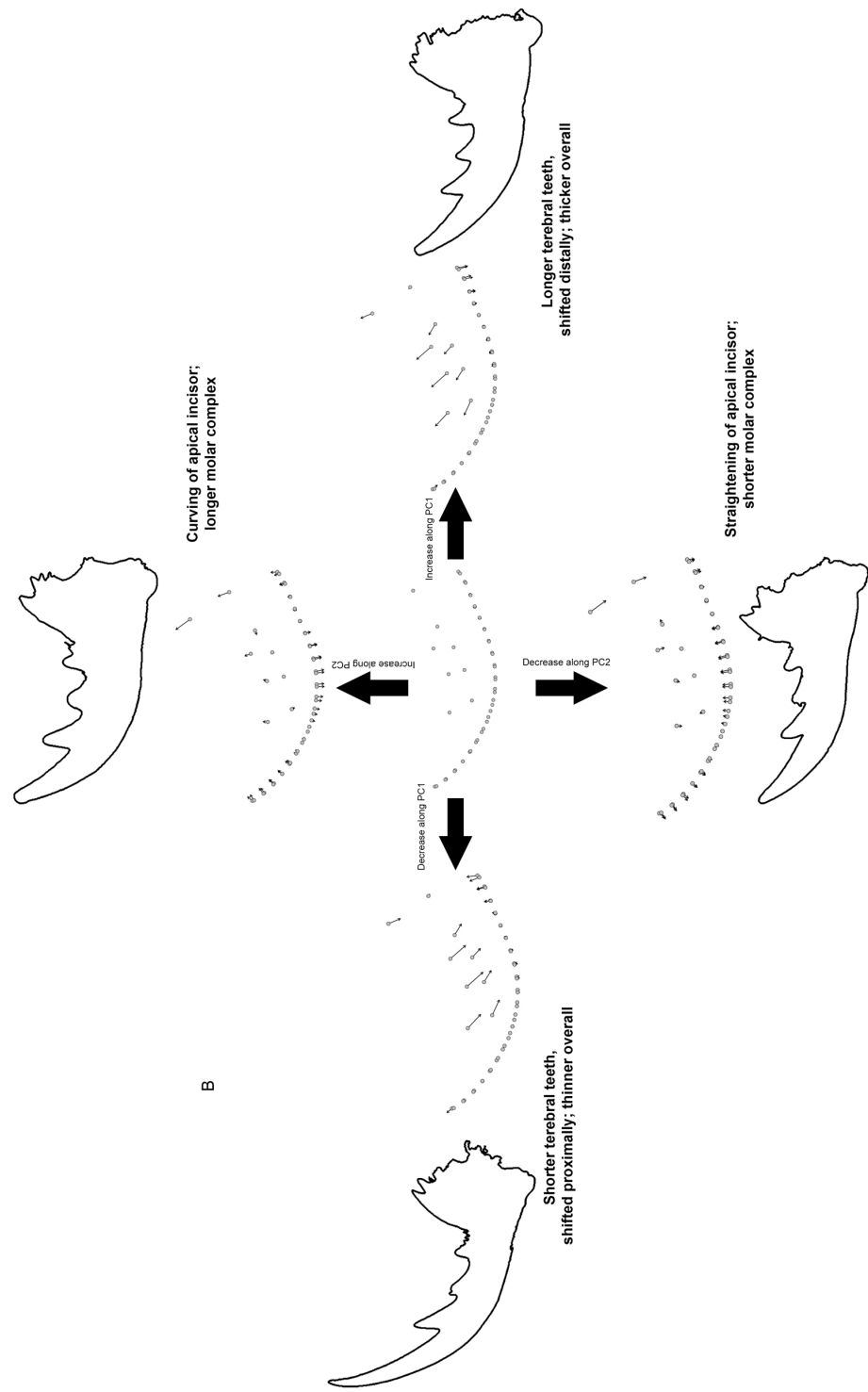


Figure 13 Based on the PCs generated previously, we were able to visualize shape change along the axes with vectors at each landmark indicating the relative landmark displacement. In the center are the mean shapes, which lie at the origins of the PC plots in Fig. 12. The surrounding plots represent the change in shape at the most positive and negative points along each axis and the specimens from **A**) North Carolina and **B**) Arizona represent various intermediates of these forms. The individual beetles whose mandibles have shapes closest to these extremes are plotted as outlines next to the vector plots.

DISCUSSION

To understand the mechanisms governing reproductive isolation in species occupying the same habitat, we sought to test two hypotheses implicating vision and morphological feedback by analyzing the behavior and mandibular morphology of two sets of co-occurring tiger beetle species. In North Carolina, *C. sexguttata* and *C. repanda* present very different elytral colorations and female coupling sulci which should make them visually and anatomically unsuitable for mating attempts between species. *C. sedecimpunctata* and *C. ocellata*, beetles from Arizona, show remarkably similar elytra but female coupling sulci still fall into different shape classifications.

