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Complex sexual selection across space and time: insights into species interactions and mating strategies

By

Lin Yan

A dissertation submitted in partial satisfaction of the
requirements for the degree of

Doctor of Philosophy

in

Environmental Science, Policy, and Management

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Damian O. Elias, Chair

Professor Eileen A. Lacey

Professor Rosemary G. Gillespie

Spring 2026

Complex sexual selection across space and time: insights into species interactions and mating strategies

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By

Lin Yan

Abstract

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Doctor of Philosophy in Environmental Science, Policy, and Management

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Professor Damian O. Elias, Chair

Sexual selection is a major driver of phenotypic diversity, shaping many of the traits through which biodiversity is most visibly expressed, including ornamentation, elaborate songs, and diverse mating strategies. Yet its influence is uneven across systems, raising a fundamental question: why does sexual selection drive extreme diversification in some lineages while remaining relatively subdued in others? Directly resolving this question is challenging, in part because of tangled causal relationships among trait evolution, mate choice, adaptations and population dynamics. However, examining systems in which sexual selection has produced extensive diversification can provide insight into the processes and consequences of these dynamics.

The paradise jumping spiders (*Habronattus*) represent one such system. This speciose and widespread genus occupies diverse habitats, from arid deserts to high-elevation forests, and exhibits striking variation in male sexually selected traits. Courtship displays vary widely in complexity across species, spanning multiple signaling modalities including visual ornamentation and substrate borne vibrations. At the same time, *Habronattus* shows a complex genetic landscape shaped by both ancient and ongoing introgression. The combination of phenotypic diversity, ecological breadth, and reticulate evolutionary history makes this genus a powerful system for investigating mating behavior, how sexual selection influences species interactions, the dynamics of species diversification, and the characteristics of sexually selected systems.

The first two chapters of my dissertation aim to understand the role sexual selection plays in species diversification using two species groups: the *Habronattus americanus* subgroup, characterized by relatively simple male courtship, and the *Habronattus clypeatus* subgroup, characterized by more complex displays. I compared outcomes and preference patterns of heterospecific matings within each group, revealing the complexity of the preference landscape within the genus and how it may contribute to speciation dynamics. While the first two chapters focus on the outcomes of existing male traits and female preference patterns, the next two chapters shift to examining the mechanisms that have shaped the emergence of mate choice and complex courtship in this system. Chapter 3 examines how female preference changes over time, providing insight into the possible ontogeny of complex courtship. Finally, Chapter 4 examines

the role of female reproductive physiology in shaping mate choice and sexual selection.

Overall, this dissertation highlights the complex nature of sexual selection. We found that sexual signals and speciation dynamics are shaped by distinct forces. In some groups (e.g. the *americanus* subgroup), a strong latent preference overrides existing preferences, suggesting a pathway to extinction through collapsed species boundaries. In contrast, in other groups (e.g. the *clypeatus* subgroup), variation in female preference is revealed when individuals are exposed to novel traits, suggesting a pathway to speciation. Further, the complex nature of sexual selection can be seen from a more proximate perspective. Changes in preferences reveal how temporal variation in female receptivity can affect selection on different components of male courtship. Further, ovarian development hints on how female mate choice is favored across a diversity of mate choice strategies. Together, these findings illustrate the many complex ways that sexual selection can operate across multiple scales to shape diversification and mating strategies.

Dedication

This work is dedicated to my grandpa. It is only a glimpse of the curiosity connection to nature that he shared with me.

Acknowledgments

I am fortunate to have truly enjoyed my time as a PhD student, thanks to the support and company of so many people. Words cannot fully express my gratitude, so I would like to instead share a few core memories that reflect how they have guided me throughout this journey.

Damian

I am very lucky to have Damian Elias as my PhD advisor. Among the many reasons why he is such a great mentor, I would like to mention two. Every time we have a spider discussion, I feel that my curiosity is always seen and supported unconditionally. I still remember, eight years ago when I was an undergraduate in the lab, Damian handed me a pair of headphones in the laser room. Through them, I heard the vibrations of a wolf spider courtship for the first time. The room was dark, with only the arena and the laser point faintly lit, and he said, now you are one of the few people who have ever listened to their courtship. That moment clicked for me, and it was just the beginning of countless inspirations from Damian in the years that followed. I would also like to mention how he creates a space that protects my well-being despite the pressures of academia and society. As an international student, many parents would envy the fact that I am able to go home and spend time with my family almost every year, while many PhD students cannot. When I am home, he often shields me from lab-related obligations so that I can spend more time with my family. He has consistently prioritized my well-being over productivity, something I did not recognize the importance of at first, but came to understand over time because of his example.

Committee members

I am also extremely lucky to have had the support of my dissertation committee members, Eileen Lacey and Rosemary Gillespie, since the very beginning of my PhD. Their wealth of knowledge has always inspired me to think about my work from perspectives I might have otherwise overlooked, and challenged me to think outside the box. My first GSI experience was during my first semester at Berkeley, and Eileen's approach to teaching set an example for me—her patient guidance really shaped how I approached teaching throughout my PhD. I also had the opportunity to GSI with Rosemary, and was able to observe the elegance of her teaching and her depth of knowledge in the classroom, in the lab, and in the field. The guidance of Frederic Theunissen and Annaliese Beery, my qualifying exam committee members, was also invaluable during the early stages of my PhD and helped shape the direction of my research.

Funding sources

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Arachnologists

I was also fortunate to have worked with amazing arachnologists through collaborations, field collection trips, and the American Arachnological Society meetings. The wealth of knowledge

of Marshal Hedin and Wayne Maddison on *Habronattus*, *Pellenattus*, the landscape, the habitat, everything about spiders, never stops amazing and inspiring me. I also got to know Marshal as a great photographer, and he showed me ways to see the beauty of the miniature world through macro lenses. I was honored to be invited on adventurous field trips and to share fun conversations and breathtaking landscapes with Marshal, Wayne, Greta Binford, G. B. Edwards, and Caleb Camilleri. Their company always makes me look forward to the next trip.

Labmates

The Elias lab has changed a lot in size since I first joined as an undergraduate, but it has always been a bubble of support and laughter, no matter how I feel before entering. It is a magical place that attracts people who are all so special, yet always make it feel like home for me in a foreign country. I would like to thank previous lab members, including Masami Amakawa for recruiting me and being the first person who introduced me to the lab; Malcolm Rosenthal and Benji Kessler for having me on their projects and introducing me to fieldwork; and Maggie Raboin, Erin Brandt, and Ignacio Escalante Meza for always being patient with my countless questions. I am also so grateful to be part of the current lab: Trinity Walls, Ciara Kernan, Jacob Gorneau, Sophie Hanson, and Noah Leith. Being in the lab with all of you makes me look forward to coming in every morning—for science discussions, venting, good food, laughter, deep dives, pranks, hiding hands and simply knowing that you are around to support me. I could not thank the undergraduate students in the lab enough for their dedication in taking care of the spiders, as well as for taking the lead on many parts of projects that I learned a great deal from, and for being instrumental in the field. I would like to give special thanks to Shiyang Wu, for pop-quizzing me every day before my qualifying exam, and to Noah Huh, for trusting my driving skills and for the insightful conversations in the mountains of Utah and California.

Fieldwork buddies

My passion for fieldwork has been one of the biggest discoveries about myself, thanks to the spiders and to the fun, supportive, and all-around amazing friends who agreed to come to the field with me. I would like to thank Aaron Balbo for taking his beloved truck to Tahoe and the Berkeley Hills, and for identifying *H. americanus* habitat far more accurately than I ever could. I would also like to thank my cohort buddy Freddy Gutierrez for the laughter he brought, and for his amazing sense of direction that saved me from walking deeper into the woods in Florida. I would also like to thank Roxie Baggott, Jacob Saffarian, and Nick Ha for making those field trips memorable.

Friends and family

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I would like to give special thanks to my parents and family members for all the documentaries they made me watch, for sneaking out with me to catch bugs at night, for putting up with my smelly, poorly maintained butterfly collections from middle school, and for always encouraging me to pursue what makes me happy. I hope they find my work interesting to read, just like those documentary stories we watched together.

Land and nature

Finally, I would like to list all the places I have visited and come to feel connected to through the spiders I worked with. Every place I visited was new to me, but weirdly enough, there was always a rock, a plant, a bird, a sunset, or the shape of a sand dune that reminded me of home, of my childhood, and made me feel connected deeply to the place and that I could belong there. The spiders have shown me the vast mountains of New Mexico, mesquite bushes full of spines in Texas, rain pouring on palm trees in Florida, rivers running through red rock valleys in Utah, windy mountain tops in Colorado, quiet salt marshes and hot springs in Nevada, cold caves hidden in the forests of Washington, falling trees in Oregon, and the cranes, grasslands, estuaries, coastlines, and redwoods of California. I have come to know these landscapes through mapping habitats, thinking about behavior, phenology, and climate, and truly experiencing how all aspects of nature are intertwined. Although each trip lasted only a few days, the spiders made me feel more like a part of the landscape than a tourist or visitor. I am forever grateful for the perspective the spiders introduced me to, allowing me to experience and connect with the natural world, and I will always think of them wherever I go next.

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Chapter 1

Latent preference for red ornamentation drives interspecific mating in nascent jumping spider species (*Habronattus americanus* group, *F. Salticidae*)

Lin Yan, Noah J. Huh, Daniel Ibañez IV, Malcolm F. Rosenthal, Marshal Hedin and Damian O. Elias

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1.1 Abstract

Heterospecific interactions between nascent species offer insights into how sexual selection shapes novel traits, illuminating patterns in species interactions and diversification. We tested female preferences between two recently diverged, allopatric species of jumping spiders: *Habronattus americanus* PLC, with red-colored males performing short multimodal displays, and *Habronattus sansoni* CC, with brown-colored males performing long multimodal displays. Mate choice experiments showed that females of both species preferred *H. americanus* males. To examine the role of red coloration, we manipulated male coloration in both species. Results indicated that red-painted *H. sansoni* CC males experienced an increase in mating success, whereas brown-painted *H. americanus* males did not show reduced success. Our study suggests that (1) strong latent female preferences can drive uni-directional introgression across species boundaries potentially leading to genomic homogenization, (2) latent preferences may override preferences for existing traits and (3) the geographic distribution of color morphs is consistent with a hypothesis of strong latent preferences across populations. Overall our study demonstrates the role that mating interactions can play in speciation dynamics.

1.2 Introduction

The prevalence of introgression across the tree of life suggests that hybridization is a key feature in speciation (Abbott et al., 2013). Speciation does not necessarily follow a simple, linear path where the evolution of nascent species is followed by the emergence of strong barriers to gene flow and subsequent complete reproductive isolation. Instead, speciation is a complex reticulate process where factors associated with hybridization (e.g. hybrid fitness, frequency of hybridization, niche availability for hybrids, etc.) can increase, stabilize or erode divergence at any stage of the speciation process (Buerkle et al., 2003; Kearns et al., 2018; Peñalba et al., 2024; Seehausen et al., 2008). To understand these complexities, it is necessary to examine the factors that promote hybridization between individuals of divergent populations.

Mate preferences are thought to be the primary pre-zygotic barrier preventing hybridization between closely related species, as intrinsic post-zygotic incompatibilities are believed to evolve later in species that recently diverged (Panhuis et al., 2001; Seehausen et al., 2014). Selection against hybrids with reduced fitness can drive mate preferences to filter out

inappropriate courters (i.e. heterospecifics) thus reinforcing assortative mating and creating barriers to hybridization. This however is not always the case. In some instances, mate preferences may increase interspecific gene flow, eroding reproductive barriers (Aubier et al., 2019; Baldassarre et al., 2014; Irwin, 2020; Servedio & Boughman, 2017). For example in manakins, mating preferences for plumage traits were shown to drive asymmetric trait introgression in a hybrid zone (Stein & Uy, 2006). These and other studies have suggested that asymmetric gene and trait introgression can blur phenotypic differences across species boundaries and potentially drive genomic homogenization (variously referred to as species collapse (Behm et al., 2010), “despeciation (Grant et al., 2004)”, or “speciation in reverse” (E. B. Taylor et al., 2005)). Importantly, while courter signals and chooser preferences (including associated sensory systems) are generally thought to be selected to be an efficient match (Gerhardt & Schwartz, 2001; Kostarakos & Hedwig, 2012), mate preferences can be complex. Research has shown that choosers can select a broad range of phenotypes including signals outside the range of natural conspecific variation including novel signals, through the expression of latent preferences (Arak & Enquist, 1993; Moehring & Boughman, 2019); spiders - Elias et al. 2006 (D. O. Elias, Hebets, & Hoy, 2006), grasshoppers - Reichert et al. 2017 (Reichert et al., 2017) and guppies - Daniel et al. 2020 (Daniel et al., 2020). In some cases, mate preferences can favor heterospecifics (Pilakouta et al., 2017).

The jumping spider genus *Habronattus* is a compelling system in which to study hybridization and mate choice. *Habronattus* is a specious group (>100 species) with evidence that a majority of species diversified in the past 2.5 MY (Azevedo et al., 2024; Bodner & Maddison, 2012). Some studies have suggested that female choice plays an important role in diversification (Blackburn & Maddison, 2014; W. Maddison & McMahon, 2000; Masta & Maddison, 2002; Peckham & Peckham, 1889). Male courtship displays vary across the genus, from simple to elaborate displays involving different ornamentation, movement, vibratory and tactile signals (D. O. Elias et al., 2012; Masta & Maddison, 2002; Rivera et al., 2021). Despite elaborate displays, male *Habronattus* frequently target their courtship to heterospecifics (Sung et al., 2025; L. A. Taylor et al., 2017). Additionally, despite trichromatic vision (Zurek et al., 05/2015) and behavioral evidence that females can detect and assess vibratory signals (L. A. Taylor & McGraw, 2013), there is evidence for incomplete assortative mating between species (Blackburn & Maddison, 2014; D. O. Elias, Hebets, & Hoy, 2006; Hebets & Maddison, 2005), ancient and recent introgression (T. C. Bougie et al., 2021; Hedin et al., 2020; Hedin & Lowder, 2009; Leduc-Robert & Maddison, 2018), and multiple hybrid zones (T. Bougie et al., 2024; W. P. Maddison & Maddison, 2016). Distinct male courtship displays with frequent gene flow across species boundaries provides a unique opportunity to test how mate choice patterns influence patterns of hybridization that can lead to genomic homogenization on one hand and diversification (i.e. adaptive introgression (Hedrick, 2013), hybrid speciation (Abbott et al., 2013)) on the other.

Among *Habronattus*, the *americanus* species complex is of interest because mate choice is likely important in determining the likelihood of hybridization because post-zygotic isolation is weak (T. Bougie et al., 2024). Members of the *americanus* subgroup have relatively simple dance and vibratory displays but marked variation in coloration (T. C. Bougie et al., 2021). While morphological data suggest that several species exist with geographic variation, genomic data suggest genetic groupings are consistent with geography and not morphology (T. C. Bougie et al., 2021). Hybrid zone studies between *H. americanus* whose parental phenotype consists of red males, and *H. kubai* whose parental phenotype consists of brown and black males, revealed a

range of intermediate and transgressive phenotypes dominated by red individuals (*H. americanus* phenotype) (T. Bougie et al., 2024). These studies suggest the possibility that morphological and genomic data are discordant because mating preferences for red coloration are driving genomic homogenization in the *americanus* subgroup. Consistent with this, the “sea of red” hypothesis posited by Bougie et al (T. Bougie et al., 2024) suggests that preferences for red phenotypes explain species distributions where taxa other than *H. americanus* are being replaced by *H. americanus* (“extinction via hybridization” (Behm et al., 2010),(Grant et al., 2004),(E. B. Taylor et al., 2005)).

To test predictions from the “sea of red” hypothesis and how mate preferences could lead to extinction via hybridization, we selected two allopatric populations (T. C. Bougie et al., 2021) representing the extremes of coloration variation in the *americanus* subgroup. *Habronattus americanus* PLC form males show red ornamentation on the anterior surface of the pedipalps and chelicerae, as well as the anterior ventrolateral side of the first femur. On the other hand, *Habronattus sansoni* CC males are brown overall with two black stripes above their principal eyes. Both populations perform vibratory displays including percussive and tremulatory elements. We first conducted con- and hetero-specific mating trials (Experiment I) to test predictions from the “sea of red” hypothesis (T. Bougie et al., 2024); if *americanus* subgroup females are biased towards red coloration, we predicted that both *H. sansoni* CC and *H. americanus* females would prefer to mate with red *H. americanus* PLC males. Next, to test whether red coloration drives the observed mating patterns, we manipulated male color in conspecific pairs (Experiment II). If red coloration is responsible, we predicted that red-colored males would be preferred over brown-colored males in both species. Lastly, we visualized the distribution of *H. americanus* and *H. sansoni* morphs using iNaturalist observations to examine whether color morph distributions were consistent with the “sea of red” hypothesis and the predictions drawn from our behavioral experiments (Experiments I and II). Overall, mate choice preferences are key to understanding the phylogenetic, geographic, and morphological relationships found in the *americanus* species group and provide insights into the role of latent preferences in speciation dynamics.

1.3 Methods

1.3.1 Animal Collection

We collected *H. sansoni* (CC morph: mostly brown with black stripes above principal eyes) from near Cedar City, Utah from late May to early June 2023. The spiders are found on aspen and/or conifer litter on exposed ground. *Habronattus americanus* (PLC Morph: red in pedipalp, leg and chelicera)(Blackburn & Maddison, 2014) were collected from South Lake Tahoe, California in mid-June of 2023. The spiders are found on conifer litter in exposed areas near sagebrush (*Artemisia*). For animal care procedures see supplementary information.

1.3.2 Courtship displays

To characterize courtship displays, we recorded male displays to a female model following standardized procedures (Girard et al., 2011; Rivera et al., 2021). Representative video vouchers of courtship displays are archived at the Macaulay Library of Natural Sounds (*Habronattus americanus* PLC: ML 488859; *Habronattus sansoni* CC: ML488860). For detailed setup see supplementary information.

1.3.3 Experiment I: Heterospecific mate choice Trials

To explore whether red-ornamented *H. americanus* males are preferred by *H. sansoni* females, we conducted heterospecific mate choice trials in a 2x2 design ($N_{\text{♀amer} \text{♂amer}}=44$, $N_{\text{♀amer} \text{♂sans}}=38$, $N_{\text{♀sans} \text{♂amer}}=31$, $N_{\text{♀sans} \text{♂sans}}=35$). For each mating trial, we placed the female in the arena and allowed her to acclimate for five minutes. We then placed the male into the arena and allowed them to freely for 15 minutes. We ended mating trials before the 15 minutes when there was either a copulation or cannibalism. After a mating trial, we measured body mass (Ohaus Analytical Plus Laboratory Scale, Wazobia Enterprise). The average age of *H. sansoni* females was 23.79 ± 2.64 (Mean $\pm$ SD) days post maturity and *H. americanus* females 24.32 ± 3.04 (Mean $\pm$ SD) days post maturity.

Using BORIS (Behavioral Observation Research Interactive Software, Olivier Friard), we measured the duration of the visual phase (see below), multimodal phase (see below), and whether a male mounted a female (copulation). For the visual phase, the behavior started when the male oriented towards the female and raised both anterior legs and ended when the male either initiated the multimodal phase or stopped displaying. For the multimodal phase, the behavior started when the male began to move its anterior forelegs while producing a vibratory signal and ended when the male attempted to mount the female, transitioned to the visual phase, was chased away by the female, or stopped courting..

To compare the mating rate between hetero- and conspecific pairs (**Fig. 1.1**), we performed a chi-square test using the R function `chisq.test()` in the *stats* package. To explore the role courtship duration plays in mating success in heterospecific and conspecific matings (**Fig. 1.3**), we used a non-parametric method by bootstrapping each subset of data stratified by copulation and pairs (AA, AS, SA, SS) and calculating the mean. This process was repeated 1,000 times and statistical significance was determined by assessing 95% confidence interval overlaps for individuals who did and did not copulate (Hall & Wilson, 1991). We did not use conventional parametric methods because the model assumptions were not met after data transformation. We did not use conventional non-parametric methods because they do not specifically draw conclusions on the mean of the subsets (Breslow, 1970; Nachar, 2008; Rosner et al., 2006). Details on the statistical significance of the bootstrapping test can be found in the Supplementary Material (**Fig. S1.3**).

