



Qualitative and quantitative comparisons of mating behaviour across multiple populations and six species of leiobunine harvestmen (Arachnida: Opiliones)

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Abstract

Mating behaviour is highly diverse in animals both among and within species. We examine variation in mating behaviour in leiobunine harvestmen, which show high diversity in genitalic traits that are predicted to correspond to patterns of behavioural diversity. We ran mating trials for six species of leiobunine from four locations, and measured body size for a subset of individuals. We described mating behaviour in detail—providing the first formal description for most species—and examined variation inter- and intraspecific in body size and behaviour. Individuals were smaller in northern populations. Furthermore, we found species- and population-specific behaviours, high variation in the timing and success of different stages of mating, and high remating rates. However, we found no correlation between behavioural and morphological variation. Leiobunine harvestmen offer an excellent system for understanding multiple mechanisms of sexual selection and geographic diversification of mating behaviour.

Keywords

mate choice, Opiliones, male–female antagonism, geographic variation.

1. Introduction

Traits involved in the coordination of mating show spectacular diversity across the animal kingdom, often representing the most divergent aspects of phenotype among closely related species and populations (Eberhard, 1985;

Andersson, 1994; Wells & Henry, 1998; Coyne & Orr, 2004; Mendelson & Shaw, 2005; Cocroft et al., 2008). Behavioural traits involved in mate attraction and assessment can be particularly variable across species and play an important role in assortative mating and the formation of reproductive isolation (Henry, 1994; Wells & Henry, 1998; Mendelson & Shaw, 2002; Cocroft et al., 2008). However, behavioural traits can also vary considerably within species (Miller et al., 1998; Simmons et al., 2001; Elias et al., 2006; Olivero et al., 2017) as a result of divergent drift or selection across geographically disparate populations. It is clear that mating behaviour can play a critical role in the formation or reinforcement of reproductive isolation (West-Eberhard, 1983; Panhuis et al., 2001; Ritchie, 2007). Therefore, behavioural variation can have important consequences for broad-scale evolutionary processes such as diversification and speciation. As a result, the connection between geographic behavioural variation and the speciation process demands examination.

Inter- and intra-specific variation in mating-related behaviour can arise through a number of processes, including accumulated differences from drift, or variation in sexual selection and/or ecological selection (Andersson, 1994; Coyne & Orr, 2004; Hankison & Ptacek, 2008; Kraaijeveld et al., 2011; Maan & Seehausen, 2011; Nosil, 2012; Scordato et al., 2014). Particularly when geographic ranges are broad, variation in ecological factors could generate significant geographic variation in mating behaviour by influencing the intensity of competition for mates, the evolution of ecologically-relevant traits that affect mating, or the development of traits that are implicated mate selection process (Lewandowski & Boughman, 2008; Cornwallis & Uller, 2010; Miller & Svensson, 2014; Machado et al., 2016). Regardless of process, the resulting behavioural variation across species and populations can be as dramatic as variation in the presence/absence of a behaviour, or as subtle as differences in the incidence or duration of a behaviour (Foster, 1999; Olivero et al., 2017).

Leiobunine harvestmen, commonly known as daddy longlegs, provide an excellent system for studying inter- and intra-species variation in behavioural and morphological traits involved in the coordination of mating. This vastly understudied group contains many species with wide geographic distributions and exhibits high diversity in genitalic traits and male armaments (i.e. pedipalps, which males use to hook the female in an ‘embrace’ prior to mating) that place the species along a spectrum of low to high intersexual

antagonism during mating (Burns et al., 2013; Burns & Shultz, 2015, 2016). In species placed on the low end of the antagonism spectrum, the tips of male penes bear specialized sacs that allow for easy delivery of a nuptial gift to the female prior to intromission; furthermore, the pedipalps are less sexually dimorphic in size (Burns et al., 2013; Burns & Shultz, 2015). In species on the high end of the antagonism spectrum, males also deliver nuptial gifts — though these gifts are lower in essential amino acids (Kahn et al., 2018) — but the specialized penile sacs have been secondarily lost; furthermore, the penes have greater biomechanical strength and females have sclerotized genitalic barricades that can block forced mating attempts (Burns et al., 2013; Burns & Shultz, 2015). Phylogenetic analyses of the distribution of sacculate and non-sacculate species indicate four to five independent transitions from an ancestral mating system of low antagonism to mating systems of high antagonism throughout the clade (Burns et al., 2012, 2013).

Despite the richness of the leiobunine system and their ubiquity across Eastern North America, very little is currently known about their mating behaviour and ecology. Previous studies have been primarily focused on inter-specific variation. In addition to detailed genitalic analysis of species in the clade (Burns et al., 2013; Burns & Shultz, 2015, 2016) and a comparative study of the chemical composition of nuptial gifts (Kahn et al., 2018), two studies provide formal descriptions/analyses of behavioural interactions in the clade. The first details transitions in the sources of selection shaping mating interactions as they progress towards copulation in *L. vittatum* (Fowler-Finn et al., 2014). The second outlines differences in mating dynamics across species in the clade that suggests inter-specific variation in selection shaping mate assessment and reproductive behaviour (Fowler-Finn et al., 2018). Combined, these two studies — in addition to work on other clades of Opiliones (Machado et al., 2015, 2016) — demonstrate that mate assessment occurs over a step-wise series of male-female interactions in clearly defined pre-copulatory, copulatory, and post-copulatory stages. Furthermore, different morphological traits predict success at various stages of mating, and the traits predicting success also differ across species (Fowler-Finn et al., 2014; Fowler-Finn et al., 2018). Despite this progress, formal descriptions of mating behaviour are completely lacking for all species in the leiobunum clade except the non-sacculate *L. vittatum* (Fowler-Finn et al., 2014). There is also no formal information on the likelihood of remating in the clade. Finally, many species cover geographic ranges large enough for

variation in mating season length or resource availability to affect the expression of sexual traits, including behaviour (e.g., Machado et al., 2016); however, no formal studies have been conducted to test or describe geographic variation in mating behaviour.