Since visual systems generally provide the most rapid source of sensory information, we predicted that the various elytral colors and patterns found in the genus *Cicindelidae* would provide adequate mate recognition cues prior to contact. Should vision be important for mate recognition, we would expect males to rarely approach heterospecifics. In the case of similar looking heterospecifics or male conspecifics, the mandibular lock-and-key hypothesis posits that mistakes in visual recognition upon the initial approach of an unsuitable mate should be quickly corrected through the morphological feedback from the coupling sulci (Freitag, 1974) or result in males not being able to maintain their grasp on the female, which may result in his seeking a mate elsewhere.

Our data do not support the mandibular lock-and-key hypothesis as it is described in the literature. Despite observing significant differences in the mandible shape between males, we do not observe the appropriate behavioral evidence. Males were able to remain mounted on both conspecific and heterospecific females, despite their vigorous attempts to remove him, making “grip” assistance unlikely. Males also attempted to insert the aedeagus into heterospecifics (and conspecific males, if present), suggesting that mandibular fit does not function in species or sex recognition.

We did provide evidence for the use of elytral coloration as a species recognition cue in the NC experiments. Male *C. sexguttata* preferred females exhibiting green elytra, regardless of whether they were conspecifics or heterospecifics. When choosing between a conspecific and heterospecific, both appearing green, males preferred the conspecific. In this case, we believe that males perceived both females as conspecifics and chose the larger of the two. Males of many insect species in promiscuous mating systems will use external indicators of fecundity, such as large body size, when choosing a mate (Bonduriansky, 2001). Though we did observe mating attempts with females appearing brown in coloration, it is likely this can be attributed to the beetles' strong response to movement cues (Lovari et al., 1992). On these fewer occasions, males likely did not investigate the qualities of the female until after contact.

C. sedecimpunctata males showed no preference for their own species, though this was expected given their similarity in appearance. These males spent nearly equal amounts of time (30-60 sec) attempting to copulate with both hetero- and conspecific females and were able to successfully insert their aedeagus with both. This successful insertion does not necessarily mean that fertilization of the eggs will occur, or even that sperm (or spermatophores) were successfully transferred (Eberhard, 1985). Some females were seen displaying behaviors consistent with ovipositing after the conclusion of the trials (Brust et al., 2012b), but the promiscuous nature of tiger beetle females made it impossible to tell how many matings had occurred prior to capture. Thus, to accurately determine viability and paternity of possible offspring, an entire generation of tiger beetles would need to be reared in a laboratory setting. Additionally, this "oviposition behavior" could have been the ejection of undesired spermatophores from the reproductive tract (Rodríguez, 2000). This is a potential component of cryptic female choice (Eberhard, 1996), that may simultaneously create sperm competition between conspecific males and prevent hybridization.

It is unclear what signals a female uses to cue spermatophore ejection. Some studies have documented the use of syn-copulatory courtship behaviors (Fielding and Knisley, 1995;

Richardson, 2013; Rodriguez, 1998), in which a male attempts to stimulate the female to accept and utilize his spermatophore after intromission has begun. These behaviors may take the form of using the legs to stroke the female or moving the genitalia in ways nonessential to maintaining appropriate contact with the female. The specifics of these actions could be used as species recognition cues, prompting ejection of the spermatophore if done incorrectly. However, there have not been enough quantitative studies to determine if the movements or sequences of movements are enough to promote female receptiveness and whether there are enough interspecies differences to be used in the identification of conspecifics.

Alternatively, females may not need to eject a spermatophore if they never permit it to be transferred in the first place. The aedeagus of male tiger beetles contains a flagellum, a structure inserted into the spermatheca duct to prepare it for sperm transfer from the bursa copulatrix and possibly remove the sperm of previous males (Schincariol and Freitag, 2012). It is possible that genital morphology may create reciprocating structures between males and females (Freitag et al., 1980; Palmer, 1976) that appropriately stimulate receptors in the female genital tract. In Lepidoptera, differential activation of specific sensory sites by dissimilar morphologies is an allows some species to remain reproductively isolated (Masly, 2012). Similar mechanisms could be used in species recognition and inadequate stimulation could inhibit spermatophore transfer or sperm migration into the spermatheca.

Given our results and the possibility of alternatives, a precise intersexual structural coupling involving the mandibles may not be necessary. Rather, it seems that diet may influence the shape of mandibles more than reproduction. Indeed, where it has been tested, tiger beetles that co-occur with other species and overlap in periods of activity show correlation between mandible sizes and prey sizes (Pearson and Mury, 1979). This makes it likely that differences in mandible shape between species may be the result of utilizing different prey bases rather than the need to interact with female coupling sulci. And although we see intraspecific sexual dimorphism in mandible shape, this could be a function of body size differences. Additionally, the lack of

successful aedeagal insertions between *C. sexguttata* males and *C. repanda* females coupled with significant size differences suggest that morphological characteristics associated with the appropriate genital contact is more important than those promoting mandible-coupling sulcus contact. Interactions between *C. sedecimpunctata* males and *C. ocellata* females further support this in that aedeagal insertions were just as common in heterospecifics with more similar physical characteristics as conspecifics.