For each species, male courtship in con- and heterospecific trials did not differ (Fig S3) thus we combined species data (**Fig. 1.2**). To compare the duration of the visual and multimodal phases of *H. americanus* and *H. sansoni* CC males used in our experiments, we built linear regression models, using data from all trials in which the male successfully mated, with male species as a binary predictor and phase duration as the response variable. Only males who successfully mated were used in this analysis since those who did not copulate did not perform the full display and are thus not representative of a “complete” courtship display. We removed 4 outlier trials with long visual courtship phases (>100 s) and another 8 outlier trials with long multimodal courtship phases (>56.25 s). All the outlier trials removed involved *H. sansoni* CC males. Note that the inclusion of these trials in the models did not change the patterns observed (see below). After checking for model assumptions, we performed a type II ANOVA to determine statistical significance using the function `Anova()` in the *car* package (Fox & Weisberg, 2018). All

statistical analyses were performed in R version 4.1.3 (R Core Team 2022). See supplementary information for details on arena setup.

1.3.4 Experiment II: Conspecific Color Manipulation Trials

We randomly assigned 38 *H. sansoni* and 45 *H. americanus* males into 2 groups: red (experimental) and brown (control). Twenty *H. sansoni* were designated for the control treatment and 18 for the experimental treatment. Twenty-three *H. americanus* were designated for the control treatment and 22 were designated for the experimental treatment. For the color manipulation, we anesthetized spiders under CO₂ under a steady stream of 0.068 Liters/minute (1 psi). Next, using a paint brush, we placed the spider into a styrofoam cut-out (1.5x1.5x2.5 cm). Under a dissection microscope (Leica M80 Stereo Zoom Microscope, Leica Biosystems), we painted over the anterior surface of the pedipalps, chelicerae and ventrolateral side of the first leg femur (Blackburn & Maddison, 2014; T. C. Bougie et al., 2021) with either red (Liquid Eyeliner Red, Handaiyan Eyeliner color01) or brown waterproof eyeliner (Liquid Eyeliner Brown, Handaiyan Eyeliner color10).

After the procedure, we waited five days for males to recover and for any volatile compounds to dissipate. Before the start of each trial, males were evaluated visually to ensure that treatments persisted. Afterwards, each male was paired with a conspecific female. All mating trials were run as described above. The substrate was changed after each mating trial to remove any chemical cues. The average age of *H. americanus* females was 70.04±22.40 (Mean±SD) days post maturity and *H. sansoni* females 67.5±18.98 (Mean±SD) days post maturity.

To compare the mating rate between treatments, we performed a chi-square test using the R function `chisq.test()` in the *stats* package (**Fig. 1.4**). To explore the role of courtship duration in mating success for our treatments (**Fig. S1.2**), we used a non-parametric method by bootstrapping each subset of data stratified by copulation and treatment and calculating the mean. This process was repeated 1,000 times and statistical significance was determined by whether the 95% confidence interval overlapped for individuals who did and did not copulate (Hall & Wilson, 1991). We did not use conventional parametric or non-parametric methods for the same reasons described above in Experiment I. Details on the statistical significance of the bootstrapping test can be found in the Supplementary Material (**Fig. S1.3**). All statistical analyses were performed in R version 4.1.3 (R Core Team 2022).

1.3.5 The geographic distribution of *H. americanus* and *H. sansoni* color morphs

Research grade observations of adult male *H. americanus* and *H. sansoni* from iNaturalist (www.inaturalist.org/) were compiled with species, coordinates and accuracy of coordinates. Note that all *H. americanus* and *H. sansoni* morphs are included in this analysis. There are nine informally described *H. americanus* morphs that vary in red ornamentation (T. C. Bougie et al., 2021) including one morph that has no red coloration (T. C. Bougie, 2022). There are currently three informally described *H. sansoni* morphs, that vary in face ornamentation and foreleg ornamentation including one morph that has red facial stripes (T. C. Bougie et al., 2021). The time range of iNaturalist observations included in this study are from May 2013 to July 2024. The presense/absence of red coloration is based on posted images. Observations where coloration could not be evaluated were discarded. We further discarded data points with a resolution lower than 1000 meters.

To visualize geographic distributions, we compiled elevation data using the *elevatr* package in R (Hollister et al., 2018). The zoom level for this procedure was set to 6. All elevations below sea level were set to NA to enhance the contrast of the visualization. The state borders were acquired by the *rnaturalearth* package (*World Map Data from Natural Earth [R Package Rnaturalearth Version 1.0.1]*, 2023) and plotted using *ggplot2*.

1.4 Results

1.4.1 Courtship display descriptions

Americanus subgroup displays can be divided into two phases: (1) visual phase and (2) multimodal phase that consists of stereotyped visual (ornaments, motion, posture) and vibratory displays (Supplementary video). Visual display phases of *H. americanus* and *H. sansoni* occur as the male approaches in a zig-zag motion, shifting his body laterally from one side to another (Blackburn & Maddison, 2015). The multimodal phase begins when the male stops moving (see above) and begins to move his forelegs back and forth, producing a vibratory multicomponent signal (Blackburn & Maddison, 2015). Two vibratory signal components are produced during this phase (**Fig. S1.1**), (1) a buzzing sound that continues throughout the multimodal phase and (2) a flicking sound that aligns with the back-and-forth movement of the forelegs. Both vibratory components form a multicomponent vibratory signal (D. Elias et al., 2006). While the motion and postural elements of the multimodal phase are qualitatively similar between species, the vibratory elements differ temporally. *Habronattus sansoni* CC males perform the multicomponent vibratory signal slower but for longer while *H. americanus* PLC males perform the multicomponent vibratory signal quicker but for a much shorter amount of time. For the purposes of this study, we quantify differences between displays for each species using data from Experiment I (see below). See supplementary information for more details on courtship displays.

1.4.2 Experiment I: Heterospecific Mate choice trials

Habronattus americanus PLC males are preferred by females of both species (**Fig. 1.1**). For *H. americanus* females, the mating rate when presented a *H. sansoni* CC male is 24% (9/38 mated), while the mating rate when presented a *H. americanus* PLC is 73% (32/44 mated) (X-squared = 17.705, df = 1, $p < 0.001$, Pearson's chi-square test). The trend of *H. americanus* males having a higher mating rate is consistent for *H. sansoni* CC females, the mating rate when presented a *H. sansoni* CC male is 31% (11/35 mated), while the mating rate when presented a *H. americanus* PLC male is 74% (23/31 mated) (X-squared = 10.386, df = 1, $p = 0.001$, Pearson's chi-square test).

Habronattus americanus PLC males display red ornamentation on their pedipalps, forelegs and chelicerae (**Fig. 1.2a**). Although *H. sansoni* CC males have a similar visual phase courtship, they do not possess red ornamentation, as they are entirely brown and black (**Fig. 1.2b**). For individuals used in this study, the mean duration of the visual phases of both species is similar (*H. americanus*: 44.16 ± 20.16 seconds, mean $\pm$ SD, $N=55$; *H. sansoni* CC: 34.58 ± 18.76 seconds, mean $\pm$ SD, $N=12$; F value = 2.3, df = 1, $p = 0.14$). *Habronattus sansoni* CC males (Mean $\pm$ SD = 33.35 ± 15.28 s, $N = 12$) perform significantly longer multimodal displays than *H. americanus* PLC males (Mean $\pm$ SD = 6.62 ± 2.22 s, $N = 55$) (F value = 172.7, df = 1, $p < 0.001$) (**Fig. 1.2**).

Males that successfully copulated in the the ♀A♂A cross (copulation: Mean±SD = 46.09±20.26 s; no copulation: 27.12±14.39 s) had longer visual phase durations but not the other crosses (♀A♂S, ♀S♂A, ♀S♂S) (**Fig. 1.3a**). Note that in the ♀S♂A pair, there is a hint that males that copulated demonstrated a longer visual phases (**Fig. 1.3a**), but this was not statistically significant using our conservative approach (**Fig. S1.3**). Males that copulated in the ♀A♂S, ♀S♂S and ♀S♂A crosses, but not the ♀A♂A cross, demonstrated a longer multimodal display duration(**Fig. 1.3b**).

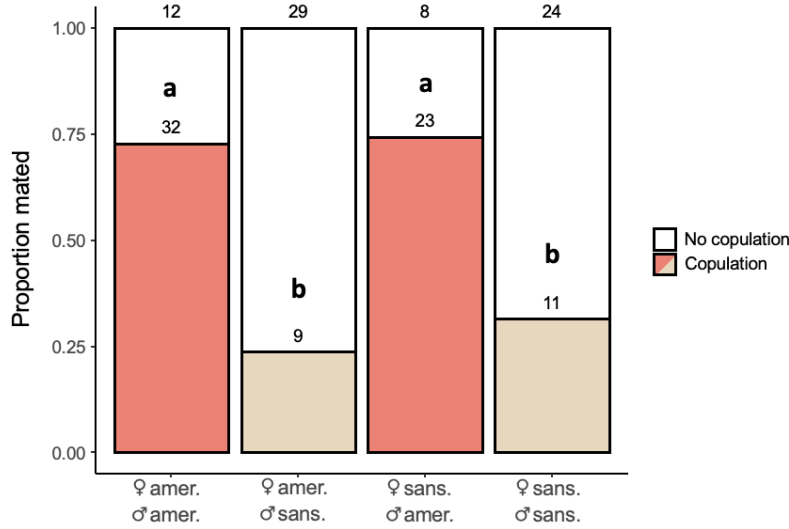


Figure 1.1: *H. americanus* males are preferred by females of both species. Bar plot representing general courtship outcome. Female and male species are labeled on the x-axis: ♂amer.: *H. americanus* male, ♂sans.: *H. sansoni* male, ♀amer.: *H. americanus* female, ♀sans.: *H. sansoni* female. Colored bars (red and brown) indicate mated pairs and white bars indicate pairs that failed to copulate. Letters indicate statistical significance (chi-square tests).

1.4.3 Experiment II: Conspecific Color Manipulations Trials

If red coloration is a major factor that determines mating, we predicted that red-painted *H. sansoni* CC males would have a higher mating rate than their brown-painted (control) counterparts, and brown-painted *H. americanus* PLC males would have a lower mating rate than their red-painted (control) counterparts. Our results show that red *H. sansoni* CC males have significantly higher mating rates than control conspecifics (45% vs 11% mated, X-squared = 4.885, df = 1, p = 0.027, Pearson's chi-square test) (**Fig. 1.4**). However, the mating rate of *H. americanus* did not differ significantly between red or brown-painted males (54% vs 48% mated, X-squared = 0.019, df = 1, p = 0.891, Pearson's chi-square test) (**Fig. 1.4**). The duration of the visual phase did not differ between pairs that did or did not copulate, but almost every male that proceeded to the multimodal courtship phase mated (**Fig. S1.2**). For the control (brown-painted) *H. sansoni* CC group, pairs that copulated (Mean±SD = 63.06±0.05s) had a longer visual phase than ones that did not copulate (25.52±16.27s) (**Fig. S1.2a**). In the case of the multimodal phase, it is similar to the result in *H. americanus* in that nearly all males that progressed to that stage copulated (**Fig. S1.2b**). We note that the number of brown-painted *sansoni* males that mated is low (2/21, **Fig. 1.4**) thus the results from this comparison should be considered preliminary. We also note that while our results show the influence of red coloration, we cannot distinguish

whether these results stem from perceived changes in hue, chroma, brightness, chromatic contrast, achromatic contrast, or a combination of these features.

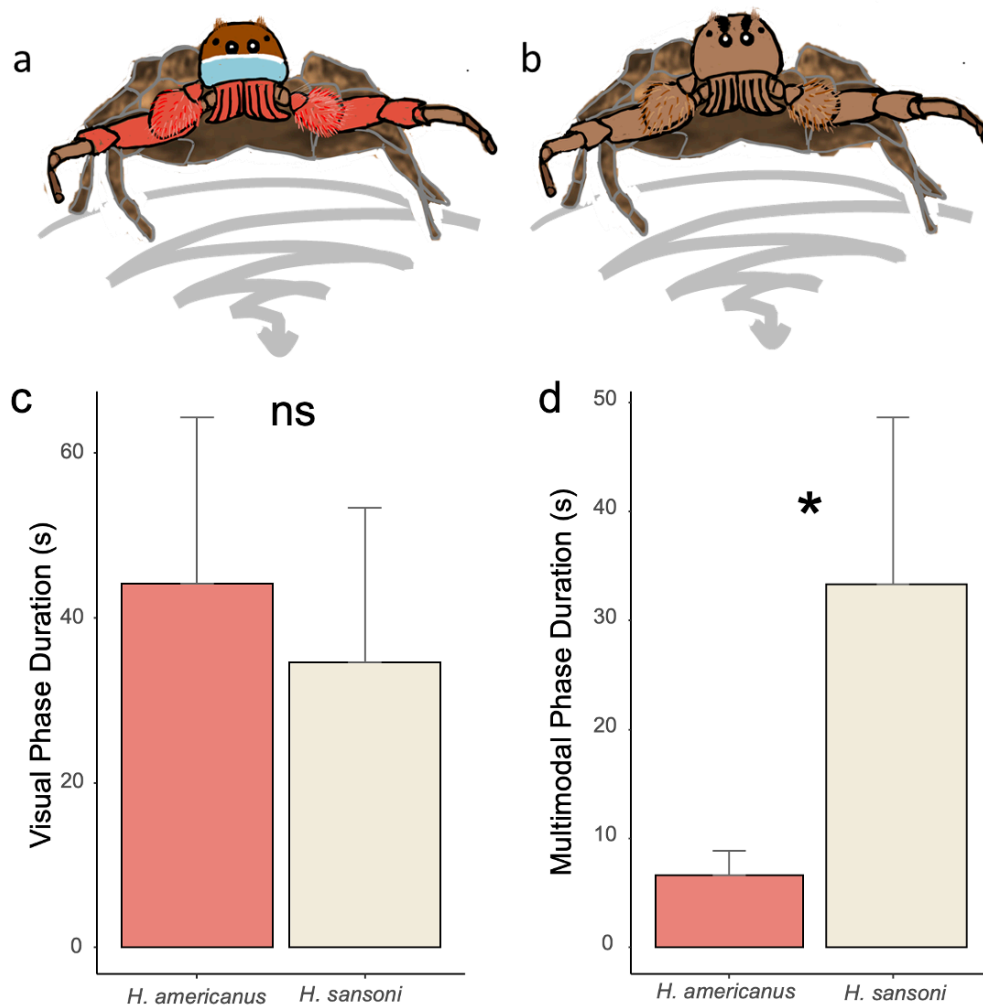


Figure 1.2 Visual phases of *H. americanus* and *H. sansoni* CC courtship do not differ in duration, but *H. sansoni* CC engage in longer multimodal phases. **a** and **b**: visual phase display posture where males of both species zig-zag (gray) towards the female. **c**: comparison of mean visual phase duration between the two species. **d**: comparison of mean multimodal phase duration between the two species. Error bars in both cases represent standard deviations.

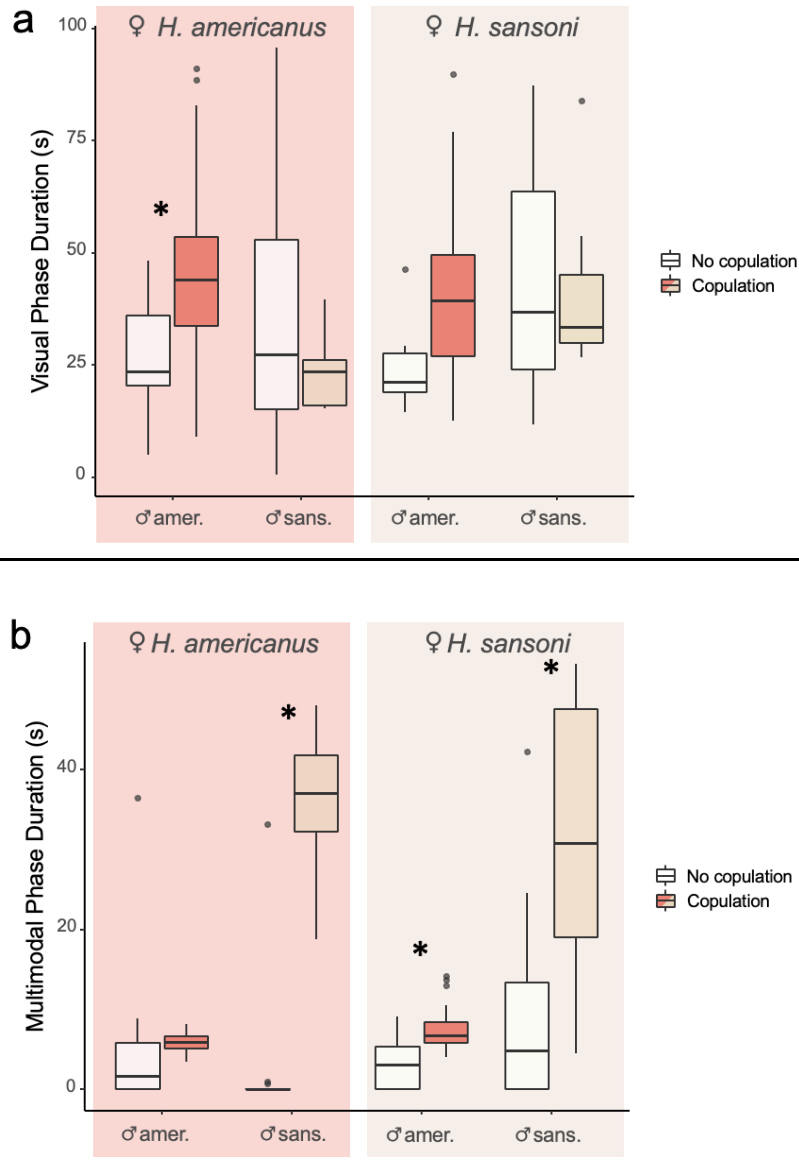


Figure 1.3: *H. americanus* males are preferred by females of both species. Male species are labeled on the x-axis: ♂amer.: *H. americanus* male, ♂sans.: *H. sansoni* male. Female species are labeled at the top of the colored boxes. Colored boxes (red and brown) indicate mated pairs and white boxes indicate pairs that failed to copulate. Asterisks indicate statistical significance (bootstrapping, **Fig. S1.3**). **a:** Boxplot comparing mean visual phase duration of mated and unmated males in each pairing group. **b:** Boxplot comparing multimodal phase duration of mated and unmated males.

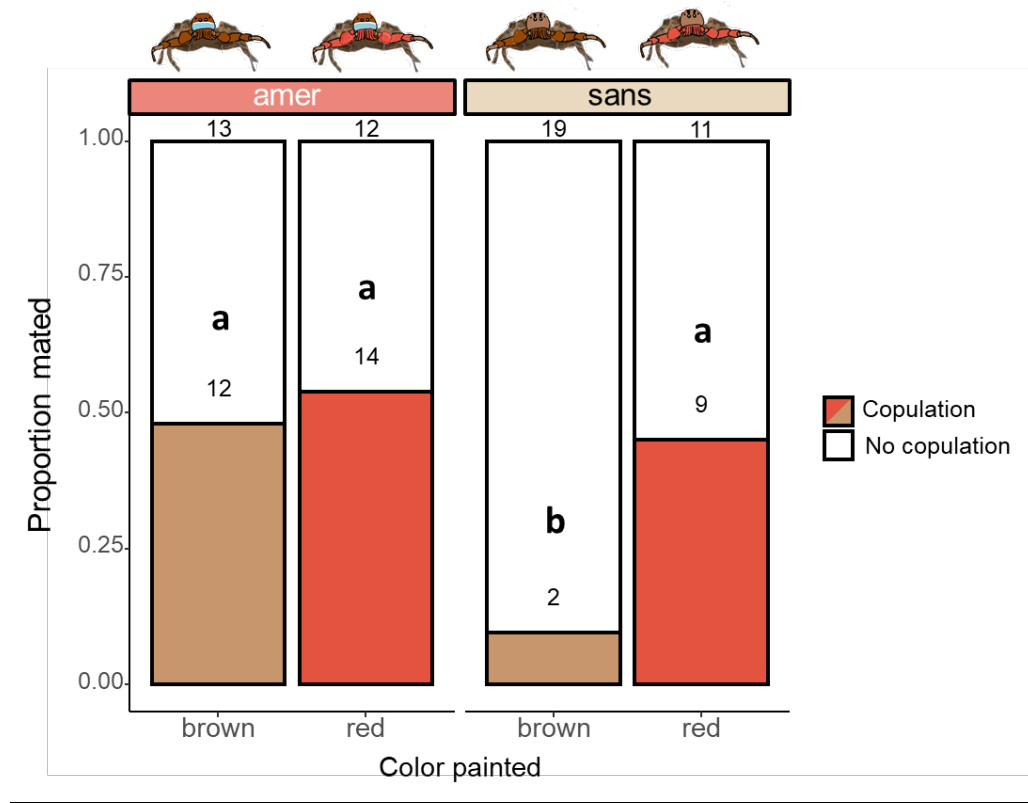


Figure 1.4. Color manipulations did not alter mating outcomes in *H. americanus* pairings but altered mating outcomes in *H. sansoni* CC pairings where females preferred red-painted males. Illustrations of color-manipulated males are on top of the horizontal bars with species identities labeled in the horizontal bars above the bar plot. For the bar plot, colored bars (red and brown) denote successful copulation and white bars denote pairs that failed to copulate. Sample sizes of each treatment are provided at the top of each bar. Letters indicate statistical significance (chi-square).

