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Partenogênese facultativa e comportamento de acasalamento no escorpião amarelo (Scorpiones: Buthidae: *Tityus serrulatus*)

Gracielle de Fátima Braga Pereira

Belo Horizonte – MG

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Partenogênese facultativa e comportamento de acasalamento no escorpião amarelo (Scorpiones: Buthidae: *Tityus serrulatus*)

AUTOR: GRACIELLE DE FÁTIMA BRAGA PEREIRA

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“Para se ter sucesso, é necessário amar de verdade o que se faz. Caso contrário, levando em conta apenas o lado racional, você simplesmente desiste. É o que acontece com a maioria das pessoas.”

Steve Jobs

Resumo

A maioria das populações do escorpião amarelo (*Tityus serrulatus* Lutz & Mello, 1922) se reproduz por partenogênese, e apenas algumas populações que se reproduzem sexualmente são conhecidas. Por muitos anos, a partenogênese foi considerada a única forma de reprodução dessa espécie. Porém, em 2009, o macho da espécie foi descrito pela primeira vez. Embora as causas da partenogênese no escorpião amarelo ainda sejam desconhecidas, pode-se questionar se as populações partenogenéticas estariam reprodutivamente isoladas das populações sexuais. Além disso, podemos perguntar se populações de *T. serrulatus* podem apresentar comportamentos diferentes durante o acasalamento, com fêmeas mais resistentes e machos mais coercivos, quando comparados a outras espécies de escorpiões em que a reprodução sexuada é obrigatória. No presente estudo, relatamos a reprodução partenogenética em fêmeas de uma população sexual, seja isolada em laboratório desde o nascimento ou coletada em estágios juvenis. Os resultados sugerem que a reprodução assexuada é facultativa nesta população. Por meio de observações comportamentais registradas em laboratório, descrevemos, pela primeira vez, o comportamento de acasalamento do *T. serrulatus* e comparamos o comportamento sexual de machos de *T. serrulatus* com fêmeas sexuais e partenogenéticas. Também investigamos se machos e fêmeas do escorpião amarelo emitem feromônios sexuais de contato e, em caso afirmativo, se sua produção e identificação ainda estão presentes em fêmeas de uma população exclusivamente partenogenética. Finalmente, descrevemos os comportamentos de competição para acasalamento entre machos de *T. serrulatus*. Concluímos que as fêmeas do escorpião amarelo não se mostraram mais resistentes ao acasalamento do que as fêmeas de algumas espécies de escorpiões que se reproduzem apenas sexualmente. Como em outras espécies de escorpiões, a coerção aparentemente não está presente, não contribuindo para a manutenção da reprodução sexuada em *T. serrulatus*. Além disso, as fêmeas partenogenéticas do escorpião amarelo podem aceitar o acasalamento com machos dessa espécie, apresentando os mesmos padrões de comportamento observados em casais com fêmeas da população sexual. No entanto, as fêmeas partenogenéticas mostraram mais resistência ao acasalamento. Nossos resultados mostraram que fêmeas e machos de populações sexuais produzem substâncias químicas de sinalização. Além disso, as fêmeas de populações partenogenéticas parecem ter perdido a capacidade de produzir feromônios sexuais, mas ainda são capazes de identificar substâncias produzidas por machos. Em relação à competição masculina, identificamos três táticas de competição: “luta entre machos”, “interrupção de acasalamento” e “dança entre machos”. Nossas observações em relação a competição nos permitem concluir que simular o comportamento feminino pode ser uma tática alternativa utilizada pelos machos para evitar o combate físico. Nesta tese, registramos características envolvendo comportamentos de acasalamento em *T. serrulatus* e discutimos possíveis fatores que poderiam levar ao isolamento reprodutivo entre populações sexuais e partenogenéticas.

Palavras-chave: Acasalamento. Competição masculina. Feromônio. Isolamento reprodutivo. Partenogênese. *Tityus serrulatus.*

Abstract

Most yellow scorpion (*Tityus serrulatus* Lutz & Mello, 1922) populations reproduce by parthenogenesis, and only a few sexually reproducing populations are known. For many years, parthenogenesis was considered the only form of reproduction of this species. However, in 2009 the male of the species was described for the first time. Although the causes of parthenogenesis in the yellow scorpion are still unknown, one can ask whether parthenogenetic populations would be reproductively isolated from sexual population ones. Moreover, we can ask if populations of *T. serrulatus* may present different behaviors during mating, with more resistant females and more coercive males, when compared to other species of scorpion in which sexual reproduction is obligatory. In the present study, we report parthenogenetic reproduction in females from a sexual population, either isolated in laboratory since birth or collected at juvenile stages. The results suggest that asexual reproduction is facultative in this population. Through behavioral observations recorded in the laboratory, we describe, for the first time, the mating behavior of the *T. serrulatus* and we compared the sexual behavior of *T. serrulatus* males against sexual and parthenogenetic females. We also investigated whether males and females of the yellow scorpion emit contact sexual pheromones and, if so, whether their production and identification are still present in females from an exclusively parthenogenetic population. Finally, we describe competition behaviors for mating between males of *T. serrulatus*. We conclude that females of yellow scorpion showed no more resistant to mating than females of some species of scorpions that reproduce only sexually. Like other species of scorpions, coercion is apparently not present, not contributing to the maintenance of sexual reproduction in *T. serrulatus.* Moreover, parthenogenetic females of the yellow scorpion can accept mating with males, showing the same behavior patterns observed in pairings with females from the sexual population. However, parthenogenetic females showed more resistance to mating. Our results showed that females and males from sexual populations produce signaling chemicals. In addition, females from parthenogenetic populations appear to have lost the ability to produce sexual pheromones, but are still able to identify substances produced by males. In relation to male competition, we identified three competition tactics: "fight between males", "mate interruption" and "dance between males". Our observations regarding competition between males allow us to conclude that simulating female behavior might be an alternative tactic used by males to avoid physical combat. In this thesis, we registered characteristics involving mating behaviors in *T. serrulatus* and discussed possible factors that could lead to reproductive isolation between sexual and parthenogenetic populations.

Keywords: Male competition. Mating. Parthenogenesis. Pheromone. Reproductive isolation. *Tityus serrulatus.*

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Prefácio

Os primeiros registros sobre o acasalamento de escorpiões ocorreram no século XIX e na primeira metade do século XX (Maccary 1810; Brongniart & Gaubert 1891, Fabre 1923), quando os pesquisadores acreditavam que a transferência de esperma era direta. Porém, na década de 1950, diversos estudos demonstraram que a inseminação nos escorpiões é realizada por meio de um espermatóforo (Bücherl 1956; Zolessi 1956; Alexander 1956, 1959), depositado em um substrato (Fig. 1a). Apenas a partir da década de 1990, análises descritivas detalhadas da corte de escorpiões tornaram-se disponíveis (Peretti 1991, 1995; Tallarovic et al. 2000). Além disso, nos últimos anos, estudos relacionados a aspectos do sucesso reprodutivo e mecanismos de seleção sexual têm ganhado foco na literatura (Peretti 1997; Mattoni & Peretti 2004; Carrera et al. 2009, Olivero et al. 2017, Vrech et al. 2019, Oviedo-Diego et al. 2020).

Os escorpiões exibem um comportamento sexual complexo e ritualizado (Polis & Sissom 1990). O cortejo e o acasalamento podem ser divididos em três fases: “iniciação”, em que ocorre o reconhecimento entre o macho e a fêmea; “dança” (conhecida como *promenade a deux*), durante a qual o casal caminha pelo ambiente, enquanto o macho busca um substrato adequado para a deposição do espermatóforo; “transferência de espermatozoides”, que consiste na deposição do espermatóforo pelo macho no substrato, e a subsequente condução da fêmea até o espermatóforo, para sua inseminação (Benton 2001). Algumas espécies também apresentam uma fase denominada “pós-transferência de espermatozoides”, na qual tanto o macho quanto a fêmea podem realizar comportamentos pós-copulatórios, como o consumo do espermatóforo (Alexander 1959; Tallavoric et al. 2000).

Tityus serrulatus, popularmente conhecido no Brasil como escorpião amarelo, é uma espécie de importância médica muito temida devido à ação neurotóxica de seu veneno (Pucca et al. 2015). Descrita em 1922 por Lutz & Mello, os primeiros exemplares foram encontrados em Belo Horizonte, Minas Gerais. Atualmente, sua presença foi registrada em praticamente todo o território brasileiro, exceto na região Norte, onde o registro se limita a alguns poucos estados (Bortoluzzi et al. 2007, Souza et al. 2009). Uma de suas características que mais chamam a atenção é a capacidade de se reproduzir por partenogênese, forma de reprodução em que uma fêmea pode originar filhotes sem a necessidade de acasalar com um macho. Por muitos anos, apenas as fêmeas de *T. serrulatus* eram conhecidas, até que em 2009 o macho do escorpião amarelo foi descrito pela primeira vez (Souza et al. 2009, Fig. 1b). A partir do conhecimento do macho de *T. serrulatus*, várias perguntas surgiram a respeito da reprodução sexuada nessa espécie. Será que fêmeas que se reproduzem sexuadamente também realizam partenogênese? E as fêmeas de populações exclusivamente femininas, aceitariam acasalar com machos e gerariam prole com origem sexuada? Existe alguma característica exclusiva no acasalamento do escorpião amarelo, uma espécie partenogenética, que difere de uma espécie que se reproduz apenas de maneira sexuada? Algumas dessas perguntas, entre outras, foram respondidas nesta tese. Aqui, nós descrevemos o comportamento de corte e acasalamento do escorpião amarelo e estudamos alguns fatores relacionados à partenogênese e seleção sexual.

O presente trabalho possui cinco capítulos; no primeiro capítulo, avaliamos se fêmeas de *T. serrulatus* de uma população sexuada também se reproduzem por partenogênese. No segundo capítulo, descrevemos a corte do escorpião amarelo e procuramos por comportamentos que poderiam favorecer a reprodução sexual dentro da espécie, uma vez que a maioria das populações se reproduzem exclusivamente por partenogênese. No Capítulo 3, avaliamos se fêmeas partenogenéticas de Belo Horizonte aceitam acasalar com machos, e, em caso afirmativo, se apresentam resistência ao cortejo masculino. No quarto capítulo, verificamos se o escorpião amarelo se comunica por feromônios sexuais de contato e, no caso da existência dessa comunicação, se fêmeas partenogenéticas ainda mantêm a capacidade de emitir ou receber estímulos. Por fim, no Capítulo 5, após observar que machos de *T. serrulatus* podem ter diferenças expressivas de tamanho corporal (Fig. 1c), nós verificamos se eles podem apresentar diferentes táticas comportamentais para conseguir acasalar com uma fêmea, que permitem que machos pequenos consigam se manter na população. Esperamos que esta tese forneça novas informações sobre a reprodução sexuada dentro de uma espécie de escorpiões cujo sexo foi um enigma por vários anos.

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Figura 1: a. Espermatóforo de *Tityus serrulatus* (Buthidae). À esquerda, espermatóforo aderido ao substrato, após a inseminação da fêmea. À direita, espermatóforo visualizado em microscópio estereoscópico (escala: 1 mm). b. Macho de *T. serrulatus* em um terrário. c. Dois machos adultos de *T. serrulatus* fixados, mostrando a diferença de tamanho entre ambos.

Chapter 1

Asexual reproduction in a sexual population of the yellow scorpion (*Tityus serrulatus,* Buthidae) as evidence of facultative parthenogenesis

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Abstract

Most yellow scorpion (*Tityus serrulatus* Lutz & Mello, 1922) populations reproduce by parthenogenesis, and only a few sexually reproducing populations are known. It has been suggested that the parthenogenesis in *T. serrulatus* is related to bacterial endosymbionts, but this hypothesis was recently refuted, so the causes of parthenogenesis in this species are still unknown. In the present study, we report parthenogenetic reproduction in females from a sexual population, either isolated in laboratory since birth or collected at juvenile stages. Twelve females collected as juveniles became adult and reproduced without contact with males (thus, through parthenogenesis) in the laboratory. Five females collected already pregnant gave birth to litters (F1) composed only by females, which is suggestive of parthenogenesis in the field. Eight F1 females from those litters subsequently reproduced by parthenogenesis in the laboratory. Another female collected already pregnant gave birth to a litter composed by males and females (F1), indicating sexual reproduction in the field.

However, one F1 female from that litter reproduced by parthenogenesis in the laboratory. These results suggest that asexual reproduction is facultative in this population.

Keywords: Geographic parthenogenesis, sex ratio, sexual reproduction

1.1- Introduction

Parthenogenesis, the development of offspring from unfertilized eggs, although present in few species, occurs in diverse invertebrate and vertebrate taxa (Simon et al. 2003). If the fertility of parthenogenetic and sexually reproducing females were the same, asexual reproduction is considered to be more efficient than sexual reproduction, since the latter involves additional costs (Maynard-Smith 1978; Kawatsu 2013), such as the production of males, whose paternal investment in the offspring is minimal (Maynard-Smith 1971). In addition, asexually reproducing animals are more efficient in the colonization of disturbed environments, since reproduction may occur without the presence of a member of the opposite sex (Cuellar 1977; Lourenço 2008). However, the evolutionary consequences of parthenogenesis may involve reproductive isolation in relation to sexual forms and the emergence of lineages with low genotype diversity, which may lead to their extinction (Simon et al. 2003). Facultative parthenogenesis, the ability to reproduce both sexually and asexually, on the other hand, brings the advantages of sexual reproduction, but with lower costs (D'Souza & Michiels 2010). Despite presenting the advantages from both forms of reproduction, facultative parthenogenesis is rare in higher eukaryotes, probably due to genetic and developmental constraints (Engelstädter 2008). Thus, the study of populations with mixed mode of reproduction may offer clues on the advantages and disadvantages of facultative parthenogenesis and its emergence from sexual reproduction (Burke & Bonduriansky 2017).

Although facultative parthenogenesis is rare, most asexual animal lineages can also reproduce sexually (Bengtsson 2009), whereas very few lineages, such as bdelloid rotifers, are exclusively parthenogenetic (Welch & Meselson 2000). In some invertebrates, sexual and asexual reproduction occur cyclically, such as in monogonont rotifers, cladoceran crustaceans, and aphids (Decaestecker et al. 2009; Loxdale 2009; Serra & Snell 2009), while in other groups, the presence of both forms of reproduction is an alternative strategy (Buřič et al. 2011). In arthropods, facultative parthenogenesis is observed in insects (Corley et al. 1999; Chang et al. 2014; Sekiné et al. 2015; Walker & Holwell 2015), crustaceans (Buřič et al. 2011) and arachnids, specifically some scorpion species (Toscano-Gadea 2005; Francke 2008; Lourenço 2008). According to criteria established by Francke (2008) for the identification of a parthenogenetic species, eleven scorpion species are currently known to reproduce asexually (Francke 2008; Ross 2010; Seiter 2012; Seiter et al. 2016; Seiter & Stockmann 2017), most through facultative parthenogenesis (Francke 2008; Seiter & Stockmann 2017). For example, although *Tityus trivittatus* Kraepelin, 1898 populations from southern Argentina appear to consist exclusively of parthenogenetic females, this scorpion has been reported reproducing sexual and asexually through parthenogenesis across several countries in South America (Maury 1970, 1997; Ojanguren-Affilastro 2005). The first evidence of parthenogenesis in scorpions came from observations regarding the sexual ratio of populations of the yellow scorpion, *Tityus serrulatus* Lutz & Mello, 1922, in which no male had been found (Piza 1940; Bücherl 1956). However, the demonstration of asexual reproduction in this species came after Matthiesen (1962) observed that captive females, isolated from other individuals, generated offspring. This was confirmed in subsequent studies (San Martin & Gambardella 1966; Matthiesen 1971). Parthenogenesis was thenceforth considered the only form of reproduction of this species, until the discovery of males from a few sexual populations in Brazil (Souza et al. 2009; Santos et al. 2014; Lima et al. 2020). Although *T. serrulatus* could be an interesting model to study parthenogenesis, the causes of asexual reproduction in this species are still unknown. A proposition of *Wolbachia*-induced parthenogenesis (Suesdek-Rocha et al. 2007) was recently refuted (Braga-Pereira et al. 2019), discarding the only proposed mechanism of asexual reproduction suggested for this species. In this study, we demonstrate through captive rearing that females from a sexual population are capable to reproduce parthenogenetically, indicating that the asexual reproduction in *T. serrulatus* is actually facultative.

1.2- Methods

We collected 174 individuals, 59 adult females, 40 adult males and 75 second to fifth instar juveniles of *T. serrulatus* in urban and suburban habitats in Itacarambi, Minas Gerais, Brazil (15.17^o S, 44.18^o W, Fig. 1a) in March 2016 and January 2017. In the second collection expedition, we recorded the animals according to the environment where it was found (urban area: backyards of houses and an abandoned sawmill; suburban area: farms and a coconut plantation). We captured the scorpions in the morning, under trunks, stones and bricks and inside termite mounds. During the night, we collected active animals using ultraviolet light. In the laboratory, we identified each specimen with a number and kept them individually in plastic containers (juveniles: 5.6 cm diameter X 6,0 cm height; subadults and adults: 8.5 cm X 6.5 cm), with a wet cotton ball to provide water and a piece of cardboard as a refuge. We fed the juveniles one cockroach (*Nauphoeta cinerea* (Olivier, 1789)) per week and the adults every 15 days, and inspected the containers four times a week to check for molting or birth events. Prey remnants were taken away one day after scorpion feeding to avoid contamination by fungi. Scorpions were kept in the laboratory at a mean temperature of 25 ± 3 °C, 58 ± 11% relative humidity and natural ambient illumination provided through a glass window, with a photoperiod varying seasonally from 10:55 to 13:20 hours of light.

After adult females (parental generation, P) gave birth, we separated the second instar juveniles (F1) from the mother after the first molt. We kept each juvenile in an individualized container, under the same conditions described above. We recorded the date of each juvenile molt until they become adult and, afterwards the date of their own offspring birth (F2, Supplemental material 1, 2). Adult individuals, both found in field and grown in laboratory, were sexed based on the width of metasomal segments and length of pedipalp segments (following Souza et al. 2009). Males that showed no unambiguous morphological differences from females were identified through their behavior when exposed to females: males exhibit tremors (“Vibración”, as in Peretti 1991) and females tend to tilt the metasoma towards the male (personal observation). Dead specimens were fixed in 80% ethanol, unless they are found in decay, when they were discarded. After the end of the study, we fixed the specimens that survived in 80% ethanol and deposited them in the Centro de Coleções Taxonômicas da Universidade Federal de Minas Gerais. We made observations between March 2016 and March 2019.

1.3- Results

Regarding the 75 collected juveniles, 44% reached adulthood, 21 females and 12 males, totaling 80 females (61%) and 52 males (39%) collected in the field on both collection expeditions. In the second collection expedition, we obtained 15 individuals, nine females (60%) and no males (0%) in urban environments (backyards of houses and abandoned sawmill), with six individuals that did not survive until the adult age (40%). A total of 49 females (46.22%) and 43 males (40.57%) were obtained in suburban environments (farms and coconut plantation), with 15 individuals that did not survive until the adult age (13.2%), totaling 107 individuals collected (Supplemental material 3).

Among the 21 females that became adult in the laboratory (and never had contact with males), 12 gave birth through parthenogenesis, once or twice (Supplemental material 1). We raised to adulthood the entire offspring (F1) of six females (P) that were collected already pregnant. Five of these females (P) gave birth to a total of 39 individuals (F1), all females, which is suggestive of parthenogenesis in the field. Eight of those 39 females (F1) reproduced by parthenogenesis in the laboratory (Fig. 1b). Another female collected already pregnant (P) gave birth to five males and nine females (F1), an indication of sexual reproduction in the field. One (F1) of those nine females reproduced by parthenogenesis in the laboratory (Fig. 1c). Furthermore, we also raised part (15 individuals, F1) of the litters born from three additional females collected already pregnant (P). Six of those F1 did not survive to adulthood. The remaining nine specimens became adult females, and five of them reproduced parthenogenetically in the laboratory (Fig. 1d). (Supplemental material 2).

1.4- Discussion

Our results demonstrate that asexual reproduction is facultative in the yellow scorpion populations in which males are found. This result reinforces our previous conclusion that the parthenogenesis in *T. serrulatus* is not induced by endosymbiont bacteria (Braga-Pereira et al. 2019), as suggested for *Wolbachia* (Suesdek-Rocha et al.

2007). This hypothesis was refuted due to negative PCR tests for *Wolbachia* in several parthenogenetic populations (Braga-Pereira et al. 2019). *Wolbachia* is an endosymbiont microorganism that reproduces mainly by vertical transmission (Hoffman et al. 1990), usually inducing parthenogenesis by converting unfertilized haploid eggs into diploid embryos via gamete duplication (Stouthamer & Kazmer 1994). Within the same lineage, we observed that one female (P) gave rise to offspring (F1) consisting of both sexes, evidence of field mating and sexual reproduction. One F1 female from this litter, which was kept isolated in the laboratory, reproduced by parthenogenesis. As far as we know, there is no report of *Wolbachia* infection suppressed in one generation and expressed in the subsequent.

Our results suggest that the absence of males may trigger the parthenogenesis in *T. serrulatus* females. The field sampling suggests that females outnumber males in the Itacarambi population, a condition that may favor parthenogenesis, as asexual reproduction may be an advantageous strategy under low male availability (Markow 2013; Burke & Bonduriansky 2019). A similar pattern was reported for *Drosophila* species that, depending on male availability, can alternately use both reproductive modes (Chang et al. 2014). In fact, females of some species can maintain their ability to reproduce asexually for generations, even after mating with males (Kramer & Templeton 2001). In populations of stick insects, which reproduce by facultative parthenogenesis, this form of reproduction can generate female-biased sex ratios, increasing mating limitation and thus leading to male scarcity in a positive feedback mechanism (Schwander et al. 2010). Within this scenario, the absence of males may lead to obligatory parthenogenesis (Schwander & Crespi 2009), an event that may have occurred in most yellow scorpion populations. Further laboratory experiments are needed to assess whether obligatory parthenogenesis is present in populations composed only by females.

Populations that reproduce only asexually have some characteristics that tend to lead a lineage to extinction (Simon et al. 2003), as the accumulation of deleterious allele combinations (Charlesworth et al. 1993), the absence of fixation of beneficial mutations (Peck 1994) and developmental constraints (Corley et al. 1999). Although we observed a slightly higher laboratory mortality rate to adulthood in individuals from urban environments, where the number of females exceeds the number of males (thus females are likely to reproduce by parthenogenesis), our small sample size prevents reliable conclusions. Thus, a comparative study specifically designed to compare mortality rates between sexual and asexual population would be welcome. Mortality rate differences apart, the ability to reproduce through parthenogenesis seems to facilitate *T. serrulatus* invasion to disturbed environments. Female-only populations have a wide geographic distribution and may have dispersed from facultative parthenogenetic populations. Originally, *T. serrulatus* was known only from Minas Gerais, a state in the Southeast region of Brazil. However, the species has been increasingly found in regions where its presence was not reported earlier (Lourenço et al. 1996; Lourenço & Cloudsley-Thompson 1999; Bortoluzzi et al. 2007; Mario da Rosa et al. 2015). Lourenço (2008) proposed that the wide distribution of *T. serrulatus* is the result of recent dispersal events, since a medically important species would be recorded as soon as it became abundant in urban areas.