In order to gain a better understanding of behavioural variation involved in reproduction in leiobunine harvestmen, the aims of the current study are multiple. We provide detailed quantitative and qualitative behavioural descriptions of mating behaviour in six species of leiobunine harvestmen (Figure 1). We then compare mating behaviour and size variation across multiple populations for five of these species. Finally, we conducted remating trials

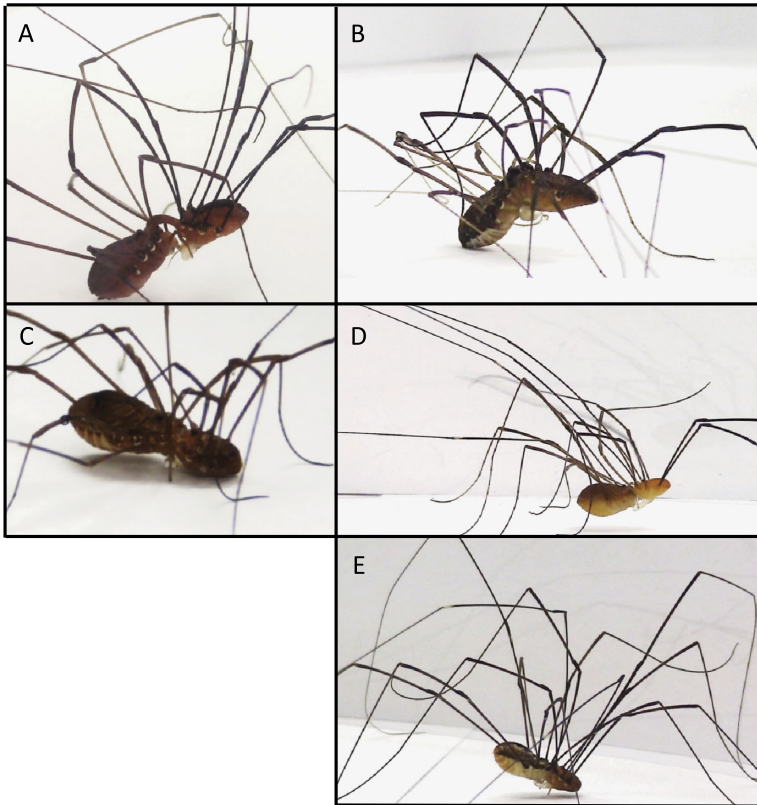


Figure 1. Mating embraces in five species of leiobunine harvestmen described in the current study: A. *Leioibunum vittatum*, B. *L. calcar*, C. *L. ventricosum*, D. *L. politum*, E. *L. aldrichi*. For each pair, the female is on the left and the male is on the right.

for four of the species to determine whether multiple mating is likely to occur.

2. Materials and methods

This study extends over four field seasons, with experiments conducted in four laboratories. We have standardized to the extent feasible across experiments such that the basic methodological approach remains similar across each experiment and we indicate minor site-specific differences in protocol throughout.

2.1. Study organisms

We studied six species of harvestmen in the genus *Leiobunum*, four of which have sacculate penes — *L. aldrichi*, *L. flavum*, *L. politum* and *L. ventricosum* — and two of which are non-sacculate — *L. calcar* and *L. vittatum*. We collected the animals from Missouri, Virginia, Wisconsin and Minnesota, with broad sympatry among many of the species (Table 1). A subset of mat-

Table 1.

Collection of organisms: locations and years of collection of the species/populations of leiobunine harvestmen used in the study.

Species	Location	GPS coordinates	Year (No. of mating trials)
Non-sacculate			
<i>L. calcar</i>	St Louis, MO	38.5461°N: 90.4337°W	2015/2016 (<i>N</i> = 49)
	MLBS, VA	38.0342°N: 78.5129°W	2014 (<i>N</i> = 9)
	KONHSA, MN	44.8118°N: 93.0294°W	2016 (<i>N</i> = 12)
<i>L. vittatum</i>	St Louis, MO	38.5472°N: 90.5439°W	2016/2017 (<i>N</i> = 20)
	Milwaukee, WI	43.0428°N: 87.5331°W	2013 (<i>N</i> = 52)
Sacculate			
<i>L. aldrichi</i>	MLBS, VA	38.0342°N: 78.5129°W	2014/2015 (<i>N</i> = 12)
	Milwaukee, WI	43.0428°N: 87.5331°W	2013 (<i>N</i> = 28)
<i>L. politum</i>	St Louis, MO	38.5472°N: 90.5439°W	2016/2017 (<i>N</i> = 6)
	MLBS, VA	38.0342°N: 78.5129°W	2014 (<i>N</i> = 15)
	Milwaukee, WI	43.0428°N: 87.5331°W	2013 (<i>N</i> = 10)
<i>L. ventricosum</i>	St Louis, MO	38.5461°N: 90.4337°W	2015 (<i>N</i> = 10)
	KONHSA, MN	44.8118°N: 93.0294°W	2016 (<i>N</i> = 27)
<i>L. flavum</i>	St Louis, MO	38.5472°N: 90.5439°W	2016 (<i>N</i> = 0)

MLBS = Mountain Lake Biological Station; KONHSA = Katharine Ordway Natural History Study Area. In 2015, *L. aldrichi* from Virginia used for size data only, and *L. politum* from Missouri were used for size data only.

ing trials from some of the locations was used in a previous study (Fowler-Finn et al., 2018), though here we incorporate an expanded sample from those populations including individuals both with and without body size and weight measurements.

Each species tended to occupy different substrata at each collection locality: *L. aldrichi* were often found on tree trunks in addition to the foliage of small trees, *L. flavum* tended to be on the forest floor amongst sticks and leaf litter, *L. politum* tended to be in low-lying vegetation in addition to low shrubs and trees and the forest floor, *L. ventricosum* on low vegetation, *L. calcar* on leaf litter and the foliage of bushes, and *L. vittatum* primarily on the trunks and foliage of trees. We identified species during collection using external morphological traits and used species-specific genitalic traits (Burns et al., 2012, 2013) to confirm identification after trials were complete.

For most species/localities, we collected animals one to three days prior to running mating trials. For *L. calcar*, we collected animals and maintained them in the laboratory for two days to three weeks before a mating trial. We housed the animals in individual containers consisting of round deli dishes 11 cm in diameter and eight cm in depth except at Mountain Lake Biological Station, VA, USA where we used $10 \times 10 \times 5$ cm plastic containers. We cut large holes in the lids of the deli dishes and stretched insect netting across the hole, thus allowing for both air flow and a substrate on which the animals could climb. We provided water ad libitum and fed the animals with varied fruits, meats and fish food upon arrival in the lab, replacing food and water as well as cleaning cages one to two times a week, or more frequently if needed.

2.2. Mating trials

We conducted mating trials in 30 cm diameter circular arenas constructed from 22 cm high acetate walls and printer paper flooring (Fowler-Finn et al., 2014; Fowler-Finn et al., 2018). We changed the paper flooring and wiped down the table and acetate paper with ethanol in between each trial to remove potential chemical cues. In order to improve visibility for video analysis, we surrounded the arena with white paper that was propped up ~ 10 cm from the arena walls.

Prior to a trial, we gently placed individuals into the arena and retained them within five cm diameter acetate barriers during a two-minute acclimation period (Fowler-Finn et al., 2018). In 2013, only females were acclimated

behind the barrier (Fowler-Finn et al., 2014). A trial started when we lifted the barriers to allow individuals to freely interact and ended when either mating was complete or the female rejected the male three times (following Fowler-Finn et al., 2014). We video recorded all trials with digital camcorders for later behavioural analyses. Note: because *L. flavum* was used only in male:male:female mating trials as part of a separate study, we include them here only for the description of mating and not for quantitative analyses or measurements.