Overall, these findings suggest that tiger beetles may use multiple strategies to maintain reproductive isolation. The mandibular lock-and-key hypothesis, as currently defined, is not a satisfactory explanation for rapid species, or even sex, recognition. Mandible shape did not appear to influence males' mating attempts. Freitag's coupling sulcus classifications are only qualitative and additional analyses are needed to quantitatively describe their shapes and determine how they interact with male mandibles. It is possible for mechanical signals to still be the primary cue for conspecific identification via the interaction between reciprocating genital structures. Because we observe color preferences, male tiger beetles likely use elytral colors to initially detect conspecific females when the coloring between conspecifics and heterospecifics is dissimilar. In this case, genitalic compatibility is a secondary barrier to hybridization to control occasional mistakes in species and sex recognition. When the coloration between to species is similar, tiger beetles rely solely physical compatibility, and possibly cryptic female preference, to reduce the incidence of hybrids.

Although we only support one of our original hypotheses, this study has still brought insight into the reproductive isolation of tiger beetles. We have provided evidence for the use of elytral coloration as a species recognition cue and rejected the lock-and-key hypothesis with regards to the mandible-coupling sulcus structure. The mandibles are used for a multitude of functions besides reproduction, including foraging and self-defense and thus, are affected by ecological factors such as prey size and density (Pearson and Mury, 1979). The aedeagus, on the other hand, is quite distinctive between species and is frequently used to identify species and

estimate phylogenetic relationships (Pearson and Vogler, 2001). Its entire function is restricted to transferring genetic material and, given the possibility of reciprocating genital structures, is a much more likely candidate for the lock-and-key hypothesis and should be subjected to additional hypothesis testing.

One of the most basic assumptions concerning co-occurring organisms is that they could not remain separate species if hybridization barriers were not present. When these barriers are not immediately evident, a closer examination of the reproductive biology is required and is critical to understanding species evolution. Tiger beetles have not been the target of intense investigation, despite their widespread occurrence and potential to serve as a model for post-copulatory hybrid prevention. Additional experiments are still needed to fully quantify the characteristics associated with the identification of appropriate mates in tiger beetles. However, we have been able to empirically refute the long-standing mandibular lock-and-key assumption and provide evidence that vision, although critical in other capacities, does not appear to influence mate selection.

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CURRICULUM VITAE

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EDUCATION

Wake Forest University ▪ Winston-Salem, NC ▪ 2015-2018

M.S. | Biology

Advisor: William E. Conner, PhD

Salem College ▪ Winston-Salem, NC ▪ 2011-2014

B.A. | Biology

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RESEARCH EXPERIENCE

UNC Center for Design Innovation ▪ Winston-Salem, NC ▪ 2013 - 2015

Internship/Research Residency Program

Kinematics of Prey Capture: Studying the relationship between flight morphology and performance in bats

PPD: Motion analysis of people with Parkinson's disease before and after improvisational dance

Group behavior in Brazilian free-tailed bats during cave emergence and return

Interpreters and Scientists Working on our Parks (iSWOOP): An educational program for park rangers at National Parks designed to enhance the informal learning experience of park visitors.

Laser Scanning: 3D point-cloud reconstructions of cave morphology of Carlsbad Caverns, Davis Cave, Ney Cave, and Frio Cave using laser scanning technology

PUBLICATIONS

Jones, T.K. and Conner, W.E. (2018). Active acoustic interference elicits echolocation changes in heterospecific bats. *Journal of Experimental Biology* (in review).

Jones, T.K. and Conner, W.E. (2018). Pre-mating reproductive isolation in tiger beetles (Cicindelidae): Vision or morphological feedback? *Journal of Insect Science* (in review).

SCHOLARLY PRESENTATIONS

Nature Inspired Flight Technology and Ideas ▪ Seattle, WA ▪ 2018
Effects of heterospecific jamming calls in bats

Society for Integrative and Comparative Biology ▪ San Francisco, CA ▪ 2018
Active acoustic interference elicits echolocation changes in heterospecific bats

Society for Integrative and Comparative Biology ▪ West Palm Beach, FL ▪ 2015
iSWOOP: Interpreters and Scientists Working on Our Parks

TEACHING EXPERIENCE

Wake Forest University ▪ 2015-2017
Teaching Assistant
Comparative Physiology

North Forsyth High School ▪ 2016
Guest Lecturer
Immunology

Winston Salem State University ▪ 2014-2015
Teaching Assistant
Introduction to Biology
Principles of Biology

GRANTS and AWARDS

Charlotte C. Magnum Student Support Program ▪ 2018
National Science Foundation Graduate Research Fellowship ▪ 2017
Graduate School Alumni Student Travel Award ▪ 2017
Elton C. Cocke Travel Award ▪ 2017
Southwestern Research Station Student Support Fund Grant ▪ 2017
Vecellio Grant for Graduate Research ▪ 2016

SCIENTIFIC SOCIETIES

Society for Integrative and Comparative Biology
International Society for Neuroethology

UNDERGRADUATE MENTORING

Sylvia Baila ▪ Effects of playback timing on behavioral outcomes in bats ▪ 2016
Christina Benedict ▪ 3D space calibration: wand vs. drone ▪ 2016
Julianne DeMarco ▪ Tiger beetle mandible morphology ▪ 2017