Furthermore, Lourenço (2008) proposed geographic parthenogenesis in *T. serrulatus,* a phenomenon in which parthenogenetic organisms have different geographical distributions than their closest sexual relatives (Vandel 1928; Tilquin & Kokko 2016). Indeed, the yellow scorpion presents traits associated to geographic parthenogenesis, such as the parthenogenetic populations with wider geographic distribution compared to sexual populations (Hörandl 2009), and high abundance in disturbed environments, either natural or those created by human action (Hoffmann et al. 2008; Vrijenhoek & Parker 2009). The explanation for these two characteristics is due to the easy dispersal of parthenogenetic organisms, since a single individual can start a new population (White 1954). However, during our field work in an area where the sexual population is present, we observed a female-biased sex-ration in disturbed environments, such as backyards and the abandoned sawmill, where vegetation was sparse and composed only of small plants and predominance of human constructions, if compared to less disturbed environments, such as farms and the coconut plantation, where we find dense vegetation, including large trees. Apparently, the both forms of reproduction of the yellow scorpion may occur in the same region, with small differences related to the habitat. This indicates that ecological conditions may favor asexual reproduction in *T. serrulatus*.

Finally, in order to understand other factors that may have led to positive selection of parthenogenesis in the yellow scorpion, it is necessary to observe some reproductive characteristics of the species. Is there any behavior in *T. serrulatus* mating that may favor parthenogenesis? For example, traits associated to sexual conflict may be a factor that favor the presence of parthenogenesis in a species, such as male coercive behaviors and female resistance responses (Burke & Bonduriansky 2017, 2019). For instance, In *Zabius fuscus* Thorell 1876 scorpions, males show little or no coercion during courtship and copulation. Females, on the other hand, though receptive to first male courtship attempts, tend to resist subsequent mating attempts (Peretti & Carrera 2005). However, parthenogenesis has never been observed in *Z. fuscus*, thus it would be interesting to verify if coercive or resistance behaviors are present in asexually-reproducing species.

Is the fertility of sexually reproducing females equal to the fertility of asexual reproducing ones? In the facultatively parthenogenetic cockroack *Nauphoeta cinerea*, developmental constraints limit the success of asexual reproduction, generating extreme clutch size variations (Corley et al. 1999). Do females of obligatory parthenogenetic populations copulate with males of sexual populations? In the facultative parthenogenetic *Drosophila albomicans*, females isolated from males for 20 years in the laboratory have reduced mating propensity, a process that may lead to obligatory parthenogenesis (Chang et al. 2014). Answers to these questions may not only help to understand the presence of parthenogenesis in the yellow scorpion, but also the factors that led to the transition from facultative to obligatory asexual reproduction.

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Figure Chapter 1

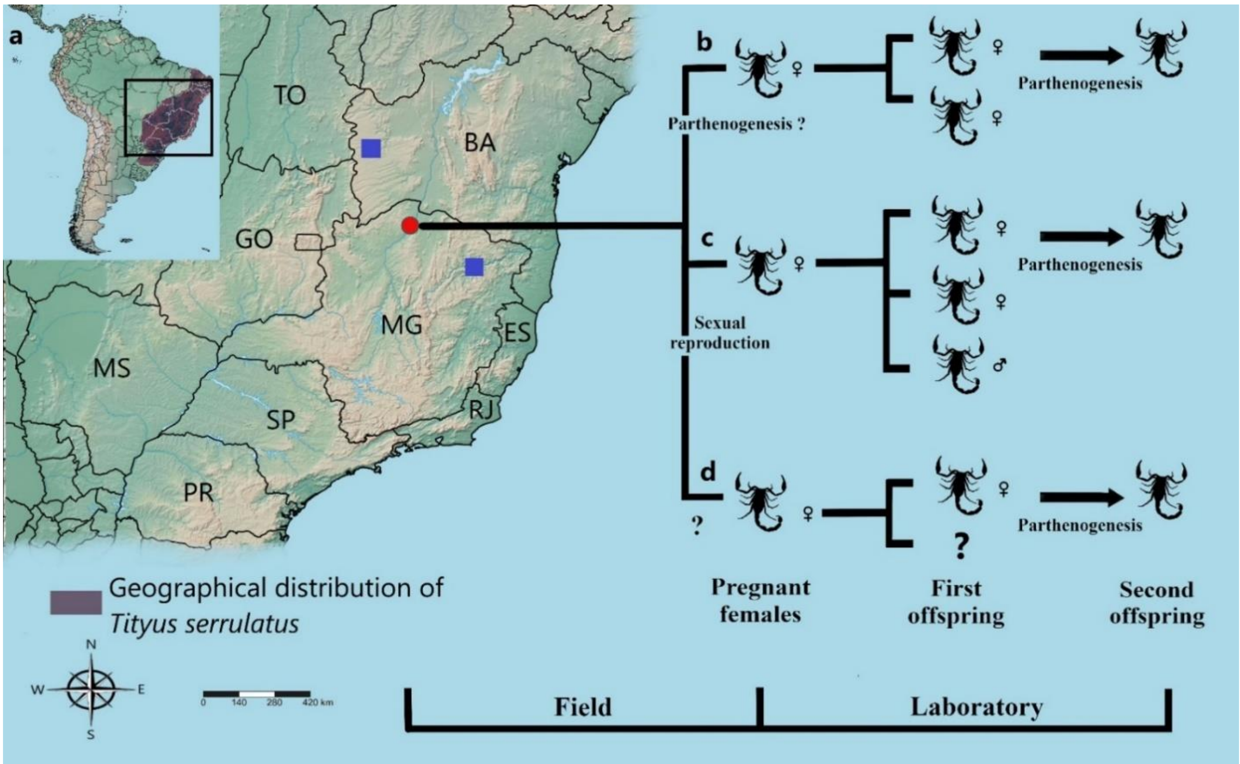


Figure 1: Reproductive history of female yellow scorpions (*Tityus serrulatus*) in laboratory: a. Geographic distribution of *Tityus serrulatus* specimens sampled in this study. The red circle represents the sexual population analyzed in the present study and blue squares show other sexual populations. b. Pregnant females gave rise to offspring containing only females; eight of them reproduced by parthenogenesis. c. Pregnant female gave rise to offspring containing male and females; one of them reproduced by parthenogenesis. d. Pregnant females gave rise to offspring containing females; five of them reproduced by parthenogenesis. Sex of the other individuals of the second offspring was not determined.

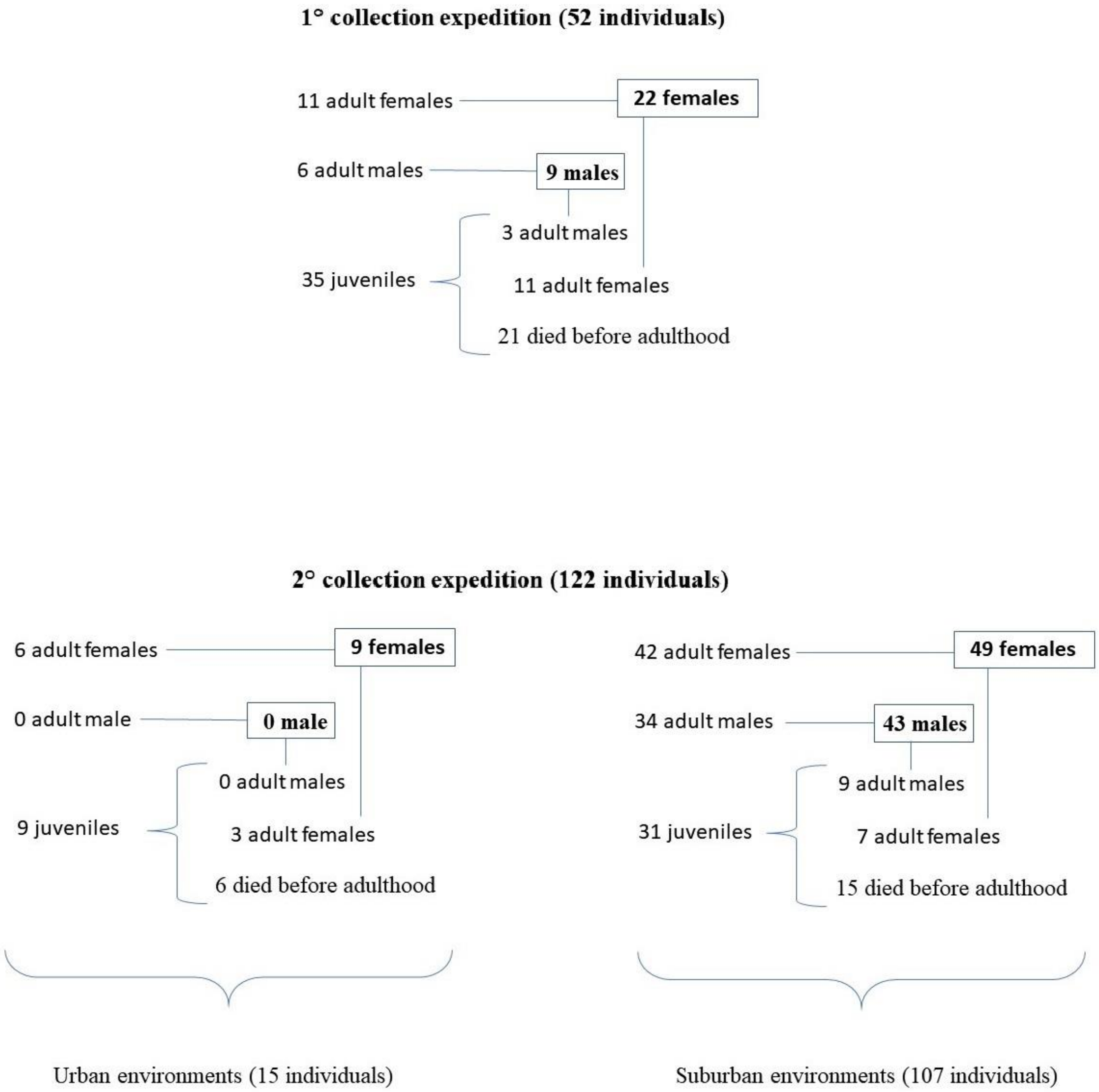
Supplemental Materials Chapter 1

Supplemental material 1: Reproductive history of female yellow scorpions (*Tityus serrulatus*), which reproduced by parthenogenesis in laboratory. Females were collected at juvenile stage, were kept until adulthood in captivity and never had contact with males.

ID	Collect	2° molt	3° molt	4° molt	5° molt	Birth of offspring
UFMG 24090	23-26- III-2016	-	-	11-X-2016	19-IX-2017	19-XI-2018
UFMG 24091	23-26- III-2016	-	-	11-X-2016	26-V-2017	26-XII-2017 15-II-2019
UFMG 24092	23-26- III-2016	-	-	3-IX-2016	15-IV-2017	26-XI-2018
UFMG 24093	23-26- III-2016	-	-	-	1-X-2017	26-XI-2018
UFMG 24094	23-26- III-2016	-	-	19-IX-2016	16-IX-2017	24-XI-2018
UFMG 24095	23-26- III-2016	-	-	1-XI-2016	3-X-2017	23-XI-2018
UFMG 24096	23-26- III-2016	23-V-2016	25-XI-2016	26-III-2017	7-III-1018	20-XI-2018
UFMG 24097	23-26- III-2016	-	-	-	5-X-2016	15-XI-2016
UFMG 24110	27-30-I-2017	26-III-2017	19-VIII-2017	27-X-2017	21-IV-2018	23-XI-2018
UFMG 24111	27-30-I-2017	-	-	-	27-II-2018	26-XI-2018
UFMG 24112	27-30-I-2017	-	-	-	9-IX-2017	20-XI-2019
UFMG 24113	27-30-I-2017	-	-	-	1-V-2018	18-XII-2018

Supplemental material 2: Reproductive history of female yellow scorpions (*Tityus serrulatus*) born in laboratory from females collected already pregnant. Abbreviations: P, females collected in the field; F1, individuals born in laboratory, from the P females; F2, offspring born in laboratory from F1 females, by parthenogenesis.

ID Mother (P)	**ID (F1)**	**Born**	**2° molt**	**3° molt**	**4° molt**	**5° molt**	**Birth of offspring (F2)**	**Siblings of F1**
UFMG 24105	UFMG 24098	18-IV-2016	28-VI-2016	26-X-2016	11-II-2017	9-III-2018	12-XI-2018	Male and female
UFMG 24106	UFMG 24099	24-V-2016	3-IX-2016	2-II-2017	5-IX-2017	22-I-2018	20-XI-2018	Only female
UFMG 24107	UFMG 24100	24-VI-2016	3-XI-2016	26-I-2017	5-IX-2017	22-I-2018	29-XI-2018	Only female
UFMG 24108	UFMG 24101	29-XI-2016	17-I-2017	9-V-2017	16-IX-2017	11-IV-2018	19-XI-2018	Only female
UFMG 24108	UFMG 24102	29-XI-2016	26-I-2017	1-IX-2017	3-I-2018	21-IX-2018	13-XI-2019	Only female
UFMG 24108	UFMG 24103	29-XI-2016	17-I-2017	19-VIII-2017	28-XI-2017	11-V-2018	26-XI-2018	Only female
UFMG 24121	UFMG 24114	9-V-2017	29-VIII-2017	22-X-2017	5-I-2018	20-VIII-2018	18-XII-2018	Only female
UFMG 24121	UFMG 24115	9-V-2017	9-VIII-2017	3-X-2017	11-I-2018	11-IX-2018	28-I-2019	Only female
UFMG 24109	UFMG 24104	20-XI-2016	26-I-2017	5-IX-2017	11-I-2018	28-IX-2018	27-XI-2019	Only female
UFMG 24122	UFMG 24116	25-XI-2017	16-I-2018	24-II-2018	20-VIII-2018	15-IV-2019	27-XI-2019	-
UFMG 24123	UFMG 24117	9-V-2017	9-VIII-2017	15-X-2017	9-I-2018	21-IV-2018	12-XI-2018	-
UFMG 24124	UFMG 24118	9-V-2017	16-VIII-2017	17-X-2017	13-XII-2017	7-III-2018	26-XI-2018	-
UFMG 24124	UFMG 24119	9-V-2017	30-VIII-2017	17-X-2017	15-XII-2017	20-V-2018	12-XII-2018	-
UFMG 24124	UFMG 24120	20-XII-2017	22-I-2018	27-II-2018	14-III-2018	26-XI-2018	5-XI-2019	-



Supplemental material 3: Individuals of *Tityus serrulatus* (Buthidae) collected in the first and second collection expeditions. The total number of individuals of each gender collected in each expedition is shown within the rectangles.

Chapter 2

Mating behavior of the facultative parthenogenetic scorpion *Tityus serrulatus* (Buthidae): do male coercive behaviors favor sexual reproduction?

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Abstract

The sexual conflict hypothesis predict that male coercion may be one of the factors that maintain sexual reproduction in facultatively parthenogenetic species, since females can be forced to copulate. In contrast, female resistance can favor parthenogenesis, since she can avoid sex by reproducing asexually. For this reason, populations that reproduce by facultative parthenogenesis may present different behaviors during mating, when compared to species in which sexual reproduction is obligatory. In this study, we describe the mating behavior of the facultative parthenogenetic scorpion *Tityus serrulatus* Lutz & Mello, 1922 and analyze whether the species has more coercive males and more resistant females, compared to exclusively sexual species, as predicted by the conflict hypothesis sexual. For this, we recorded the mating of *T. serrulatus* in the laboratory and compared it with some behaviors described for other buthid species. Of 114 pairings studied in captivity, 24 resulted in complete mating. We have found 46 behavioral acts, which we divide into ten behavioral categories. In a first pairing, only 27.03% of females with unknown status were receptive to mating, but 87.5% of virgin females were receptive. Females showed no more resistant to mating than females of scorpions that reproduce only sexually. Males show insistence and sexual stimulating behaviors, similar to those observed in other, exclusively sexual buthids. Like other species of scorpions, coercion is apparently not present, not contributing to the maintenance of sexual reproduction in *T. serrulatus*. Our results are not consistent with the predictions of the sexual conflict hypothesis.

Keywords: Coercion, female resistance, sexual stimulation

2.1- Introduction

Sexual conflict, in which males and females have different strategies for optimizing fitness, is a potential consequence of sexual reproduction (Parker 1979; Parker & Partridge 1998). In sexually-conflicting mating systems, males tend to exhibit behaviors to coerce female mating acceptance, sometimes even harming the female (Parker 1979; Arnqvist & Rowe 2005). Females, on the other hand, may exhibit resistance to mating (Alexander et al. 1997). This male-female conflict might be particularly intense in facultatively parthenogenetic species, in which the coexistence of sexual reproduction and parthenogenesis can be maintained by antagonistic coevolution between male coercion and female resistance (Kawatsu 2013). Male coercive behavior could promote the persistence of sex, since coercive males may inhibit the spread of parthenogenesis by forcing females to reproduce sexually (Dagg 2006; Kawatsu 2013; Burke et al. 2015). In response to male coercion, more resistant females can be selected, which favors parthenogenesis (Burke et al. 2015; Burke & Bonduriansky 2019).

Parthenogenesis has been reported in several species of arachnids, specifically in mites, harvestmen, spiders and scorpions (Oliver 1971; Tsurusaki 1986; Edwards et al. 2003; Francke 2008). Among scorpions, the earliest reported, and the better-known parthenogenetic species is the Brazilian yellow scorpion *Tityus serrulatus* Lutz & Mello, 1922 (Matthiensen 1962). Parthenogenesis has been considered the only reproductive form of this species for many years, until Souza et al. (2009) firstly reported a sexually-reproducing population and described the male. Afterwards, only two other sexual populations have been found (Santos et al. 2014; Lima et al. 2020), despite the species wide distribution in Brazil. Most importantly, although the specific parthenogenesis mechanisms of the species are still unknown, it has been shown that females from one sexual population can facultatively reproduce either sexually or asexually (Braga- Pereira & Santos in press).

Scorpions exhibit complex and ritualized sexual behavior, during which the male deposits a spermatophore on the substrate and conducts the female to it (Alexander 1959; Polis & Sissom 1990). As in other animals that transfer sperm through externally deposited spermatophores, coercion during copulation is supposedly limited or nonexistent in scorpions (Peretti 2001; Peretti & Carrera 2005). Coercive behaviors from the male should be particularly ineffective in the group, since females are well equipped to respond to male aggression using their chelate pedipalps and venomous sting (Peretti 2001). However, males of a few scorpion families, such as Bothriuridae, have been reported to exhibit potentially coercive behaviors during sperm transfer, such as holding the female by the chelicerae or legs (Peretti 1997a). Coercive behaviors have also been reported in Buthidae, the yellow scorpion family, such as males hardly and quickly pulling reluctant females towards the spermatophore (Peretti 2001). Females can resist mating through a variety of behaviors, such as moving in the opposite direction to the male, not showing any body movement, preventing the male from holding her pedipalps, threatening or even biting the male, closing the genital opening during sperm transfer, among others (Peretti 2001; Peretti & Carrera 2005). Virgin females tend to be less resistant (Peretti & Carrera 2005) because virgin females must guarantee at least one copulation during their lifetime (Kokko & Mappes 2005). Thus, female receptivity is expected to decrease with successive inseminations, and females with wider previous insemination history are generally more aggressive (Peretti 2001). Since *T. serrulatus* females can reproduce by parthenogenesis, male coercive and female resistance (mainly from virgin females, since resistance is expected in previously inseminated females) behaviors should be more frequent in this species, compared to exclusively sexually-reproducing species.

Coercive behavior is not the single possible response of male scorpions towards resistant females, as stimulatory or appeasement behavior have also been described in the group (Peretti 2001, Peretti & Carrera 2005). For instance, males can perform cheliceral massage or rub the female body with their legs during the dance phase, when the male attempts to conduct the female through the environment and search for a suitable place to deposit the spermatophore (Polis & Sissom 1990; Peretti 2001; Peretti & Carrera 2005). These same behavioral acts have been reported during the sperm transfer, possibly serving as a copulatory courtship tactic (Peretti 1997a). In the case of *T. serrulatus*, since females are capable of parthenogenesis, male stimulatory (if not coercive) behavior should be frequent during pairing in order to convince females to reproduce sexually.

In this study, we describe the mating behavior of a facultative parthenogenetic population of *Tityus serrulatus* for the first time, and compare its mating sequence to two related species, *Zabius fuscus* (Peretti 1991, 2001; Peretti & Carrera 2005) and

Tityus bahiensis (Outeda-Jorge 2010). The latter are well studied species regarding their mating behavior and, as far as currently known, reproduce only sexually. Thus, our comparison is focused mainly on potential male coercive or stimulatory behavior, and female resistance. Based on recently theoretical and empirical developments (Dagg 2006; Kawatsu 2013; Burke et al. 2015), we expect a higher frequency of coercive/stimulatory and resistance behaviors in *T. serrulatus*, compared to other buthid species. Additionally, we also evaluate whether virgin females are more prone to accept copulation, if compared to already inseminated females.

2.2- Methods

Specimen collection and rearing

We collected 45 females (37 adults and eight subadults) and 38 males (33 adults and five subadults) of *T. serrulatus* in urban and suburban areas of Itacarambi, Minas Gerais (15.19S, 44.20W, Fig. 1a), in March 2016 and January 2017. We searched for the scorpions in the morning under trunks, rocks and brick piles and inside termite mounds. We also collected active specimens at night using ultraviolet light. We brought the individuals to the laboratory and kept them for at least 60 days before the observations. We recorded each individual with a number and kept them individually in plastic cages (8.5 cm diameter X 6.5 cm height) containing a wet cotton ball to provide water and a piece of cardboard as a refuge. We fed them one cockroach (*Nauphoeta cinerea*) per week; scorpions rejected cockroaches when fed more frequently. Scorpions were kept in the laboratory at 25 ± 3 °C and 58 ± 11% relative humidity and 12 hours photoperiod. Subadults were kept in containers until adulthood and were used for observations at least 60 days after their last molt. We deposited voucher specimens in the arachnid collection of the Centro de Coleções Taxonômicas da Universidade Federal de Minas Gerais.