2.3. Remating trials

For three species from Mountain Lake Biological Station — *L. aldrichi*, *L. politum* and *L. calcar* — we ran individuals in a second mating trial zero to four days after the first trial (seven to eight trials per species). For a fourth population — *L. vittatum* from Wisconsin — we ran four remating trials eight to 13 days after the first trial. Finally, we ran only a single remating trial for *L. ventricosum*. In each remating trial, individuals were paired randomly with a different individual than the one encountered in their first mating trial.

2.4. Morphological analyses

At the conclusion of each day during which trials had been run, we weighed all individuals to the nearest 0.0001 g using a Mettler Toledo analytical balance. We preserved specimens in 70% ethanol for morphological analyses. We used cephalothorax width, measured at the widest point on the carapace between legs two and three, as a measure of body size (Fowler-Finn et al., 2014, 2018). Like most arachnids, body size is fixed at adulthood in Opiliones. We oriented individuals in a standardized position under a Leica 205 C microscope fitted with a Leica MC170 HD microscope camera at 4× magnification (SLU) or Olympus SZX10 microscope (Macalester College, *L. ventricosum* and *L. calcar* from MN). Images were captured and measured using Leica imaging software. We also measured male pedipalp femur length, a trait that influences mating dynamics in some species (Fowler-Finn et al., 2014, 2018). We first removed the right pedipalp (or the left when the right was damaged) and laid it in a stereotyped manner on a slide covered with a cover slip. We then took pictures and measured the length from the longest point-to-point distance diagonally across the pedipalp femur using the same techniques and software as for body size measurements (Fowler-Finn et al., 2014, 2018). For each morphological trait, we took two

pictures per individual — removing and then reorienting the body/pedipalp in between the two photos — and measured both images twice. The final measurement was taken from the mean of means of the two measurements for the two images. All individuals performing measurements were trained in the same way by KDFE, and repeatability estimates of measurements exceeded 0.98 ($p < 0.05$) for all data sets.

2.5. Behavioural video analysis

Following Fowler-Finn et al. (2014, 2018), we examined a standard set of behaviours: if a mating attempt occurred, if the attempt was successful, if the female resisted the first attempt by a male, if mating occurred, if postcopulatory contact occurred for \geq five seconds (see behavioural descriptions below in ‘General patterns of mating’; Figure 2). We also quantified the timing of: first contact to first attempt, attempt to successful embrace, attempt to failure of the attempt, length of intromission, length of postcopulatory contact (Figure 2). For samples sizes measured for each behaviour, see Table 3.

2.6. Statistical analyses

To test for species and geographic variation in the presence/absence of behavioural traits, we analysed the full data set from all species and all populations together in nominal logistic models. Each of the behavioural responses (yes/no for attempt, successful embrace, female resistance, mating, and postcopulatory mate guarding) were considered as the response variables, and species and population were main effects. We used JMP v12 for the analyses.

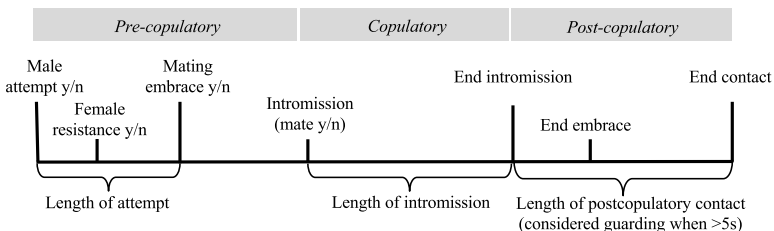


Figure 2. Timeline of the mating sequence in *leiobunine harvestmen*, with major behaviours quantified in this study indicated with hash marks. Behaviors are broken into pre-copulatory, copulatory, and post-copulatory stages of mating.

3. Results

3.1. Morphological variation — body size, weight, sexual dimorphism and palp:body measurements

For all species, the body size ratio between males and females was close to 1. Furthermore, the body size was largest in southern populations and smallest in northern populations (Table 2; Figure 3). Across species, we found that females were consistently heavier than males (Table 2; Figure 3). However, the male:female weight ratio was variable across both populations and species (Table 2; Figure 3). The smallest differences between male and female weight were found in *L. calcar*, and the largest in *L. ventricosum* and *L. vittatum* (Table 2; Figure 3). Finally, the male palp:body size ratio was greatest in *L. vittatum* and *L. politum* (Table 2).

3.2. General patterns of mating

All species studied followed a similar sequence of events during mating interactions (Figure 2). Initial contact appeared to be primarily accidental,

Table 2.

Morphological variation. Variation in male:female size ratios for weight and body size, as well as the male pedipalp length to body size ratio, in various species and population of leionunine harvestmen.

Species	Population	Male/female weight	Male/female body size	Pedipalp length/body size
Non-sacculate				
<i>L. calcar</i>	MO	0.65	1.00	0.48
	VA	0.69	1.04	
	MN	0.72	1.03	0.49
<i>L. vittatum</i>	MO	0.44	0.94	
	WI	0.54	0.97	0.81
Sacculate				
<i>L. aldrichi</i>	VA	0.54	0.97	0.51
	WI	0.62	0.97	0.48
<i>L. politum</i>	MO	0.63		
	VA	0.50	0.95	0.85
	WI	0.50	0.93	
<i>L. ventricosum</i>	MO	0.48	0.91	
	MN	0.45	0.91	0.45

Blanks indicate cells for which we do not have data.