1.4.3 The geographic distribution of *H. americanus* and *H. sansoni* populations

Geographic distributions of *H. americanus* and *H. sansoni* populations from iNaturalist data suggest that the red coloration dominates across the distribution of both species (**Fig. 1.5**). Non-red coloration appears in three pockets, one in Northwest US/Southern Canada, one in the Intermountain West (Utah, Colorado, S Wyoming, N Arizona, N New Mexico), and a small isolated population in Northeastern California (**Fig. 1.5**). The geographical distribution of red coloration is consistent with the “sea of red” hypothesis (T. Bougie et al., 2024).

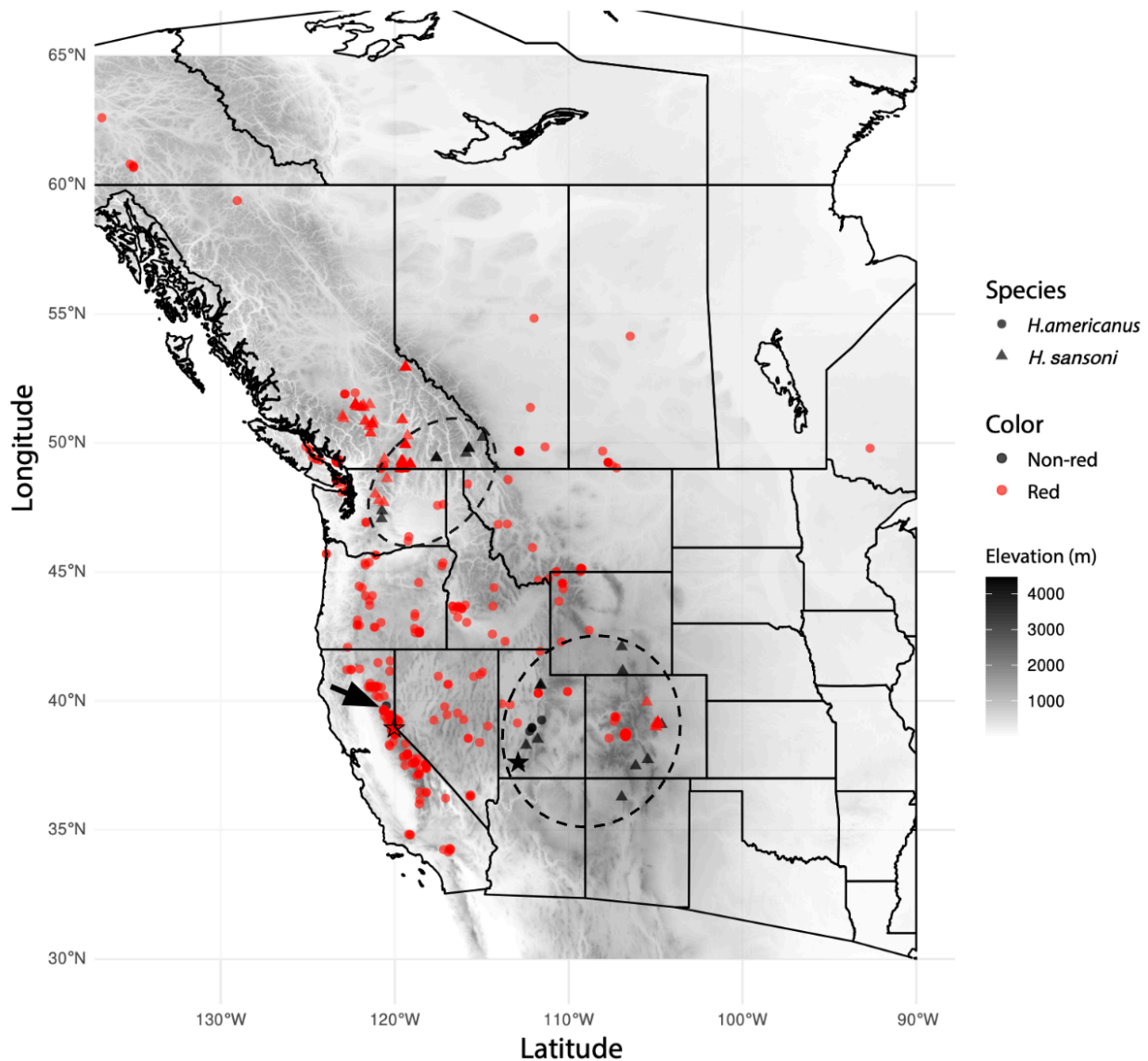


Figure 1.5. Red coloration dominates populations of *H. americanus* and *H. sansoni*. Shade of the map indicates elevation and state borders are shown in black solid line. Red points indicate observed male with red coloration and black points indicate males without any red coloration. Circle points indicate *H. americanus* males and triangle points indicate *H. sansoni* males. The *H. americanus* PLC form and *H. sansoni* CC form used in this study is marked with red and black stars, respectively, on the map. Two black circles with dashed line indicate pockets with non-red males observed. The black arrow points to the Portola population of non-red males.

1.5 Discussion

In this study we investigated mating in the *Habronattus americanus* subgroup using an experimental design that simulated secondary contact between allopatric populations of *H. americanus* and *H. sansoni* CC. One primary goal was to understand the behavioral patterns that may be driving diversification patterns observed in this species complex (T. C. Bougie et al., 2021; Hedin et al., 2020; Leduc-Robert & Maddison, 2018). We found that the displays of *H.*

americanus and *H. sansoni* CC differed in some respects (ornamentation color, multimodal phase) but not in others (visual phase). For conspecific *H. americanus* crosses, successful males had longer visual phases compared to unsuccessful males and successful males had longer multimodal phases for both con- and heterospecific *H. sansoni* CC crosses as well as heterospecific *H. americanus* cross. Interestingly, our results showed that females of both species preferred *H. americanus* PLC males. To test whether red coloration drove the observed mate choice patterns, we conducted a color manipulation experiment and found that coloring *H. sansoni* CC males red significantly increased mating success while coloring *H. americanus* PLC males brown had no effect. Together these results suggest strong latent preferences for red coloration in *H. sansoni* CC and that secondary contact could lead to hybridization and introgression with a biased directionality (*H. sansoni* to *H. americanus*), consistent with the “sea of red” hypothesis (T. Bougie et al., 2024). On the other hand, our results suggest weak to no preferences for red coloration in *H. americanus* and instead, that mate choice in *H. americanus* is based on a more complex combination of visual (posture, color) and vibratory information.

1.5.1 Signal Function and Signaling Tradeoffs

In spiders, sexual signals may provide information about mate quality and species identity, attract attention, prevent aggression, and/or stimulate females to mate (Lallo & Uetz, 2024; Uhl & Elias, 2011). Visual phases may function to attract attention (Hebets & Papaj, 1/2005) contrasting with the background to potentially prevent aggression (Girard et al., 2018; Hiermes et al., 2015; Ord et al., 2011; Ramos & Peters, 2017; Yeager & Barnett, 2021). Our results suggest that the visual phase is particularly important for *H. americanus*. It is possible that red ornaments in *H. americanus* function to stand out from the brown background of the forest floor (Arenas et al., 2014; Lovell et al., 2005). While this may be the case, all mate choice trials in this experiment were conducted in the same uniform environment. One possibility is that red coloration might be an “aesthetic” preference providing no other function than being attractive (MacLaren, 2024; Prum, 2012). In heterospecific crosses, this may be an example of a hidden aesthetic preference (red coloration in *H. americanus* PLC males) (Miller, 2001) overriding existing preferences (Burley & Symanski, 1998) (longer multimodal phase in *H. sansoni* CC males, see discussion below).

Multimodal phases may also function to provide information about mate quality and species, attract attention, prevent aggression, and/or function to overload the sensory system of females (Hebets & Papaj, 1/2005; Uhl & Elias, 2011; Yan et al., 2024; Zeng et al., 2018). Our data indicate that multimodal phases are likely important for both *H. americanus* and *H. sansoni* CC. In *H. sansoni* CC conspecific trials, females prefer males that produce longer multimodal courtship. Longer courtship duration, especially multimodal courtship, has been shown to predict mating success in various groups (Briceño & Eberhard, 2000; Canale et al., 2013; Parri et al., 2002) including jumping spiders (Sivalingham et al., 2010) potentially as indicator traits due to the high metabolic cost of acoustic signals (Hebets & Papaj, 1/2005; Kuhelj et al., 2015). Interestingly, although *H. sansoni* CC males emphasize this aspect of their display and females prefer longer multimodal displays, the presence of red overrides this preference. This is further supported by our findings that red-painted *H. sansoni* CC are more likely to mate than brown-painted *H. sansoni* CC. Our results suggest a tradeoff between the duration of multimodal courtship and red coloration with *H. sansoni* CC males spending a significantly larger amount of time performing multimodal courtship. This may indicate cost based tradeoffs. While our study focuses on the role of female preferences, males may also assess cues of female receptivity and respond accordingly. Currently, we cannot distinguish the contributions of male assessment.

1.5.2 Function of red coloration

Red coloration has been shown to play an important role in sexual selection in many animal systems such as guppies, house finches, sticklebacks and lizards (Barker & Milinski, 1993; Healey et al., 2007; G. E. Hill, 1990; Kodric-Brown, 1985) as well as jumping spiders (Cross et al., 2020; L. A. Taylor & McGraw, 2013; Tedore & Johnsen, 2012). Preferences for red coloration may be driven by mechanisms such as perceptual/sensory bias (Rodd et al., 2002; Ryan & Cummings, 2013) and its links to mate quality (G. E. Hill, 1990; Kodric-Brown, 1989; L. A. Taylor et al., 2011). Preferences for red coloration could also arise from enhanced contrast (achromatic and chromatic) with the signaling environment (Arenas et al., 2014; Girard et al., 2018; Glenszczyk et al., 2021), even in animals that cannot perceive red (Girard et al., 2018; Glenszczyk et al., 2021). Red is also associated with important food items in a wide range of taxa such as fish (Rodd et al., 2002), birds (G. E. Hill, 1996; Schaefer & Schaefer, 2006; Teichmann et al., 2020) and primates (Skalníková et al., 2020) and may be a signal of food gathering ability (i.e. carotenoid based coloration). The acquisition of carotenoids and its links to, among other things, parental care (Linville et al., 1998; Rincón-Rubio et al., 2023; Segura & Mahler, 2019), body condition (Barber et al., 2000) immune competence (Alonso-Alvarez et al., 2004; Peters et al., 2004; Weaver et al., 2018) have been posited as explaining the use of red ornaments in a variety of taxa. However, the fact that *H. sansoni* CC females prefer a red trait that does not exist in the conspecific males suggests that it arose through a perceptual bias for red. Further experiments that examine females' response to red coloration outside of mating contexts (e.g. predation) are necessary to test this hypothesis.

Red sexual ornaments have been described in several species of jumping spiders. Interestingly, despite its prevalence in many groups, evidence for the use of red coloration in mate choice has been weak. Red only improves mating success under sunlight in *Habronattus pyrrithrix* (L. A. Taylor et al., 2014b; L. A. Taylor & McGraw, 2013); *Saitis barbipes* lacks red photoreceptors necessary to perceive red contained in the red ornaments of males (Glenszczyk et al., 2021); contrast seems to matter more than red coloration *per se* in *Maratus volans* (Girard et al., 2018); male *Lyssomanes viridis* with more red in their chelicerae tend to lose fights (Tedore & Johnsen, 2012). While many salticids are dichromatic (De Voe, 1975), *Habronattus* spiders are able to see red using a retinal filter (Zurek et al., 05/2015) and red coloration is common in males of many *Habronattus* species (e.g. *H. americanus*, *H. pyrrithrix*, *H. virgulatus*, *H. coecatus* etc.). The presence of red secondary sexual characters and their position on body parts that are displayed to females suggests their function in courtship displays. Interestingly, many *Habronattus* male juveniles also have a red-colored stripe below the principal eyes (=clypeus) which in most species disappears at maturity (L. A. Taylor et al., 2014a). The presence of red across *Habronattus* species suggests that the salience of red coloration has driven the evolution of ornaments (sexual or otherwise) multiple times.

Our study supports the hypothesis that a general preference for red coloration in males exists in the *Habronattus americanus* subgroup. A mutual preference for red coloration in females across the *americanus* subgroup would predict that red ornamentation would dominate in any population where the trait is present and over multiple generations of hybridization and introgression, populations lacking red would eventually be taken over by populations with red males. The current distribution of *H. americanus* and *H. sansoni* populations support this pattern with the majority of the distribution of *H. americanus* and *H. sansoni* consisting of males with red ornamentation (**Fig. 1.5**). Geographic distribution maps suggest that the few non-red

populations of *H. americanus* and *H. sansoni* group into three “islands”, one located in Utah, Colorado and southern Wyoming, one in northern Washington and neighboring British Columbia and a smaller pocket located in northeastern California. As posited by the “sea of red” hypothesis (T. Bougie et al., 2024), it is as if populations with red secondary sexual traits flood the landscape and non-red populations are the last isolated islands in this sea of red.

1.5.3 Implications for speciation dynamics

In the literature, the behavioral mechanisms driving hybridization, particularly widespread hybridization such as that observed in the *americanus* subgroup (T. C. Bougie et al., 2021; Buerkle et al., 2003), have been attributed to “errors” in mate choice driven by habitat degradation (particularly anthropogenic disturbances) and its effects on the ability to distinguish between species (Grabenstein & Taylor, 2018; Seehausen, 2006). Our results point to an alternative mechanism for widespread hybridization based on a strong preference that is outside the range of conspecific variation. Mate preferences outside the range of natural variation, as shown in the current study, may be the result of (1) permissive or (2) latent preferences. Permissive preferences refer to a “wide” fitness peak in a preference function. For example, female Hawaiian crickets are thought to select variants of male songs because females will approach male signals with a wide variety of frequencies (R. Zhang et al., 2023). While permissive preferences emphasize the range of preference functions as they relate to existing trait variation, latent preferences emphasize preference for traits that are not expressed. Latent preferences occur due to “hidden” fitness peaks in preference functions that are not explored because courters do not express those traits. Perceptual biases and neophilia fall under this framework (Basolo, 1995; Daniel et al., 2020; Gray et al., 2016; McCLINTOCK & Uetz, 1996). Perceptual biases in the context of mate choice refer to biases for certain features that evolved outside reproductive contexts (e.g. foraging, predation) or for features that more effectively stimulate an organism's sensory systems (Ryan & Cummings, 2013). Neophilia is a preference for a trait that is unfamiliar/novel. Compared to perceptual biases that emphasize the trait and the perceptual system of the chooser, neophilia emphasizes escape from habituation (Daniel et al., 2020). Given the major differences between coloration patterns, we suggest that *H. sansoni* CC preferences for red represent a latent preference. With our design, we cannot disentangle whether the preference for red in *H. sansoni* females is driven by perceptual bias or neophilia. Further experiments with additional color manipulations can provide more insights. Based on the widespread presence of red coloration in males in *Habronattus*, including in non-sexual traits (L. A. Taylor et al., 2014a), it is more likely that perceptual biases drive the pattern observed.

One surprising result from our study is that painting *H. americanus* brown did not affect the likelihood of mating. These results suggest the intriguing possibility that once expressed and spread in a population/species, strong preferences for red can become relaxed. The same pattern was also found in Poeciliid fishes (Basolo, 1998). In this example, preference for swordtails are found in female platyfish although male platyfish are swordless (Basolo, 1990). Preference for swords was found to be weaker in swordtails compared with swordless platyfish (Basolo, 1990). Reduced selection makes it possible for choosers to select mates on a combination of traits as opposed to one strongly preferred trait. Intriguingly, looking across different *H. americanus* populations, the variation in the use of red ornaments is much greater than the color variation seen in any of the other *americanus* subgroup members. In two populations of *H. americanus*, red ornamentation appears to be nearly absent (T. C. Bougie, 2022). The disappearance of strong preferences for red may be the result of antagonistic coevolution similar to arguments posited for explaining the xenophilic/neophilic preferences in the *H. pugillis* group (D. O. Elias, Hebets, &

Hoy, 2006; Hebets & Maddison, 2005). In those studies, females of some populations (Santa Rita Mountains) preferred males that used vibratory signals (Atascosa Mountains) not expressed in their own local populations of males (D. O. Elias, Hebets, & Hoy, 2006). In this scenario, males evolve novel exploitative signals, females subsequently evolve resistance to their own males, but not to novel traits expressed in other populations that the females have not been exposed to but which share perceptual biases. Similar to this study, manipulating signals produced by local males did not affect mating success while manipulating the signals by the non-local populations eliminated the preference for non-local males (D. O. Elias, Hebets, & Hoy, 2006). Another possibility is predation pressures are higher on red individuals leading to a weakened preference for red assuming some genetic linkage between the red trait and preference for red. This hypothesis was posited as explaining reduced preferences for swords in swordtail fish (Johnson, 2003). Alternatively, the red coloration of *H. americanus* is indeed important for female choice but our choice of red color masked the natural coloration that females strongly preferred. It is important to note that the females used in the conspecific color manipulation experiment were older than those used in the heterospecific mate choice experiment. It is possible that some of our results may reflect shifts in female preferences as they age, for example our observed differences in mating rates observed between the two experiments. Given these potential differences, we caution against directly comparing results from the heterospecific mating trials (Experiment I) with the conspecific color manipulation trials (Experiment II). Future work in the *americanus* subgroup that manipulates females' ability to assess coloration and explores age related changes to mate choice are necessary to explore these possibilities.

Nevertheless, our results suggest that strong preferences for novel traits can override existing preferences for traits expressed by conspecifics and will likely have strong impacts on the evolutionary trajectory of the populations in question. Two previous studies point to two possible evolutionary trajectories. First, Bougie et. al (T. Bougie et al., 2024) documented an existing hybrid zone between *H. americanus* and *H. kubai*, which may point to what may happen in a timeline where two populations, one with a strong, pre-existing preference for a trait exhibited by the other come into secondary contact. In this study, Bougie et. al documented a genetically homogeneous "hybrid swarm" with more hybrid than parental phenotypes but where red phenotypes of one population (*H. americanus*) occurred at much higher frequency than the phenotype of the other (*H. kubai*). Furthermore, this study suggested that non-red phenotypes were localized to microhabitats (beneath shrubs) that were shadier (T. Bougie et al., 2024), consistent with findings that the processing of red is only possible in bright light environments (L. A. Taylor & McGraw, 2013). This study exemplifies a scenario where a strong preference from one population drives widespread directional introgression, potentially resulting in a period of lineage "fusion" and the potential extinction of *H. kubai* (extinction by hybridization (Rhymer & Simberloff, 1996)).