Observation of sexual behavior

We performed behavioral observations from May 2016 to November 2017 inside. glass containers (25 cm diameter X 19 cm height) as arenas. To emulate the types of substrate available for the species in its habitat, the arena contained earth at the bottom, together with a rock, a piece of termite mound, a piece of a roof tile, and a branch positioned through the diameter of the terrarium (Fig. 1b). Observation arenas were kept at same temperature and humidity as the specimens' individual containers. We carried out the observations at night (from 21:00h to 00:00h), when the species is active (Mineo et al. 2009). In order to sample the greatest possible number of behavioral acts of the species, we used each individual in three different pairings; as the number of females collected was greater than the number of males, some females were used twice. . Females that were apparently pregnant were not selected for pairing. The time between the use of the same animal in one experiment and the next was at least 30 days and no specific male-female pair was repeated.

We randomly selected female-male pairs, placed them in the mating arena and waited up to 40 minutes for male-female interaction to start. Once specimens started interacting, we observed them until all sexual behaviors ended. The arena was illuminated with red light at the beginning of the observation section, but we switched to white light after the initiation of the mating sequence in order to facilitate the observation. Although the white light inhibited the behavior of the animals before the initiation of courtship, our previous observations showed that the change of light did not alter their behavior afterwards. We recorded the behavior of the specimens using a

Canon EOS Rebel T5 camera, together with photos taken with a Microsoft Lumia 640 mobile phone and written notes.

Behavioral repertory

We considered “complete pairing” the parings in which the female has been inseminated and “incomplete pairing” those which had any sexual behavior but finished before insemination. Pairings in which there was no interaction between males and females were not considered in the analyses. We characterized the behavioral repertory using as reference ethograms of related species *Z. fuscus* (Peretti 1991) and *T. bahiensis* (Outeda-Jorge 2010) and modified behavioral categories according to our observations. Given the extensive number of behavioral acts involved in mating, we classified them in up to ten categories. Throughout the text, behavioral acts are presented between quotation marks, and behavioral categories in italics. In the case of exclusive behavioral acts of *T. serrulatus*, we use chi-square test or Fisher's exact test, depending on the total frequency in each category, to check whether the frequency (presence or absence of a behavior during pairing) is different between complete and incomplete pairings.

Female resistance

To analyze whether *T. serrulatus* females are more resistant to mating than other species of buthids that reproduce only sexually, we classified female receptivity patterns (based on criteria established by Peretti 2001; Peretti & Carrera 2005) as Positive (female accept to mate), Intermediate (females show some type of resistance during mating), and Negative (female rejects the male). As *T. serrulatus* females with offspring on the back are resistant to mating (personal observation), we observed two pairings with a female with offspring, under the same conditions described above, in order to standardize our classification of female resistance behaviors.

Adult females could have been collected as virgins, have been inseminated previously (having given birth or not) or reproduced by parthenogenesis, thus we refer to them as "unknown status". Females who have become adults in the laboratory were referred to as “virgin status”. Previous studies on non-parthenogenetic species showed that approximately 80% of virgin females show Positive receptivity, and that receptiveness decreases according to the number of previous inseminations (Peretti 2001; Peretti & Carrera 2005). Thus, if *T. serrulatus* females are more resistant than females of species that reproduce only sexually, a relatively lower frequency than 80% of virgin females in Positive status is expected. For females of unknown status and who have agreed to mate, regardless of the species resistance level, a lower frequency of individuals in a Positive status is expected when compared with virgin females, since it is possible that the females have mated in the field. Therefore, we focused mostly on virgin females to analyze the resistance of *T. serrulatus* to mating.

Male sexual stimulation and coercion

To access whether males of *T. serrulatus* present potentially coercive behavioral acts more often than non-parthenogenetic species, we recorded whether males threaten resistant females in a way that could cause bodily harm to the female (such as venom inoculation). We also evaluated whether males hard and quickly pull reluctant females towards the spermatophore (as observed in *Z. fuscus*), since this behavior may indicate a certain degree of coercion (Peretti 2001). We analyzed whether there are possible male insistent behaviors, such as constantly trying to touch a reluctant female until her pedipalps are taken (Peretti 2001). It is expected that coercive or insistent behaviors will be performed when the female is in the Intermediate or Negative status. Finally, we checked if *T. serrulatus* males exhibited sexual stimulation or potential copulatory courtship behaviors (such as cheliceral massage and rubbing with legs before or during the insemination).

We tested whether coercion, insistence or stimulus behaviors occurred at different frequency (presence or absence of each behavior during pairings) in complete and incomplete parings. We performed these comparisons using chi-square test or Fisher's exact test, considering a significance level of 5%. We made all analyses using R version 4.0.2 (R Development Core Team, 2020).

2.3- Results

Behavioral repertory

We staged 114 pairings, from which 24 (21.05%) ended with spermatophore deposition and insemination. Courtship activity was observed in 69 (76.66%) incomplete pairings. In the remaining 21 failed pairings (23.33%), females and/or males walked around the arena without contact with each other or remained stationary where they were placed. We recorded 46 behavioral acts (Supplementary Material 1, 2) of successful mating, grouped in ten behavioral categories (Table 1, Figs. 2,3).

Most behavioral acts reported herein have been recorded previously in other scorpion species. However, we observed a new behavioral act in which the male or the female stretches the metasoma horizontally and defecates (Fig. 2a, b. Supplementary material 3) during the *environment exploration* and, sometimes, the *couple presentation*. In this behavior, the individual continues with the metasoma horizontally for some time and walks rubbing the anal region on the substrate. In some cases, the male may defecate and, when rubbing the anal area on the substrate after defecating, he exhibits courtship behaviors, such as vibration. We also observed simultaneous defecation of both sexes in the same pair in one incomplete pairing. We considered “defecate” as a sexual behavior because it took place only after *couple presentation*, in which male and female started interacting in the terrarium. Among the 69 incomplete pairings, 12 individuals defecated (five females and seven males), while only two individuals defecated (one female and one male) in the 24 complete pairings. The difference in defecation behavior between the two groups was not significant (Fisher's exact test, p= 0.5075).

Female resistance

We observed the following behavioral acts according to female receptivity (Supplementary material 4): Positive status— in the Initiation phase, female does not avoid male behaviors (does not completely stop - “absence of movement” and does not walk in the opposite direction- “escape”). The male does not need to insist on the female during the courtship. Intermediate — female shows resistance during mating, presenting “absence of movement” or “escape”. She can also present “slow metasoma swing”, in which the female curves the metasoma and moves it slowly over the body, apparently with little aggression. The female may also exhibit resistance during the dance phase, demonstrating a low movement response when guided by the male. In Intermediate status, a female can accept or refuse mating. Negative: female presents “fast metasoma swing”, in which the female keeps the metasoma curved over the body and sways it quickly, being able to move aggressively towards the male. The female may also perform “clubbing”, moving the metasoma and striking the male's metasoma. In response, the male moves the female metasoma using his own metasoma as a shield. In some cases, if the male insists on the courtship, she tries to sting him ("threaten to sting"). Pairings with females in Negative status never reach the dance stage.

Among the eight virgin females, seven (87.5%) accepted to mate in the first pairing, but only one of them (12.5%) accepted to mate in the second pairing. Of the seven virgin females that mated in the first pairing, one was in an Intermediate status, while the others did not offer any resistance, presenting themselves in the Positive status. The virgin female that mated in the second pair was in the Intermediate status both in the first and in the second pairing. Thus, a total of six virgin females (75%) were in the Positive status, a percentage close to that observed for other species. Among the 37 unknow status females, 14 (37.84%) accepted to mate (eight mated in the first pairing, one in the second pairing, three in the third pairing). Two females mated twice, in the first and second pairings and in the first and third pairings, totaling 27.03% females that mated in the first pairing. Among all complete pairings of unknown status females, regardless of whether it was the first, second or third pairing, in 62.5% of the pairings the females were in Intermediate status and 37.5% in Positive status. "Threaten to sting", the most aggressive behavior observed, were performed by only two females (1.75%) among the total pairings.

Coercion and sexual stimulation

We did not observe males of *T. serrulatus* showing coercive behavior. However, *male insistence* behaviors were present in pairings with females of Intermediate or Negative status (Fig. 2e. Supplementary material 1, 4). *Male insistence* behavior is treated here as a behavioral category with two behavioral acts: the male pulls the female while she remains still, or holds on the female legs or metasoma while she is walking, and is dragged by her. *Male insistence* was observed in 41.67% of complete pairings and 13.04% (Fig. 4) of incomplete ones, a statistically significant difference (chi-square test, X-squared = 11.552, p= 0.00068).

The behavioral category *male insistence* has been reported for other species, but probably including behavioral acts not observed in this study, such as “touch" or “*agarre*”. The latter was considered an initial body subjection behavior (Peretti 2001). In our study, we separate behaviors into different categories because *male insistence* does not occur with females with Positive receptivity, unlike *touch*. We classified as *touch* movements performed by pedipalps, mainly from the male, in various regions of the body of the opposite sex, such as metasoma, legs and pedipalps. This movements are smooth and do not move the body of female. Meanwhile, in the behavioral act “male pulls the female”, the male uses the pedipalps and shakes the female, pulling parts of the body, such as the legs and may even move the female from the place where she is standing. The act is usually followed by “male anchor in female”, as the male is still pulling parts of the body of female, while she moves.

Regarding potentially stimulating behavioral categories, *rubbing metasoma* consists in males and females rubbing their anal region in various parts of the body of the partner (Fig. 2d. Supplementary material 1, 2). This behavioral category was observed in 63.16% of the complete pairings and 42.03% of the incomplete pairings (Fig. 4), but the difference between them was not statistically significant (chi-square test, X-squared = 1.8911, p= 0.1691). Two additional sexually stimulating behavioral acts, “cheliceral massage” and “rubbing with legs”, were observed in all complete pairings during the Dance phase, sometimes together with “spermatophore deposition” and “positioning of the female”. However, these behavior categories were not observed during “female insemination”, when males pull the female over the spermatophore and keep her still, until the female starts the “pedipalp rattle”. With this last behavior, the couple separates their pedipalps and moves away from each other, and the female may threat the male with the metasoma if he does not release her pedipalps. The absence of any behavior other than holding the female pedipalps during insemination may indicate the absence of copulatory courtship in *T. serrulatus*.

2.4- Discussion

Our observations showed that the mating sequence in *Tityus serrulatus* is similar to the reported for other buthid species. Although *male insistence* behavior apparently favors female acceptance, coercive behaviors from the males were not particularly frequent in our observations, compared to the reported for exclusively sexually-reproducing species. Additionally, the timing of stimulus behaviors from the male are not suggestive of copulatory courtship, and females did not seem more resistant than those of other species. Thus, as will be discussed below, our results are not consistent with the predictions of the sexual conflict hypothesis, which posits that facultative parthenogenesis may be maintained by sexual conflict (Burke & Bonduriansky 2019).

In this study we report males and females defecating after the first contact in the arena. We think this behavioral act could be related to pheromone release, as observed in other arthropods such as cockroaches (Persoons et al. 1979) and *Drosophila* flies (Mercier et al. 2018). In fact, male scorpions of some families have glands at the end of the metasoma that release contact pheromones during mating (Peretti 1997b; Teruel et al. 2015) However, if the defecating behavior in *T. serrulatus* is related to courtship, apparently its function is not related to mating success, since we found no significant difference in the frequency of this behavior between complete and incomplete pairings.

We believe the function of this behavioral act deserve further attention, mostly based on chemical analyses of feces released during pairings. Studies like this, associated to behavioral experiments might contribute to our understanding of the role of sexual communication on the maintenance of parthenogenesis in the species, since pheromone signaling can be altered in populations that reproduce asexually (van der Kooi & Schwander 2014).

Female resistance

Our observations showed that *Tityus serrulatus* females are apparently not particularly resistant to mating, if compared to exclusively sexually reproducing species. The frequency of “threaten to sting”, the most aggressive behavior performed by the female, was very low (1.75%) and, when it occurred the male protect himself using the metasoma or escaped from the female. In most cases, the low receptivity of the female in courtship is demonstrated by the “absence of movement”, “escape”, or “slow metasoma swing”. Most importantly, most virgin females did not show resistance behaviors, mating with the first male they encounter.

Different from virgin females, females collected as adults (most of them probably have already mated in the field) exhibited resistance to mating with higher frequency. Adult female scorpions may be less receptive according to their previous mating experience or when they are pregnant (Peretti 2001; Peretti & Carrera 2005). Nevertheless, a female being resistant only from the second mating onwards may contribute little to the success of parthenogenesis, since females will be able to store and subsequently use the sperm from the first mating. Female scorpions can store sperm for late fertilization, producing multiple offspring from a single mating (Kovoor et al. 1987; Polis & Sissom 1990; Lourenço 2000). For parthenogenesis to be frequent, it would be necessary for the female to be resistant from the first encounter with a male and not only in the subsequent ones.

Male insistence and copulatory courtship

Males of *T. serrulatus* exhibit insistence behaviors as much as males of other species, who may constantly try to touch reluctant females until pedipalps are taken (Peretti 2001). In addition, insistent behaviors described here have probably been observed but reported differently in other species, as mentioned above. So, insistence is not an exclusive behavior of parthenogenetic species, and is probably always performed by males facing resistant females. Males of the yellow scorpion who use *male insistence* has more success in the mating among resistant females. Meanwhile, the stimulating *rubbing metasoma* showed no difference in frequency between complete and incomplete pairings, suggesting it is apparently not important behavior to mate with a reluctant female. So, our results indicate that insistence, an agonistic behavior, appears to be more effective in *T. serrulatus* than *rubbing metasoma*, a stimulatory behavior. However, other stimulus behaviors were observed in *T. serrulatus*, which does not allow us to assure that insistence is more successful in convincing a resistant female than stimulus behaviors.

We did not observe new stimulus behaviors in the courtship of *T. serrulatus* when compared to closely related species that reproduce only sexually. As *Z. fuscus* and *T. bahiensis* (Peretti 1991; Outeda-Jorge 2010), *T. serrulatus* exhibit “cheliceral massage” and “rubbing with legs”. Both behaviors are present in all stages of the *T. serrulatus* courtship, except during “female insemination”, indicating the possible absence of copulatory courtship. However, “cheliceral massage” was observed concomitantly with “spermatophore deposition” and “positioning of the female”, two behavioral acts that immediately precede insemination. Since those behavioral acts have been observed in almost every pairing, stimulus behaviors seem to be fundamental to the mating success. So, in addition to the insistence by the male, stimulatory behaviors could also induce resistant female to mating, favoring the sexual reproduction.

Contrary to what was previously proposed in this study, *T serrulatus* can exhibit even fewer behavioral acts that could force the female to mate, especially during insemination, if compared to other species. For example, *Tityus melici* Lourenço 2003, a species phylogenetically close to *T. serrulatus* (Souza et al. 2009), might show coercive behaviors during female insemination (personal observation, based on two laboratory-staged pairings), similar to what has been reported for *Z. fuscus* (Peretti 2001). Insemination in *T. melici* is marked by tugging and pushing of the female over the spermatophore and finish with the male forcing the female over the spermatophore by pressing the anterior part of his body over the female. In *T. serrulatus*, the male only guides the female over the spermatophore, not exhibiting behaviors that could be interpreted as compelling the female to position herself over the spermatophore and not showing any behavior during insemination. However, it is unknown whether *T. melici* also reproduces by parthenogenesis. To advance our understanding of the relationship between parthenogenesis and mating behavior in scorpions, it would be necessary to increase our knowledge on courtship behavior in species that reproduce through facultative parthenogenesis.

In this study, we described for the first time the mating of the Brazilian yellow scorpion, as a first step towards the study of the relationship between sexual conflict and parthenogenesis in scorpions. We concluded that virgin females of *T. serrulatus* do not offer resistance to mating, thus facilitating sexual reproduction. In addition, insistent and sexual stimulus behaviors can help to maintain sexual reproduction in the species.

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Table Chapter 2

Table 1: Behavioral category performed by males and females of *Tityus serrulatus* (Buthidae) during courtship and mating.

Phase	**Behavioral category**	**Description**	**Duration** (mean ± SD)
Initiation	Environment exploration	Male and female do not show evident sexual behavior. The couple has not yet identified the partner or, if both had already met in the terrarium, there was little sexual behavior. In some courtships, after male and female found and separated again, male or female defecate in the terrarium.	5.38 ± 0.37 minutes
Initiation	Couple presentation	Physical contact between the male and the female. The encounter appears to be accidental, tending the female to reach the place where the male is, since the females walker than the males. Males start courtship upon contact with female, vibrating the body, a behavioral act exhibited until the beginning of the dance phase. Male and/or female inclines the metasoma sideways to the body toward the side the partner is in. Occurs pulsation of the metasoma of the male and/or female, a behavioral that may be exhibited until the post-transfer phase. In addition, the male expands the pectens, and moves them sweeping the substrate.	40.8 ± 41.4 seconds
Initiation	Rubbing metasoma	Male rubs his metasoma into the female metasoma, and vice versa, especially in the anal region. In addition, male and female may rub the metasoma into different parts of each other, such as pedipalps, prosoma and mesosoma, and simultaneously rub the anal region in the oral region of the other.	27.0 ± 30.6 seconds
Initiation	Male Insistence	Male tends to insist on the female to remain in the courtship, a behavioral act that occurs when the female is not receptive. Male pulls the metasoma, the legs and the pedipalps of the female, which resists. If the female is in motion, the male hangs on the legs and metasoma of the female, that drags him around.	22.2 ± 16,2 seconds
Initiation	Touch	Touches made by the pedipalps, mainly of the male, in several regions of the body of the partner, such as metasoma, legs, pedipalps and chelicerae. When grasper one of the pedipalps of the female, the male rotates his body until they both stand facing the chelicerae, in a position ready to begin dancing. At the end, the male protrudes the chelicerae, maintaining them until the separation of the couple. Opening and closing movements of chelicerae can be performed. *Touch* can occur simultaneously with *Rubbing metasoma*.	1,03 ± 0,59 minutes
Dance – *Promenade à Deux*	Dance - Module I	Male holds, with his own pedipalps, the pedipalps of the female. Male can release and pick up one of the female pedipalps, but rarely gives up the two pedipalps simultaneously. The couple walks in the terrarium, where the male leads the female, pulling or pushing. The couple can hold pauses, during which the male stops almost all activity, but still holding the pedipalps of the female. When stopping, the couple may be with the chelicerae near or far	5,68 ± 8,49 minutes

Dance – *Promenade à Deux*		from each other. There are successive movements of the approximation and distance from the pair, in which, in the approach, the male moves the leg I in the genital opening of the female and rubs the chelicerae in the chelicerae of the female or in the base of the pedipalps. During the movement of approximation and away, the male inclines the metasoma ahead, and moves it smoothly.	
	Dance-Module II	The stops occur only with the chelicerae close to each other, and the approximation and distance movements are accompanied by intense movements of the male metasoma, which tend to be circular and sides. The movement of the male legs I in the genital opening of the female and the cheliceral rubbing also tend to be more intense. Couple moves less through the terrarium compared to phase I.	2,65 ± 1,81 minutes
Sperm Transfer	Spermatophore Deposition	Male releases the spermatophore through the genital opening, and adheres it to the substrate. While the male makes the deposition, he raises and oscillates the metasoma intensely, performing touch on the female's chelicerae with his own chelicerae and movement the legs I on the genital opening of the female.	10,8 ± 14,4 seconds
Sperm Transfer	Insemination	Male pulls the female over the spermatophore. She remains a few seconds with the genital opening on the spermatophore spermatic duct, until sperm transfer occurs.	9.0 ± 3.6 seconds
Pos-Sperm Transfer	Post-transference	Composed of several behavioral acts. Although it is a very diversified phase, some behavioral acts tend to occur few times between different couples. In this phase, the separation of the couple occurs and the male releases the pedipalps of the female. Some males consumed the spermatophore, either without removing it from the place of deposition, or when it had walked with the same by the terrarium.	2,28 ± 1,89 minutes

Figures Chapter 2

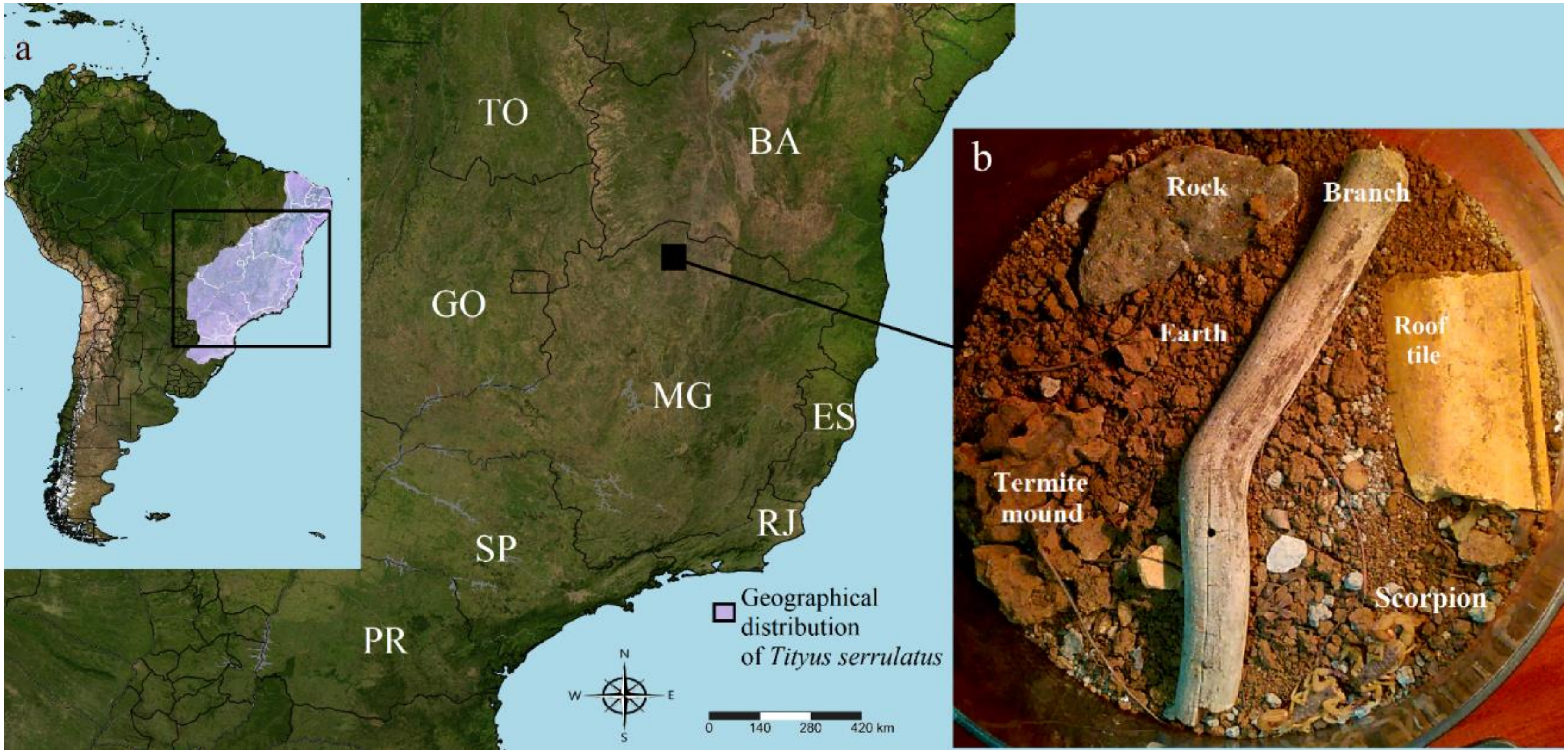

Figure 1: a) Geographic distribution of *Tityus serrulatus* (Buthidae) and location of the population used to describe mating behavior. b) Terrarium used for the study of mating behavior of *Tityus serrulatus*.