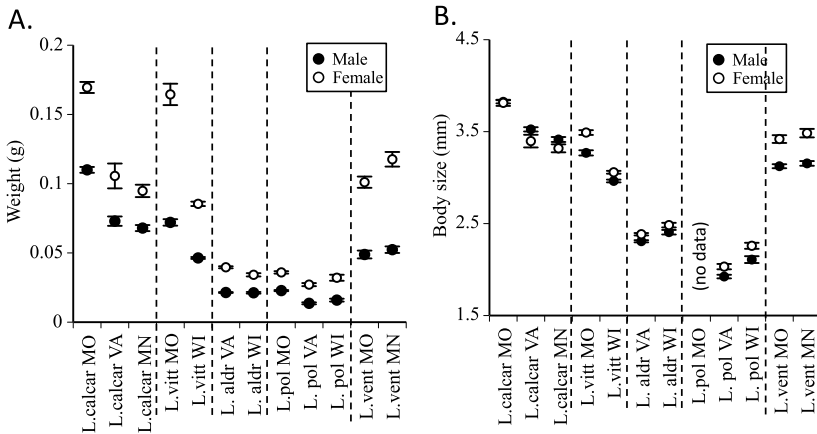


Figure 3. (A) Weight and (B) body size variation across species and populations of leiobunine harvestmen. Samples sizes per population (male weight, female weight, male CW, female CW) are as follows: *L. calcar* MO ($N = 45: 42: 46: 45$), *L. calcar* VA ($N = 9: 7: 14: 8$), *L. calcar* MN ($N = 11: 6: 12: 7$), *L. vittatum* MO ($N = 20: 20: 20: 19$), *L. vittatum* WI ($N = 52: 52: 45: 52$), *L. aldrichi* VA ($N = 108: 59: 91: 51$), *L. aldrichi* WI ($N = 28: 28: 28: 25$), *L. politum* MO ($N = 10: 5, 0, 0$), *L. politum* VA ($N = 15: 14: 15: 15$), *L. politum* WI ($N = 11: 11, 0, 0$), *L. ventricosum* MO ($N = 16: 16: 16: 16$), *L. ventricosum* MN ($N = 27: 27: 26: 23$).

although we observed a couple of exceptions in *L. ventricosum* mating trials, in which males walked straight towards the female very quickly prior to making physical contact with her. Most commonly, initial contact was followed immediately by an attempt by the male to secure the female in a ‘mating embrace’ (Figure 1): the male and female orient face-to-face with the male pedipalps hooked behind the coxae of the female’s second legs, and the female pedipalps and chelicerae were free to contact the male genitalic operculum and genitalia (except in *L. calcar*, see below).

After securing a female in a mating embrace, the male everts his penis, which everts via hydraulic pressure during mating and retracts within the abdominal cavity while not mating. Prior to insertion of the penis into the opening of the genital operculum, the male may insert his penis first into the female’s oral opening to deliver a nuptial gift, with delivery facilitated by penial sacs in some species. The precise timing of these events is difficult to discern in most species because of the speed at which it occurs and the proximity of the female mouth and genitalia. Furthermore, in both sacculate and non-sacculate species, the delivery of the nuptial gift can occur during copulation. While the male genitalia is everted, females touch and stroke the

inflated haematadocha — the hydrostatically inflated sac at the base of the penis — with their pedipalps and chelicerae.

Most species have a single insertion and end the embrace shortly after insertion is complete. The embrace ends when one of the individuals breaks away, although it is often difficult to discern which one initiates the disengagement. Sometimes the male pulls up and out of the embrace or the female shakes the male to end the embrace. Postcopulatory contact or guarding by the male can follow copulation.

3.3. Species descriptions and population variation

3.3.1. *Leiobunum vittatum*

Mating in *L. vittatum* has been previously described (Fowler-Finn et al., 2014); we provide a brief description here with the main focus on identification of inter- and intra-species variation in mating behaviour. The entirety of the mating sequence in *L. vittatum* is the longest of the species studied, sometimes lasting three or more hours (Figure 4A), and is characterized by drawn out struggles between the male and female prior to the mating embrace, as well as multiple penile eversions and insertions.

After contact, nearly all males attempt to copulate. Females exhibit strong resistance to male attempts in the form of: running away, body bobbing, and assuming a face down position that appears to prevent the male from being able to hook his pedipalps behind the coxae of the female's second legs (Fowler-Finn et al., 2014). After the male secures the female in a mating embrace, there is a short delay before he everts his penis, which often follows tapping of the male genital operculum by the female with her pedipalps and chelicerae (Fowler-Finn et al., 2014). Next, several eversions and partial intromissions (which could be either into the female's oral opening or genital operculum) occur, during which females use their pedipalps to grasp behind two prominent protrusions on the inflated haematodochal sac (Fowler-Finn et al., 2014). The incomplete insertions are followed by a final moment of what appears to be full copulation. Afterward the male and female disengage from the mating embrace: they either separate—typically with the female walking off—or remain in contact (60% or more of trials) with the male contacting the female on multiple body parts including wrapping his legs around hers.

3.3.1.1. Population variation. The primary qualitative difference between the two populations was leg wrapping behaviour. In the Wisconsin population, the males always wrapped their third pair of legs around the femurs

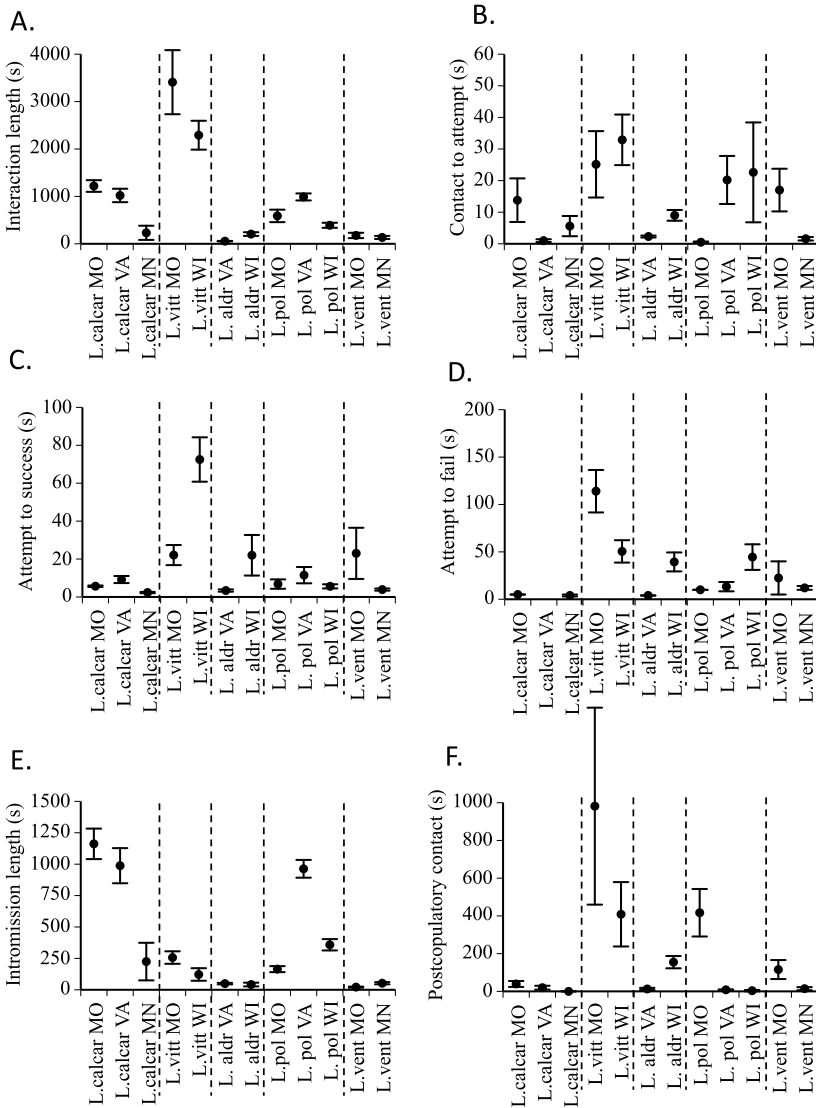


Figure 4. Variation in the length of various behaviours and stages of mating interactions in different species and populations of leiobunine harvestmen. For sample sizes, see Table 3.

of the females’ second pairs of legs to achieve the embrace, and the leg wrapping appears to be necessary in order to properly secure the female (Fowler-Finn et al., 2014). In the Missouri population, leg wrapping did not

Table 3.