Our manipulative experiment however points to a scenario where this strong preference is ephemeral as painting *H. americanus* brown had no effect on mating. In other words, the presence of red coloration in males weakens the preference for red thus making the strong preference for red transient. This relaxed selection for red males might give opportunities for other color morphs to evolve. The evolutionary trajectory emerging from this may be exemplified by a study by Blackburn and Maddison (Blackburn & Maddison, 2014) who documented widespread gene flow between different morphs of *H. americanus*. Blackburn and Maddison (2014) documented phenotypically divergent populations of *H. americanus* (three

morphs) in a small area with high genetic similarity but with evidence for isolation by distance likely caused by drift. This study would exemplify a scenario where a strong preference for a simple trait has been weakened, potentially through the evolution of female habituation (Brown, 1979; Daniel et al., 2019; Dong & Clayton, 2009) or counterbalancing natural selection pressures leading to the evolution of a variety of secondary traits as seen across *H. americanus* males (Blackburn & Maddison, 2014; T. C. Bougie et al., 2021). Female habituation and preference for novel traits have been shown to drive genetic diversity in guppies (Daniel et al., 2020), and the same mechanism might have given rise to the ornament diversity in the *americanus* subgroup. For these females, preferences would be based on the evaluation of multiple signals as suggested by our conspecific *H. americanus* mating data. This may signal a “fission” epoch in speciation dynamics (Meier et al., 2023). Importantly for this study, unlike adaptive radiations such as cichlid fish in the African Rift Lakes, Darwin’s Finches in the Galapagos, and Hawaiian Silverswords, diversification in most *Habronattus* species groups is likely not driven by ecological processes (but see (Hedin & Lowder, 2009)). The *Habronattus* system may illuminate diversification dynamics driven primarily by sexual selection, as suggested by previous authors (Masta & Maddison, 2002). It is important to note that while there is no direct evidence of a current hybrid zone between *H. americanus* and *H. sansoni*, genomic evidence suggests that they have interbred in the past given the limited genomic differentiation between all members of the *americanus* subgroup (T. C. Bougie et al., 2021; Leduc-Robert & Maddison, 2018). Additionally, the existence of several hybrid zones for other *americanus* subgroup members (T. Bougie et al., 2024) suggests that hybridization in the subgroup produces viable and fertile offspring. Future work is needed to verify this hypothesis.

1.6 Conclusion

The present study suggests that perceptual biases could be a mechanism that leads to hybridization among closely related species. Furthermore, our study suggests that some preferences may be so strong as to override existing mate choice patterns and in extreme situations drive lineage fusion. This phenomenon could be ephemeral however, and provide opportunities for novel traits (and potentially species) to evolve through adaptive introgression (Pardo-Diaz et al., 2012; Parsons et al., 1993; Schilthuizen et al., 2004; Staubach et al., 2012; Whitney et al., 2006) and the enhanced evolvability of hybrids (Grant & Grant, 2019). Similar to the spectacular diversifications of African Cichlids and Hawaiian Silverswords which had its roots in ancient hybridization events (Barrier et al., 1999; J. Y. Choi et al., 2021; Meier et al., 2017, 2023; Rhymer & Simberloff, 1996), extensive cyclical hybridization in *Habronattus* (Leduc-Robert & Maddison, 2018) may be an important contributor to rapid diversification and the spectacular diversity of displays found across the genus (T. Bougie et al., 2024; D. O. Elias et al., 2005, 2012; Hebets & Maddison, 2005; Rivera et al., 2021; Sun et al., 2021; L. A. Taylor et al., 2011).

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Chapter 2

Heterospecific mate choice reveals hidden and variable female preferences in jumping spiders

Lin Yan and Damian O. Elias

2.1 Abstract

Sexual selection is expected to act as a barrier to heterospecific mating by reinforcing species-specific mate preferences. Mate preferences however can in some cases facilitate heterospecific mating and hybrid speciation. The jumping spider genus *Habronattus* is speciose and has diversified under sexual selection with evidence for introgression in several lineages. We simulated secondary contact of two allopatric but closely related species with complex male courtship displays: *Habronattus formosus* and *H. gilaensis*, to understand heterospecific interactions. Males of both species exhibit courtship behaviors that differ in some respects but not others. We found that females of both species mated indiscriminately with conspecific and heterospecific males. Female preference toward courtship duration alone did not differ between species pairs, however, more complex models revealed that female preferences were more variable between individuals when interacting with heterospecific than with conspecific males. We also observed a increased latency to copulation in heterospecific pairings. These results suggest that, despite apparently indiscriminate mating behavior, females make quicker mating decisions when interacting with conspecifics, likely due to stable preferences for traits expressed by conspecifics. Our findings highlight the complex role of female preferences in shaping mating outcomes and maintaining species boundaries and suggest a behavioral mechanism for diversification even with “leaky” species boundaries.

2.2 Introduction

The biological species concept implies strict reproductive isolation. Sexual selection, especially mate choice, is often expected to act as a mechanism for reproductive isolation by reinforcing species-specific mate preferences and acting against heterospecific mating (Kyogoku & Wheatcroft, 2020) due to reduced hybrid offspring fitness (Burke & Arnold, 2001). Pre-copulatory mate choice preferences can effectively prevent heterospecific mating and promote assortative mating, while post-copulatory mechanisms, including hybrid incompatibility and cryptic female choice, further reduce hybridization. However, in nature, heterospecific interactions and hybridization are common, particularly among recently diverged species where reproductive incompatibility remains incomplete (Abbott et al., 2013). Similarly, sexual selection does not always reinforce isolation; in certain ecological and biological contexts, sexual selection may instead facilitate heterospecific mating and create opportunities for hybridization, introgression, or even collapse of species boundaries (Kagawa, 2026; Rosenfield & Kodric-Brown, 2003). In fact, recombination of genetic material from heterospecific sources creates novelty more quickly than mutation (Abbott et al., 2013), thus providing variation for selection to act on, especially when given ecological opportunities (Grant & Grant, 1994). Examples of

heterospecific interactions and sexual selection mechanisms contributing to speciation can be found across animal systems including plants, insects, fish, birds and more (reviewed in (Mendelson & Safran, 2021; Servedio & Boughman, 2017). Understanding how sexual selection and mate choice functions as a barrier or a bridge between species is thus a major goal in evolutionary biology.

Pre-copulatory sexual interactions between heterospecifics are especially critical in recently diverged systems, where insufficient time has passed for genetic incompatibilities to accumulate. Although assortative mating and sexual character displacement often serve to maintain or strengthen species boundaries, the effectiveness of these processes varies across systems, depending on a variety of factors including the signal modalities used and courtship signal complexity. In some cases, when traits used in mate choice (i.e. signals, preferences) overlap between con- and heterospecifics, hybridization may occur. For example, traits and preferences shaped by a common sensory bias (Rodd et al., 2002) (a bias from the sensory system evolved from contexts other than sexual selection) may promote heterospecific mating. In some cases, traits and preferences shaped by sensory bias may outcompete conspecific signals if the heterospecific individual exhibits an “overexpressed” trait (Shaw, 1995). A classic example occurs in the fish genus *Xiphophorus*, where females of swordless species prefer males bearing a sword (Basolo, 1990). In other cases, if a costly trait for a conspecific can be exploited or expressed with a lower cost in heterospecifics (Jones & Hunter, 1998; Pfennig, 2000), hybridization may be favored. Spadefoot toad females may mate with a lower-quality conspecific male to avoid heterospecific mating with males whose signals resemble those of high-quality conspecifics (Pfennig, 2000).

Heterospecific encounters frequently occur during secondary contact, providing ways to examine how mate choice operates when individuals encounter novel sexual signals outside the context of conspecific interactions. Novel traits provide a unique opportunity to evaluate preference dynamics and their potential impacts on species boundaries. A study in electric fish showed that a novel communication channel can fuel rapid diversification that outpaces ecological divergence (Arnegard et al., 2010). The outcome of exposure to novel mating traits also depends on the landscape of existing preference and function of each signal, especially when signals are complex and/or multimodal (Hebets & Papaj, 1/2005). For species pairs whose courtship displays and preferences have been stabilized through generations of selection, encountering heterospecific displays that differ in some respects but overlap in others may reveal hidden preference structures that are not evident during conspecific interactions (Yan et al., 2025). Mate preferences may function similarly when encountering novel traits or may expose latent biases or variation that has not been expressed in species-specific contexts (Gray et al., 2016). If hidden preferences are common, heterospecific interactions may generate greater variability in mating outcomes, potentially increasing opportunities for hybridization and influencing diversification processes. Alternatively, exposure to novel traits might reveal variability and plasticity of existing preferences (Welsh et al., 2026; R. Zhang et al., 2023) that was not revealed due to factors such as limited variation in conspecific male signals. Exploring how exposure to novel traits shapes mating decisions is therefore essential for understanding behavioral mechanisms that contribute to speciation dynamics, and to predict species dynamics following secondary contact.

Jumping spiders in the genus *Habronattus* provide an ideal system for exploring these questions. Species within this group exhibit a spectrum of courtship complexity, from unimodal displays with single components to multimodal displays with several coordinated signal components involving visual ornamentation, dances, and vibratory signaling. This speciose group contains more than 100 species, yet has diversified relatively recently, driven by sexual selection (Masta & Maddison, 2002). Molecular and field-based evidence suggests that its diversification is accompanied by ancient and ongoing hybridization and introgression events in species groups across the genus (T. Bougie et al., 2024; Leduc-Robert & Maddison, 2018). Heterospecific mating patterns in *Habronattus* have been explored in two groups, the *pugillis* and *americanus* group. In the *pugillis* species complex, heterospecific mating rates were explored between females of one population to males from two populations with divergent courtship displays (D. O. Elias, Hebets, Hoy, et al., 2006; Hebets & Maddison, 2005). Experiments showed that females from one population preferred males from another population over their own (Hebets & Maddison, 2005). These mating patterns are likely driven by more complex vibratory signals expressed by the preferred species (D. O. Elias, Hebets, Hoy, et al., 2006). In reciprocal heterospecific mate choice experiments in the *americanus* subgroup between one species with red coloration and a short vibratory display (*H. americanus*) and another with brown/black coloration and long vibratory displays (*H. sansoni* CC), females of both species preferred *H. americanus* (Yan et al., 2025). Signal manipulation experiments demonstrated an overriding effect of female preference for red coloration expressed by heterospecifics over vibratory displays expressed by conspecifics in *H. sansoni* CC (Yan et al., 2025).

In this study, we simulate secondary contact between two closely related but allopatric populations of two species in the *Habronattus clypeatus* species group that have relatively complex displays compared to the other *Habronattus* species groups where heterospecific interactions have been previously explored. *Habronattus formosus* and *H. gilaensis* males both perform long courtship displays with various coordinated visual and vibratory signals (Rivera et al., 2021) (**Fig. 2.1**). Their courtship displays are comparable in some ways and distinct in others (see below). By quantifying mating outcomes, latency to copulation, and preferences, we evaluate whether complex courtship reinforces species boundaries or facilitates hybridization. We predict that, given the complex landscape of male courtship traits and the sharing of some components between species, females will prefer conspecific males given that the male courtship is complex. This contrasts with patterns observed in species groups with simpler courtship (*H. americanus* group), where female preference functions may be more easily biased by novel traits (Yan et al., 2025). Examining heterospecific mating patterns across *Habronattus* will illuminate the role that signal complexity and preferences play in diversification dynamics.

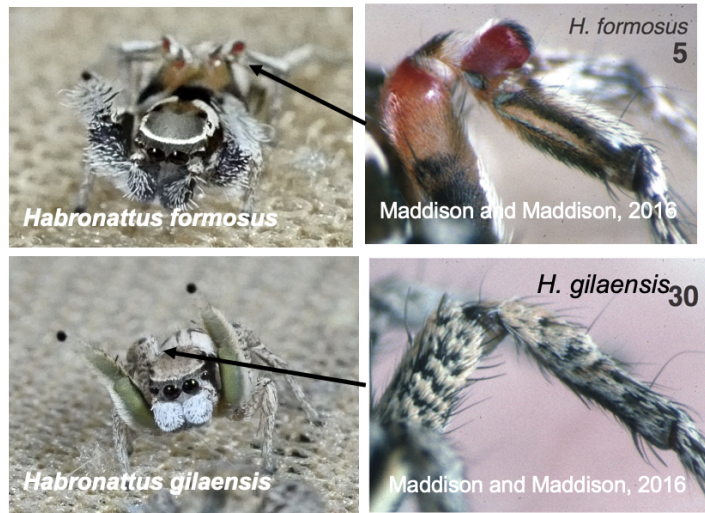


Figure 2.1. Focal species of the study during courtship display and their 3rd leg ornamentations.

2.3 Methods

2.3.1 Animal collection and care

All female spiders were field collected as penultimate to ensure no prior mating experience. All male spiders were collected from the field as adults or penultimate and reared in the lab until sexual maturity. *Habronattus formosus* were collected near Victorville, California, and *Habronattus gilaensis* were collected near Silver City, New Mexico in March 2022. All spiders are individually housed in 3x3x5 cm plastic containers (AMAC Plastic Products) with crinkled paper at 25 degrees Celsius with a 12:12 hour light cycle. Each spider was provided weekly with a drop of water and 4-6 fruit flies. Sexual maturity was visually checked twice a week. Maturity of males is determined by coloration and maturity of females is determined by visible epigynum.

2.3.2 Courtship trial setup

All mating trials are conducted between early May and early July of 2022. Some males were collected as adults ($N = 108$) thus their ages at the time of testing are unknown; males collected as penultimate instars were 32.29 ± 8.84 (mean $\pm$ sd) days old at testing. Females were all collected as immatures and were 33.57 ± 10.29 (mean $\pm$ sd) days post-maturity during trials. All trials were run at a temperature of 35.91 ± 1.18 (mean $\pm$ sd) degrees Celsius. We used a 2x2 factorial design for heterospecific and conspecific preference trials followed procedures described in (Yan et al., 2025).

2.3.3 Male courtship in the study species

Habronattus formosus and *H. gilaensis* are both in the *clypeus* species group. Thus their courtship displays contain similarities as previously described in the general courtship of the group (Rivera et al., 2021). The introductory phase of their courtship involves extended forelegs and rotation of the pedipalps, approaching the female in a zigzag motion. No vibratory signal is detected in this phase of courtship. The subsequent phase of courtship involves vibratory signals coordinated with visual displays and are different in the two species. *Habronattus formosus* males have a modified third leg patella and tibia with triangular protrusions and red coloration

(**Fig. 2.1**). Males lift their bodies and raise the ornamented third leg tibia in between shorter vibratory displays. *Habronattus gilaensis* males, however, do not have any modification on the third leg tibia (**Fig. 2.1**), and instead of lifting their body and third leg, *H. gilaensis* males have longer and more intense vibratory signals.

2.3.4 Statistical analyses

All statistical analysis were conducted in R (version 4.1.3). Mating rates (percentage of mated individuals) between conspecific and heterospecific pairs were compared using Pearson's Chi-squared tests. To examine whether courtship duration (introductory and multimodal phases) differed between males that copulated versus those that did not, and whether this effect varied across pair types, we fitted linear models including copulation outcome (yes/no), pair type, and their interaction as fixed effects. Model selection was performed using Akaike's Information Criterion (AIC), and the model with the lowest AIC was retained.

To evaluate the effect of overall male courtship performance on mating outcome, we analyzed conspecific and heterospecific interactions separately. Within each group, we performed principal component analysis (PCA) on standardized (centered and scaled) courtship variables to reduce dimensionality and minimize collinearity among predictors (**Fig. S2.1** and **S2.2**). We then trained random forest classifiers (Liaw & Wiener, n.d.) using principal components (PCs) as predictors and copulation outcome (yes/no) as the response variable. For each group, we generated 1,000 resampled datasets, with 70% of observations used for training and 30% for testing. For each model, we recorded the out-of-bag (OOB) error rate (error rate of a model on new data not used in the training process) (Liaw & Wiener, n.d.) and identified the top three most important PCs based on permutation importance. We compared OOB error rates between models trained on conspecific versus heterospecific interactions to assess differences in predictive performance. Consistency in the top contributing PCs across iterations was used to evaluate variability in predictors of mating success. Copulation latency for each pair that mated was compared using a linear model containing pairs as categorical variable, followed by a posthoc test using estimated marginal means (emmeans) with Tukey correction.

2.4 Results

2.4.1 No preference for con- or heterospecific males

We found no statistically significant differences in mating rates between heterospecific versus conspecific pairs (**Fig. 2.1**) (**Table S2.1**)

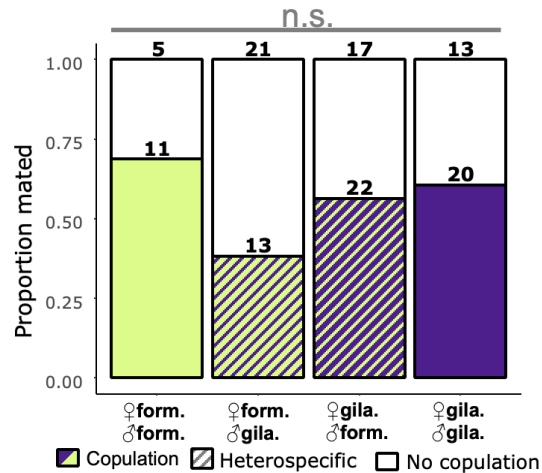


Figure 2.2. Similar mating rates in heterospecific and conspecific pairs. Sample sizes are labeled above each block. The green background represents pairs with *H. formosus* female, and the purple background represents pairs with *H. gilaensis* females. Solid colored bar represents conspecific mated pairs. Shaded bar columns represent heterospecific pairs.

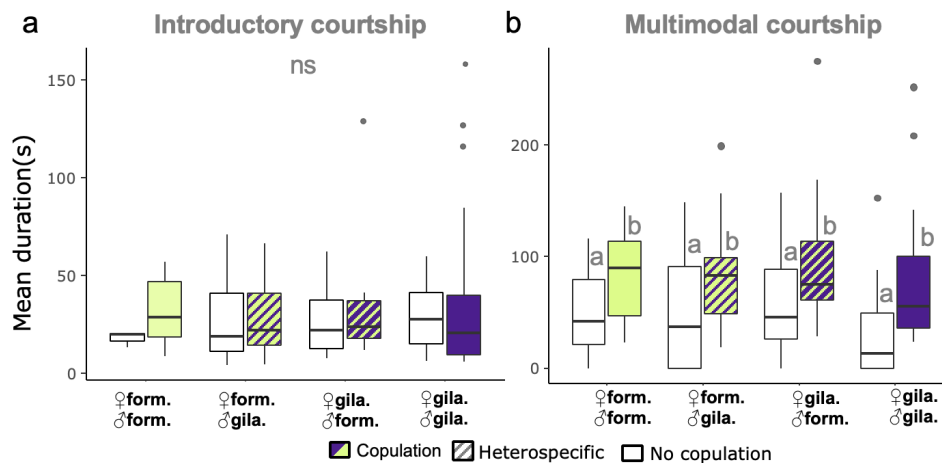


Figure 2.3: Courtship duration does not differ in predicting male courtship success between pairs. **a:** mean duration of introductory courtship between mated and unmated males in each pair. **b:** mean duration of multimodal courtship between mated and unmated males in each pair. Green background represents pairs with *H. formosus* female, and purple background represents pairs with *H. gilaensis* females. Colored bar/box represents mated pairs. Shaded box columns represent heterospecific pairs.