Figure 2: Categories and behavioral acts observed in the courtship and mating of *Tityus serrulatus* (Buthidae). a. *Environment exploration* – "Defecate" (male, arrow indicates feces). b. *Environment exploration* – "Defecate" (female). c. *Couple presentation* – "Inclination of metasoma". d. *Rubbing metasoma* – "Rubbing with metasoma". e. *Male insistence* – "Male pulls the female". f. *Touch* – "Body grip".

Figure 3 Categories and behavioral acts observed in the courtship and mating of *Tityus serrulatus* (Buthidae). a. *Dance – module I*. b. *Dance – module II*. c. *Spermatophore deposition*. d. *Insemination* (arrow: flagellum of spermatophore). e. *Pos- transference* (arrow: spermatophore after insemination). f. *Pos- transference* – “Spermatophore consumption” (arrow: adhesive substance, where the spermatophore was adhered).

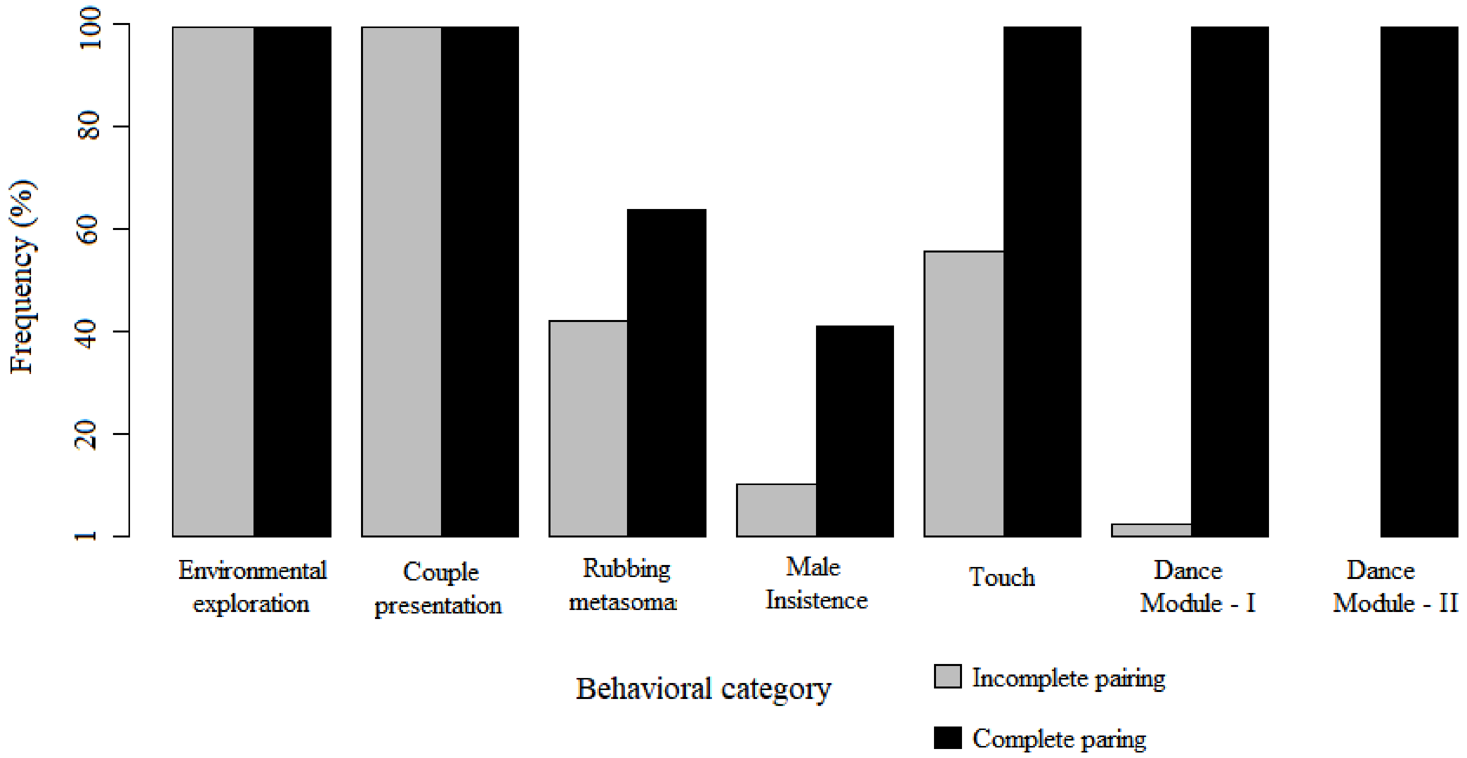

Figure 4: Frequency of behavioral categories observed in "incomplete pairing" and "complete paring" in *Tityus serrulatus* (Buthidae).

Supplementary Materials Chapter 2

Supplementary material 1: Behavioral acts performed by males and females of *Tityus serrulatus* (Buthidae) during courtship and mating. Abbreviations: EE, *Environment exploration*; CP, *Couple presentation*; RM, *Rubbing metasoma*; MI, *Male insistence*; T, *touch*; D1, *Dance -module I*; D2, *Dance-module II*; SD, *Spermatophore deposition*; I, *Insemination*; PT, *Post-transference*.

Behavioral acts	**Description**	**Sex**	**Category**	**References**
Individual displacement	Locomotive movements, mainly forward, slow or fast.	♀ ♂	EE	*Desplazamiento individual*- Peretti 1991 *Deslocamento individual*- Outeda-Jorge 2010
Individual pause	Absence of locomotive movements, with immobility of the individuals.	♀ ♂	EE	*Detenimiento individual* Peretti 1991 *Pausa individual*- Outeda-Jorge 2010
Defecate	The metasoma stretches horizontally, and the individual defecates.	♀ ♂	EE	
Encounter	Male encounter with female.	♀ ♂	CP	*Encontro*- Outeda-Jorge 2010
Escape	Individual escapes in response to behavior of the opposite sex.	♀ ♂	CP, RM, MI, T	
Absence of movement	Female remains completely still, with the body shrunk, without showing any movement.	♀	CP, RM, MI, T	
Mutual distance	After encounter, male can release female and one move away from each other	♀ ♂	CP	*Afastamento mútuo*- Outeda-Jorge 2010
Vibration	Vibratory movements of the body, especially the pedipalps	♂	EE, CP, RM, MI, T D1 PT	*Vibración*- Peretti 1991 *Tremores*- Outeda-Jorge 2010
Pectens movement	Pectens are widely spread and sporadically sweep across the substrate	♂	All categorys	*Movimientos de peines*- Peretti 1991 *Movimento de pentes*- Outeda-Jorge 2010

Behavioral acts	**Description**	**Sex**	**Category**	**References**
Body touch	With pedipalps, individual slightly touches the body parts of the other	♀ ♂	T	*Tanteo de la hembra / Tanteo del macho*- Peretti 1991 *Sondagem do macho*- Outeda-Jorge 2010
Body grip	Male grabs female in various body parts, mainly legs and metasoma	♂	T	*Toma de región peribucal*- Peretti 1991 *Tomada de prossoma / Tomada de mesossoma / Tomada de metassoma / Tomada de pernas*- Outeda-Jorge 2010
Cheliceral protraction	Male protrudes the chelicerae	♂	T, D1, D2, SD, I, PT	*Protração de quelíceras*- Outeda-Jorge 2010
Cheliceral movement	After protraction, male opens and closes the chelicerae, moving the chelicerae apart	♂	T, D1, D2, I SD, PT	*Movimientos de quelíceros del macho*- Peretti 1991 *Movimento de quelíceras*- Outeda-Jorge 2010
Pedipalp grip	The male grasps the female in a pedipalp to pedipalp grip	♀ ♂	T, D1	*Toma de pinzas*- Peretti 1991 *Tomada de pedipalpos pelo macho*- Outeda-Jorge 2010
Loosening and taking pedipalps	Male releases one of the female's pedipalps and holds it again	♀ ♂	T, D1	*Soltura de pedipalpos pelo macho*- Outeda-Jorge 2010
Conduction	Joint locomotor movements of male and female, walking through the terrarium	♀ ♂	D1, D2	*Desplazamiento conjunto*- Peretti 1991 *Condução*- Outeda-Jorge 2010
Pause	Male and female, in pedipalp grip, stop moving They may be with cheliceras near or far	♀ ♂	D1, D2, I	*Detenimiento conjunto*- Peretti 1991 *Pausa*- Outeda-Jorge 2010
Cheliceral massage	Male touches, with his chelicerae, the chelicerae and the bases of the female pedipalps	♂	D1, D2, SD	*Roce con quelíceras*- Peretti 1991 *Roçar com quelíceras*- Outeda-Jorge 2010
Rubbing with legs	Movement of the first pair of legs of the male, touching the substrate or anterior ventral region of the female prosome	♂	D1, D2, SD, PT	*Roce con patas*- Peretti 1991 *Tateamento com pernas*- Outeda-Jorge 2010
Metasoma oscillation	Male moves the metasoma back and forth, sideways and circularly. It occurs at the end of the Dance phase, just before spermatophore deposition.	♂	D2	*Oscilación del metasoma*- Peretti 1991 *Oscilação do metassoma*- Outeda-Jorge 2010
Leg tremor	Male shakes legs, usually the fourth, while depositing spermatophore.	♂	SD	*Tremor de pernas*- Outeda-Jorge 2010

Behavioral acts Description Sex Category References

Behavioral acts	Description	Sex	Category	References
Inclination of metasoma	Individual tilts the metasoma sideways to its body and coincides with the side on which the another is positioned. The motion may be dorsal. Can be performed simultaneously between male and female.	♀ ♂	CP	*Arqueo del metassoma de la hembra / Arqueo del metassoma del macho*- Peretti 1991 *Inclinação do metassoma da fêmea / Inclinação do metassoma do macho*- Outeda-Jorge 2010
Fast metasoma swing	Individual moves fast the metasoma, curved over the body, from side to side, showing a lot of aggression. Can perform the behavior while moving towards the partner. Can be performed simultaneously between male and female.	♀ ♂	CP, PT	*Balanço do metassoma da fêmea / Balanço do metassoma do macho*- Outeda-Jorge 2010
Slow metasoma swing	Individual moves slow the metasoma, curved over the body, from side to side, showing little aggression. Can be performed simultaneously between male and female.	♀ ♂	CP, PT	*Balanço do metassoma da fêmea / Balanço do metassoma do macho*- Outeda-Jorge 2010
Clubbing	Individual, usually the female, strikes the other's metasoma. This uses its own metasoma with a shield.	♀ ♂	CP, PT	*Bate metassoma*- Outeda-Jorge 2010
Threaten to sting	Female threatens to sting the male, tries to reach the mesosome. Male protect yourself using the metasoma as a shield.	♀	CP, PT	
Metasoma pulsation	Individual curves the metasoma forward, exerting rapid flexions with the last segments and the telson.	♀ ♂	All categorys	
Female swaying	Female holds the legs fixed to the substrate and moves the body from side to side.	♀	EE, CP, PT	*Gingada da fêmea/ Movimento do corpo da fêmea*- Outeda-Jorge 2010
Rubbing with metasoma	Individual rubs metasoma on various parts of the body of the partner	♀ ♂	RM	*Roçar metassoma no corpo*- Outeda-Jorge 2010
Rubbing anus/anus	Both individuals rub anal region in the anal region of the other	♀ ♂	RM	*Arqueo conjunto de metassomas*- Peretti 1991 *Roçar metassoma*- Outeda-Jorge 2010
Rubbing anus/chelicerae	Both individuals rub anal region in the oral region of the other	♀ ♂	RM	
Male pulls the female	Male pulls many parts of the female's body, which remains stationary, without showing interest in mating	♂	MI	
Male anchor in female	Male anchors to female, holding legs or metasoma, while female walks through the terrarium, without showing interest in mating	♂	MI	

Spermatophore deposition	The male presses his genital opening against the substrate and sticks the base of the spermatophore to the substrate.	♂	SD	*Depósito del espermatóforo*- Peretti 1991 *Deposição do espermatóforo*- Outeda-Jorge 2010
Metasome stretch	Elevation and oscillation of the male metasoma, with posterior stretching, occurring during spermatophore deposition. The metasoma is erect, perpendicular to the body.	♂	SD	*Elevação do metassoma*- Outeda-Jorge 2010
Positioning of the female	The male assists the female in positioning her genital aperture over the spermatophore.	♀ ♂	I	*Ubicación de la hembra*- Peretti 1991 *Posicionamento sobre espermatóforo*- Outeda-Jorge 2010
Female insemination	Female remains embedded on the spermatophore, allowing sperm uptake.	♀	I	*Acción sobre el espermatóforo*- Peretti 1991 *Inseminação*- Outeda-Jorge 2010
Pedipalp rattle	Male and female rattle pedipalps, still united, until their separation.	♀ ♂	PT	
Separation	Individuals separate but remain in the same place.	♀ ♂	PT	*Separação*- Outeda-Jorge 2010
Pause around spermatophore	After separation, individuals stand around the spermatophore.	♀ ♂	PT	
Individual moving away	Individual withdraws from the place where the spermatophore was deposited.	♀ ♂	PT	*Afastamento da fêmea / Afastamento do macho*- Outeda-Jorge 2010
Individual removes the other	Individual makes moves, aggressive or not, to remove each other from spermatophore site.	♀ ♂	PT	
First pair movement	Male moves the first pair of legs in his ventral region, probably to release the spermatophore flagellum.	♂	PT	*Movimientos del primer par de patas*- Peretti 1991 *Movimentos de perna I*- Outeda-Jorge 2010
Walking with spermartophore	Male removes spermatophore from substrate with chelicerae and walks with him through the terrarium.	♂	PT	
Spermatophore consumption	Male consumes spermatophore.	♂	PT	*Consumo do espermatóforo*- Outeda-Jorge 2010
Cheliceral retraction	Male shrinks the chelicerae, which return to normal position.	♂	PT	*Retração de quelíceras*- Outeda-Jorge 2010

Supplementary material 2: Behavioral categories and acts performed by males and females of *Tityus serrulatus* (Buthidae) during courtship and mating. Link: https://youtu.be/5dow38jWTYE

Supplementary material 3: Defecate behavioral act observed during the mating of *Tityus serrulatus* (Buthidae). Link: https://youtu.be/I1v4kWjmzdc

Supplementary material 4: Behavior of *Tityus serrulatus* (Buthidae) females according to sexual receptivity (positive, intermediate and negative) and behavior of males in relation to female receptivity. Link: https://youtu.be/xxU5i_Q9u18

Chapter 3

Sex of asexuals: response of parthenogenetic females of the yellow scorpion *Tityus serrulatus* (Scorpiones: Buthidae) to courting males

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Abstract

Tityus serrulatus Lutz & Mello, 1922 has, as one of its most striking features, the ability to reproduce asexually. For many years, parthenogenesis was considered the only form of reproduction of this species. However, in 2009 the male of the species was described for the first time. With the discovery of a sexual population, one can ask whether parthenogenetic populations would be reproductively isolated from sexual population ones. In this study, we compared the sexual behavior of *T. serrulatus* males against sexual and parthenogenetic females. We staged pairings of males from a sexual population from Itacarambi, northern Minas Gerais (Brazil), with females from the same location and females from a parthenogenetic population in Belo Horizonte (Minas Gerais). We conclude that parthenogenetic females of the yellow scorpion can accept mating with males, showing the same behavior patterns observed in pairings with females from the sexual population. However, parthenogenetic females showed more resistance to mating. The mating duration with parthenogenetic females is significantly longer than the mating with sexual females, mainly in the Initiation, species and sexual recognition phase. Our results suggest that parthenogenetic populations are not completely isolated reproductively from sexual ones, though with vestigialization of sexual behavioral traits in parthenogenetic females.

Keywords: Female resistance, mating behavior, vestigialization

3.1- Introduction

Phylogenetic evidence suggests that most species or populations that reproduce by parthenogenesis, in which embryos develop from unfertilized eggs, evolved recently from sexual ancestors (Butlin 2002). Some parthenogenetic populations are isolated from their sexual ancestors for many years, and it is controversial whether these linages that reproduce only by parthenogenesis should be considered independent species or considered the same species as their parents (Soltis et al. 2007). Birky and Barraclough (2009) established criteria for considering parthenogenetic lineages as independent and, therefore, belonging to a different species than their ancestral. One of these criteria is reproductive isolation. So, studying differences in courtship behavior between sexual and parthenogenetic populations can help to understand whether both are in speciation.

In sexual populations, courtship and mating behavior are under strong sexual selection pressure and for this can evolve rapidly (Barlow 2002); however, in populations that reproduce by parthenogenesis, traits that were related to sexual reproduction are no longer under selection (Normark 2003). Thus, these traits can be reduced or disappear entirely in asexual lineages, in a process called “vestigialization” (Fong et al. 1995). Vestigialization of sexual traits can be observed in many arthropods, as *Drosophila mercatorum* Paterson & Wheller, 1942, in which parthenogenetic females showed reduced mating propensity towards conspecific males, compared to sexual females (Carson et al. 1982). Asexual females of the bush cricket *Poecilimon intermedius* Fieber, 1853 exhibit no phonotaxis towards singing of closely related interespecific males, although spermatophores is transferred and sperm successfully enter the female genital opening when males and females are exposed in captivity (Lehmann et al. 2011). Parthenogenetic females of *Timema* Scudder, 1895 stick insects have modified sperm storage organs, produce altered pheromone and lost ability to fertilize their eggs (Schwander et al. 2013).

In scorpions, parthenogenesis has already been confirmed in eleven species. (Francke 2008; Ross 2010; Seiter 2012; Seiter et al. 2016; Seiter & Stockmann 2017). The earliest reported, and the better-known parthenogenetic species is the Brazilian yellow scorpion *Tityus serrulatus* Lutz & Mello, 1922 (Matthiensen 1962). For a long time, *T. serrulatus* was considered an exclusively parthenogenetic species composed of female-only populations (de Toledo Piza 1939; Bücherl 1956; Lourenço 1981). However, sexual populations have been discovered, and the male of the species has been described recently (Souza et al. 2009; Santos et al. 2014; Lima et al. 2020). Sexual populations of *T. serrulatus* are rare, in comparison with exclusively female populations, which are registered in a large part of the Brazilian territory (Souza et al. 2009). Due to their high distribution, compared to sexual populations, parthenogenetic populations may be the product of ancient colonization events, and therefore have been devoid of sexual reproduction for some time, being in the absence of sexual selection. So, it is expected that there will be changes in sexual behavior of parthenogenetic females, compared to sexual females.

Specific recognition in scorpions occurs through small variations in mating behavior, as the courtship involves a complex series of stereotyped behaviors, most of which are conserved among taxa (Polis & Sissom 1990, Peretti 1993). Differences in specific recognition may involve frequency in certain sexual behaviors, such as mating-stimulating behaviors (Peretti 1993). In general, scorpion courtship can be divided into four phases: Initiation, the stage in which specific recognition occurs; Dance (*promenade à deux*), in which the male seeks an adequate place for sperm deposition; Sperm transfer, in which the male deposits the spermatophore on a substrate and the female is inseminated; post-transfer, with sexual behaviors that occur after couple separation (Alexander 1959, Tallarovic et al. 2000, Benton 2001). The courtship of *T. serrulatus* is similar to the reported for other scorpion species (Chapter 2). As described for other species (Peretti 2001, Peretti & Carrera 2005), virgin females of *T. serrulatus* are more receptive to mating, and males do not use coercive behaviors to mate with reluctant females, with insistence and sexual stimulation behavior predominating (Chapter 2). One of the most interesting characteristics of sexual populations is that parthenogenesis is present and occurs on an optional basis (Braga-Pereira & Santos in press). Facultative parthenogenesis, the capacity to reproduce both sexually and asexually, is considered an evolutionary step in the transition from sexual reproduction to obligatory parthenogenesis (Normark 2003, Schwander et al. 2010). So, if females from exclusively parthenogenetic populations show evidence of vestigialization behavior in relation to sexual populations, parthenogenetic strains may be specifying in relation to their sexual ancestors.

In the present study, we test if females collected in an exclusively parthenogenetic population of *T. serrulatus* are more resistant to mate with males from a facultative parthenogenetic population. Specifically, we compare the mating behavior sequence of mating pairs composed of asexual and facultative parthenogenetic females in order to verify whether those populations are reproductively isolated at the behavioral level.

3.2 - **Methods**

Collection and rearing

We collected 47 females (37 adults and ten juveniles) and 45 males (33 adults and 12 juveniles) of *T. serrulatus* in urban and suburban regions of Itacarambi, Minas Gerais (15.19S, 44.20W), in March 2016 and January 2017. We search the scorpions in the morning, under trunks, rocks, bricks and roof tiles and inside termite mounds. During the night, we collected active animals using ultraviolet light. We also collected or received as donations from colleagues 37 *T. serrulatus* females (29 adults and eight juveniles) from urban areas in Belo Horizonte, Minas Gerais (19.92S, 43.94W), during 2016 and 2017 (Fig. 1). Heretofore, we refer to females from the former locality as "sexual females", and "parthenogenetic females" to the latter ones.

We brought the individuals to the laboratory and kept them for 60 days before the observations. We recorded each individual with a number and kept them individually in plastic cages (8.5 cm diameter X 6.5 cm height), with cotton absorbed in water and a piece of cardboard as a refuge. We fed them with one cockroach (*Nauphoeta cinerea*) per week. Scorpions were kept in the laboratory at a mean temperature of 25 ± 3 °C, 58 ± 11% relative humidity and 12 hours photoperiod. We used only adult individuals in the observations. Subadults were kept in containers until adulthood and were used for observations at least 60 days after their last molt. We deposited voucher specimens in the arachnid collection of the Centro de Coleções Taxonômicas da Universidade Federal de Minas Gerais.