Sample sizes for total mating trials run, trials in which we measured the length of different stages in mating, and trials in which the presence/absence of behaviours was measured for each population of five species of leibunine harvestmen.

Species	<i>L. calcar</i>			<i>L. vittatum</i>		<i>L. aldrichi</i>		<i>L. politum</i>			<i>L. ventricosum</i>	
	MO	VA	MN	MO	WI	VA	WI	MO	VA	WI	MO	MN
Total trials run	49	14	12	20	52	12	28	12	25	12	16	27
Interaction length	34	9	6	11	21	8	7	0	12	7	8	23
Contact to attempt	37	9	10	19	50	10	26	6	15	0	10	26
Attempt to success	35	9	6	18	16	8	7	5	12	7	8	24
Attempt to fail	2	0	3	1	25	0	19	1	3	3	2	2
Intromission length	35	9	6	11	24	8	7	5	12	0	0	22
Postcop contact	34	9	7	11	24	8	7	5	12	0	0	23
Attempt y/n	48	9	12	20	52	12	28	7	15	11	10	27
Success y/n	37	9	10	19	50	12	25	6	15	11	10	26
Resist y/n	37	9	9	19	50	10	25	6	15	0	10	26
Mate y/n	49	9	12	20	52	12	28	6	15	10	10	27
Guard y/n	34	9	12	11	24	8	7	5	12	0	8	23

occur in every mating pair, and was not necessary for achieving the mating embrace.

In terms of quantitative variation, female resistance was more frequent in the Wisconsin population, with a corresponding lower success rate of males for achieving the mating embrace; however, mating rates were near 50% for both populations because a lower proportion of males that were successful in achieving the embrace in the Missouri population ended up mating. Additionally, the Wisconsin population took longer to achieve the mating embrace, but failures were much shorter; and both intromission and the total interaction length were shorter in the Wisconsin population (Figure 4).

3.3.2. *Leiobunum calcar*

The mating sequence in *L. calcar* typically lasts around 20 minutes, though this duration is highly variable across populations (Figure 4A). The majority of males attempt to secure the female in a mating embrace, with very few failed attempts (Figure 5A). Males shake extremely rapidly by undulating their body so quickly that it is impossible to measure the speed with a regular speed camera. This shaking appears to assist in proper positioning of the male to secure the female with his pedipalps, which have an additional

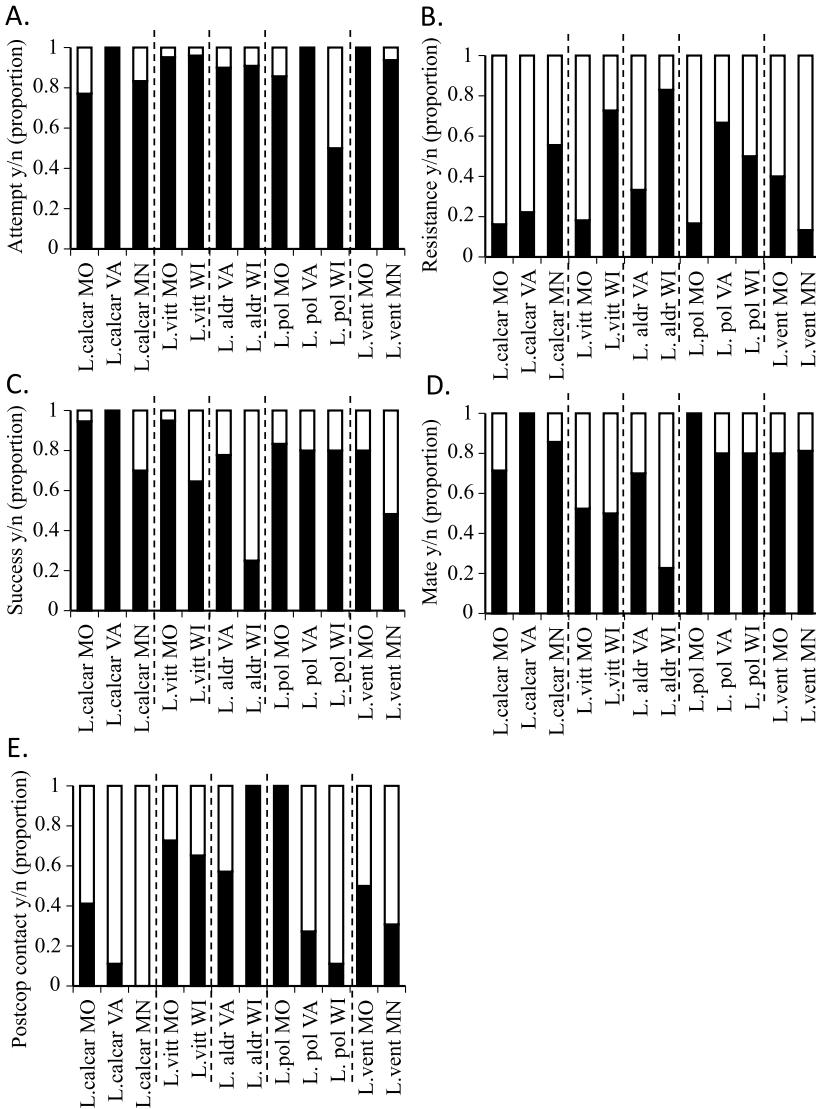


Figure 5. Variation in the proportion of mating trials that demonstrated each of the behaviours indicated across different species and populations of leiobunine harvestmen. For sample sizes, see Table 3.

protrusion on their pedipalpal femurs called an apophysis, which is unique among the species studied. During this rapid shaking, the females remain relatively still, and female resistance was low (Figure 5B). When a female

did resist, she typically ran from the male before he was able to attempt to achieve the mating embrace. The positioning of the female pedipalps during mating is unique among the species studied: female pedipalps are held either out to the side or above the male's carapace, likely because of how the male pedipalps articulate with the female in the embrace. For example, in the Mountain Lake Biological Station population (for which we quantified this behaviour), in seven of 15 trials in which mating occurred, the female pedipalps were clearly seen above the male carapace; in eight trials, the pedipalps were out to the side and not touching the male genitalia; in only one trial did the female pedipalps come into contact with the haematodocha of the male genitalia.