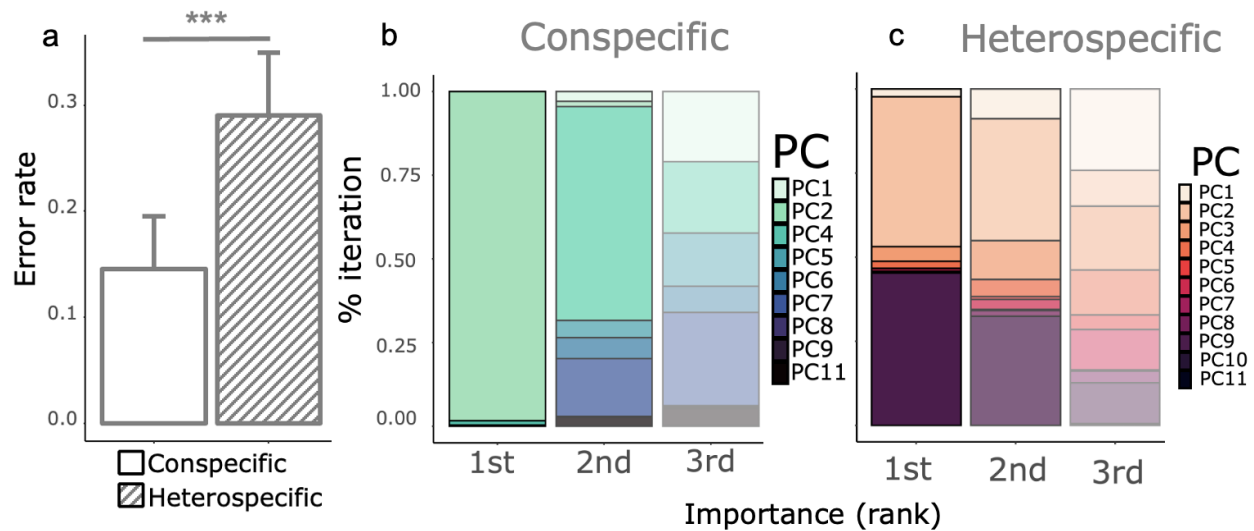


Figure 2.4. Models trained on data from conspecific interactions perform better than those trained on data from heterospecific interactions. **a:** bar plot of OOB error rates across 1,000 iterations of random forest model trained on different subsamples. Error bars represent standard deviation. **b** and **c:** ranks of top three most important principal components (PC) in each iteration of model trained on conspecific datasets (**b**) and heterospecific datasets (**c**). Sizes of bars of different color represent the frequency of a given PC receiving the rank labeled on the x-axis.

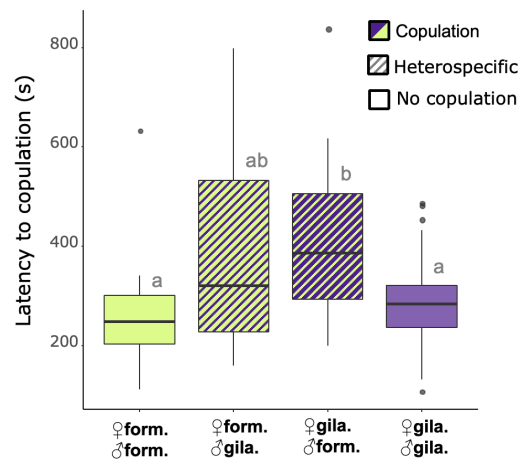


Figure 2.5. Longer latency to copulation in heterospecific pairs. Letters indicate statistical significance. For the color and pattern representation, see the legend in **Figure 1**.

2.4.2 Similar preference evaluated by raw courtship durations between con- and heterospecific pairs

We then asked whether duration of either or both phases of courtship predict mating success, and whether this pattern is consistent in heterospecific and conspecific pairs. For introductory courtship, all candidate models have a higher AICc than the null model (**Table S2.2**), suggesting neither pair type nor the mean duration of introductory courtship explained copulatory success (**Fig. 2.3a**). For multimodal courtship, the best model only includes copulation but not pair or the interaction between pair and courtship duration (**Table S2.3**). Posthoc test shows that the duration of multimodal courtship is significantly longer ($df = 104$, $t.ratio = -3.397$, $p.value =$

0.0010) for males that successfully copulated with the female (emmeans = 85.7 seconds) compared to the unsuccessful males (emmeans = 49.9 seconds); this pattern is consistent across all pairs (**Fig. 2.3b**). This demonstrates that duration of the multimodal phase but not the introductory phase of male courtship is an important predictor for copulation success. This pattern is consistent across all pairs, regardless of species or con- and heterospecific interactions.

2.4.3 More variation in preference in hetero- than conspecific pairs

Our study reveals a consistent pattern in both conspecific and heterospecific interactions in terms of duration of courtship phases and copulatory success. To examine how other courtship variables contribute to success (**Fig. S2.1**), we approximated success of conspecific and heterospecific pairs using a random forest model, including multiple measures of courtship phases beyond mean duration. We found that in 1,000 iterations of random forest models trained on resampled datasets, the OOB error rate when tested by unseen data is significantly higher in models trained with heterospecific pairs (mean $\pm$ sd = 0.290 $\pm$ 0.059) than models trained with conspecific pairs (mean $\pm$ sd = 0.145 $\pm$ 0.049) (**Fig. 2.4a**). This suggests that preferences in heterospecific pairs are less predictable than conspecific pairs when modeled using a supervised random forest model. Further, for each iteration of the RF model, we identified the top three most important variables in model accuracy using permutation. In line with previous findings, the top three variables are more consistent across all iterations of models that are trained on conspecific datasets (**Fig. 2.4 b-c**), compared with models trained on heterospecific datasets.

2.4.4 Delayed copulation in heterospecific pairs

Following the less predictable choice pattern in heterospecific pairs, we also observed a difference between pairs in latency to copulation. The conspecific pairs had similar latency to copulation, however, the heterospecific pair of *H. gilaensis* female and *H. formosus* male have a significant delay in copulation compared with conspecific pairs of both species. The heterospecific pair of *H. formosus* female and *H. gilaensis* male did not show a statistically significant difference in latency to either conspecific pairs or the opposite heterospecific pair (**Fig. 2.5**).

2.5 Discussion

We found that *Habronattus formosus* and *H. gilaensis* females mated indiscriminately between species even though males show complex, divergent, species-specific courtship. In examining the male courtship traits that predict mating success in con- and heterospecific matings, we found no differences between the mean courtship time in successful versus unsuccessful males in the early stages of their displays. We did find, however, that at later stages, successful males courted more than unsuccessful males, although no differences were observed between successful con- and heterospecific pairings. Exploring courtship traits further, our modeling analyses revealed that in conspecific pairings, the same traits tended to predict mating success in different iterations of our mating models, while in heterospecific pairings, different traits predicted mating success across iterations of our mating models. These results suggest that in situations mimicking secondary contact, while females may mate indiscriminately across species, females base their mating decisions on a more predictable set of male traits in conspecific interactions, while females base their mating decisions on a variety of mating traits in heterospecific interactions.

Finally, our results examining latency to copulation revealed that conspecific matings happened significantly faster than heterospecific matings.

Across *Habronattus*, widespread evidence exists regarding the presence of both ancient (Leduc-Robert & Maddison, 2018) and current genetic (T. Bougie et al., 2024; T. C. Bougie et al., 2021) introgression driven by cross-species matings. Our experiments were designed to mimic secondary contact between closely related species with divergent courtship phenotypes to understand the behavioral and evolutionary dynamics that may emerge when two closely related species contact each other. Our results show that while females are choosy (only 40-70% of mating interactions resulted in successful copulations), there were no significant differences between the mating rates of con- versus heterospecific crosses. The fact that heterospecific pairs exhibit similar mating rates as conspecific pairs suggests that heterospecific mating will likely occur upon secondary contact. This pattern is consistent with phylogenetic evidence indicating a history of introgression across the genus *Habronattus* including *H. gilaensis* and other closely related species (Leduc-Robert & Maddison, 2018). If hybridization occurs upon secondary contact in *Habronattus*, its future effects on speciation dynamics will depend on the fitness of hybrids. If hybrids are unfit, species boundaries are predicted to be reinforced, despite heterospecific mating (M. L. Arnold & Hodges, 1995; Mikkelsen & Irwin, 2021). If hybrids are viable, it would lead to consistent gene-flow between populations (Dufresnes et al., 2021) and the potential formation of a hybrid swarm (Seehausen et al., 2008). Previous work in the *H. americanus* subgroup, where cross-species mating is biased in one direction, suggests that heterospecific mating may lead extinction via hybridization. (T. C. Bougie et al., 2021; Yan et al., 2025).

Habronattus formosus and *H. gilaensis* courtship displays have some shared traits (i.e. intro motifs, thump-scrrape motifs), do not share others (presence of third leg ornaments, coloration patterns), and differ in the overall prevalence of others (buzz motifs) (**Fig. 2.1**). In order to simplify our mate choice analyses, we divided the displays into two categories: introductory courtship that are broadly similar between species (although the ornamentation between the two species differ) and multimodal courtship which generally differ between species (Rivera et al., 2021). For introductory courtship, mean duration did not differ between successful and unsuccessful males. Introductory displays are similar across closely related *Habronattus* species and likely act as an efficacy-based attentional signal, priming the female before the transition to multimodal courtship (Hebets & Papaj, 1/2005). For multimodal courtship, mean duration differed between successful and unsuccessful males, suggesting that those traits influenced mating decisions. Multimodal courtship in *H. formosus* and *H. gilaensis* involves coordinated sequences of visual and vibratory components (Rivera et al., 2021), and likely evolved to advertise male quality (Blackburn & Maddison, 2015).

To explore courtship traits more fully, we approximated female preferences using random forest models. Random forest analyses use a supervised machine learning algorithm to simulate decision trees (i.e. female preferences in our case) allowing us to explore both the variables that are important in our preference models as well as the accuracy in the models by observing the error rate between different iterations of the model. In our case, if the error rate is low, it would suggest that the courtship variables identified by our preference models are consistent, suggesting that the females in our data set used specific traits in mating decisions. On the other hand, if the error rate is high, it would suggest that the courtship variables identified by our preference models inconsistently predict mating success, suggesting variation in the traits females use to make their mating decisions.

We observed that conspecific mating interactions had low error rates, suggesting that choosers had preferences for specific courter traits and thus that there was coevolution of courter traits and chooser preferences in conspecific *H. gilaensis* and *H. formosus* interactions. Preferences for traits that courtiers are exposed to are under continuous selection and are thus likely to co-evolve. The coevolution of specific courtship traits and chooser preferences is generally predicted to occur over time and lead to choosers assessing specific courtship traits that accurately reflect heritable viability (Candolin, 2003). This has been generally supported by genetic and behavioral evidence across a variety of systems and taxa (Brennan & Prum, 2015; Wiley & Poston, 1996).

Preferences for traits that courtiers have never been exposed to, on the other hand, would not be under selection, thus it is difficult to predict what will happen when choosers are exposed to novel courtiers (Broder et al., 2021). In such scenarios, the ability to physiologically detect novel signals is critical. This is likely to happen in the case of secondary contact between closely related species, since there are commonalities between courtship repertoires and sensory systems used to assess signals. Second, how choosers respond to novel courtiers is important. Our results suggest that in the *clypeatus* species group, females respond to novel courtiers in a range of different ways and do respond to novel traits, likely due to latent preferences. The high error rate from models trained on heterospecific interactions suggests large-scale variation in the traits that choosers are using to select mates with because variation in chooser preferences are exposed when choosers are presented with novel signals, as would be the case in the initial stages of secondary contact. One possibility that emerges from this exposure of variation of female preferences in zones of secondary contact could be the evolution of novel traits.

Finally, our results show that the latency to copulation for conspecific crosses is significantly shorter than for heterospecific crosses. Copulation latency is typically shorter for higher quality males (Sanghvi et al., 2025), and therefore, the longer latency observed in heterospecific pairs may still reflect female preference for conspecific males, even if this preference is not evident in overall mating rates. Extended assessment during courtship may also impose costs (reviewed in (Scharf et al., 2013)): for females, increased predation risk and lost opportunities to encounter higher-quality mates (Maklakov & Arnqvist, 2009; Pomiankowski, 1987); and for males, increased energetic investment in prolonged courtship (Martin & Hosken, 2004). This suggests that, overall, interactions with longer latencies to copulations would not be favored. Additionally, preliminary data suggest that *clypeatus* group females only mate once. Latency to copulation in the context of this study is thus likely critical to mating dynamics in the field. In the absence of density or demographic differences, conspecific matings are likely to dominate as they happen more quickly and successfully mated females will not mate again.

Overall, our results suggest a scenario of stable, yet leaky species boundaries when species experience secondary contact. This would be consistent with genomic data that reveals a long history of genetic introgression in the *clypeatus* group (Leduc-Robert & Maddison, 2018) without evidence of complete species mixing as has been found in other *Habronattus* species (T. C. Bougie et al., 2021). However, when heterospecific mating does happen, hybrid offspring may perform intermediate or transgressive courtship (Kagawa & Takimoto, 2018), providing a genetic basis for novel courtship traits potentially leading to speciation, given the right ecological opportunities.

In a recent modeling study, Kagawa (2026) showed that hybridization between genetically distinct populations (like in the *clypeatus* group) would give rise to variation in mate preferences and sexual displays, altering sexual selection landscapes thus creating opportunities

for speciation as previously unoccupied preferences and trait combinations are explored. This and other studies in *Habronattus* suggest that in a given population, latent female preferences are common and when females are exposed to novel variation (i.e. heterospecific courtship), variation in their preferences are exposed often leading to hybridization. Heterospecific mating gives rise to more variation in sexual displays and preferences, thus creating opportunities for rapid speciation (Kagawa, 2026). We suggest that this mechanism is widespread in *Habronattus* and is responsible in part for the rapid diversification seen here. We also suggest that the long history of introgression across *Habronattus* could underlie the presence of latent female preferences.

Our study demonstrates the complexities inherent in mate choice and how these complexities may reveal themselves during secondary contact. In a classic view of sexual selection, co-evolved signals and mate preferences work in concert to establish assortative mating and reinforce species boundaries. Assortative female preferences at the population level, however, may not preclude hybridization if individual females mate with males with novel traits. A novel yet effectively “neutral” trait (at the population level) may create leaky species boundaries that create opportunities for novel traits (or trait combinations) to evolve (Kagawa & Takimoto, 2018). While ecological opportunities, demographic and population density as well as the relative strength of latent preferences will ultimately determine whether species go “extinct” through genetic homogenization (e.g. hybrid swarm; (T. Bougie et al., 2024; Yan et al., 2025) or whether genetically distinct species persist with gene flow, as demonstrated in the present study, the ultimate outcome is increased genetic variation. The resulting variation in courtship displays and preferences may in turn act as a positive feedback loop for behavioral and genetic variation that leads to rapid diversification (R. F. Schneider & Meyer, 2017). We suggest that a wider exploration of preference landscapes (including latent preferences) may be fruitful for understanding species diversification, particularly in rapidly diversifying species or species phylogenetic evidence for genetic introgression.

2.6 Acknowledgments

We would like to thank Trinity Walls and Shiyang Wu for assistance during specimen collection, Shiyang Wu and Jaewon Lee for assistance in analysis of courtship behavior, and all members of the Elias lab for assistance with animal care. We are grateful to Marshal Hedin and Noah Leith in helpful discussions regarding the framing of the work and insights on interpretation of the results.

Chapter 3

Timing matters: age-dependent female receptivity and preference patterns

Lin Yan and Damian O. Elias

3.1 Abstract

The timing of reproductive processes can influence how mating interactions shape reproductive strategies. We examined how female age correlates with receptivity and preference in the jumping spider *Habronattus formosus*, a system characterized by elaborate male courtship and strong female choice. To test how receptivity changes across the post-maturation period, we paired females of different ages with males and quantified male courtship displays and mating outcomes. Females were not receptive immediately after maturation and instead exhibited receptivity primarily 2–3 weeks after maturation. We further found that the full male courtship predicted mating success in older females, whereas only the second stage of the courtship predicted success in younger females. The delay in female receptivity is consistent with expectations for strong female choice systems, where females may have a greater opportunity to evaluate male courtship before mating. These results highlight the importance of age-dependent changes in female reproductive state and suggest that the timing of receptivity may shape how different components of male courtship influence mating success.

3.2 Introduction

Sexual selection is a fundamental evolutionary process that shapes a significant portion of biodiversity (Andersson, 1994). Intra- (mate competition) and inter-sexual selection (mate choice) are the two primary mechanisms through which sexual selection typically operates and is thought to be dependent on (and in turn shape) an organism's physiology, behavior, ecology, and evolution (S. J. Arnold, 1983; Hunt et al., 2009). One important yet often understudied aspect of sexual selection is the timing of reproductive behaviors like receptivity which is hypothesized to, among other things, influence the intensity and efficacy of intra- and intersexual selection. For example, when receptivity is predictable and/or synchronized the strength and effectiveness of intrasexual competition is increased as animals are able to increase their fitness by restricting access to mates (Grafen & Ridley, 1983; Vellnow et al., 2020). This often leads to evolution of traits and behaviors associated with competition such as weapons and mate guarding (Daupagne et al., 2023; Hau et al., 2017). Alternatively, when receptivity is unpredictable and/or asynchronous the benefits of restricting access to mates is reduced often increasing the strength of intersexual selection thus leading to the evolution of traits used in mate assessment such as courtship signals.

A significant complication may also exist if the expression of reproductive traits and behaviors are not static and instead shift through time, likely influencing sexual selection dynamics (Carleial et al., 2024). Across animals, there is extensive evidence of age-related effects on reproductive behavior (Cotton et al., 2006; Kelly, 2018; Richard et al., 2005; Šmejkal et al., 2021). In reindeer, older males' reproductive effort is higher for older dominant males,

likely because they reduce their effort in periods where females are not in oestrus, while young males maintain their effort across the breeding season even when females are not in oestrus (Tennenhouse et al., 2012). Age has been shown to impact choosiness and preference (Ah-King & Gowaty, 2016; Anjos-Duarte et al., 2011; Cotton et al., 2006; Dougherty, 2023; Ligout et al., 2012; Sarmiento-Ponce et al., 2021; J. M. Schneider et al., 2016; Tanner et al., 2019; Umbers et al., 2015; Wilgers & Hebets, 2012), the two major components of mate choice (G. G. Rosenthal, 2017). Sand goby females for example are increasingly choosy as the breeding season progresses (Forsgren, 1997). Age has also been seen to have the opposite effect on choosiness. For example, ovoviviparous cockroach females exhibit reduced choosiness and require shorter courtship durations as they age (Moore & Moore, 2001). Younger female crickets in habitats with high male density exhibit the most choosiness (Atwell & Wagner, 2014). Second, age has been shown to have effects on mate preferences. Coral damselfish maintained the same level of choosiness but reversed their preference functions when they got older (Wacker et al., 2016). Cichlid females shifted their preferences based on proximity to spawning (i.e. fertilization) and the presence of male hormones in the water from smaller to larger males (Kidd et al., 2013). Similarly, in crickets, younger females expressed strong preferences for attractive calls while older females did not (Gray, 1999). In another species of cricket (*Gryllus assimilis*), age explained 64% of the variation in the mate-finding behavior (i.e. phonotaxis) of females (Pacheco et al., 2013). While theory predicts that females should be more likely to accept mates as they age due to the increasing costs of mate searching and the urgency of reproduction (Cotter et al., 2011), studies suggest that shifts in mate choice may also stem from changes to sensory processing capabilities in choosers as they age (Kodric-Brown & Nicoletto, 2001; Ronald et al., 2012).

The Salticidae (jumping spiders) are a highly speciose family known for their diverse sexually selected traits and mating strategies. Like many arthropods, at the terminal molt, jumping spiders develop to adulthood as their adult reproductive anatomy: pedipalps and epigyna for males and females respectively, is expressed along with other secondary sexual characters. For many jumping spider species, males guard nests of soon to mature females (co-habitation) attempting to restrict access to other males (R. R. Jackson, 1980, 1986; R. R. Jackson & Hallas, 1986; Mendez et al., 2017). Males successfully defending terminal molt females gain fitness benefits as females are receptive immediately after maturation (D. O. Elias et al., 2014; McGinley et al., 2015). In other species, including species from the genus *Habronattus*, the subject of this study, co-habitation has not been observed (personal observation). *Habronattus* males are known for their complex multimodal displays that include color, motion, and vibratory songs (T. Bougie et al., 2024; D. O. Elias et al., 2003, 2012; D. O. Elias, Hebets, Hoy, et al., 2006; W. Maddison & Hedin, 2003; W. Maddison & McMahon, 2000; W. Maddison & Stratton, 1988; Rivera et al., 2021; L. A. Taylor et al., 2011). Female *Habronattus* tend to have relatively low mating rates (Blackburn & Maddison, 2015; Brandt et al., 2020; D. O. Elias et al., 2003, 2005; D. O. Elias, Hebets, & Hoy, 2006; L. A. Taylor & McGraw, 2013) and evidence suggests that courtship displays are essential to mating success (D. O. Elias et al., 2005; D. O. Elias, Hebets, & Hoy, 2006).