Observation of sexual behavior

We made observations from May of 2016 to November of 2017. We did two experiment sets; one in which we paired males with sexual females (intra-population mating) and another with males with parthenogenetic females (inter-population mating). We used glass containers (25 cm diameter X 19 cm height) as arenas for these observations, with earth as substrate, a rock, a piece of termite mound, a piece of roof tile, and a branch going through the diameter of the terrarium (Supplementary material 1). We use two terraria with similar conditions, one for observations with sexual females and another for observations with parthenogenetic females, in order to avoid possible pheromones released by sexual females that could influence the identification of parthenogenetic females by males (see chapter 4). Mating observations were done at same temperature and humidity mentioned above, always at night (21:00h to 00:00h), when the species is active (Mineo et al. 2009).

We randomly selected female-male pairs and placed them in a mating arena and waited for the initiation of the mating sequence for up to 40 minutes. Mating sequences initiated during that period were then recorded until all sexual behaviors ended. We tested 100 mating pairs with sexual females and 100 mating pairs with parthenogenetic females and used each specimen, males and females from both populations, in two or three pairings. Males were also used in two or three pairings for each experiment set. Among the subadult specimens, eight molted as adult males in the laboratory. Thus, for the first pairing we randomly assigned four of them to parthenogenetic females and the other four to sexual females. Although specimens were used repeatedly in different pairing observations, no exact male and female combination was repeated. We started the observations under red light; but turned the room illumination to white light after the beginning of the mating sequence, in order to facilitate the observations. Our previous observations show that, although white light inhibited the specimen’s behavior before the copulation, the change of ambient light after the initiation of the mating sequence has no effect on their activity. Specimen behaviors were recorded with photos taken with a Microsoft Lumia 640 mobile phone, videos from a Canon EOS Rebel T5 camera, and written notes.

To check if the parthenogenetic females got pregnant after mating, we kept them under the same environmental and feeding conditions mentioned above for specimens rearing. We check the females three times a week for two years to record possible births. We record the time between mating and the birth of juveniles. In addition, we raise the offspring of two females until adulthood, keeping them in separate containers and under the same environmental conditions, in order to identify the gender of the individuals, since the same is not possible with young. We fixed the offspring from other females in 95–100% ethanol at -20 °C.

Female resistance

Our hypothesis of reproductive isolation between parthenogenetic and sexual populations predict that parthenogenetic females should be more resistant to mating than sexual females. Thus, we compared the frequency of male acceptance by the females in both groups. Males were considered as accepted when the mating sequence reach the last stage, with female insemination. In addition, we checked whether parthenogenetic females show the same frequency of receptivity status as females from sexual populations. Three status of female receptivity have been proposed for Buthidae scorpions. Positive status, when females accept mating with males, without any sign of resistance, is usually observed in approximately 80% of virgin females. In the Intermediate status, females accept copulation but show some type of resistance during mating. Finally, females in the Negative status reject mating, after many attempts at courting the male (Peretti 2001, Peretti & Carrera 2005).

Receptiveness in scorpions decreases according to the number of previous inseminations (Peretti & Carrera 2005). This is not possibly for parthenogenetic females, as they do not mate; this female are by definition virgins, even though they might have given birth previously. However, it is possible that female receptivity decreases according to the number of reproductive events caused by parthenogenesis. In this context, adult collected females may have originated offspring in the field and their receptivity may be different from that of a female that had no offspring. Sexual females collected as adults may be virgins, or may have been previously inseminated (having given birth or not) or may have reproduced by parthenogenesis. Thus, they could show different receptivity status. So, only parthenogenetic and sexual females who became adults in the laboratory (thus, surely virgins) were used for receptivity comparison. We compared the frequency of receptivity status between sexual and parthenogenetic females through the Fisher's exact test, considering a significance level of 5%.

Behavioral repertory, mating sequence and data analyses

To assess whether there are differences in sexual behaviors between intra-population mating and inter-population mating, we compared the behavioral repertory between mating observations using as reference a previous ethogram of *T. serrulatus* mating sequence (Chapter 2, Table 1). Braga-Pereira & Santos (Chapter 2) recorded 46 behavioral acts, classified into ten behavioral categories (Table 1, Fig. 2, 3). We compared if the sequence of transitions of the categories are the same, as well the duration of each category, using a first order Markov chain built with the software

Jwatcher 1.0 (www.jwatcher.ucla.edu), which was analyzed with the TraMineR package (Gabadinho et al. 2011). We evaluated if the behavioral sequence followed or not a random pattern using a chi-square test (significance level of 5%), where a matrix for observed frequencies was compared with a previously built random matrix for expected frequencies. Matrices were represented using flow diagrams. To compare the duration of each behavioral category between intra-population mating and inter-population mating, we use a mixed linear model, since the measurements are repeated (use of the same individual in different pairings). We used duration of the categories as response variable and intra-population and inter-population mating as explanatory variable.

It is possible that males of *T. serrulatus* have a greater difficulty in guiding females of a size relatively larger than his, requiring more time during the mating, especially in the Dance phase. For this, the differences in mating duration may not be related to parthenogenesis, but to differences in relation to the size of the females. Parthenogenetic *T. serrulatus* females tend to be smaller than sexual females (Braga-Pereira et al. in prep.). If size influences mating duration, we expect females from parthenogenetic populations to have shorter mating durations, since they are smaller. To assess whether size influences mating time, we photographed the individuals of sexual population on graph paper and measured their carapace length using the ImageJ software (Rasband 1997-2018). Among the individuals that copulated, we analyzed whether the size of the male relative to the female influenced the duration of Initiation, Dance, and Sperm transfer phases, using Pearson product-moment correlation coefficient. We made all analyzes using R version 4.0.2 (R Development Core Team, 2020).

3.2- Results

Female resistance

Among the pairings performed in this study for each experimental set, parthenogenetic females of *T. serrulatus* agreed to mate in a similar proportion to sexual females, with no significant difference frequency (X-squared: 0.0301, p=0.8622). Males mating (with spermatophore deposition and possibly female insemination) with parthenogenetic females in 20 of 100 pairings (20%) and with sexual females in 22 of 100 pairings (22%). In a single pairing with a parthenogenetic female the male was unable to release the spermatophore during its deposition. We exclude this pairing from the analyzes. Of the 20 parthenogenetic females that mated, 12 gave birth after mating, with an average of 309.46 days (standard deviation = 108.71) between mating and birth. Regarding the offspring of the two parthenogenetic females whose post-embryonic development was monitored, one was made up of 11 individuals, reaching adulthood five individuals. The other offspring consisted of 6 individuals, reaching adulthood only one individual. We identified all individuals as females.

Parthenogenetic females showed the same receptive behaviors observed by Braga-Pereira & Santos (Chapter 2) for sexual females: *Positive*: females did not avoid male courtship behaviors (did not completely stop and did not walk in the opposite direction) as *touch* and *rubbing metasoma*, and males showed no behavioral acts that could be interpreted as insistent or coercive. *Intermediate*: females showed resistance during mating, remaining still and motionless or walking in the opposite direction from the male. She could also move the metasoma in a curved position, over the body itself, slowly. *Negative*: females did not accept mating. She moved the metasoma quickly in a curved position over its own body. In some cases, she hit her own metasoma on the male's metasoma and more rarely, attempting to sting the male.

Although the mating frequency was similar between the two experimental groups, parthenogenetic virgin females were more resistant than sexual virgin females. Among the 10 virgin sexual females tested in a first pairing, nine mated (eight females in the positive status and one in the intermediate status), and a single female also mated in the second paring (in the intermediate status in both pairings). Of the eight parthenogenetic females, four mated in the first pairing (one in the positive and three in the intermediate status) and four females did not mate in any pairing. So, of the females that became adults in the laboratory and did not have previous offspring, 80% of sexual females were in the positive status, compared to only 12.5% of parthenogenetic females, a significant difference between the two groups (Fisher's exact test, p-value: 0.002879).

Behavioral repertory and mating sequence

Behavioral repertory was analyzed for 19 of 22 successful intra-population and 17 of 20 inter-population successful mating. Six mating sequences were discarded because we did not get videos with sufficient quality to record the duration of behavioral acts. We found the same 46 behavioral acts described by Braga-Pereira & Santos (Chapter 2) for *T. serrulatus,* in both populations. In both experiment sets, the Initiation phase was marked by alternation between the behavioral categories, while the other phases (Dance, Sperm-transfer, Post-transfer) tend to be unidirectional (intra-population: X^2_{81}= 1418.57, p-value <0.001, fig. 4a. Inter-population: X^2_{81}= 1637.57, p-value <0.001, fig. 4b). However, behavioral sequences showed a few differences between the experiment sets. For example, in sexual populations, *male insistence* sometimes returned to the *couple presentation*, with no such transition in inter-population mating. Total mating duration of inter-population mating was significantly longer than intra-population mating ($X^2_1 = 4.01$, $p<0.05$, Fig. 5b). Except for the *dance-module I* category, the duration of all behavioral categories was significantly longer in the inter-population mating (X2= 4.01, p< 0,05. Fig. 5a).

Sexual females of T. *serrulatus* are larger than parthenogenetic. Sexual females have carapace length (mean ± standard deviation) 7,69 ± 0,84 mm, (range = 5.36 - 9.84), males have 7,99 ± 1,21 mm (range = 5.76 - 10.45) and parthenogenetic females have 6,69 ± 0,57 mm, (range = 5.25 – 7.05). The duration of Initiation and Sperm transfer phases were positively correlated with size ratio (p-value = 0.04673 / cor = 0.4614487, p-value = 0.03446 / cor = 0.4869978, respectively). The duration of the Dance phase was not significantly correlated with size ratio (p-value = 0.2914 / cor = -0.2553469) (Fig. 6).

3.3- Discussion

Our observations indicate that parthenogenetic females of *T. serrulatus* accept to mate with males in the same frequency that sexual females but are more resistant to mating. The courtship was similar in intra and inter-population pairings, but time spent in mating with parthenogenetic females is greater than the time spent with sexual females, differing mainly in the Initiation and transfer of sperm. Thus, as will be discussed below, our results indicate that the parthenogenetic females of *T. serrulatus* have traces of vestigialization, in relation to sexual populations.

We observed the same behavioral units in intra and inter-population mating of *T. serrulatus*. However, mating with parthenogenetic females took longer than with sexual females, except in *dance- module I*. In the sexual population, the mating duration is longer when females are relatively larger. Since parthenogenetic females are smaller, mating would be expected to be faster compared to sexual females. Therefore, size is not the factor related to the difference between our experiment sets.

The behavioral category *environment exploration* occurred with a longer duration in observations with parthenogenetic females, indicating that the couple took longer to meet or recognize each other. This was probably because parthenogenetic females passed through a long period of relaxed sexual selection pressure over male recognition channels. For instance, parthenogenetic females apparently have reduced pheromone emission capacity, or even produce no pheromones at all (Chapter 4.). Loss or modification of pheromones has been reported in parthenogenetic strains of other species, such as in females of spiny leaf stick insect *Extatosoma tiaratum* Macleay, 1826, which have alterations in pheromonal signals, becoming imperceptible to males (Burke et al. 2015). In our observations, the reduced pheromone emission was not strong enough to influence the frequency of mating with parthenogenetic females, possibly because of the restricted space within the terraria. However, the absence or reduction of pheromones can lead the individual to delay identifying the partner, requiring more time during the recognition phase.

The Initiation phase, when species and sexual recognition occurs (Benton 2001), can be longer in parthenogenetic females of *T. serrulatus* because they show greater resistance to courtship. Although the frequency of intra-population mating is similar to the frequency of inter-population mating, females from parthenogenetic populations showed greater resistance to mating when compared to females from the sexual population. Most of the virgin parthenogenetic females had intermediate or negative status, the male needing to invest more in the courtship, differently from virgin sexual females, who were mainly in positive status. Consequently, males showed higher investment in insistence and sexual stimulation behaviors, such as *metasoma rubbing* (Fig. 5a). As in other species of scorpions, males of *T. serrulatus* showed little or no coercive behaviors aimed at forcing resistant females to mate (Peretti 2001, Chapter 2). For this reason, female receptivity is essential for successful mating. If parthenogenetic females are less receptive, males need to be more insistent for mating to occur. If the female is in the negative status, mating does not occur, with the females usually chasing the males away.

An addition evidence on the low receptivity of parthenogenetic females is the return to the early stages of the courtship. As mentioned above, the whole mating sequence is similar between intra and inter-population pairings, but with a few specific differences (Fig. 4). Possibly the most important difference is that males in the Dance phase can perform a behavioral category from the previous (Initiation) phase when courting parthenogenetic females. This is, indicating that females are more resistant and requiring larger male investment in stimulus behaviors (*rubbing metasoma*) to encourage the female to continue mating.

The Dance phase is the moment when the male search for a suitable substrate for the deposition of the spermatophore (Benton 2001). The only behavioral category that showed no differences between intra and inter-population mating was *dance-module I*, which is characterized as the phase in which the couple move more through the terrarium than *dance- module II* (Chapter 2). In *dance-module I*, the couple has a greater movement through the terrarium, probably because the male is still looking for a suitable substrate for spermatophore deposition. In *dance-module II*, the couple remains in the same place, indicating that the male has already found the appropriate substrate. As there is no significant difference between the duration of the *dance- module I* between intra and inter-population mating, it is probably that the duration of this category is more related to the environment than to the interaction between males and females, being influenced by the difficulty of the male in find a suitable substrate for sperm deposition.

During the Sperm transfer, males took longer to deposit the spermatophore and to inseminate parthenogenetic females. The longer time spent by the male may be due to the lesser collaboration of the female during the Sperm transfer phase, while the longer insemination time may be related to resist mating by closing the genital operculum during Sperm transfer (Peretti 2001). In our observations, we were unable to observe the fit of the spermatophore in the female genital opening, and therefore we do not know whether sperm transfer was successful in parthenogenetic females. So, the time difference between intra and inter-population mating during female insemination may also be related to low female receptivity. The resistance of females during the Sperm transfer phase, as well as in the Initiation phase, may indicate that parthenogenetic females have reduced propensity to copulation, as well as *D. mercatorum* (Carson et al. 1982), which could be a first step towards the process of speciation between sexual and parthenogenetic populations.

In this study we conclude that parthenogenetic females of *T. serrulatus* have traces of vestigialization, in relation to sexual populations, since females from exclusively female populations are more resistant during mating. However, although parthenogenetic females of *T. serrulatus* have mated with males, we do not know if fertilization occurred. For example, parthenogenetic females of *Timema* stick insects have changes in spermatheca morphology and the ability to fertilize eggs, in relation to sexual sister species (Schwander et al. 2013). As in *Timema*, parthenogenetic females of *T. serrulatus* may have changes in their reproductive structures that prevent or hinder the formation of embryos by sexual reproduction. Based on our results, we cannot affirm whether the offspring of parthenogenetic females that mated are of sexual origin, or if fertilization did not occur and the females continued to reproduce by parthenogenesis, since our sample of juveniles raised to adulthood is very small. So, paternity tests of offspring born after mating can indicate whether there is reproductive isolation between sexual and parthenogenetic populations of the Brazilian yellow scorpion.

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Table Chapter 3

Table 1. Behaviors performed by males and females of *Tityus serrulatus* (Buthidae) during mating. The terms and definitions are based on scorpion literature for some species of Buthidae (Peretti, 1991, Outeda-Jorge 2010; adapted from Chapter 2).

Phase	**Behavioral category**	**Description**
Initiation	Environment exploration	Male and female do not show evident sexual behavior. In some courtships, after male and female found and separated again, male or female defecate in the terrarium.
Initiation	Couple presentation	Meet of the male and the female. Males starts courtship upon contact with female, vibrating the body. Male and / or female inclines the metasoma sideways to the body toward the side the partner is in. Occurs pulsation of the metassoma of the male and / or female. Male expands the pectens, and moves them, sweeping the substrate.
Initiation	Rubbing metasoma	Male rubs his metasoma into the female metasoma, and vice versa. In addition, male and female may rub the metasoma into different parts of each other.
Initiation	Male Insistence	Male tends to insist on the female to remain in the courtship, occurring when the female is not receptive. Male pulls the metasoma, the legs and the pedipalps of the female, which resists. If the female is in motion, the male hangs on the legs and the metassoma of the female, that drags it.
Initiation	Touch	Touches made by the pedipalps, mainly of the male, in several regions of the body of the partner. When grasper one of the pedipalps of the female, the male rotates his body until they both stand facing the chelicerae, in a position ready to begin dancing. At the end, the male protrudes the chelicerae.
Dance – *Promenade à Deux*	Dance - Module I	Male holds, with own pedipalps, the pedipalps of the female. The couple walks in the terrarium, where the male leads the female, pulling or pushing. There are successive movements of the approximation and distance from the pair, in which, in the approach, the male moves the leg I in the genital opening of the female and rubs the chelicerae in the chelicerae of the female. Male inclines the metasoma ahead and moves it smoothly.

Phase	Behavioral category	Description
Dance – *Promenade à Deux*	Dance-Module II	Approximation and distance movements are accompanied by intense movements of the male metasoma. The movement of the male legs I in the genital opening of the female and the chelicerae rubbing also tend to be more intense. Couple moves less through the terrarium, compared to phase I
Sperm Transfer	Spermatophore Deposition	Male releases the spermatophore through the genital opening, where it adheres to the substrate. While make the deposition, the male raises and oscillates the metasoma intensely, executing touches on the female's cheliceras, with their own cheliceras.
Sperm Transfer	Insemination	Male pulls the female over the spermatophore; it remains a few seconds with the genital opening on the spermatophore spermatic duct, until sperm transfer occurs.
Pós-Sperm Transfer	Post-transference	Composed of several behavioral acts. In this phase the separation of the couple occurs, in which the male releases the pedipalps of the female. Some males consumed the spermatophore.

Figures Chapter 3

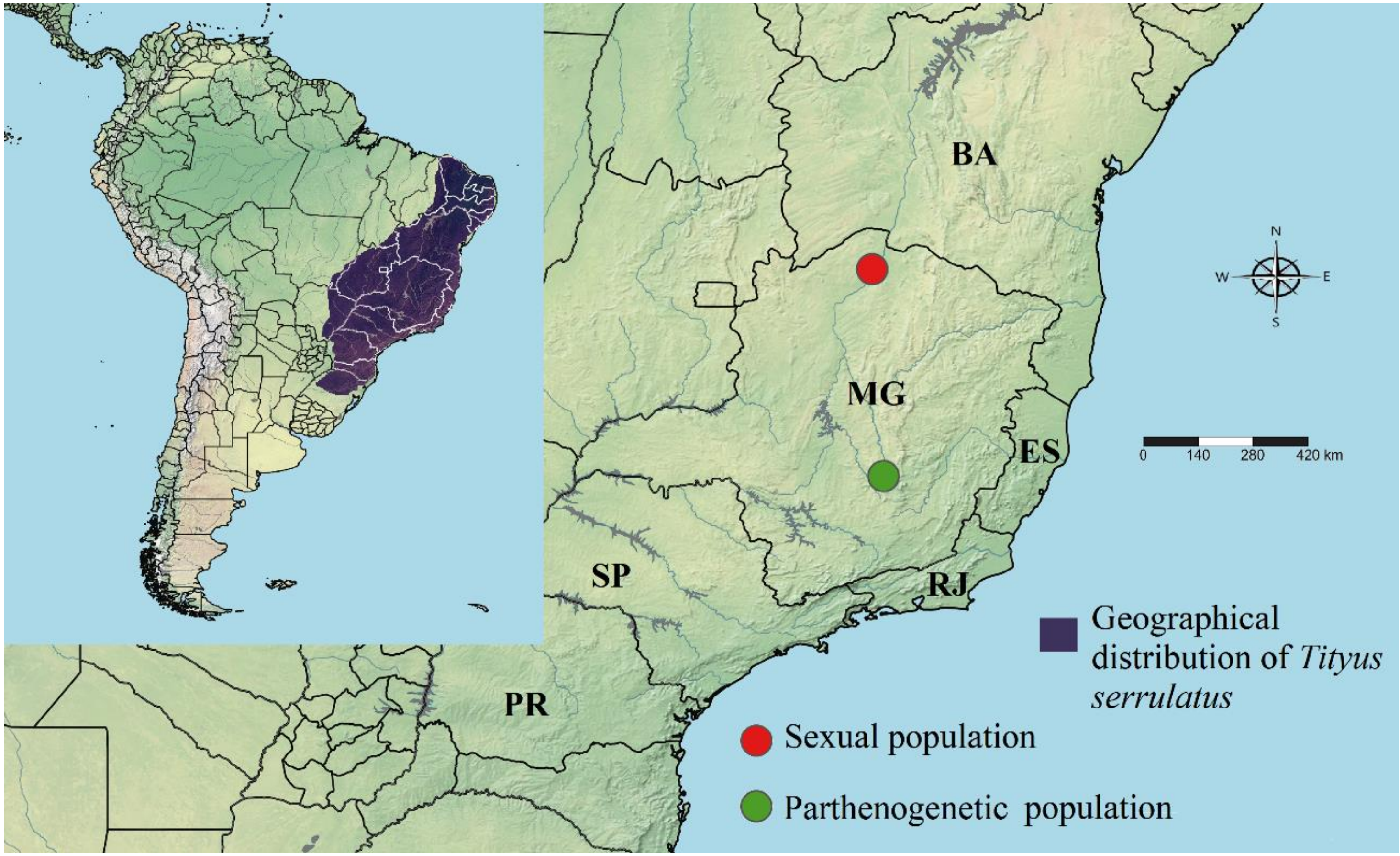


Figure 1: Geographic distribution of *Tityus serrulatus* (Buthidae) and source location of specimens used in this study.