After achieving the mating embrace, the male curls the ends of his third legs around the abdomen of the female (Figure 1), forming a loose basket around her; he then squeezes his legs around her while lightly vibrating his body. Eversion and insertion occur immediately after the male secures the female, and both the male and female angle their faces upward so their bodies are not parallel to the substrate or each other. Males insert only once, and the females remain relatively still during intromission, which is the longest of the species (Figure 4A). During the first portion of the insertion, males will rapidly wave their third legs above the female, and the males do not fully touch their haematodocha to the females' genital opening, as observed in other species; thus, the sclerotized portion of the penis remains visible. During the latter part of insertion, the males appear to have decreased hydrostatic pressure in their limbs, and the haematodocha is fully inflated and in direct contact with the female genital operculum.

The interaction typically ends with the male lifting up and out of the embrace. Males appear capable of preventing females from ending the interaction, as female movement does not seem to be able to dislodge the male from the embrace. As the pair is disengaging from the embrace and separating, the tip of the male penis can be seen still inside the female genital opening, which does not occur in the other species studied. Postcopulatory contact is very rare in *L. calcar* (Figure 5E).

3.3.2.1. Population variation. We observed a few qualitative differences between the mating behaviours exhibited in the Virginia and Missouri populations. In Virginia, males typically wrapped their third legs around the females' fourth legs and appeared more drained at the conclusion of the mating interaction. Finally, at the conclusion of the mating embrace, Vir-

ginia males lifted up their bodies and walked away, whereas Missouri males stayed in place, with the females walking away.

Quantitative differences among populations were subtle. While attempt rates were generally high, they were somewhat variable, with the lowest number of attempts in the Minnesota population (Figure 4). Resistance was lower in Virginia than the other two populations, and, correspondingly, attempts in successful trials were longer (Figure 4). Finally, intromission was considerably shorter in the Minnesota population compared to the other two populations (Figure 4).

3.3.3. *Leiobunum ventricosum*

The mating sequence in *L. ventricosum* is relatively short, lasting on average two to three minutes (Figure 4A). The majority of *L. ventricosum* males attempt to mate with variable success across populations (Figure 5). When a male attempts the mating embrace, he rapidly puts himself into position facing the female and hooking his pedipalps behind her second legs' coxae, during which time the female remains still. Female resistance is fairly low and consists primarily of the female spreading her legs out and assuming a head down position, which makes it difficult for the male to secure the embrace.

After achieving the mating embrace, the male immediately everts and inserts his genitalia; he then curls the ends of his third legs to grasp any of the female's legs that he contacts. Sometimes, male *L. ventricosum* will even grasp their own back legs if they come in contact with them during the embrace. While grasping the female, the male waves his first pair of legs above the female rapidly. During the insertion of the male genitalia, the female places her pedipalps on either side of the haematodocha, which is fully inflated and can be seen pumping. While female contact with the haematodocha is common in most species and also found in *L. ventricosum*, female *L. ventricosum* do not move their pedipalps, instead leaving them hanging down prominently on either side of the haematodocha. However, the female rubs the top of the haematodocha with her chelicerae, running them along the top towards her mouth.

After a minute of intromission (on the short side for the species studied; Figure 4E), the male angles the back end of his body upwards and pulls out of the mating embrace, and the female remains still with her body slightly elevated. At this point, sometimes the mating interaction ended, but there were frequent remating events that occurred within a single interaction in

this species. One of two behavioural sequences occurred when remating happened. The male remained with his pedipalps behind the coxae of the female's second pair of legs, but his body lifted up and out of the mating position before lowering himself and remating with the female. Or, the pair contacted each other again shortly after separation from the mating embrace and remated.

3.3.3.1. Population variation. The two populations of *L. ventricosum* studied here exhibited very similar mating behaviour, but there were some quantitative differences. Success rates were lower in the Minnesota population (Figure 5C), although it took longer to achieve a successful mating embrace in the Missouri population (Figure 4). Also, when post-copulatory contact occurred (as happened in fewer than 50% of trials for both populations; Figure 5E), it was shorter in the MN population (Figure 4F).

3.3.4. Leiobunum politum

The mating sequence in *L. politum* typically lasts on average six or seven minutes to a little over 15 minutes, depending on population (Figure 4A). In two of three populations, nearly all males attempt to secure females in a mating embrace (Figure 5A). It is notable that in *L. politum* most first attempts are not successful, as females often run away before a male can successfully secure them; however, the majority of males are successful upon their second attempt (Figure 5C). To secure a female in a mating embrace, males undulate their bodies to position themselves to place their pedipalps behind the coxae of the female's second legs, while the female remains relatively still. The motion of males is similar to that seen in *L. calcar* except greatly reduced in speed. In fact, *L. politum* appears in the field and in mating trials in the lab to generally move more slowly than other species in the clade observed for this study (except for the Missouri population, see below in population comparisons).

The mating embrace is quickly followed by eversion and subsequent insertion, with male-female pairs remaining relatively parallel to the ground during insertion. The male haematodocha is fully inflated and extended and lacks the "handles" observed in *L. vittatum* and *L. calcar*. A pumping motion within the haematodocha is clearly observed as it elongates and shortens rhythmically. Females run their chelicerae along the top of the haematodocha and rest their pedipalps on either side of the haematodocha, occasionally moving the pedipalps back and forth in short bouts. Throughout insertion,

the pair cycles through periods of relative calm broken up by periods of brief struggles. During the calm periods, both male and female remain still other than the pumping motion of the male genitalia and movements of the female chelicera and pedipalps. Males also stick their third pair of legs in the air, and wave them in a circular motion, sometimes over the top of the female. The third leg motions are similar to, but much slower than, the motions observed in *L. calcar*. When the male's third legs contact the female's or his own limbs, he will lightly grasp those legs, similar to what occurs in *L. ventricosum*. The embrace ends typically by the female disengaging and running off. Postcopulatory behaviour was nearly absent in *L. politum* (Figure 5E).

3.3.4.1. Population variation. Qualitatively, the Virginia and Wisconsin populations were very similar to one another in mating behaviour. However, the Missouri population of *L. politum* exhibited behaviour that was distinct. Most notably, they appeared to move significantly faster than the Wisconsin and Virginia populations, and their resistance behaviour was distinct: females would commonly flip over the male in a summersault in order to evade male attempts at securing a mating embrace.

We found significant quantitative variation among populations. Attempts and thus mating rates were lowest in the Wisconsin population, although the success rates of attempts were similar across all populations (Figure 5). Intromission was much longer in the Virginia population than the other two, and the total interaction time was also longer in the Virginia population (Figure 4). Attempts after initial contact were almost immediate in the Missouri population, and intromission was shortest in the Missouri population. Long postcopulatory contact (longer than all other species except *L. vittatum*) was present in all trials in the Missouri population (Figure 5E, Figure 4F); in contrast, post-copulatory contact was rarely observed in the Virginia or Wisconsin population, and when it occurred it was brief (Figure 5E, Figure 4F).