In this study, we examined the relationship between age, receptivity, and female courtship preferences in *Habronattus formosus*, a member of a species group with well-described complex courtship (*clypeatus* group; (Rivera et al., 2021)). Given the absence of mate guarding and co-habitation, we hypothesized that *H. formosus* females would not be receptive immediately after sexual maturation. Further, we predicted that preference patterns would shift

with age, with part of the courtship predicting mating in younger females and the whole courtship predicting mating in older females. We define age 0 as the day that a female spider is found with the presence of an epigynum for the first time, since the presence of epigynum anatomically enable the female to copulate with a male. By exploring reproductive timing and its relationship to age in this species, we aimed to understand how sexual selection may be operating in this system and how it may change over time.

3.3 Materials and Methods

3.3.1 Specimen Collection and Housing

Penultimate female and adult male *Habronattus formosus* were collected in Victorville, California in June 2022. Spiders were individually housed in 3x3x5 cm plastic containers (AMAC Plastic Products) with crinkled paper as environmental enrichment. One drop of water was provided per container each week along with 4-6 fruit flies or small crickets. Spiders were housed in the lab at 25 degrees Celsius with a 12:12 hour light cycle. Maturity checks on penultimate females were performed at least every 2 days to track maturity dates. Female sexual maturity was assessed morphologically via the presence of a female's epigynum, regardless of behavior.

3.3.2 Age assignment and mating trials

All mating trials were conducted between April and July 2022. Using a random number generator drawing from a uniform distribution in R, we assigned each female to an age (immature or 1 to 30 days post-maturation) at which the trial would be conducted. For each female, a randomly chosen male was identified the day before the designated date for the trial. Spiders were fed the day before the trial. All males were only used once when possible (n=6/113 males used twice).

On the day of the trial, the female from each pair was allowed to acclimate in the experimental arena for a minimum of 5 minutes. The arena was made by stretching printer paper on a circular embroidery hoop (11cm diameter). A reflective piece of tape was placed in the middle of the arena to facilitate the recording of vibratory signals (Girard et al., 2011). An acetate ring (9cm diameter, 4.5 cm in height) was placed on top of the arena floor, with Vaseline applied on the interior of the ring to prevent the spiders from escaping. The arenas were illuminated by two LED light panels (160 LED CN-160, Neewar) and heated by a heat lamp (LF-13 Professional Series Dimmable Clamp Lamp, Zoo Med Laboratories) to ensure a controlled temperature as temperature can influence mating patterns (Brandt et al., 2018).

After 5 minutes of female acclimation, the male was dropped into the arena. Trials were run for 15 minutes, or until spiders copulated or one was cannibalized (n=1). Trials were recorded with a mirrorless camera (Panasonic LUMIX GH4 4K Mirrorless Digital Camera) with a macro lens (Olympus M.Zuiko ED 60mm F/2.8 Macro). Vibratory signals were recorded using a laser vibrometer (Polytec PDV100). After the trial, both spiders were weighed using an Ohaus Analytical Plus scale. Mating trial videos were further analyzed using BORIS (Friard & Gamba, 2016). For each video, courtship duration, female aggression and copulation were marked. For

courtship, we broke down the courtship display of males into two parts. During part 1, the introductory phase, the male sidles around the female in circles and gradually gets closer without any vibratory signals (Rivera et al., 2021). During part 2, the multimodal courtship phase, the male approaches the female in a straight line with various vibratory and visual displays. See detailed descriptions of the components in the multimodal phase of the courtship in (Rivera et al., 2021). Not all males that display part 1 courtship advance to part 2, providing an important source of variation in male responses to females.

3.3.3 Statistical analysis

All statistical analyses were conducted in R version 4.1.3 (R Core Team 2022). We used a logistic regression to model female receptivity at different ages (days post maturity, age 0 is the date that females matured) with copulation success as a binary variable. To compare how female preference patterns might differ in younger and older females, we again used logistic regression with copulation success as a binary response variable, and binary age group and courtship measurements (mean duration of introductory or multimodal courtship, see above) as predictor variables. We defined “young” (N = 63) and “old” (N = 34) females based on the threshold identified by the logistic regression model of female receptivity. We removed two outliers that had a mean introductory courtship duration of more than 150 seconds (more than 3 standard deviations above the mean). We selected the best model based on the lowest AIC scores, and significance levels of predictors were determined by type III ANOVA because of the presence of interaction terms.

3.4 Results

3.4.1 Female receptivity

Immature females never copulated with males. Nine (45%) of 20 males, however, did court immature females (9/20). In contrast, mature females mated during 32 (40%) of 80 trials. The earliest copulation occurred on day 10 after the final molt. The 50% likelihood for copulation occurred at 19.4 (95%CI = 16.3, 22.6) days post maturation (**Fig. 3.1a**).

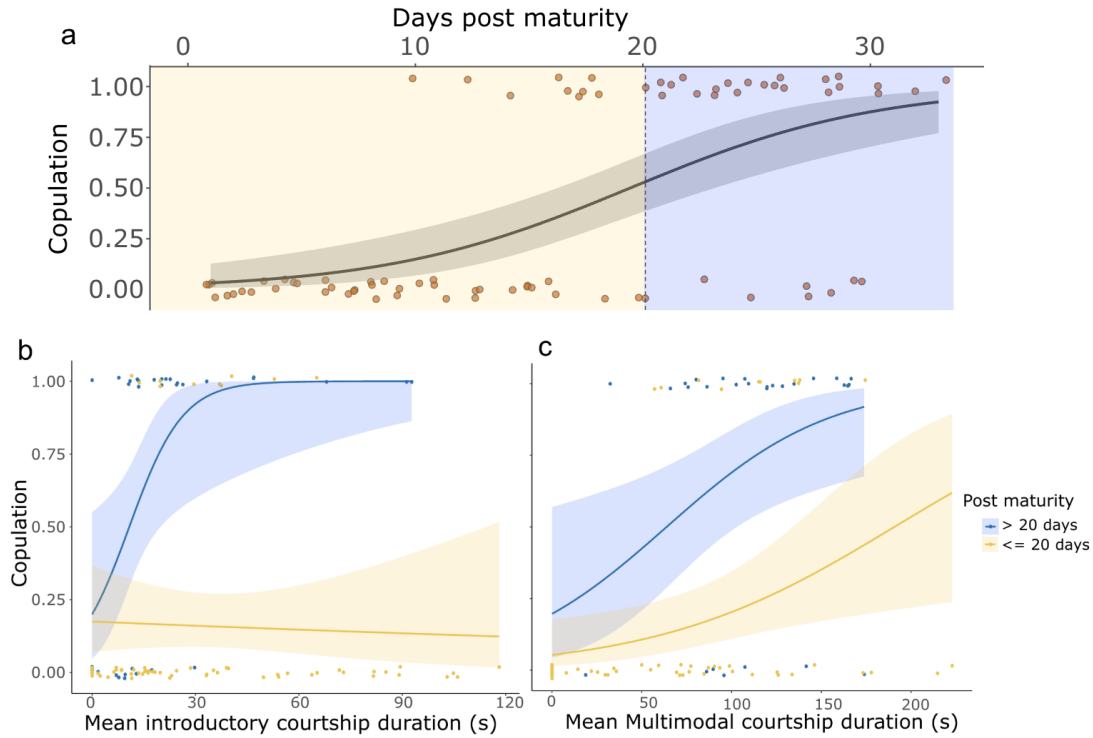


Figure 3.1. Female receptivity and preference across age. **a:** Logistic regression model showing receptivity and age. **b** and **c:** Logistic regression models of how male courtship display phases (**b:** introductory display phase, **c:** multimodal display phase) predict copulation success for young (yellow) and old (blue) *H. formosus*. All colored points are jittered along y axis for visualization and 95% confidence intervals shown by the ribbon.

3.4.2 Female preference patterns and age

We examined the relationship between introductory and multimodal courtship phases and mating across two age groups, young and old and found that preference patterns do change with age in *H. formosus*. The final model included age group, mean duration of introductory and mean duration of multimodal courtship phases, and the interaction of age and the mean duration of introductory courtship. The best model indicates that longer multimodal courtship plays a significant role in predicting copulation in both young and old females (Likelihood ratio chi-square (LR Chisq) = 15.2109, df = 1, $p < 0.0001$) (**Fig. 3.1c**, **Table S3.1**). However, the interaction between age and duration of introductory display also contributes significantly to the model that predicts copulation success (LR Chisq = 11.8248, df = 1, $p = 0.001$). It further indicates that the introductory courtship predicts copulation differently in younger and older females: longer introductory courtship predicts copulation in older but not younger females (**Fig. 3.1b**).

3.5 Discussion

In this study we found that *Habronattus formosus* were not receptive immediately after maturation and instead, females became receptive two to three weeks after they matured to adulthood (**Fig. 3.1a**). Furthermore, we observed a shift in male courtship traits that predicted

mating success as females aged. The entire complex male courtship (visual and multimodal phase) display predicted mating success in older females while only later parts of the courtship (multimodal phase) display predicted mating success in younger females.

3.5.1 Evidence for delayed receptivity.

As opposed to the predictable patterns of receptivity found in some jumping spider species where receptivity occurs immediately after maturation (D. O. Elias et al., 2014; Hoefler, 2007), we found that female receptivity was unpredictable. No females were receptive in the first 10 days after maturation after which females became receptive over a period of about two weeks. Multiple factors may contribute to the variability in ages at which females first become receptive, such as variation in nutrition intake, genotypic differences, etc., and may not be adaptive. Regardless, the unpredictable gap between maturation and receptivity may provide females a way to prevent male manipulation/harrassment through an increase in costs and reduction of benefits to guarding females until they are receptive. This may provide opportunities for intersexual selection through female mate choice. In this scenario, males would invest in searching for receptive females and use elaborate courtship displays to access mating opportunities. The absence of mate guarding and male-male aggressive displays, in addition to the presence of complex multimodal courtship in *H. formosus*, are all consistent with this hypothesis. A delay in female receptivity after sexual maturation has been documented in a few other spider species in which female mate choice is important for reproductive interactions (Klein et al., 2012; Uetz & Norton, 2007), providing additional support for a connection between variable female maturation and elaborate male courtship displays.

3.5.2 Delayed receptivity and complexity of courtship behavior.

One potential consequence of this type of delay between maturation and receptivity is selection for greater complexity of male courtship displays. Delayed maturation may allow females the opportunity to sample courtship across multiple males, thereby allowing more informed choice of the males with which a female mate with, since males still court females even when they are not receptive (Tuni & Berger-Tal, 2012). From the perspective of the females, the ability to both sample male densities and a large distribution of male courtship traits before they mate could potentially increase a female's fitness if they were able to plastically respond to the availability of males as well as the male traits they experienced (Kappeler et al., 2023). It has been shown that redback black widows can respond to cues of male densities (Kasumovic et al., 2008; Kasumovic & Brooks, 2011; Lymbery et al., 2025; Scott et al., 2020).

3.5.3 Comparisons with other species.

Our finding that *H. formosus* females have a delayed receptivity aligns with anecdotal evidence in other spider systems in which female choice plays a dominant role. Some wolf spider females adjust their future mate choice patterns depending on courtship they observed as juveniles (Hebets, 2003). These studies show that spiders can adjust their mate choice patterns depending on what they experience. Intriguingly, experimental studies on mate choice behavior in the wolf spider genera *Schizocosa* (e.g. (Hebets et al., 9/2013; Norton & Uetz, 2005; M. F. Rosenthal et al., 2018; Uetz & Norton, 2007)), the peacock jumping spiders (Maratus (Girard, 2017; Girard et al., 2018)), and other *Habronattus* (Brandt et al., 2020; D. O. Elias et al., 2005; D. O. Elias, Hebets, & Hoy, 2006), species/genera renowned for the complexity in their courtship displays, conduct experiments 2-3 weeks after maturation. While anecdotal, this does

suggest a possibility that in spider species with complex courtship, there is a “critical period” where mating preferences develop. Future work examining this hypothesis, as well as what occurs during this period would help elucidate how mating preferences form. We suggest that delayed receptivity (and associated learning/experience-dependent mate choice) may be a feature of systems with diverse and complex communication during sexual selection events. In *Habronattus* species, preliminary observations suggest that females are predominantly monogamous. Delayed receptivity may reduce the costs associated with monogamy by allowing enough time to sample potential mates. Once again, similar findings in *Schizocosa* (Norton & Uetz, 2005) and *Maratus* (Girard et al., 2015) spiders suggest that this may be a feature of systems where females mate once and have complex communication. Future work should explore this hypothesis across different systems.

3.5.4 Temporal variation in female preferences.

When examining the relationship between male traits and mating success, we observed that in *H. formosus*, younger females selected males based only on a part of their display (multimodal courtship) while older females selected males based on the entire display (introductory courtship + multimodal courtship) (**Fig. 3.1b** and **3.1c**). The courtship display of *H. formosus* males follows a sequential pattern, progressing from an introductory display to multimodal displays showing a series of stereotyped motifs (Rivera et al., 2021). While successful matings involved all display stages, we found that the duration of the introductory displays did not influence copulation decisions in younger females, whereas both the introductory and multimodal display phases influenced mating success in older females. This suggests that older females assess the entirety of the display and thus incorporate more information into their decision-making processes than younger females do.

While many studies have shown that experience affects mate choice (Dukas & Bailey, 2024; Hebets, 2003; Li et al., 2018; Stoffer & Uetz, 2023), the differences in female preference pattern observed in the present study are not due to prior mating experience, as all females were collected from the field as immatures and had never mated before the trials. This suggests the possibility that the shifts we observed were related to cognitive and/or physiological that occur with age (Kelly, 2018; Ronald et al., 2012; Rowell et al., 2021). Our findings may further suggest that younger females might not have the capacity to integrate information about the entire display like older females do. While some studies suggest that older females exhibit decreased sensory capacity in mating contexts (Ronald et al., 2012), this decline is typically observed after peak reproductive age. In contrast, our findings suggest that female preference patterns may continue to develop after sexual maturity, rather than simply declining with age. We suggest that this variation in female preferences could favor the evolution of more complex signaling like that found in *Habronattus* displays. While some theoretical models have suggested that over evolutionary time, courtship signals should evolve to be less complex (Iwasa & Pomiankowski, 1994), other authors have suggested that variation in female preferences may drive the evolution of more complex signals (Candolin, 2003; Jennions & Petrie, 1997; Reichert et al., 2017). We suggest that age-based variation in preferences may favor complex signals. Our results further highlight the importance of female age when measuring preference, as preferences may vary depending on their relationship to peak reproductive timing and shifts in the balance of selective forces.

Overall, our results suggest that reproductive timing is essential to understanding mating behavior. The timing between sexual maturity (morphological evidence) and sexual receptivity (behavioral evidence) may be associated with patterns of inter- and intra-sexual selection, and as a result, which traits or trait combinations are likely to evolve. We suggest that increases in the timing between sexual maturity and sexual receptivity are likely to favor the evolution of complex signals and/or preferences. Finally, we suggest that variation in the timing between sexual receptivity and sexual maturity across a population may favor the ability to plastically respond to relevant variation in potential reproductive partners. Regardless, understanding the timing of receptivity and how age impacts mate choice patterns is a critical, largely understudied aspect of mate choice. These types of studies are both needed to understand the dynamics of mate choice and to interpret behavioral data.

3.6 Acknowledgments

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Chapter 4

The relationship between oogenesis and reproductive timing in jumping spiders may facilitate female mate choice

Lin Yan and Damian O. Elias

4.1 Abstract

The physiological processes underlying reproductive timing, such as receptivity, are unknown in most species. To explore this, we examined the relationship between female receptivity and ovarian physiology in jumping spiders. Using two jumping spider species that show distinct mating strategies, *Phidippus otiosus* and *Habronattus formosus*, we associated female receptivities at different ages with micro-CT imaging of female ovaries. We found that *P. otiosus* females are receptive immediately after sexual maturation, compared to *H. formosus* who shows a delay in female receptivity after sexual maturation. If ovarian development is associated with receptivity patterns, *P. otiosus* females should thus have more developed oocytes than *H. formosus* females at a given time point. Surprisingly we saw opposite patterns in ovarian development than expected relative to the timing of receptivity. We discuss the processes underlying variation in reproductive timing and suggest that the timing of oogenesis could facilitate differences in female mate choice in the two species.

4.2 Introduction

While sexual selection research has a rich history in elucidating the relationships between male sexual traits, physiology, and performance (reviewed in (Andersson, 1994; Lailvaux & Irschick, 2006)), relatively less attention has been given to the role female reproductive physiology plays in mediating the mechanisms of sexual selection operating in a particular mating system (Ah-King, 2022; Hare & Simmons, 2019). Reproductive fitness in sexually reproducing organisms is directly related to gamete development (ovaries/eggs in females) and access to mating opportunities mediated through adult reproductive morphology (i.e. genitalia), receptivity patterns, and the traits used to restrict access to mates and/or secondary courtship traits that are assessed via mate choice. Timing of female receptivity has been hypothesized as a key determinant of a population's environmental potential for polygamy (Emlen & Oring, 1977) and thus a process that separates competition-driven (intrasexual) and mate choice (intersexual) - driven systems. In competition-driven systems, receptivity is hypothesized to be relatively predictable as it increases the benefits of behaviors/traits functioning to restrict access to mates (Grafen & Ridley, 1983; Vellnow et al., 2020). In mate choice-driven systems, receptivity is hypothesized to be relatively less predictable thus increasing the costs in competitive behaviors and favoring behaviors/traits that are assessed by choosers. In other words, receptivity is thought to determine whether competition or mate choice are the primary drivers of sexual selection.

While the timing of receptivity is variable across species, in general it has been assumed across animal systems that the timing of receptivity generally correlates with the timeline of adult maturation, physiology, ovarian development, and fertilization (Manning, 1967; Míčková et al.,

2024; Ringo, 1996; Rooker & Gavrillets, 2020; Tomihara et al., 2026; Walton et al., 2020) and that receptivity patterns can be good proxies for sexual selection mechanisms, as discussed above, but this may not always be the case (Sawrey & Dewsbury, 1985). In this study, we explored the relationship between ovarian development, adult maturation, and receptivity in two jumping spider species with two distinct mating strategies. We hypothesized that differences in receptivity patterns are related to patterns of ovarian development such that in a species where receptivity is synchronized to adult maturation, female spiders have advanced ovarian development relative to species where receptivity occurs several weeks after adult maturation.

Ovaries in spiders are paired internal organs located at the ventral part of the abdomen that produce egg cells (oocytes). While poorly studied overall, studies in *Loxosceles intermedia* and *Latrodectus hesperus* (Morishita et al., 2003; Ouriel, 2016) suggest that eggs begin as undifferentiated oocytes attached to the epithelial wall of the ovary on a pedicle stalk (Morishita et al., 2003). During the vitellogenic phase in development (Ouriel, 2016), likely triggered by juvenile hormone (JH) analogs (Bonaric, 1988; Nicewicz et al., 2021; Trabalon et al., 1992; Z. Yang et al., 2022), oocytes (and the ovary itself) increase in size as the cytoplasm expands due to the accumulation of fine-grained protein granules (yolk) and lipid droplets that accumulate around the nucleus (Morishita et al., 2003). At the end of the vitellogenic stage, yolk granules nearly fill the entirety of the cytoplasm and the developed oocyte is many times larger (Morishita et al., 2003). Next, during the post-vitellogenic phase (Ouriel, 2016), the oocyte changes in shape through a rearrangement of the yolk mass before being released into the ovarian lumen as the pedicle releases the developed oocyte (Morishita et al., 2003). A continuous distribution of oocyte sizes can be observed within the ovary, with the largest oocytes likely being the most developed (Y.-S. Choi & Moon, 2003; Morishita et al., 2003; Pourié & Trabalon, 2003). Fertilization occurs during egg laying (oviposition) when mature oocytes move through the oviduct and past the sperm storage organs (Dallai et al., 2012). For spiders, copulation occurs prior to fertilization and sperm is stored in paired seminal receptacles (spermatheca) connected to the epigynum, the female's genital opening (Foelix, 2011). The epigynum only appears when a female matures to adulthood, although the ovaries are present at earlier life stages (F. Zhang et al., 2021). For spiders, female reproduction is thus related to the development of oocytes (with vitellogenesis being a key stage), female maturation (i.e. the appearance of the female copulatory opening), mating (including when a female is willing to accept mates [choosiness] and the male trait values preferred by females [preference]), and oviposition/fertilization, likely mediated through JH analogs. Each of these can occur at different times and the timing and associated tradeoffs between each of these events are likely important for successful reproduction and may vary relative to the type of sexual selection acting on the system. One open question is how the progression of oocyte development as females age is related to sexual selection mechanisms.