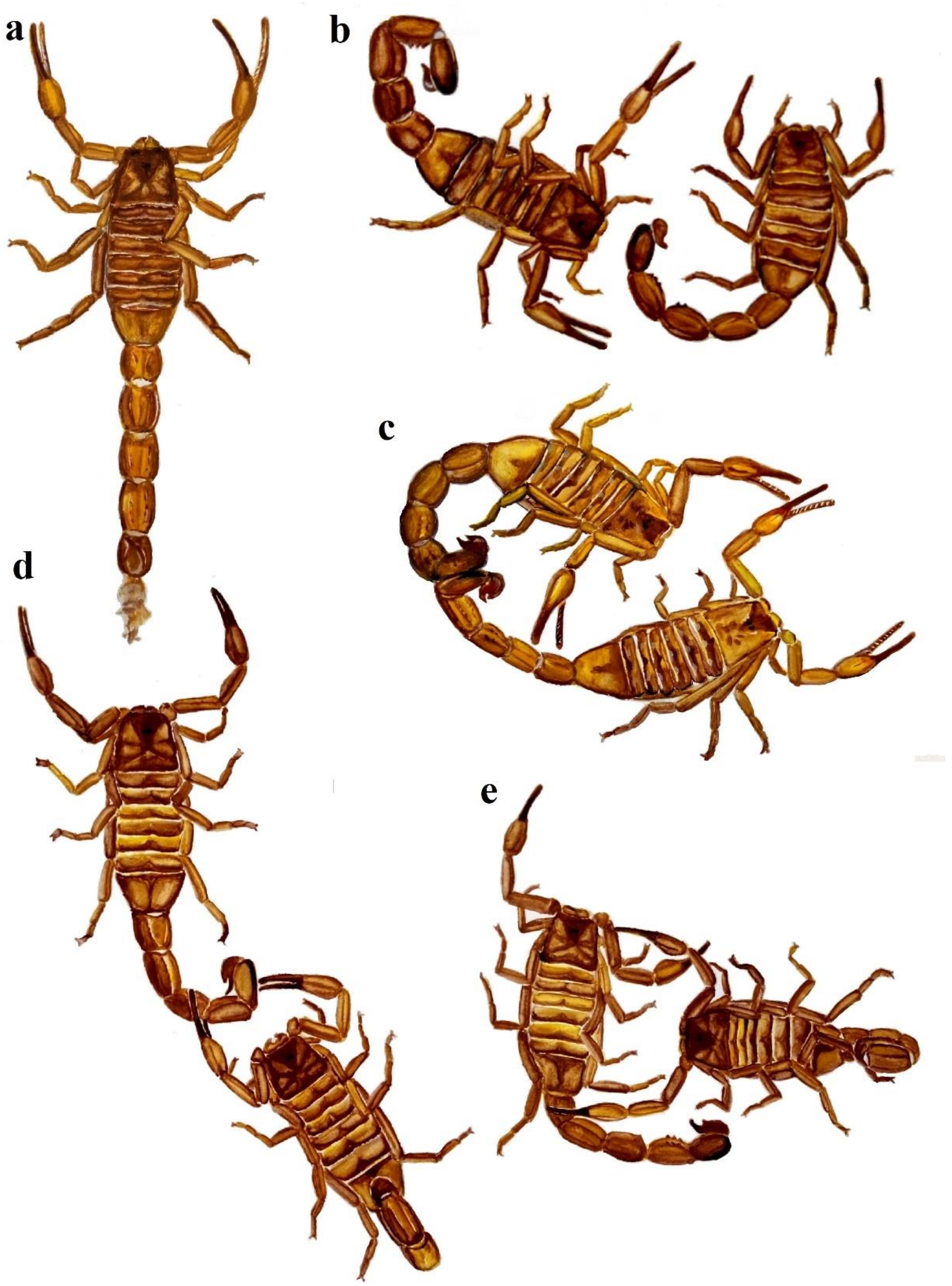


Figure 2: Behavioral categories observed in the courtship and mating of *Tityus serrulatus* (Buthidae). a. *Environment exploration*- Individual defecates. b. *Couple presentation*- Female tilts metasoma, while male courts her. c. *Metasoma rubbing*- Male and female rub each other's anal region. d. *Male insistence*- Male grabs the female's metasoma while she moves away from him. e. *Touch*- Male touches several parts of the female.

Figure 3: Behavioral categories observed in the courtship and mating of *Tityus serrulatus* (Buthidae). a. *Dance – module I*- Male and female walk through the terrarium. b. *Dance – module II*- Male touches the female chelicera with his own chelicerae. c. *Spermatophore deposition*- Male deposits spermatophore on substrate. d. *Insemination*- Female positions herself over the spermatophore e. *Pos- transference*- Male consumes spermatophore.

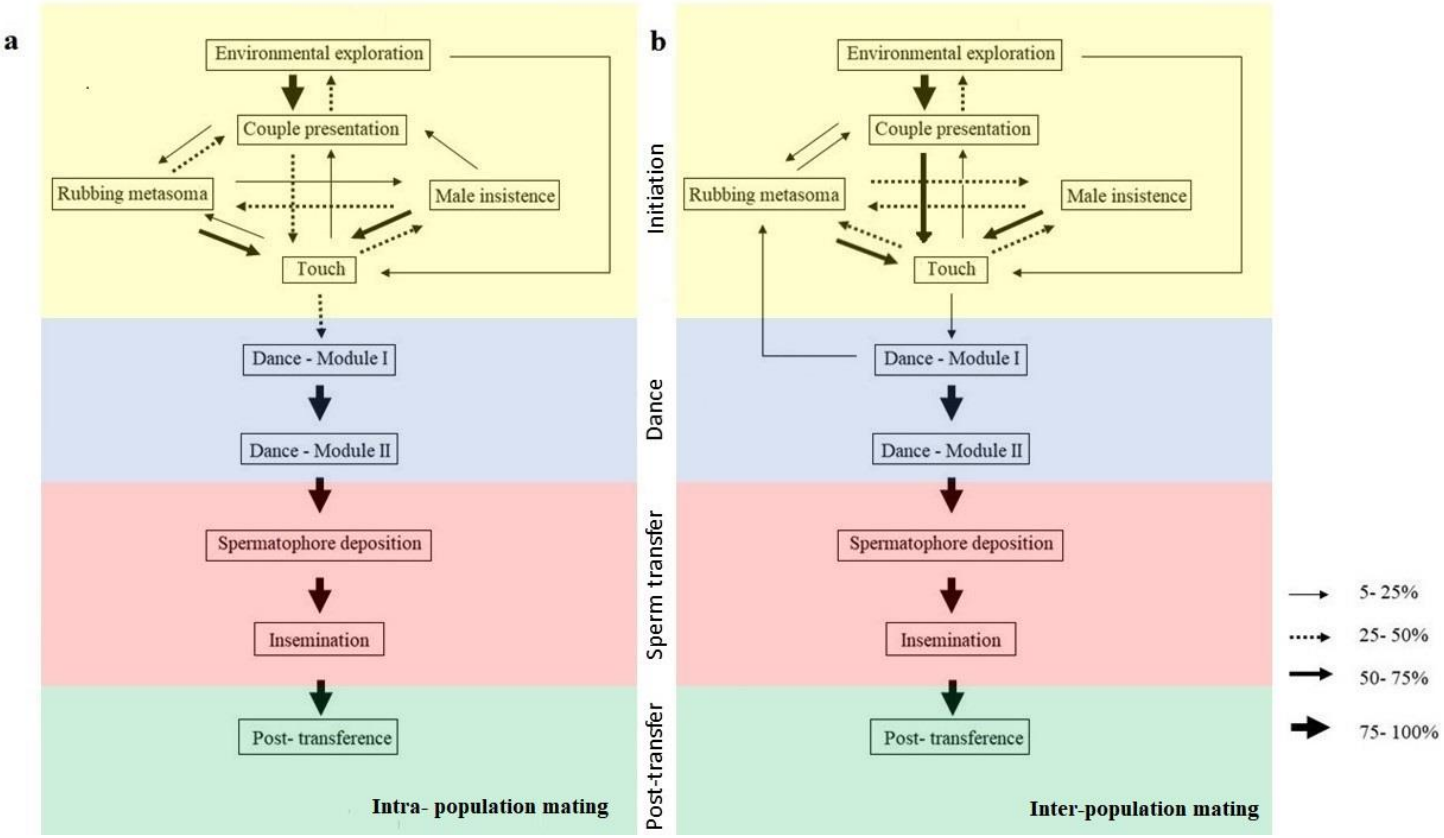


Figure 4: Ethogram showing the mating sequence of *Tityus serrualtus* (Buthidae), pairing males with sexual (a) and parthenogenetic (b) females. Mating phases and categories are based on Chapter

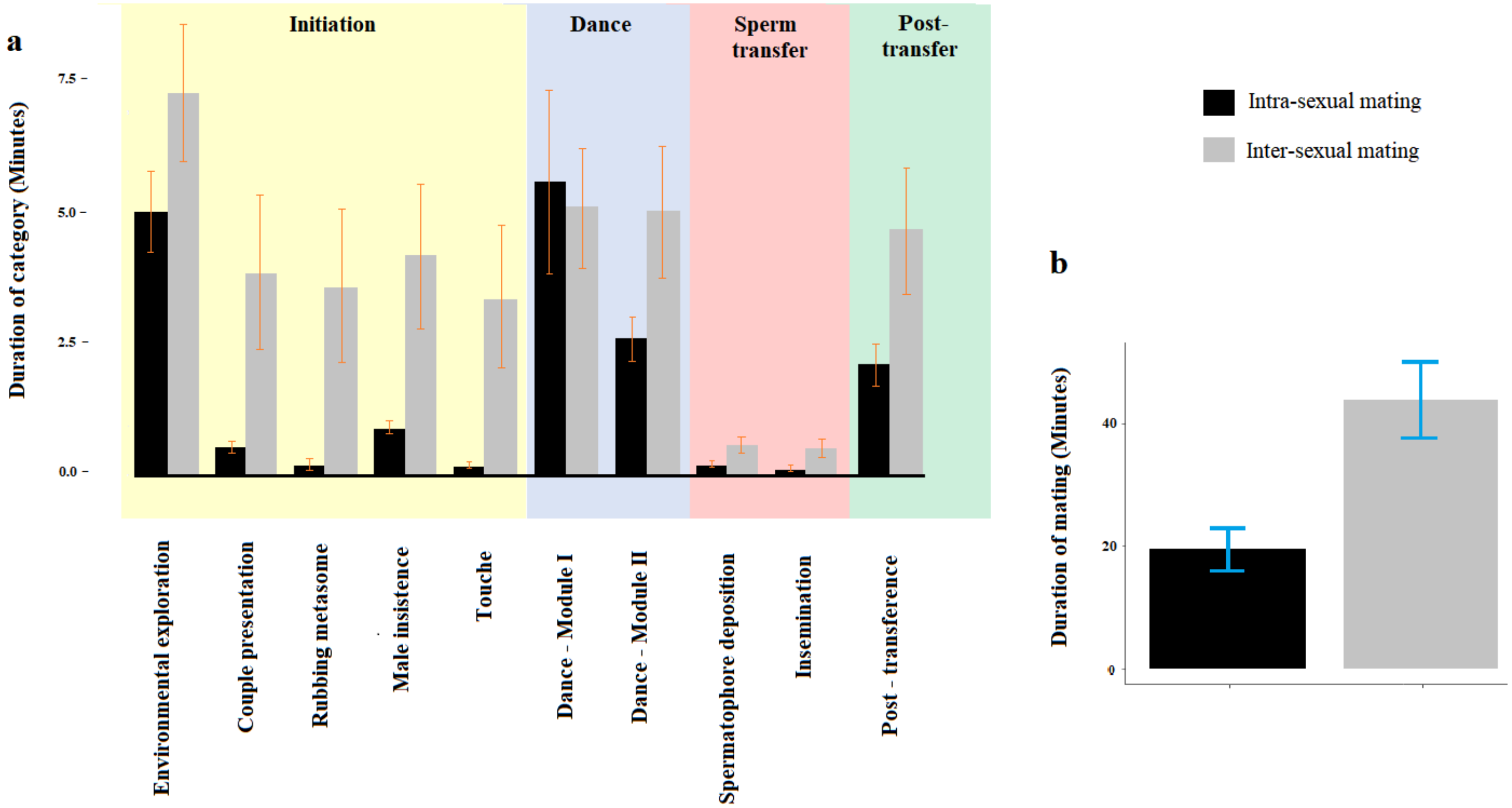


Figure 5*:* Mean and standard deviation of duration of **a)** each behavioral category and **b)** total duration of *Tityus serrulatus* (Buthidae) mating sequencing in intra and inter-population pairings.

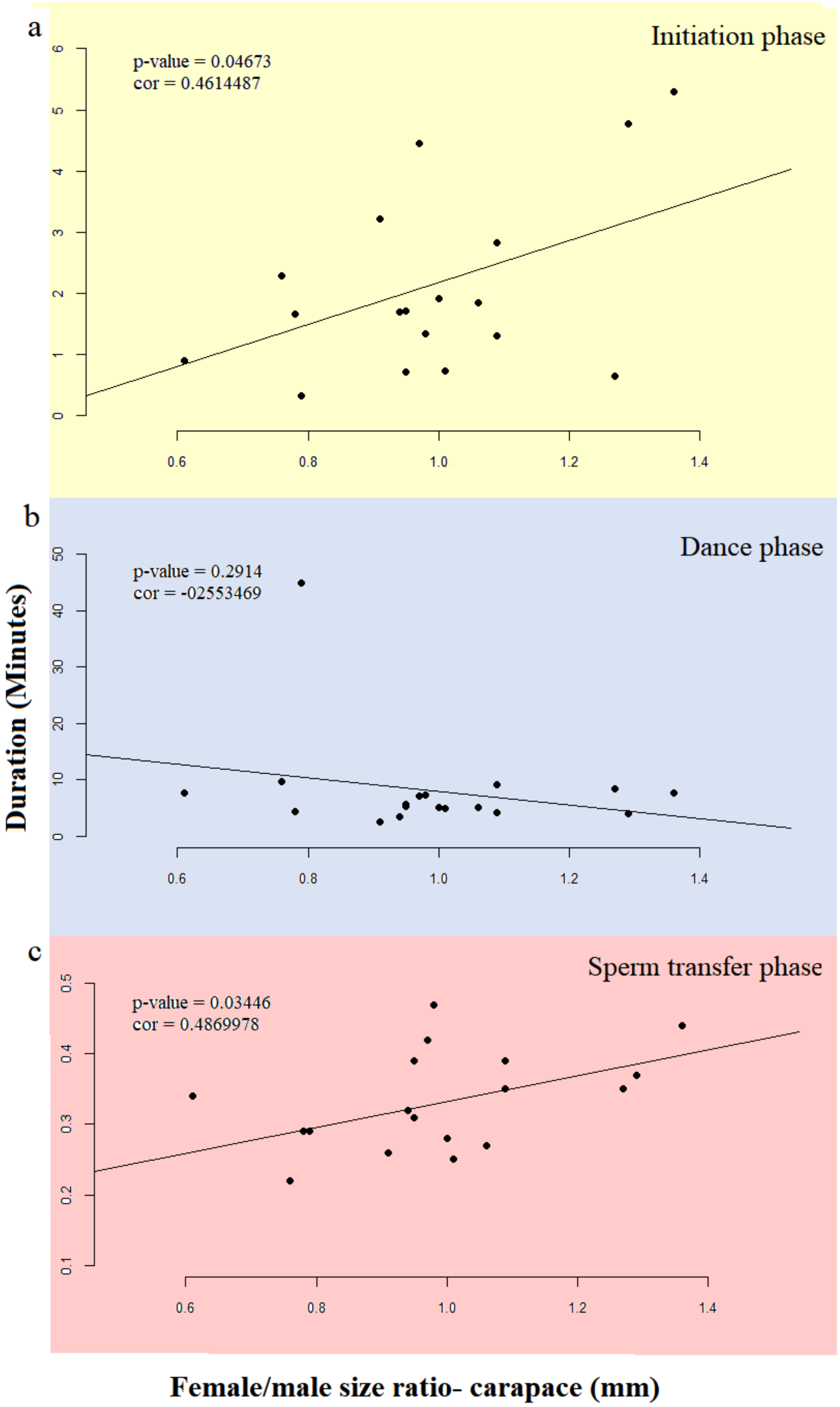
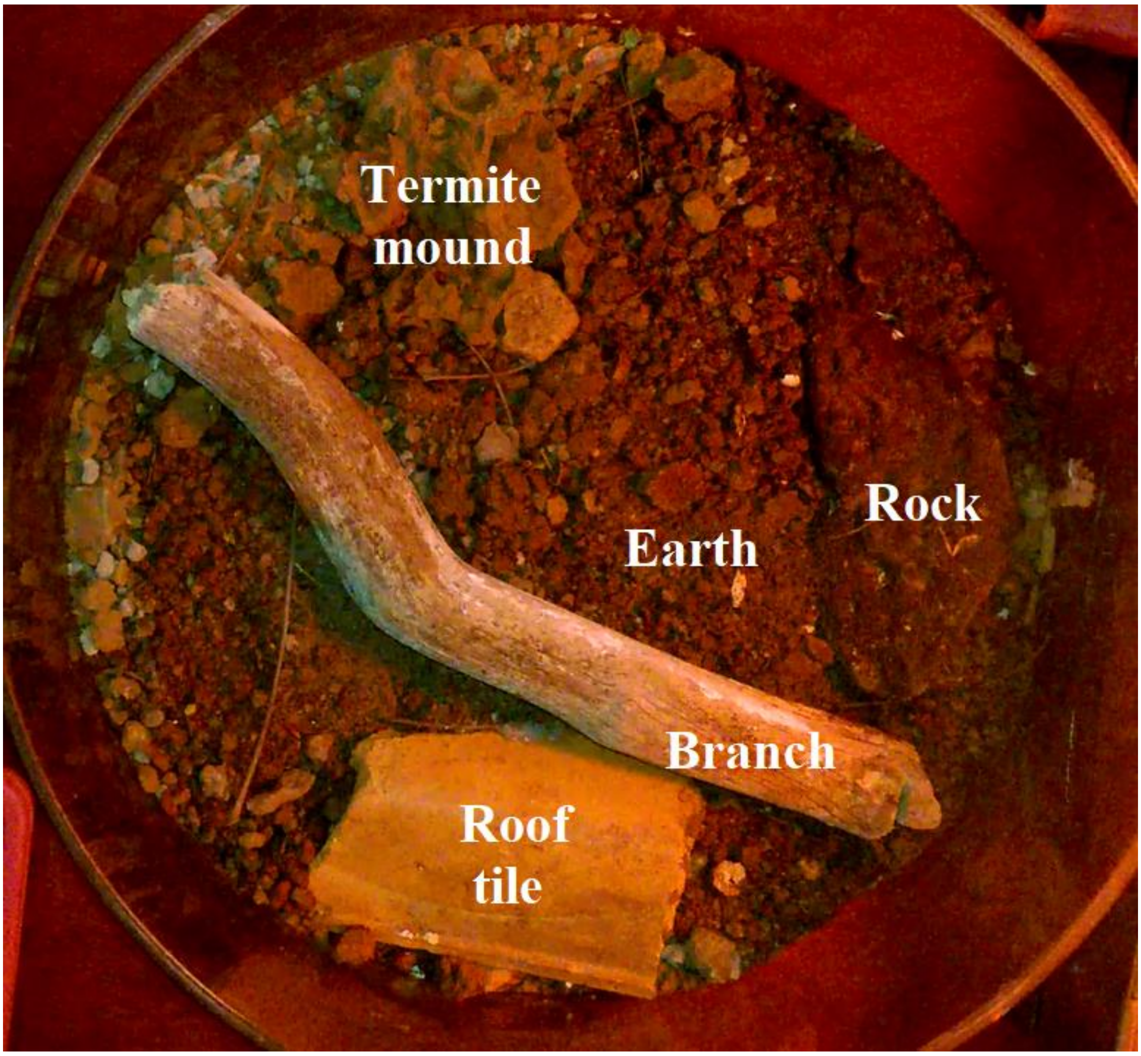


Figure 6: Effect of sexual female/male carapace width ratio on the duration of mating phases of *Tityus serrulatus* (Buthidae).

Supplementary Material Chapter 3



Supplementary material 1: Terrarium used in this study for recording of mating behavior of *Tityus serrulatus* (Buthidae).

Chapter 4

Lost smell: chemical communication in sexual and parthenogenetic populations of the yellow scorpion *Tityus serrulatus* (Scorpiones: Buthidae)

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Abstract

Characteristics related to sexual reproduction are usually considered under relaxed pressure and prone to disappearance in parthenogenetic organisms. Sexual pheromones, chemicals emitted to signal the opposite sex, are essential in the reproduction of several arthropods and is an example of a lost sexual trait in asexual lineages. In this study, we investigated whether males and females of the yellow scorpion *Tityus serrulatus* (Buthidae) emit contact sexual pheromones and, if so, whether their production and identification are still present in females from an exclusively parthenogenetic population. We kept individuals of both sexes inside containers with filter paper on the bottom, and subsequently exposed the filter paper to individuals of the opposite sex. We then recorded whether the experimental specimen responds to chemicals with previously describe sexual behaviors. In order to confirm whether the behaviors exhibited by the females are related to the identification of sexual pheromones, we placed parthenogenetic and sexual female pairs in terraria previously used for mating and recorded the behavior of these females. Our results showed that females and males from sexual populations produce signaling chemicals. In addition, females from parthenogenetic populations appear to have lost the ability to produce sexual pheromones, but are still able to identify substances produced by males. Also, females inserted in mating arenas respond to chemical signals by aggressive behavior towards each other. This outcome was observed in all female pairings, regardless of their population (sexual or parthenogenetic) of origin. We can conclude that females from exclusively parthenogenetic populations show signs of incipient reproductive isolation from sexual populations, a process usually regarded as leading to the fixation of obligatory parthenogenesis.

Keywords: Relaxed pressure, reproductive isolation, sexual behavior

4.1- Introduction

In populations that reproduce sexually, sex-related behaviors and characteristics tend to evolve rapidly (Barlow 2002). Nevertheless, sex-related traits, including physiological, morphological and behavioral, can be lost when they are no longer under selection, as occurs in populations that reproduce by parthenogenesis (Normark 2003, Van der Kooi & Schwander 2014). The decay of sexual traits in parthenogenetic populations occurs especially in mate attraction and location; the loss of these characteristics, together with barriers that prevent the fertilization of eggs, may prevent sexual reproduction, if males emigrate and invade an asexual population (Van der Kooi & Schwander 2014).

The divergence in systems involving pheromones can generate reproductive isolation and speciation in insects (Grimaldi & Engel 2005) and sexual pheromones can be modified or lost in populations that reproduce asexually (Kremer et al. 2009; Schwander et al. 2013; Tabata et al. 2012; 2017). Van der Kooi & Schwander 2014, in a review with 93 animal lineages characterized by asexual reproduction, mention that female-linked sexual traits, like pheromones, show evidence for decay in the majority (82%) of asexual lineages. The production of pheromones requires a high energy cost, and is avoided when no longer needed, as occurs with sexual females of mealybugs, which stops the production of pheromones after copulating with males, thus avoiding the costs of continuous production of the chemical substance (Tabata et al. 2017). In addition, sexual pheromones can be used by parasites and predators to find their prey, which causes their production to be negatively selected in populations where sexual selection is relaxed (Zuk & Kolluru 1998).

Sexual communication through chemicals is widespread in the animal kingdom (Wyatt 2003). Several animals use pheromone to locate partners, sexual recognition and choice of individuals for copulation (Johansson & Jones 2007; Wyatt 2009). In many insects, communication by pheromones is the first step towards sexual interaction (Gullan & Cranston 2000). Females usually emit the chemical signal that will attract males, the opposite is less common, and these signals are under strong selective pressure, since they are a major factor in the male search (Greenfield 2002, Tabata et al. 2012).