3.3.5. Leiobunum aldrichi

The mating sequence in *L. aldrichi* is one of the shortest of the species we studied, typically lasting one to three and a half minutes, which is similar to the length of interactions in *L. ventricosum* (Figure 4A). Nearly all males attempt to mate, and the incidence of resistance by females differs substantially between the two populations studied (Figure 5). Resistance by the females included both running and/or bobbing behaviour, and any unsuccessful attempts were very brief. Prior to achieving the embrace, males shake the females vigorously and females remain still.

After achieving the mating embrace, the male everts and then inserts his penis in quick succession. The male and female remain relatively parallel to the substrate during intromission, and the haematodocha — which lacks handles — appears fully inflated for the duration of intromission. The pair remains fairly still during intromission and no notable behaviours occur other than a pumping motion of the male genitalia and female stroking of the haematodocha with her pedipalps and chelicerae.

Postcopulatory contact after the conclusion of the embrace is common (Figure 5E). When postcopulatory contact does not occur, the female leaves the male where he remains in place. When postcopulatory contact does occur, the male remains near the female, touching her legs with his pedipalps and chelicerae on her femurs until he finds the female's second leg (an elongated pair involved in gathering sensory input). Males that initially grab legs other than the second leg continue searching until they find the second leg. They then run the female's leg through their chelicerae and pedipalps, appearing to nibble on it. In some cases, the male will then violently and quickly shake up and down in bouts of 1–5 shakes or more while still holding the female's second leg in his chelicerae. Note: this shaking behaviour has also been observed between interacting males in the field and laboratory (Fowler-Finn pers. Obs.).

3.3.5.1. Population variation. We observed no obvious qualitative differences between mating trials of different populations but did find significant quantitative differences. The Virginia population had higher rates of attempts and successful embraces, as well as lower rates of female resistance to attempts (Figure 4). In Virginia, males also attempted more quickly, secured the females more quickly, and were rejected more quickly in unsuccessful trials (Figure 5). While the length of intromission was similar across populations, the total length of contact between males and females was significantly shorter in the Virginia population (Figure 4). Finally, in the Wisconsin population, males guarded in 100% of the trials in which mating occurred, versus only approx. 60% in the Virginia population, and Wisconsin males also guarded for significantly longer (Figures 4 and 5).

3.3.6. L. flavum

Our description of *L. flavum* mating interactions is based on male-male-female trials, and so we provide only a qualitative description here. Similarly to *L. ventricosum*, *L. flavum* mates multiply. However, unlike *L. ventricosum*

or any other species documented here, in between the mating bouts, the male disengages from the mating embrace, then climbs onto the female, rotating his body to face in the same direction as the female. While on top of the female, he shakes, and then re-engages in the face-to-face embrace. This sequence can be repeated 1-3 times and has been observed both in the field and the laboratory.

3.4. Combined analyses

Most behaviours were influenced by species, population, or a combination of the two factors (Table 4). However, the incidence of attempts, and the length of failed attempts did not correspond with either factor (Table 4). All other behaviours measured were influenced by both factors, except that resistance was only dependent on population (Table 4).

3.5. Primary differences among species

The most distinctive attempts to achieve the mating embrace were *L. vittatum*'s long struggles with high female activity and resistance, and *L. calcar*'s rapid undulations and very low female resistance. The most distinctive behaviours during the embrace include: *L. calcar* males holding the female pedipalps on top of their body or to the side so that she has no access to the haematodocha and vibrating their bodies shortly after achieving the embrace; leg wrapping behaviour in *L. vittatum*; and males waving their third legs and grasping the female legs in *L. politum* and *L. ventricosum*. In terms of intromission, the following behaviours stood out: *L. vittatum* exhibited a long

Table 4.

Behavioural variation: variation across species and populations in the likelihood of various behaviours being exhibited during mating interactions in leiobunine harvestmen.

		df	χ^2	<i>p</i>	<i>N</i>	
Attempt y/n	Species	4, 7	10.8	0.0289*	251	Highest in MO
	Pop	3, 7	3.4	0.3346		Highest in MO
Success y/n	Species	4, 7	9.7	0.0455*	230	Highest in MO
	Pop	3, 7	13.6	0.0035***		Lowest in WI
Resist y/n	Species	4, 7	3.2	0.5281	216	Highest in WI
	Pop	3, 7	41.2	<0.0001***		Lowest in VA/MO
Mate y/n	Species	4, 7	13.6	0.0087***	250	Lowest in WI
	Pop	3, 7	9.8	0.0202*		
Guard y/n	Species	4, 7	24	<0.0001***	153	Highest in WI
	Pop	3, 7	16.5	0.0009***		Lowest in VA/MN

delay between the embrace and intromission, and *L. calcar* had the longest intromission of the species studied whereas *L. ventricosum* and *L. aldrichi* had the shortest intromissions. Finally, in the postcopulatory phases, the most distinct behaviours included: lengthy guarding by *L. vittatum* versus a complete lack of guarding in *L. calcar*; biting the female's second leg and violent shaking in *L. aldrichi*, and multiple mating in *L. ventricosum* and *L. flavum*.

3.6. Remating trials

Eight of eight female *L. aldrichi* re-mated; seven of eight *L. politum* re-mated, seven of eight *L. calcar* re-mated; two of four *L. vittatum* re-mated; and, one of one *L. ventricosum* re-mated (with the two trials being on the same day).

3.7. Variation in presence/absence of behaviours across species and populations

We found significant variation among species in the presence/absence of all behavioural traits except resistance and found significant population variation in all of these same behaviours except attempts (Table 4). Attempts and success rates were highest in MO (Table 4). Resistance was highest, success was lowest, and guarding was highest in WI (Table 4).

4. Discussion

We provide detailed descriptions of mating behaviour in six species of leiobunine harvestmen, with the mating behaviours of five species being described formally for the first time. We also compared mating behaviour in five of the species across four geographic locations. Leiobunine harvestmen exhibit highly diverse mating behaviours in pre-, peri-, and post-copulatory mating stages, and significant variation in mating dynamics across species and populations. We also found high rates of remating, suggesting a highly polygamous nature of the clade. Finally, we documented substantial variation in size and weight across species and populations, with individuals tending to be larger in body size in more southern populations in non-sacculate species, and the opposite pattern for sacculate species.