The Salticidae (jumping spiders) are a highly speciose family known for their diverse sexually selected traits and mating strategies, offering an excellent framework for linking behavioral variation with ovarian physiology. In this study, we selected two jumping spiders from genera that exhibit distinct mating strategies, *Phidippus* and *Habronattus*. Using case studies from these two genera, we examined the relationship between female reproductive physiology (oogenesis) and female receptivity to understand whether ovarian physiology is closely linked to behavioral receptivity.

In *Phidippus* systems, female receptivity is predictably tied to maturation to adulthood which occurs over a relatively short amount of time (Hoefler, 2007). Males of several *Phidippus* species have been observed to cohabit with subadult females (R. Jackson, 1978; R. R. Jackson, 1986) and defend nests against male competitors (Edwards, 1990; Hoefler, 2007). Males compete against other males using a series of relatively simple aggressive displays that include visual and vibratory signals (D. O. Elias et al., 2008, 2014; R. Jackson, 1978; R. R. Jackson, 1982). Larger (Hoefler, 2007; Kasumovic et al., 2009) and resident males (Kasumovic et al., 2010) that signal more (D. O. Elias et al., 2008) are more likely to successfully defend a nest. In *P. clarus*, males who cohabited with a female mated successfully 85% of the time (D. O. Elias et al., 2014). While unmated and guarded females mated with prospective mates at similar rates, guarding significantly decreased the likelihood that a female would mate multiply (D. O. Elias et al., 2014). For unguarded females, females were more likely to mate with males with longer legs that signaled at higher rates (D. O. Elias et al., 2010; Sivalingham et al., 2010). Male-male competition and not female choice has thus been inferred as the primary mechanism of sexual selection in *Phidippus*.

In *Habronattus* systems, on the other hand, female receptivity is not tied to maturation to adulthood and instead occurs in a span of 2-3 weeks after maturation (Chapter3). *Habronattus* males have not been observed cohabiting with subadult females and evidence suggests the absence of male competitive behaviors (Blackburn & Maddison, 2015). Instead, males have evolved complex multimodal displays that include color, motion, and vibratory songs (T. Bougie et al., 2024; D. O. Elias et al., 2003, 2012; D. O. Elias, Hebets, Hoy, et al., 2006; W. Maddison & Hedin, 2003; W. Maddison & McMahon, 2000; W. Maddison & Stratton, 1988; Rivera et al., 2021; L. A. Taylor et al., 2011). Female *Habronattus* tend to have relatively low mating rates (Blackburn & Maddison, 2015; Brandt et al., 2020; D. O. Elias et al., 2003, 2005; D. O. Elias, Hebets, & Hoy, 2006; L. A. Taylor & McGraw, 2013) and evidence suggests that courtship displays are essential to mating success (D. O. Elias et al., 2005; D. O. Elias, Hebets, & Hoy, 2006). Female choice has thus been inferred as the primary mechanism of sexual selection in *Habronattus*.

In this study, we specifically focused on *Phidippus otiosus* and *Habronattus formosus*. Based on field observations (Edwards, 1990; D. E. Hill, 1978), *P. otiosus* males guard and cohabit with penultimate females. Preliminary evidence suggests they engage in male-male contests and exhibit relatively simple courtship towards females. Conversely, in *H. formosus*, males engage in complex multimodal courtship (Rivera et al., 2021). Preliminary evidence also suggests that males do not engage in stereotyped contests (unpublished data). The contrasting mating strategies of these two species, provides a unique opportunity to examine the timing of female receptivity and ovarian physiology. Specifically, we examined the timing of oogenesis (measured by ovary size and presence of yolk granules) and mating receptivity. An earlier study demonstrated that *H. formosus* females are not immediately receptive after reaching sexual maturity. Instead, the peak increase in receptivity comes 2-3 weeks after (Chapter3). We predicted that *Phidippus otiosus* females would become receptive shortly after molting to adulthood, in line with the presence of mate-guarding behavior. Next, we explored whether the timing of female receptivity is related to oogenesis, a proximate indicator of a female's overall physiological status (Hoffmann, 2018; Rodrigues et al., 2008). We predicted that, despite different timing in receptivity in the two species, increases in ovary size and the presence of yolk granules would match receptivity patterns, with *P. otiosus* females showing larger ovaries and

more yolk granules (i.e.earlier oogenesis) compared to *H. formosus* females at a given point in time. Understanding the relationship and timing between ovarian physiology and mate choice is critical to understanding the potential physiological drivers behind progression of critical reproductive behaviors.

4.3 Materials and Methods

4.3.1 Specimen Collection and Housing

Penultimate females and adult male *Phidippus otiosus* were collected in Gainesville, Florida in August 2023. They were individually housed in 6x6x8 cm plastic containers with mesh squares as environmental enrichment. The containers housing males were wrapped with brown paper to prevent interactions with other males. Three drops of water were provided per week along with 1-2 crickets for food. Spiders were housed in the lab at 25 degrees Celsius with a 12:12 hour light cycle. Maturity checks on penultimate females of both species were performed at least every 2 days to track maturity dates. Maturity was assessed with the presence of a female's epigynum. Penultimate females and adult male *Habronattus formosus* were collected from Victorville, California in June 2022, as part of another study on reproductive timing and mate preferences (Chapter3). For collection and housing details of *Habronattus formosus*, please see (Chapter3).

4.3.2 Age assignment and mating trials

All mating trials involving *Phidippus otiosus* were conducted between September 2023 and March 2024.

For these trials, the arena used a circular embroidery hoop (15 cm diameter) with an acetate ring of 14cm in diameter and 10.5cm in height, with Vaseline applied. Similar mating trials were run on *Habronattus formosus* as part of another study (see above, Chapter3).

4.3.3 CT prep, scanning and quantification

After mating trials for both *P. otiosus* and *H. formosus* (Chapter3), all female individuals were sacrificed within 24 hours after the conclusion of the mating trial and preserved in 100% ethanol for at least 48 hours for fixation. After fixation, the abdomen was separated from the prosoma and spinnerets removed for faster stain penetration. Samples were then transferred into a 0.3% Phosphotungstic Acid (PTA) staining solution on an orbital shaker (VWR OS-500) at speed 3 for two weeks to a few months, depending on the size of the spider. Penetration of stains was visualized during scans, and incomplete samples were kept on the shaker until fully stained. The majority of scans were performed at the Center for Molecular and Genomic Imaging (CGMI) facilities at UC Davis, while a few were performed at the Advanced Light Source (ALS) at Lawrence Berkeley National Lab (beamline 8.3.2).

For the CGMI scans, specimens were fixed in a centrifuge tube and scanned in the staining solution (0.3% PTA made by diluting 1% PTA with absolute ethanol) using MicroXCT-200 (Carl Zeiss X-ray Microscopy). All *Habronattus formosus* specimens were scanned using 4x objectives and *Phidippus otiosus* using 0.4x objectives, due to the drastic difference in specimen sizes. The resolution of *H. formosus* females ranges from around 3-4 $\mu\text{m}/\text{pixel}$ and 9-10 $\mu\text{m}/\text{pixel}$ for *P. otiosus*. All scans were visually examined to ensure that shrinkage was minimal. For ALS scans, X-ray images were generated over the course of two to three scans for each specimen using a 4x objective lens (*H. formosus* only). Specimens were placed within a pipette tip containing 0.3% PTA for scanning. The pipette tip was affixed to a wooden rod and sealed on

either end with parafilm. ~1750 unique images were generated for each specimen respectively and used to form reconstructed 3D images with a voxel size of 1.605 μm . Examination of scans using both methods showed that oogenesis patterns were qualitatively similar. For all scans, we measured (1) ovary size as a proxy for oogenesis, given observed increases in oocyte size during the vitellogenesis stage in oocyte development (Y.-S. Choi & Moon, 2003; Garcia et al., 2025) and (2) the presence of yolk granules as an indicator of ongoing vitellogenesis. The digital volume was visualized using Dragonfly version 2021.3 (Object Research Systems), and the abdomen and ovary were manually labelled using the 3D paintbrush tool. As each voxel is a cube with a known edge length (voxel size), the volume value given by Dragonfly represents the sum of the area of the voxels selected.

4.3.4 Statistical analysis

All statistical analyses were conducted in R version 4.1.3 (R Core Team 2022). We compared the size of the ovaries of the two species across ages using linear regression, with relative ovary size (ratio of the abdomen) as the response and age+species as predictor variables. We removed an outlier measurement of a *H. formosus* female (more than 10 standard deviations from the mean). In order to incorporate ovarian measurements of immature females, we arbitrarily assigned the age of -5 days for all females for the analysis but their exact age of the female is unknown because imaging ovaries is a terminal procedure. We used type II ANOVA to assess the significance of predictors.

4.4 Results

4.4.1 Female receptivity

Immature females *P. otiosus* never copulated with males, despite all males paired with immature females courted (N=6). Mature *P. otiosus* females mated 83% (19/23) of the time. *P. otiosus* females were receptive early, with the earliest copulation occurring at day 0. The 50% likelihood for copulation occurred at 2.08 (95%CI = -5.46, 9.63) days post maturation (**Fig. 4.1**). Data from a previous study is used to plot for *H. formosus* showing that the earliest copulation occurred at day 10, and 50% likelihood for copulation occurred at 19.44 (95%CI = 16.33, 22.56) (Chapter3).

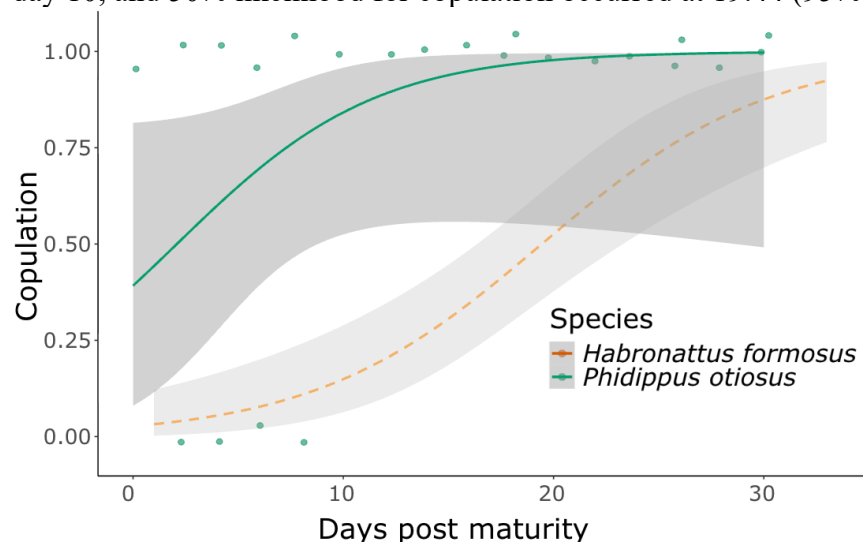


Figure 4.1. Logistic regression model showing receptivity and age in *Phidippus otiosus* (green). Regression line for *Habronattus formosus* (orange) plotted using data from (Chapter3). Colored points are jittered along y axis for visualization and 95% confidence intervals are shown in grey.

4.4.2 Female oogenesis

To map the process of oogenesis to female receptivity and preference patterns, we used microCT to image the ovaries of females used in behavioral trials (Chapter3). We predicted that patterns of receptivity would match ovary size and vitellogenesis. Unexpectedly, although *P. otiosus* females are receptive earlier than *H. formosus*, their ovaries were not proportionally larger than those of *H. formosus*. Although the relative ovary size increases as females age ($p = 0.0007$), it does not significantly vary between the two species ($p = 0.91543$). Despite similar ovary size post-maturation, scans of ovaries reveal a different oogenesis pattern between the two species (Fig. 4.2b and Fig. 4.3).

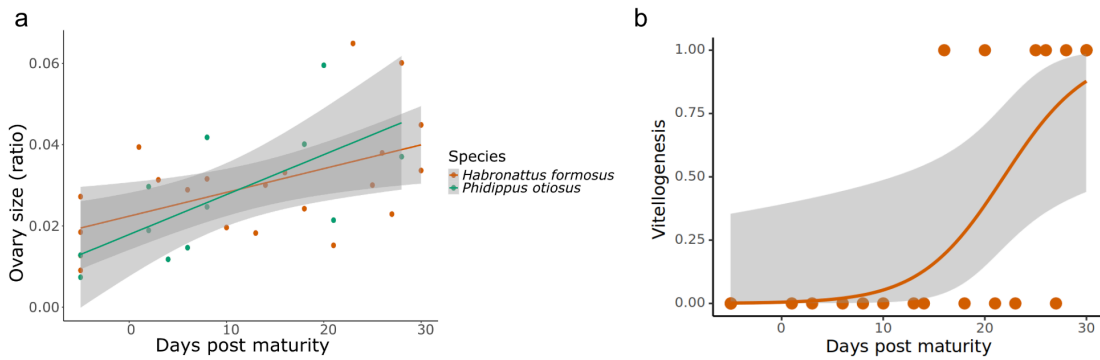


Figure 4.2. Oogenesis and age. **a:** Linear regression model of ovary size and age in *Phidippus otiosus* (green) and *Habronattus formosus* (orange). 95% confidence interval indicated in grey. **b:** logistic regression of age and vitellogenesis presence in the ovary in *Habronattus formosus*. 95% confidence intervals in grey.

The process of vitellogenesis can be visualized by the accumulation of heavily stained granules inside the oocytes. We predicted that the presence of vitellogenesis would follow receptivity patterns with vitellogenesis present earlier in *P. otiosus* females relative to *H. formosus*. While we observed vitellogenesis in older *H. formosus* and not younger *H. formosus* (Fig. 4.2b), we did not observe vitellogenesis in *P. otiosus* females of any age. Instead, *P. otiosus* females show more early stage oocytes as females get older (Fig. 4.3), thus the observed increase of ovary size.

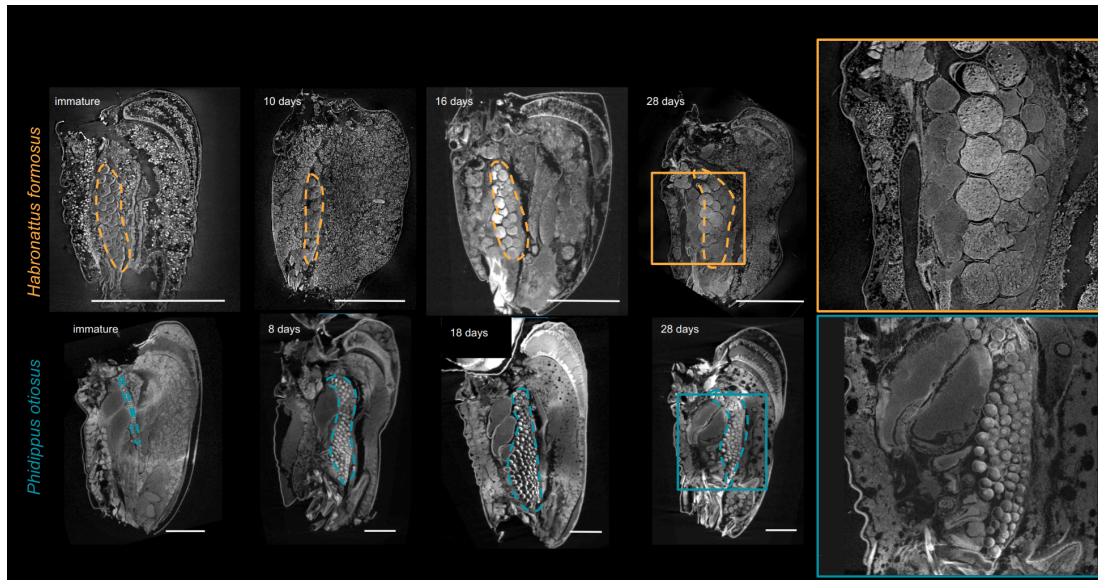


Figure 4.3. Visualization of oogenesis and age in *Habronattus formosus* (orange) and *Phidippus otiosus* (green). Ovaries of both species at 28 days are zoomed in to show the differences in vitellogenic oocytes in *H. formosus* and pre-vitellogenic oocytes in *P. otiosus*. Oocytes are outlined by dashed lines of respective colors. The immature *H. formosus* scan was collected at the Advanced Light Source (LBNL), and all other scans were collected at UC Davis. All scans were visualized in Dragonfly. Scale bar = 1mm.

4.5 Discussion

We examined two jumping spider systems, *P. otiosus* and *H. formosus*, to explore the relationship between female reproductive timing and physiology. Our results show major differences in the timing of female receptivity between the two systems.

Phidippus otiosus females were receptive almost immediately after maturation, contrasting with delayed receptivity in *Habronattus formosus* females (Chapter3) (**Fig. 4.1**). When examining ovary development, we thus expected larger, more developed ovaries in the earlier receptive *P. otiosus* relative to the later receptive *H. formosus*. Surprisingly, we found that *P. otiosus* ovaries were not relatively larger when compared to *H. formosus* (**Fig. 4.3**). *H. formosus* ovaries were the same size as *P. otiosus* (**Fig. 4.2a**) and were relatively more developed when females were receptive compared to when *P. otiosus* was receptive (**Fig. 4.2b**). This demonstrates that female receptivity is not necessarily associated with oogenesis in the same way across systems.

P. otiosus males have been observed to cohabitate with immature females in the field (Edwards, 1990; D. E. Hill, 1978) in order to restrict mating access and those observations are in line with the receptivity patterns reported in this study. Early receptivity would increase the benefits of male defense and thus it would be expected that males would invest in traits such as weapons, signals of competitive ability, assessment of female age, and mate searching (D. O. Elias et al., 2014; Fahey & Elgar, 1997; Tuni & Berger-Tal, 2012). In general, these observations are in line with studies in the congener *Phidippus clarus* (Hoefler, 2007), and often cited examples of female defense polygyny where males restrict access to females and females benefit from mating with males that are able to successfully defend those access points (Dodson & Beck, 1993; Erez et al., 2005; Fahey & Elgar, 1997; Fernández-Montraveta & Cuadrado, 2003). It is generally assumed that in these competition-driven systems, females do not actively choose mates,

avoiding the costs of mate choice (e.g. assessment costs, male harassment, etc). Our observations of *P. otiosus* ovarian physiology point to a more complicated picture, however. In jumping spiders, fertilization does not occur at the time of copulation. Instead, females can store sperm for an extended period, with fertilization occurring only during oviposition (Herberstein et al., 2011). As a result, delayed oogenesis compared with receptivity, as seen in *P. otiosus*, does not impact reproductive success, but instead, provides a longer time window before oviposition for females. We suggest that this time window could be used to find additional mates (Eberhard, 1996). By assessing and mating with multiple males, females can increase genetic diversity of offspring (Rovelli et al., 2015) and/or bet-hedge against lower quality or infertile males (Sutter et al., 2019). Polyandry, especially in the case of *Phidippus otiosus*, can further be beneficial due to the high fecundity typical of the *Phidippus* genus (Sivalinghem et al., 2010). For our study, we kept females and males separately housed and as a result, we cannot rule out the possibility that delayed oogenesis is due to the lack of cohabitation with a male.

H. formosus females on the other hand have not been observed to cohabitate. Their delayed receptivity relative to maturation was hypothesized to be a mechanism to allow females the time to sample courtship across multiple males (males court unreceptive females) before they mated (Chapter3). Given the observations that *Habronattus* females do not multiply mate (unpublished data), the time window due to delayed receptivity was hypothesized to allow for a critical period where mating preferences could develop to ensure optimal mating decisions (Chapter3). In this system, receptivity appears to generally be associated with vitellogenesis as we originally predicted. This suggests that by the time females are receptive, females would not have to wait to fertilize their eggs.