As in insects, scorpions are also able to detect chemical substances, including sexual pheromones, and for this use the pectens, chemoreceptor structures located in the ventral region of the mesosome (Polis & Sissom 1990, Gaffin & Brownell 1992, Trabalon & Bagnères 2010). Studies show that males of some species of scorpion exhibit mating behavior, as “tail-wagging” and “juddering or vibration”, when exposed to substrates previously occupied by females, indicative of sexual contact pheromones (Steinmetz et al. 2004, Taylor et al. 2012, Pordeus et al. 2019). In the Buthidae, the presence of chemical signaling was registered in two species, *Centruroides vittatus* Say, 1821 (Steinmetz et al. 2004) and *Tityus pusillus* Pocock, 1893 (Pordeus et al. 2019). Despite this, studies involving the detection of possible chemicals produced by males and detected by females are scarce in the literature. Here, we tested the presence of pheromones in the parthenogenetic species *Tityus serrulatus* Lutz & Mello 1922 and its implication in asexual reproduction.

T. serrulatus, popularly known as yellow scorpion, is a species highly distributed in Brazilian territory. Most populations reproduce by parthenogenesis, with few sexual populations known where the male is present (Souza et al. 2009, Santos et al. 2014, Lima et al. 2020). Even in sexual populations, parthenogenesis can be observed in facultative way (Braga-Pereira & Santos in press). However, it is unknown whether parthenogenesis has evolved to an obligatory form in populations where only females are present, making both forms reproductively isolated. Females from an asexual population accept mating with males in captivity, though showing more resistance than females from a sexual population, especially in the Initiation phase (Chapter 3). The Initiation phase is particularly important in this respect of being when species recognition occurs (Benton 2001). Nevertheless, male-female contact may be facilitated in laboratory conditions, due to restricted space within experimental arenas. Thus, the importance of chemical signals in mate location in this species remains unknown, and particularly if those signals would be effective with females from asexual populations.

Although it is unknown whether *T. serrulatus* communicates by chemical signals, some observations indicate that males produce sexual pheromones and females respond to that signal. In 2017, we erroneously introduced two sexual females in a terrarium where a *T. serrulatus* mating had occurred previously, and the newly introduced females showed aggressive behavior towards each other. Females curved the metasoma over the body and rocked it quickly, both hitting the metasoma on the other female's metasoma simultaneously. This observation was unexpected, since *T. serrulatus* females from parthenogenetic populations are kept in boxes containing hundreds of individuals and aggressive behaviors are not observed, except in food deprivation (personal observation). However, the females we introduced erroneously were previously fed. So, from that evidence, we evaluate whether *T. serrulatus* males and females communicate sexually through pheromones. We then test whether females from asexual populations have diminished or lost ability to produce and detect sexual pheromones.

4.2- Methods

Collection and rearing

We obtained adult and subadult males and females (heretofore “sexual females”) from a facultative parthenogenetic population located in urban and suburban regions of Itacarambi, Minas Gerais (15.19S, 44.20W) in January 2017 and February 2020. Adult and subadult parthenogenetic females were obtained through collection from a female-only population in Belo Horizonte, Minas Gerais (19.91S, 43.93W) (Fig.1a), throughout 2017 and February 2020. We search the scorpions in the morning, under trunks, rocks, bricks and roof tiles. During the night, we collected active specimens using ultraviolet light. The specimens were individually recorded with a number and kept in plastic cages (8.5 cm diameter X 6.5 cm height), with cotton absorbed in water and a cardboard refuge. We fed them with one cockroach *(Nauphoeta cinerea*) per week. Scorpions were kept in the laboratory at a mean temperature of 25 ± 3 ° C, 58 ± 11% relative humidity and 12 hours photoperiod. All individuals used underwent a period of 30 days of acclimatization before starting the observations.

Behavioral assays

We used only adult individuals, either collected in different instars. We performed two sets of observations, each to test whether both sexes produce and detect pheromones, from February to May 2020. We used 20 parthenogenetic females, 20 sexual females and 20 males, totaling 20 experimental replicates of each. In both sets, we selected individuals at random. To obtain possible contact pheromones, each individual was kept for 24 hours in a circular plastic recipient (8.5 cm diameter X 6.5 cm height) with a filter paper on the bottom. The filter paper was then positioned at one end of a rectangular plastic recipient (13.5 cm length X 8.5 width X 4.5 cm height), and a randomly selected specimen of the opposite sex were released at the other end. We observed the experimental specimen for 20 minutes. If the specimen did not come into contact with the filter paper after that period, we gently guided it to the paper and observed its conduct for 10 minutes. For the control treatment, we performed the same procedures using filter papers not exposed to any individual. We recorded specimen behavior using written notes and videos and photos taken with a Samsung J8 mobile phone. To identify possible sexual behaviors from the experimental specimens, we used the behavioral repertoire described by Braga-Pereira & Santos (Chapter 2) as a guide, focusing on male and female behaviors in the Initiation phase. All observations were performed between 21:00 and 23:00, the period of highest activity (Mineo et al. 2009), under the same ambient conditions in which specimens were kept in the laboratory. After each experimental session, we discarded the filter paper and soaked the containers in water and detergent for 24 hours, followed by successive washing with ethanol and water and the containers were dried at room temperature. We manipulate the filter papers with tweezers, which were washed with soap and water for each different filter paper.

In the first experiment set, we tested pheromone release by sexual females by analyzing whether males react to a surface previously occupied by a female. We carried out the same test with parthenogenetic females, in order to detect whether they maintain pheromone production. Each experimental session consisted in a male exposed to a filter paper that came into contact with a sexual female (Fig.1b), a male exposed to a non-contact filter paper (control) (Fig. 1c), and a male exposed to a filter paper that came into contact with a parthenogenetic female (Fig. 1d), simultaneously. All the males we used in this study were submitted to the three treatments, on different experimental session.

In the second experiment set, we analyzed whether males produce pheromones by observing sexual females' behavior when exposed to a surface previously occupied by a male. On each experimental session, a sexual female was exposed to filter paper that came into contact with a male (Fig. 1e) and a sexual female was exposed to a non-contact filter paper (control) (Fig. 1f), simultaneously. The same test was performed with parthenogenetic females, in order to identify whether they maintain the ability to detect pheromones (Fig. 1g, h).

We consider as a positive response to the filter paper the presentation of at least one behavior from the Initiation phase of the ethogram by the experimental specimen. In the first experimental set, we tested for a difference in the frequency of behaviors (presence or absence) of males that came into contact with filter paper exposed to a sexual female and males that came into contact with filter paper exposed to a parthenogenetic female. In the second experimental set, we verified whether the frequency of sexual females who had contact with filter paper exposed to a male and showed sexual behavior was similar to the frequency of parthenogenetic female who presented sexual behavior in the same conditions. For this, we used a generalized estimation equation (GEE) model, in which the presence or absence of behaviors was used as a response variable and the female classification (sexual or parthenogenetic) was used as an explanatory variable. We did all the analyses using R version 4.0.2 (R Development Core Team, 2020).

Observations with two females

To verify if the fighting behavior between females, as mentioned above, is frequent, and if it also occurs with parthenogenetic females, we conducted the behavioral experiment described below. We did the observations in glass containers (25 cm diameter X 19 cm height) with a substrate composed by earth, a rock, a piece of termite mound, a piece of roof tile, and a branch going through the diameter of the terrarium. At each experimental session, the container was used for a mating observation, after which we removed the mating pair and spermatophore. After 10 minutes, we introduced two females, and recorded their behavior for up to 30 minutes. We did three treatments: sexual female x sexual female, parthenogenetic female x parthenogenetic female, sexual female x parthenogenetic female. We made three experimental replicates of each treatment. Each experimental replica was made after a different previous mating event. Each female specimen was used only once, and the experimental specimens were never used in the previous mating session. Some females were pregnant, both parthenogenetic and sexual. As a negative control, we exposed each of the female pairs in a container with the same characteristics, but where no mating occurred previously with a new content in the terrarium and the container being washed with soap and water (Fig. 2). The observations were made under red light and same environmental ambient conditions in which specimens were kept in the laboratory. We recorded the behavior of the experimental specimens through written notes and photos and videos on Microsoft Lumia 640 mobile phone.

4.3- Results

We recorded two male behaviors that suggest female detection through pheromones: front to back body vibration ("vibration") and lifting of the metasoma with pulsating movements of its apical segments ("metasoma pulsation"). Females showed two behaviors suggestive of sexual partner detection, the "metasoma pulsation", as in the males, and "female swaying", wherein the specimen moves the body from side-to-side whole legs are kept in contact with the substrate (Supplementary material 1). All scorpions that exhibited sexual behavior initiated the behavior in the first five minutes of the experiment. None of these behaviors were observed during exposure to the control filter paper.

Males showed sexual behavior when they came into contact with filter paper exposed to sexual females, but not with parthenogenetic females. Females, both sexual and parthenogenetic, showed behavior when they came into contact with filter paper exposed to males. In the first experiment set, 17 of the 20 males (85%) showed the behaviors described above when exposed to filter paper previously occupied by sexual females. No male showed any of those behaviors when exposed to filter paper previously occupied by parthenogenetic females, with significant difference between groups ($p < 0.05$, Fig. 3a). In the second experiment set, nine out of the 20 sexual females (45%) and seven of the 20 parthenogenetic females (35%) showed mating behaviors when exposed to the male-occupied filter paper, a difference statistically not significant ($p = 0.5195$, Fig 3b)

No individual who was gently guided to the filter paper exhibited any sexual behavior after contact with the paper. Among the guided males, three were guided to the control filter paper, one was guided to the paper exposed to a sexual female, one was guided to the paper exposed to a parthenogenetic female and also to the control filter paper. Among parthenogenetic females, two were guided to the control filter paper, four were guided to the paper exposed to the male and one was guided in both roles. Among sexual females, four were guided to the control filter paper and one was guided to the paper exposed to the male.

Observations with two females

In all treatments, females walked around the terrarium and met. Female showed aggressive behaviors towards each other when introduced in the containers in which previous mating occurred (Supplementary material 2). They curved the metasoma over the body and moved it quickly. They tried to hit the metasoma on the body or the metasoma of the other female; the opponent then protected itself with its own metasoma, like a shield. In some experimental replicas, one female held the other's metasoma with the pedipalps, simultaneously. Some females tried to sting the opponent, and in an experimental replica with two parthenogenetic females, one female was stung, causing loss of hemolymph. The aggressive behaviors were more expressive in pregnant females; these usually start the fight and show faster movements. However, when exposed to the terrarium where there was no mating before, no female showed aggressive behavior, including pregnant females. In this case, when meeting, the females touched themselves with the pedipalps and depart after the meeting.

4.4- Discussion

In this study, we show that both females and males from a sexual population of *T. serrulatus* apparently communicate through chemical contact substances, which elicit behaviors characteristic of the initial stages of mating. Our results also show that females from a parthenogenetic population have lost or reduced pheromone production, but maintained the capacity to detect and react to male contact substances. Thus, as will be discussed below, exclusively parthenogenetic populations may be in the early stages of reproductive isolation from sexual populations, which may lead to obligatory parthenogenesis fixation.

Regarding the sexual population, both males and females exhibited mating behaviors when exposed to substrates that came into contact with members of the opposite sex. This indicates that both can signal their presence to potential partners through contact chemical substances. Nevertheless, males showed higher frequency of response than females. This suggests that, as reported for other arthropods, *T. serrulatus* females are likely to be the main signalers, while males are mostly the receptors (Greenfield 2002).

The "vibration" behavior exhibited by males in our observations is clearly sexual behavior. “Vibration” is a typical sexual behavior exhibited by males of several species of scorpions and it is probably related to the detection of sexual substances released by females (Polis & Sissom 1990, Brownell & Gaffin 2001). On the other hand, we are not sure if the behaviors exhibited by females are exclusively sexual behaviors, since female behavior during mating is performed in response to a male behavior (personal observation), such as *Touch* (see *Touch* in Chapter 2), becoming less evident, since male are not present. In the “female swaying”, the female approaches the ventral region with the surface on which she is positioned and swing the body laterally, which can increase the contact of the pectens with the substrate. Therefore, “female swaying” can be also present when in contact with chemical substances released by prey and may not be exclusively sexual behavior.

Our results suggest that *T. serrulatus* females from the asexual population have reduced or no capacity to produce contact pheromones, since all males exposed to filter paper previously put into contact with those females exhibited no mating behavior. It is possible that *T. serrulatus* produces volatile pheromones, like other species of scorpions (Romero-Lebrón et al. 2019), but the production of these pheromones may also have been lost in parthenogenetic females. The production of pheromones requires a high energetic cost in many arthropods (Harari et al. 2011, Tabata et al. 2017) and maintaining its production in parthenogenetic females would probably be an unnecessary energy expenditure, since males are absent in populations where they are found. In addition, it is possible that female pheromones also attract predators and parasites, as occurs in many species of arthropods (Colazza et al. 1997, Zuk & Kolluru 1998, Franco et al. 2008). Therefore, parthenogenetic females of *T. serrulatus* may have lost or reduced pheromone production due to costs involved in their production and possible attraction of predators and parasites.

Despite the absence of detectable chemical signaling from parthenogenetic females, they still mating with males when paired in terraria; the encounter of males with females in captivity is facilitated by the confined environment, but has a longer duration when the female is parthenogenetic, indicating that the absence of pheromones can make the female's location difficult (Chapter 3). In the same way, parthenogenetic bushcricket females *Poecilimon intermedius* Fieber, 1853, despite have lost the ability to respond to sound signals emitted by males, accept mating when confined with males of related sexual species; however, no mating has been recorded in natural conditions since the couple does not meet (Lehmann et al. 2011). Some characteristics linked to mating, as courtship behaviors, can be maintained when not exposed to selection, even in species with extensive decline in some sexual traits (Van der Kooi & Schwander 2014), as in the case of linages that do not produce pheromone, but still mate. Nevertheless, the preserved characteristic would be under relaxed selection, resulting in the accumulation of neutral mutations (Hall & Colegrave, 2008; Lahti et al. 2009). This process neutral mutation accumulation should proceed slowly, resulting in the loss of the characteristic in the long run (Hall & Colegrave 2008). As exclusively parthenogenetic linages of *T. serrulatus* are unlikely to mate in a natural environment, it is likely that traits linked to courtship will also disappear over time, leading to the obligatory parthenogenesis.

Despite the loss of sexual pheromone production, parthenogenetic females showed to be able to detect male chemical signals and respond to them exactly as the sexual females. Loss of signal reception, however, is also common in parthenogenetic populations, as occurs in bushcricket, whose sexual females receive sounds emitted by males. Nevertheless, parthenogenetic females have reduced auditory structures and do not respond to male singing (Lehmann et al. 2007, 2011). As parthenogenetic females of *T. serrulatus* have not lost the ability to identify substances produced by males, it is possible that the detection of pheromones can be advantageous for the scorpion, being associated, for example, to find prey, and for this reason it has been maintained. In cases of pleiotropy, in which the same gene is expressed in several processes, certain characteristics remain, since the genetic information is still maintained (Fong et al. 1995). Therefore, it is possible that the detection of male pheromones has been maintained if the receptors that detect pheromones are the same as prey detection.

The aggressive behavior between two *T. serrulatus* females also suggests that both sexual and parthenogenetic females have the ability to detect sex-related chemicals. In laboratories that produce anti-scorpion venom serum, where parthenogenetic females of yellow scorpion are raised in boxes containing hundreds of individuals, some pregnant or with brood, aggressive reactions between them are not observed (personal observation). The aggressive behaviors of experimental females are probably not related to competition for food, since all specimens were previously fed, including those in the control group. Pregnant females of *T. serrulatus* showed greater aggressiveness, compared to apparently non-pregnant females. Pregnant scorpion females are generally resistant to mating and may behave aggressively when they encounter males (Peretti 2001). In our experiment, chemicals released by males during mating may have been identified by these females, who have shown themselves to be aggressive towards other females, due to an error in gender identification due to the presence of male pheromones. Gender identification errors are common in arthropods when pheromone is present in the environment (Vanacker et al. 2003, Ruther & Steiner 2008). However, non-pregnant females also showed aggressive behavior, although less intense. An alternative explanation would be competition for males, as they are outnumbered by females (Braga-Pereira & Santos in press) or nonexistent in populations of *T. serrulatus*. Although less common than the opposite, female's competition for mating opportunities may happen when sex ratio are female-biased or when male quality is variable (Andersson 1994, Rillich et al. 2009 Fritzsche et al. 2016). For instance, females of *Gryllus campestre* Linnaeus, 1758 compete with each other through aggressive behaviors in the presence of sounds produced by males (Rillich et al. 2009). Regardless of why females fight, both reproductive forms have the ability to identify chemicals released during mating, being another indication that confirms the maintenance of this characteristic in parthenogenetic females.

In this study we conclude that individuals from a sexual population of *T. serrulatus* use sexual contact pheromones to signal possible sexual partners for mating. Meanwhile, parthenogenetic females of the yellow scorpion lost or reduced the production of pheromones, and this loss may be related to high costs involving the production of signaling substances. However, parthenogenetic females maintain the ability to detect chemical substances produced by males, which may indicate that pheromone reception channels may be the same as for prey identification. The loss or reduction of pheromone production by females allows us to conclude that females from parthenogenetic populations may be beginning to isolate themselves from sexual populations, which may lead to the fixation of obligatory parthenogenesis in *T. serrulatus*.

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Figures Chapter 4

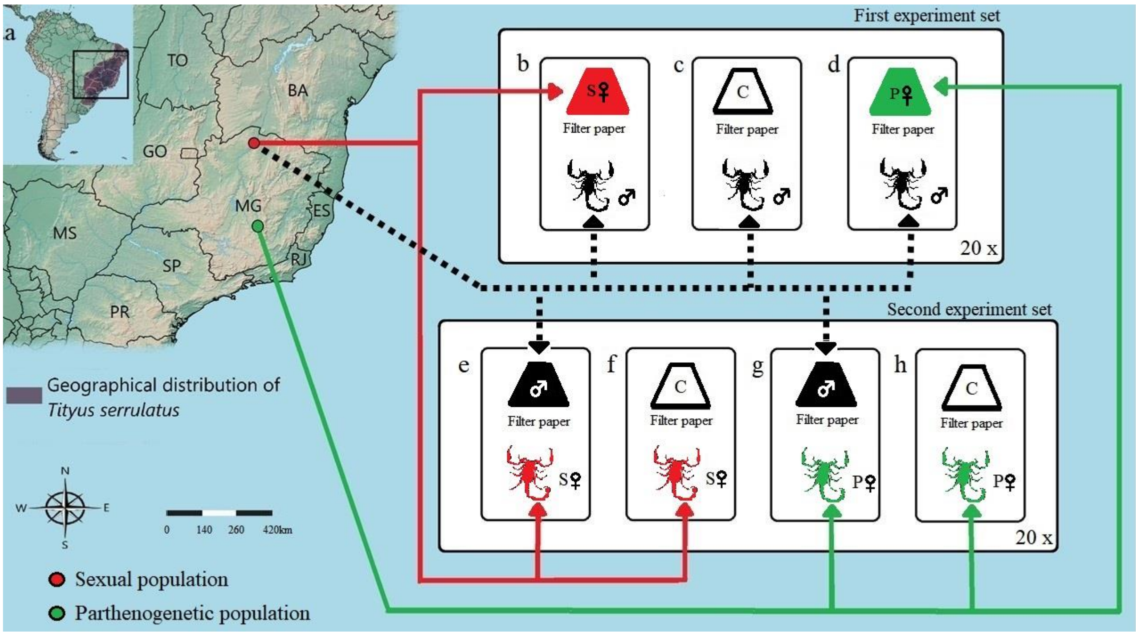

Figure 1: a) Geographic distribution of *Tityus serrulatus* (Buthidae), location of source populations studied, and experimental design used to detect male and female response to chemical signals. First experimental set: b) Male exposed to filter paper that was in previous contact with a sexual female. c) Male exposed to control filter paper. d) Male exposed to filter paper that was in previous contact with a parthenogenetic female. Second experimental set: e) Sexual female exposed to filter paper that was in previous contact with a male. f) Sexual female exposed to control filter paper. g) Parthenogenetic female exposed to filter paper that was in previous contact with a male. h) Parthenogenetic female exposed to control filter paper. S: Female sexual. P: Parthenogenetic female.

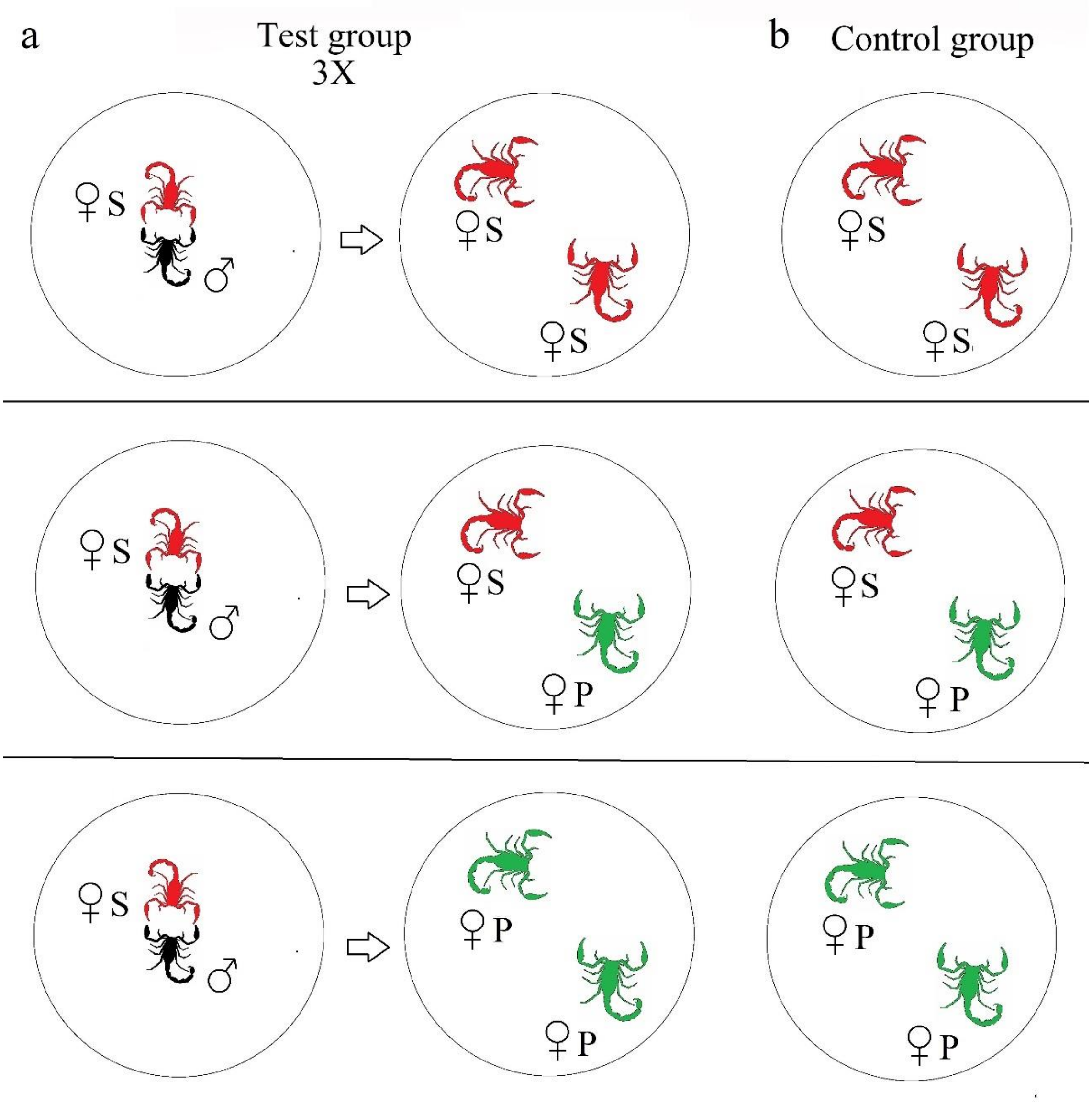

Figure 2: Experimental design to test the response of pairs of *Tityus serrulatus* females to chemical signals left in terraria where mating has previously occurred. a) After mating, the couple was removed and two females were placed inside the terrarium, which possibly contained sexual pheromones. b) Two females were exposed to a terrarium where no prior mating occurred. S: Female sexual. P: Parthenogenetic female.