Mating interactions across all species were characterized by a highly conserved sequence of interactions (Fowler-Finn et al., 2014, 2018), starting with the mating embrace and involving tactile exchanges between the female pedipalps and chelicerae, and the male genitalia (with the exception of

L. calcar, for which males appear to hold the female pedipalps away from the male genitalia). However, species exhibited distinct pre-, peri-, and post-copulatory behaviours as well as variation in the length of these stages. Some of these behaviours—for example, *L. aldrichi* males biting the second leg and shaking vigorously—may function in the context of female cryptic choice (Eberhard, 1996). Others — long copulation in *L. calcar* or post-copulatory contact in *L. vittatum* — may function to increase sperm transfer or reduce competition with other males (e.g., Alcock, 1994; Simmons, 2001; Laird et al., 2004; Machado & Macías-Ordóñez, 2007; Harts & Kokko, 2013; Sato et al., 2017), guard the female from future potential mates (Boggs, 2018), or decrease the chances the female will mate again quickly (Parker, 1970; Simmons, 2001; Linn et al., 2007). The significant variation in the dynamics and length of different stages of mating interaction suggests that species may vary in the strength of mate competition, cryptic choice, or other aspects of selection that occur during mating interactions. Mate assessment can occur across multiple stages of mating interactions in other taxa (Andersson & Simmons, 2006; Kvarnemo & Simmons, 2013; Fowler-Finn et al., 2014), and so these behavioural patterns suggest that leiobunine harvestmen could serve as an excellent system for understanding diversification across mating stages.

While behavioural diversity was high, some mating behaviours were shared among species. Both species in the early season clade (Burns et al., 2012) — *L. flavum* and *L. ventricosum* — exhibited multiple mating embraces and copulations, which are behaviours we have also observed in these species on multiple occasions in the field (Fowler-Finn, pers. obs.). *Leiobunum politum* and *L. calcar* are in sister clades (Burns et al., 2012) and males in both species wave the third leg in circles above the female and undulate while securing the female in a mating embrace. Increased sampling comparing sister species could reveal further behavioural homologies that are phylogenetically informative (Prum, 1990). Interestingly, despite the variation we observed across species, no clear qualitative or quantitative patterns in behaviour emerged distinguishing sacculate and non-sacculate species. However, the non-sacculate species — *L. vittatum* and *L. calcar* — were the only two species to possess ‘handles’ on the hemata and angled their bodies towards each other during copulation, though this did not correspond to any clear behavioural differences in the female interactions with the male genitalia during copulation.

In addition to showing significant variation among species, we also found qualitative and quantitative differences in behaviour across populations. Most notably in *L. politum*, in which the Missouri population differed enough from the other two populations — showing prolonged post-copulatory contact and more intense interactions with distinctive front flips the females used to evade mating attempts — that we suggest this population should be more closely examined to determine if it constitutes a distinct species. Shoemaker et al. (2017) noted that penis anatomy is variable within *L. politum*, with distinct sacs visible in specimens from Maryland, but reduced to the point that they may be absent entirely in Minnesota and likewise called for additional effort around species delimitation in this lineage (Shoemaker et al., 2017). However, nearly all behaviours we measured varied geographically, which could arise from multiple sources of selection or drift. While we did not test reproductive isolation among populations, geographic variation in behaviour can affect patterns of reproductive isolation (e.g., Miller et al., 1998).

The patterns of variation in body size were intriguing. While non-sacculate species had smaller individuals in northern populations — a pattern consistent other terrestrial arthropods (Horne et al., 2015) — sacculate species had smaller individuals in more southern populations. While the cause of the seemingly opposite patterns in body size variation across latitudes is unknown — and a larger sample of populations might reveal a different pattern — the observed variation suggests the potential for ecological factors to generate geographic variation in behaviour. Ecological factors affecting size — e.g., temperature (Atkinson, 1994; Forster et al., 2012), food availability (Diamond & Kingsolver, 2010) and season length (Chown & Gaston, 2010) — could affect the development of traits involved in the coordination of mating and consequently sexual selection and mating behaviour. Furthermore, other ecological factors could affect mating behaviour: the length of the mating season can influence the intensity of competition for mates (Emlen & Oring, 1977; Machado et al., 2016); food availability can influence the willingness of females to engage in mating interactions (Rowe et al., 1994); and, a multitude of other ecological factors that can vary geographically can influence the expression of sexual traits and the action of sexual selection (Miller & Svensson, 2014; Machado et al., 2016). Geographic variation in behaviour could also result from sampling populations at different points in the mating season, as many animals show seasonality in mating behaviour (Qvarnström et al., 2000; Kahn et al., 2013; Cirino &

Miller, 2017). In a separate study, we found that as little as two weeks can affect mating dynamics (e.g., likelihood of mating, levels of aggression, length of interactions; Fowler-Finn, Boyer, Ikagawa, Kahn and Larsen, unpublished data). Testing this idea would require a deeper understanding of the phenology of multiple populations, as detailed information on seasonality is almost entirely lacking across this group.

Consistent with findings that many species of Opiliones are polygynandrous (Machado & Macías-Ordóñez, 2007), we found high rates of remating in the species tested. Multiple mating by females is generally common in nature (Taylor et al., 2014), and can have consequences for selection shaping male competition (Kvarnemo & Simmons, 2013). Multiple mating allows for the potential for sperm competition (Parker, 1970; Kvarnemo & Simmons, 2013), as well as cryptic female choice during or after copulation (Eberhard, 1996). Harvestmen sperm are immotile (Alberti, 1995), stored in small female sperm receptacles at the tip of the ovipositors, and fertilize eggs as they are oviposited (Moya et al., 2007); thus, sperm competition would likely favor increased sperm transfer, which could be achieved through longer copulation and/or post-copulatory contact (e.g., Alcock, 1994; Simmons, 2001; Harts & Kokko, 2013; Sato et al., 2017), both of which are variable within and across leiobunine species. Remating could also generate conflict between males and females, as a female could benefit from acquiring more nuptial gift without much benefit to the male. Nuptial gifts are rich in amino acids (Kahn et al., 2018), and it has been suggested that female *L. vittatum* may prolong mating for longer with unwilling males in order to procure more nuptial gift (Fowler-Finn et al., 2014).

To summarise, this study demonstrates high inter- and intra-species variation in a multitude of aspects of mating behaviour and dynamics in the leiobunine clade of harvestmen. Size variation along a latitudinal gradient suggests that ecological variation could generate geographic variation in mating behaviour. Despite high variation in mating dynamics and behaviours across species, we did not identify any aspects of mating that differed between sacculate and non-sacculate species. Finally, high polygamy coupled with interesting peri- and post-copulatory mating behaviours sets the stage for interesting dynamics involving cryptic choice and sperm competition. The leiobunine clade offers a system rich with behavioural adaptations for mating and a wide range of behavioural phenomena shaped by sexual selection that could be studied within a geographic context to better understand the role of ecology in the evolution of mating behaviour.

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