Oogenesis is a highly energy-intensive process (Laino et al., 2013; Romero et al., 2019; Tsukimura, 2001), making its timing relative to maturation, mating, and fertilization crucial for balancing energy allocation between reproduction and other activities. The association between receptivity and oogenesis has been found to be variable, even between closely related species. While female receptivity is associated with ovarian development in the onion fly *Delia antiqua* (McDONALD & Borden, 1995) and two *Drosophila* species (Manning, 1967), mating receptivity is not associated with oogenesis in the house fly *Musca domestica* (Adams & Nelson, 1990). We found similar variation in our study across the two different mating systems.

One of the well-studied families of regulators for reproduction in arthropods, juvenile hormone (JH), plays a key role in reproductive timing and oogenesis. While JH has been extensively studied in insect oogenesis and receptivity (Manning, 1967; Santos et al., 2019), evidence suggests its presence and function in arachnids as well. JH receptors have been identified in spiders (Nicewicz et al., 2021), and JH analogs have been implicated in spider vitellogenesis (Bonaric, 1988). The close association between JH and spider ovaries is further supported by the high expression of JH biosynthesis-related genes in ovarian tissues of female spiders (Z. Yang et al., 2022). While directly measuring JH levels has proved challenging in spiders (Sawadro et al., 2017), future experiments manipulating JH and observing its effects on receptivity and oogenesis would be an exciting new direction to understand the regulators behind mating strategies.

Our study suggests that receptivity is not necessarily linked with ovarian physiology and that the timing between maturation, receptivity, mating, and fertilization can all vary and potentially lead to a variety of mating systems and behaviors. One intriguing possibility that emerged from our data is that the timing of ovarian development may serve to increase access to mate choice. In the case of *P. otiosus*, early receptivity and a mismatch with ovarian

development favors more time between copulation and fertilization, as ovaries need time to develop, potentially increasing the likelihood of multiple mating and post-copulatory mate choice. In the case of *H. formosus*, time is needed for females to become receptive but once females become receptive, their ovaries are well developed. The time needed between maturation and receptivity would allow time for mate choice preferences and the sensory/cognitive mechanisms used to assess courtship displays to develop. In this system, time also emerges as a mechanism to avoid male monopolization (Chapter3). In both *P. otiosus* and *H. formosus*, time emerges as a way of providing females opportunities to maximize their ability to choose. In many competition-driven systems, male aggression in the context of mate guarding prevents (or at least influences) access to choice (Hindmarsh Sten et al., 2025; Tokarz, 1995; Wong & Candolin, 2005; Y. Yang & Richards-Zawacki, 2021). For example, less attractive males guard the female from attractive males in birds (Dowling & Webster, 2017), male guarding prevents extra pair copulation in marmots (Cohas et al., 2006), and females do not choose preferred brightly colored males in the presence of male competition in damselfish (Wacker et al., 2016). The time needed for oogenesis may counteract behaviors evolved to restrict access to mates. On the other hand, in more mate choice dominant systems, more time between maturation and receptivity decreases the likelihood of monopolization and mate guarding by males.

Female reproductive physiology has long been a black box in mate choice research. The few studies that have explored this topic have shown, not surprisingly, the breadth of adaptations possible and the importance of female physiology to mate choice processes (Cordero-Molina et al., 2023; Kekäläinen & Evans, 2017; Orbach et al., 2021; Sato et al., 2021; Simmons & Fitzpatrick, 2019). In the case of these two case studies, female reproductive physiology reveals how sexual selection may favor female mate choice, even in systems with mate guarding and strong competition to restrict access to mates. Much more work is needed, particularly in arthropods, but it promises to illuminate our understanding of sexual selection and mating behaviors (Ah-King, 2022).

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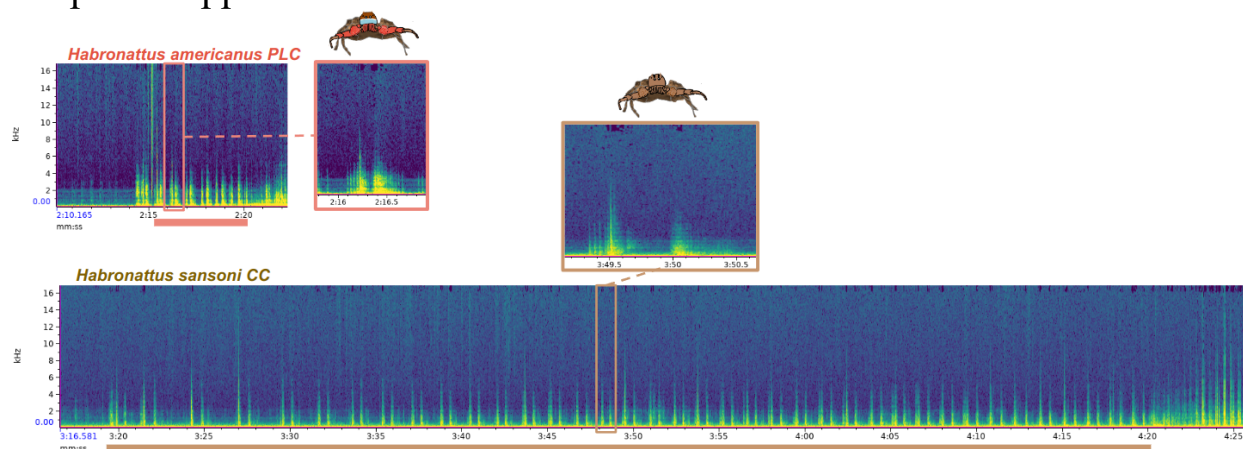
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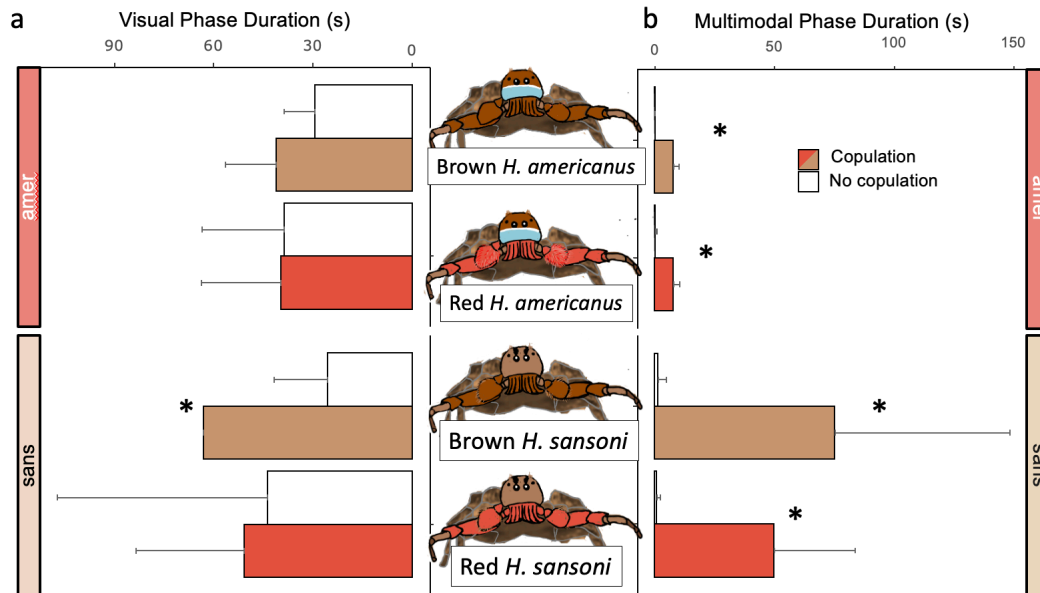
Appendices

Chapter 1 Appendix

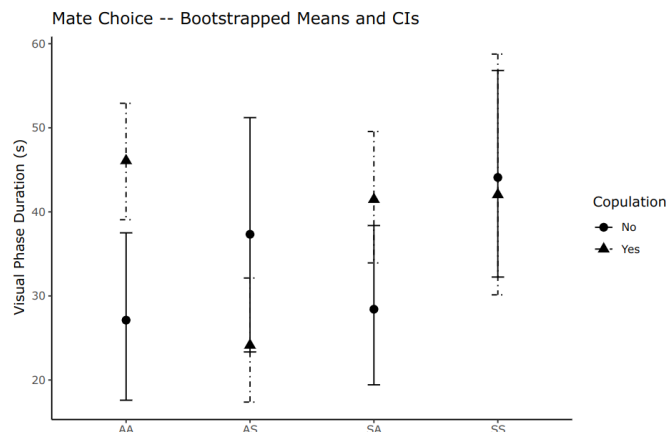


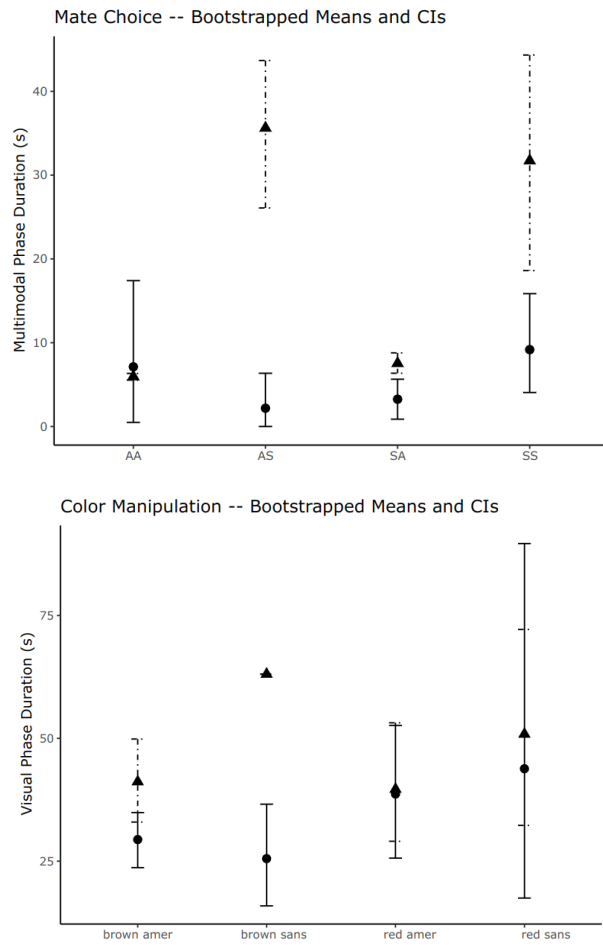
Supplementary Figure S1.1: An example spectrogram of the multimodal display of *H.*

americanus PLC and *H. sansoni* CC males. The duration of the full display is shown by the color bar, and a single signal unit of each display is shown in the inset. The displays are visualized using RavenPro with a window size of 512 samples.



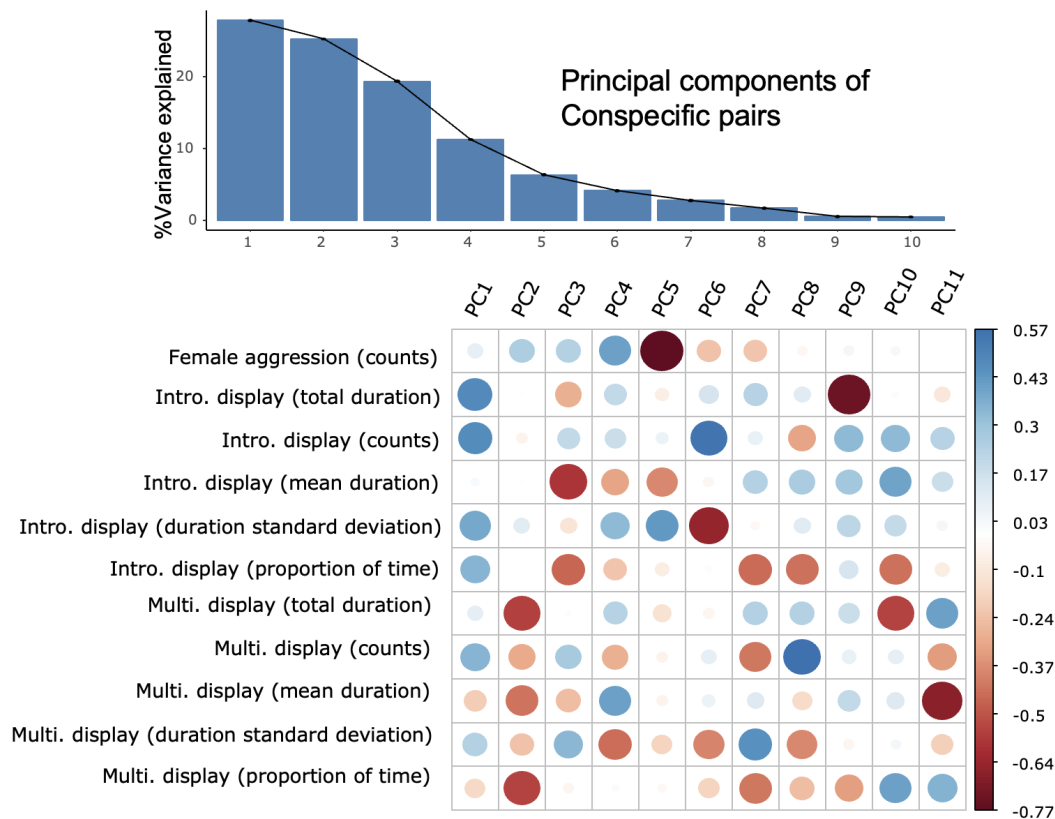
Supplementary Figure S1.2. In color manipulation trials, *H. americanus* males that performed longer multimodal phases were more likely to copulate while brown painted. *H. sansoni* CC males that performed longer visual and multimodal phases were more likely to copulate and red painted *H. sansoni* CC males that performed longer multimodal phases were more likely to copulate. Duration of courtship phases (visual (**a**) and multimodal (**b**) display) in color manipulation experiments. Colored bars (red and brown) represent pairs that copulated and white bars represent pairs that did not copulate. The white bars are very narrow in plot b since most spiders that performed multimodal courtship mated. Species identity is labeled on the side of each subplot. The illustration of each species and color painted is in the middle. Significant differences are indicated by asterisks (bootstrapping, Fig. S1.3).



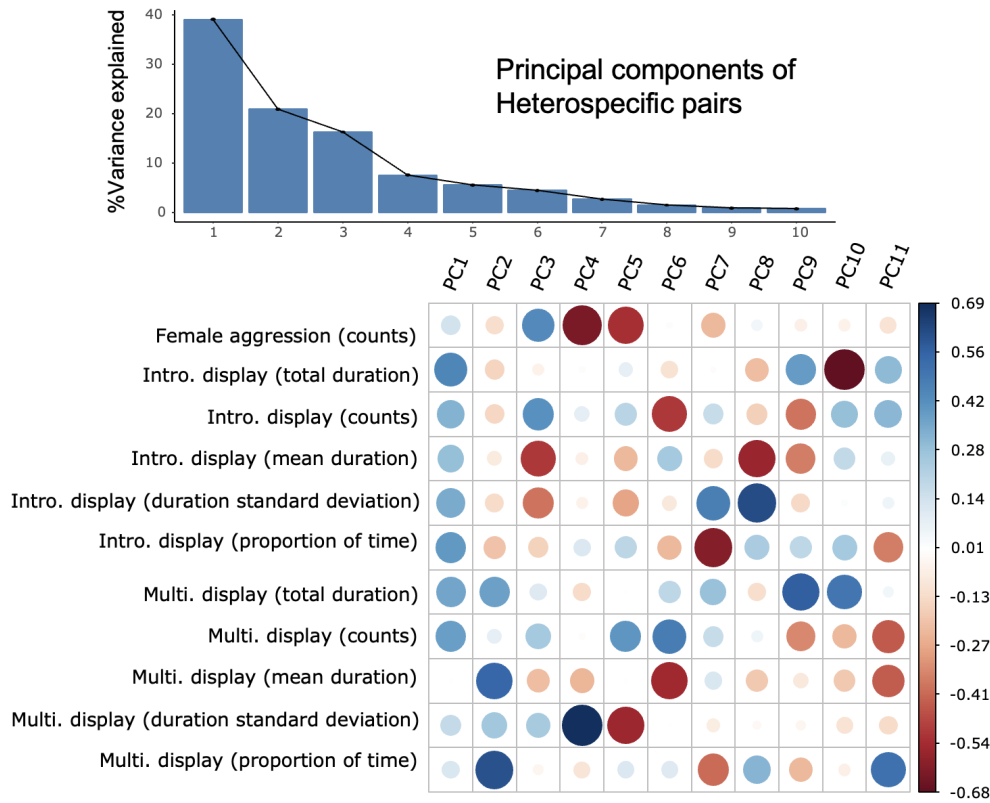


Supplementary Figure S1.3: Bootstrapped mean of visual and multimodal phase durations. The dot indicates the bootstrapped mean and bar indicates 95% confidence intervals. Data from copulated males are represented by triangles and dashed lines, while unsuccessful males are represented by circles and solid lines.

Chapter 2 Appendix



Supplementary Figure 2.1. Principal components of courtship measures in conspecific pairs. The upper panel shows the percentage of total variance explained by each principal component (PC), and the lower panel shows the loadings of variables on each PC. Circle size and transparency indicate the magnitude of each loading.



Supplementary Figure 2.2. Principal components of courtship measures in heterospecific pairs. The upper panel shows the percentage of total variance explained by each PC, and the lower panel shows the loadings of variables on each PC. Circle size and transparency indicate the magnitude of each loading.

Supplementary Table S2.1. Chi-squared test of heterospecific mating rates

	♀ form. ♂ form.	♀ form. ♂ gila.	♀ gila. ♂ form.	♀ gila. ♂ gila.
♀ form. ♂ form.	—			
♀ form. ♂ gila.	$\chi^2=2.928$ $p=0.087$	—		
♀ gila. ♂ form.	$\chi^2=0.297$ $p=0.586$	$\chi^2=1.731$ $p=0.188$	—	
♀ gila. ♂ gila.	$\chi^2=0.057$ $p=0.811$	$\chi^2=2.518$ $p=0.113$	$\chi^2=0.014$ $p=0.905$	—

Supplementary Table S2.2. Model selection for introductory courtship duration. Intro_mean is the mean duration of introductory courtship, pair is the mating pairs and cop is whether copulation occurred.

model	df	logLik	AICc	Δ AICc	weight
intro_mean~1	2	-450.514	905.1	0.00	0.731
intro_mean~cop	3	-450.513	907.3	2.12	0.254
intro_mean~pair+cop	6	-450.168	913.2	8.04	0.013
intro_mean~pair*cop	9	-450.820	917.5	12.37	0.002

Supplementary Table S2.3. Model selection for multimodal courtship duration. Vib_mean is the mean duration of multimodal courtship, pair is the mating pairs and cop is whether copulation occurred.

model	df	logLik	AICc	Δ AICc	weight
vib_mean~cop	3	-570.775	1147.8	0.00	0.904
vib_mean~pair+cop	6	-569.851	1152.6	4.77	0.083
vib_mean~1	2	-576.352	1156.8	9.04	0.010
vib_mean~pair*cop	9	-569.742	1159.4	11.58	0.003

Chapter 3 Appendix

Supplementary Table S3.1. Model selection for logistic regression that predicts copulation. Intro_mean is the mean duration of introductory display, vib_mean is the mean duration of multimodal courtship, old is the age group (young/old) and copulation is a binary variable of whether copulation occurred.

model	df	logLik	AICc	Δ AICc	weight
copulation~vib_mean+old*intro_mean	5	-35.848	82.4	0.00	0.616
copulation~old*vib_mean+old*intro_mean	6	-35.805	84.5	2.19	0.206
copulation~old*vib_mean*intro_mean	8	-33.632	84.9	2.55	0.173
copulation~vib_mean+intro_mean+old	4	-41.761	92.0	9.60	0.005
copulation~old	2	-49.641	103.4	21.05	0.000
copulation~vib_mean	2	-50.187	104.5	22.15	0.000
copulation~1	1	-61.508	125.1	42.70	0.000