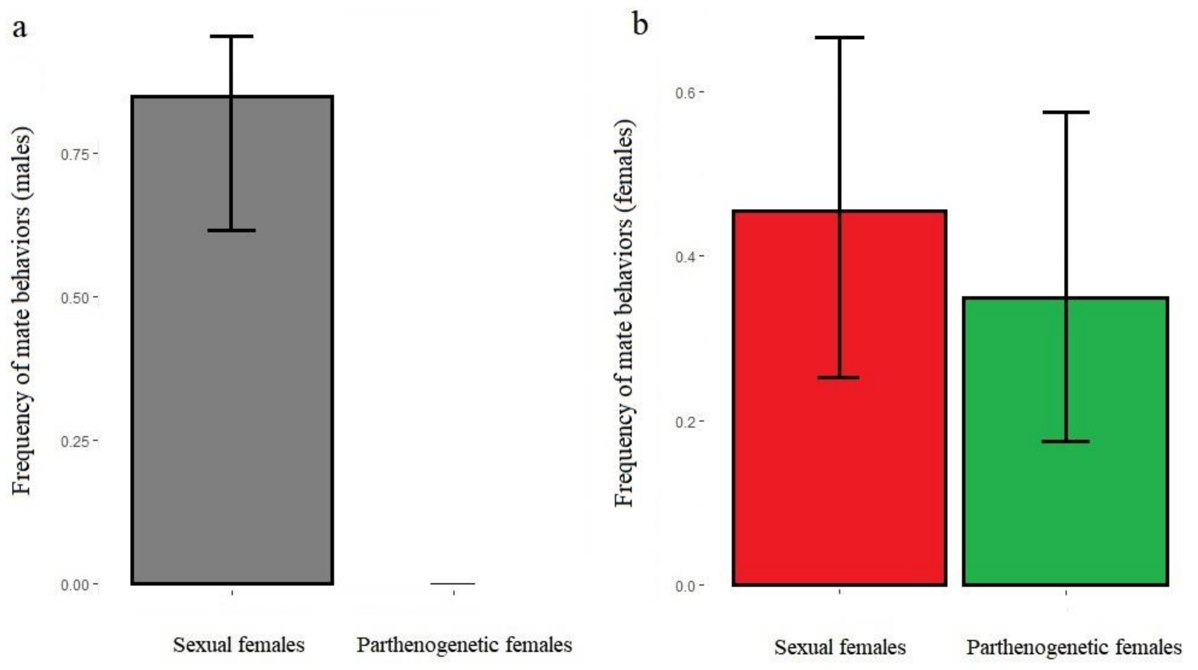

Figure 3: Mean and standard deviation of the frequency of individuals of *Tityus serrulatus* showing mating behaviors when exposed to a filter paper previously occupied by an individual of the opposite sex. a) Frequency of males that exhibited mating behavior when exposed to filter paper previously exposed to sexual and parthenogenetic females. b) Frequency of sexual and parthenogenetic females that showed mating behavior when exposed to a filter paper previously exposed to a male.

Supplementary Materials Chapter 4

Supplementary material 1: Behaviors presented by females and males of *Tityus serrulatus* (Buthidae) when exposed to a filter paper that had previously been in contact with an individual of the opposite sex. Link: https://youtu.be/llgQCVXaPSk

Supplementary material 2: Behavior of *Tityus serrulatus* (Buthidae) females when exposed in an arena where a mating of individuals of the same species occurred previously. Three treatments are shown: sexual female x sexual female, parthenogenetic female x parthenogenetic female, sexual female x parthenogenetic female. Link: https://youtu.be/We4i8M3b0mI

Chapter 5

Male competition in the yellow scorpion *Tityus serrulatus* (Scorpiones: Buthidae): different behavior tactics for getting a mate

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Abstract

Competition for reproductive partners in animals occurs mainly among males. In addition to direct physical combat, males can use several tactics before and after mating, to assure egg fertilization by his own sperm. Non-dominant males in particular can use alternative tactics to avoid physical combat with stronger opponents and ensure access to females. In this study, we describe competition behaviors between males of the Brazilian yellow scorpion *Tityus serrulatus* Lutz & Mello 1922 in captivity, and test whether opponent size difference influences the result of male competition. We set up 50 experimental replicates in which two males and one female were placed together in a terrarium. Males were previously measured, in order to evaluate whether mating success, indicated by successful spermatophore deposition and female insemination, is correlated to the male size in relation to his opponent. We identified three competition tactics: "fight between males", "mate interruption" and "dance between males". The latter was the only behavior whose frequency was related to the male size difference, tending to occur mostly when both contenders are similar in size. Besides that, male size apparently does not influence the mating success. Our results allow us to conclude that simulating female behavior might be an alternative tactic used by males to avoid physical combat. Same-sex sexual behavior can be beneficial for reducing bodily harm caused by aggressive behavior, but can also induce the opponent to deposit its spermatophore away from the female. As a result, a weaker contender may guarantee access to the female, without taking the risks of physical confrontation.

Keywords: Aggressive behavior, male-male interaction, same-sex sexual behavior, sexual selection

5.1- Introduction

Competition for reproductive partners occurs mainly among males (Darwin 1871, Andersson 1994). In populations with male-biased operational sex-ratio, sexual selection tends to favor adaptations to fight, such as aggressive behaviors or well-developed morphological weapons (Emlen & Oring 1977, Correa et al. 2003). Thereby, secondary sexual characteristics related to combat between males are under sexual selection (Emlen et al. 2005; Schutze et al.2007; Innocent et al. 2007). These characteristics encompass not only differences in body size, but also the presence of extreme dimorphism between males of the same species (Cook et al. 1997, Correa et al. 2003). However, even smaller and not well-armed males show several strategies, different from direct physical combat, to ensure access to females (Gross 1996, Cook et al. 1997, Correa et al. 2003).

Alternative mating tactics can be adopted among individuals of the same species, or by a same individual in different contexts (Waltz & Wolf 1984, Martin & Taborsky 1997, Correa et al. 2003). Competition for sexual partners can include various behaviors, such as female coercion, attempts to find females before the rivals, nuptial gifts, infanticide, among others (Andersson & Iwasa 1996). Although competition for sexual partners is the most studied sexual selection mechanism, and alternative mating tactics has been described mainly in insects and crustaceans (Shuster 1987, Cook et al. 1997, Correa et al. 2003, Andersson & Simmons 2006), little is known about tactics used among scorpions before mating.

Scorpions have indirect sperm transfer, which is performed through a spermatophore fixed over the substrate (Alexander 1959, Polis & Sissom 1990). Mating is similar between species and can be divided into three phases. The first phase, denominated Initiation, is marked by the sexual recognition. In the second phase, known as Dance (*promenade à deux*), the male guides the female to an adequate substrate for spermatophore deposition. Finally, in the third phase the Sperm transfer occurs when male deposits the spermatophore and leads the female to it (Alexander 1959, Polis & Sissom 1990). Some species also show a fourth phase, the post-sperm transfer, in which the consumption of the spermatophore by the male or the female may occur (Tallarovic et al. 2000). In this study, we analyze possible alternative mating tactics in the yellow scorpion, *Tityus serrulatus* Lutz & Mello, 1922, a species widely distributed in Brazil.

The yellow scorpion reproduces mainly by parthenogenesis (Matthiesen 1962). Few bisexual populations have been described and, in at least one of those, parthenogenesis occurs facultatively (Souza et al. 2009, Santos et al. 2014, Lima et al. 2020, Braga-Pereira & Santos in press). Although females are more abundant than males in that facultatively asexual population (Braga-Pereira & Santos in press), many females are found with young or about to give birth during the reproductive season (personal observation). Since scorpions are viviparous animals that show maternal care until the young have their first molt, and pregnant and young-carrying females are usually not receptive to mating (Lourenço 2000, Peretti & Carrera 2005). Consequently, a lower availability of receptive females can favor competition between males.

Males of *T. serrulatus* was originally described as larger and more robust than females (Souza et al. 2009), but male body size is highly variable within the facultatively asexual population, even with individuals smaller than the females (personal observation). This variation in male size may be related to the instar in which they become adults. Most individuals reach adulthood on the sixth instar, but some can reach maturity on the fifth or, more rarely, the seventh instar (Braga-Pereira et al. in prep.). An important implication of body size variation in male scorpions is that it may have an influence on competition for sexual partners. For example, larger males of *Euscorpius flavicaudis* are more successful in mating with reluctant females and to guard their refuges (Benton 1992). However, being a dominant male requires high investment; thus, individuals in competitive disadvantage can manage to improve their reproductive success by avoiding competition and adopting alternative, low-cost tactics (Eberhard 1982, Taborsky 1998, Emlen 1997)

The objective of this study is to analyze if there is sexual competition between males of *T. serrulatus* and if the male body size influences the mating success. Furthermore, we explore the importance of alternative behavioral tactics by males in competitively weaker males.

5.2- Methods

Collection and rearing

We collected adult and subadult females and males from a facultative parthenogenetic population of *T. serrulatus* in urban and suburban regions of Itacarambi, Minas Gerais (15.19S, 44.20W) in Mach 2016 and January 2017. Specimens were collected under trunks, rocks, bricks and roof tiles during the morning, and with ultraviolet light at the night in a coconut field. All specimens were individually identified with a number and kept in plastic cages (8,5 cm diameter X 6,5 cm height), with a wet cotton ball to provide humidity and a cardboard shelter. We fed them with one cockroach (*Nauphoeta cinerea*) per week. Most importantly, specimens did not accept prey offered more than once a week, indicating that possible aggressive behaviors during observations were not related to hunger. Scorpions were kept in the laboratory at 25 ± 3 °C, 58 ± 11% relative humidity and a 12 hours photoperiod. All individuals underwent at least 30 days of acclimatization before starting the observations.

Observation of sexual behavior

We performed behavioral observations from December 2017 to May 2018 and October 2018 to March 2019. We used subadult scorpions collected in the field that became adult in the laboratory, as well as individuals born in captivity. Thus, only virgin individuals participated in the observations. The individuals were used only once, except for those who had no interaction with another individual in the first experiment, being used again in a second experiment, with a minimum period of 15 days. To assess specimen size, we measured the length of the carapace of the exuviae released in their last molt. Observations were performed in a plastic container (16 cm length X 12 cm width X 12 cm height) with the bottom covered with earth, a branch and a stone. Immediately before starting the observations, we spray water on the earth to keep the environment humid. We changed the substrate of the arena and washed with water and detergent the container after each replicate, allowing the container to dry at ambient temperature.

At each experimental replicate, we randomly choose two males and one female and marked one of the males with a spot of acrylic paint on the back of the mesosoma. After 30 minutes, we placed each male on one end of the terrarium and, then we put the female in the middle. We set up 50 experimental replicates and made observations until sexual behaviors were no longer displayed. Observation terraria were kept at same temperature and humidity under which specimens were kept and were illuminated with red light. We carried out the observations at night (from 21: 00h to 00: 00h), when the species is active (Mineo et al. 2009). We recorded the specimens' behavior using a Canon EOS Rebel T5 camera, together with photos taken with a Microsoft Lumia 640 mobile phone and written notes.

Data analyses

We obtained the frequency (presence or absence) of possible behavioral tactics observed. We also obtained the percentage of males that perform each behavior, according to their size (larger or smaller male) and, of these, the percentage of males that have mating success. We considered successful at mating the males that deposited the spermatophore and conducted the female to it. In addition, we obtained the frequency of females that copulated with the two males.

In order to evaluate the association between the male size and the mating success, a generalized linear model (GLM) was performed using a binomial distribution. The mating success was the response variable and the length of the male and female carapace were the fixed effects. We included the female size to check a possible effect of the female size over the mating success in order to confirm any possible interaction between fixed effects.

Additionally, we analyzed the association between the difference in male size within each replicate and the frequency of each behavioral tactic using a binomial distribution GLM. In order to test for correlation between different sexual tactics, the Phi coefficient was estimated for each pair ("mate interruption" x "fight between males", "mate interruption" x "dance between males" and "dance between males" x "fight between males"; see the tactics below). We performed the statistical analyses using R v. 4.0.2 (R Development Core Team, 2020). and Ggplot 2 for plot the graphics (Wickham 2016).

5.3- Results

Of the 50 experimental replicates, we discuss below and analyzed results from only 46, because three resulted in no mating behavior and in another the female killed and consumed one of the males at the beginning of the experiment. Four females (8.69%) mated with the two males; although in which replicate the spermatophore of the second male did not adhere to the substrate. That same male was stung by the opponent before his mating attempt (see below). Six females (13.04%) do not accept mating with any of the males, even though they have been courted by at least one of them. All the females that mated accepted the first male that courted her or that defeated an opponent. We observed three behavior tactics used by males, which we named “fight between males”, “mate interruption” and “dance between males” (Supplementary material 1).

Fight between males

“Fight between males” was present in 21 experimental replicates (45.65%), in which males attempt to sting each other with the metasoma, or defend themselves with their own metasoma, using it as a shield. To avoid being stung by of the opponent, the attacked male usually held the opponent’s metasoma with the pedipalps. One individual threw the opponent up with the metasoma. It is a behavior that can be observed at the beginning of the experiment or after other interactions between individuals. In an experimental replicate, a male was stung by the opponent and remained motionless for a few hours, while the opponent mated with the female. In three cases, the stung individual died. Two individuals who managed to dance with the female after being stung were unable to fix the spermatophore on the substrate. In 19 out of the 21 fights between males we were able to identify which male started the fight; nine were initiated by the larger male and 10 by the smaller one (Fig. 1). In addition, in 14 replicates one of the males danced with the female after the fight, seven of them were the larger male (Fig. 2).

Mate interruption and Threesome dance

“Mate interruption” occurred in 73% of the replicates and was performed by both males in a same replicate, during the Dance and Sperm transfer phase. One male (the intruder) tries to separate the couple by pulling the metasoma and the legs of the female or of the other male, vibrating the body during the attempt. Meanwhile, the mating male tries to push the intruder with the metasoma. Larger males tried to interrupt mates in 20 experimental replicates (43.47%), without success in 13 attempts. Smaller males attempted to interrupt mates in 18 replicates (39.13%), with nine failed attempts (Fig. 2). “Mate interruption” can progress to a behavior we named “threesome dance”, in which males and the female walk through the terrarium with the pedipalps connected (Fig. 3a), while one male pushes the other's metasoma with his own metasoma. Meanwhile, the female can rub the metasoma on both males during the Dance. From “threesome dance”, female or one of the males can leave the dance. When the female leaves the “threesome dance”, the males can fight or dance with each other (Fig. 2).

Dance between males

We observed “dance between males” (mean: 47:46 /sd: 27:31 minutes) in 17 of the 46 experimental replicates (36.96%). In this tactic, both males enter “*promenade à deux*”, where one of them behaves like a female (passive male), while the other deposits the spermatophore (active male) (Fig. 3b). Initially, males keep the metasoma curved on their own body and swing from side to side, appearing aggressive. During the dance, males can alternate which one behave passively. Seven larger males behave like a female in the dance between males and four of those mated with the female later. Ten smaller males behave like a female and five of these mated with the female afterwards (Fig. 1). So, after spermatophore deposition by one of the males, 52.94% of the males that did not deposit the spermatophore (passive male) were able to subsequently mate with the female.

Female behavior

Females apparently do not choose males, as they mate with the first one who courts her. Females that are resistant to the courtship of the first male are also resistant to the courtship of the second male. While males perform interactive behaviors between them ("fight between males" and "dance between males"), females usually stand somewhere in the terrarium. Some females walked through the terrarium, walking next to the males who were performing "dance between males", being able to touch the couple with the pedipalps or rub their metasoma on the male's body. Even with the female's interaction, the males continue to dance with each other, but eventually they can touch the female's legs and pedipalps.

Data analyses

The absolute size of the male in relation to the opponent was not correlated with the success in the spermatophore deposition with the female, as suggest the association analysis (z-value= 0.096, p =0.924). Nevertheless, the pairs of males used in each experimental replicate have small differences in size, as can be indicated by small differences in carapace length (Supplementary material 2).

Our results suggest that the “dance between males” tends to occur when the males have similar size, but the other tactics are apparently not related to the difference in size between males. The differences between the male size in each pair were not associated with “fight between males” (z-value= -1.043, p=0.297) or “mate interruption” (z-value=0.878, p=0.380) occurrence, but was significantly and negatively associated with the occurrence of “dance between males” (Fig. 4, z-value= -2.253, p=0.0242). Interestingly, we found no evident association of the size difference between males and the occurrence of a specific or multiple events in each replicate, except between tactics “fight between male” and “mate interruption”, in which a moderate and significant correlation was observed between them (phi=0.342, p=0.002, Fig. 5). We found no significant correlation between “mate interruption” and “dance between male” (phi=0.080, p=0.588), “fight between male” and “dance between male” (phi=0.016, p=1.00).

5.4- Discussion

Herein, we observed three tactics used by males of *T. serrulatus* when facing a competing male. In addition to aggressiveness, males of the yellow scorpion may also exhibit same-sex sexual behaviors. Apparently, “fight between males” and “mate interruption” is not related to the size of the opponent, but “dance between males” tends to occur when the males have similar size. The size of the male in relation to the size of the adversary did not influence the success of mating, factor that may be related to the small difference in the size of the males in our study. Next, we will discuss the male behavioral tactics observed in this study, with their possible implications.

In our experiment, we recorded “fights between males” after several interactions between individuals. “Fight between males” of the yellow scorpion probably involves high energy cost, as in other arthropods (Hack 1997, Kemp & Wiklund 2001). Furthermore, physical combat can result in injuries or even death, as observed in our experiment. In addition to the energy needed for the fight and the damage caused by it, indirect costs involving the production of venom and spermatophore may also be present in the “fight between males”: the venom, when used against the opponent, must be produced again to feed and defend the scorpion; the spermatophore is wasted and does not inseminate the female, since it does not adhere to the environment when it is deposited by the male who suffered the injury. Both the production of venom of scorpions and spermatophore requires high energy expenditure (Evans et al. 2019, Vrech et al. 2019). Despite high costs involving this aggressive behavior, the fight was present after several interactions between individuals, being a frequent behavior. In most observations one of the males was able to dance with the female afterwards, indicating that the fight between the males can be an advantageous strategy, despite being expensive.

Our results showed that the frequency of “fight between males” is correlated with “mate interruption”, probably because attempts by a male to interrupt can lead to aggressive responses from the male who in “*promenade à deux”* with the female. In addition to Dance, we observed that males of *T. serrulatus* also trying to separate the couple during the Sperm transfer. Attempts at copulation interruption has been reported in other arthropods, and body structures may be under sexual selection for avoiding male intruders (Contreras-Garduño et al. 2007, Katsuki et al. 2014, Keena & Sánchez 2018, Russell et al. 2018). So, the scorpion's metasoma may also be under selective pressure, as it is used against the intruder male during attempts to separate the couple. In approximately half of the “mate interruption” events, the male who suffered the interruption attempt was able to deposit the spermatophore and to conduct the female to it, without having to engage in other disputing behaviors for the female. Apparently the " mate interruption " is not very effective, as many males have managed to continue with the female despite attempts by the intruder male. However, it is possible that it will interfere with the insemination when it lasts until the transfer of sperm. As we did not see the female genital opening during the observations, it was not possible to verify whether insemination in fact occurred.

The tactic “dance between males” can have the advantage of reducing bodily harm caused by aggressive behavior, as males that submit themselves to the dance could avoid being stung by the opponent. Males of some insect species mimic females during conflicts with dominant males, reducing the risk of injuries (Peschke 1987; Iguchi 1996). In addition, “dance between males” might benefit the subordinate male, as the opponent became unable to inseminate the female after spermatophore deposition. This kind of submissive behavior, when a male assumes the role of a female and does not offer resistance, is usually seen as adaptive (Scharf & Martin 2013). In our observations, the passive male had the opportunity to mate with the female after the active male had deposited the spermatophore. If in a natural environment the female still remains available in the place where the males interacted, this behavior will tend to remain in the population.

Probably, active yellow scorpion males are at a disadvantage with the loss of the spermatophore, but passive males can also be at a disadvantage if they are unable to copulate with the female in dispute. “Dance between males” has two major disadvantages. While both males spend a lot of time dancing, the female can move from where the males are interacting and none of them can mate with her. Furthermore, a third male can mate with the female. In the shrimp *Rhynchocinetes typus* Edwards 1837, while dominant males fight over a female, a smaller male manages to mate (Correa et al. 2003). As “dance between males” tends to occur with males of similar sizes, two large males of *T. serrulatus* can waste time in this behavior, while a small male can mate with the female. Costs are also associated with same-sex sexual behavior, mainly related to the energy and time spent, which could be invested in mating (Scharf & Martin 2013). So, “dance between males”, although it can provide reproductive benefits for one of the competitors, is probably an inefficient strategy for the males that dancing in a natural environment.

“Dance between males” took longer than “*promenade à deux”*, which occurs between male and female (approximately eight minutes, chapter 2). In other animals, usually the time spent on same-sex sexual behavior is shorter than heterosexual behavior, probably due to lack of receptivity of the males who act as a female or from the eventual identification of the partner as a male, by the active male during the courtship (Vanacker et al. 2003, Wen et al. 2012, Scharf & Martin 2013). We believe that “dance between males” took longer than “*promenade a deux”* because at the beginning of the “dance between males” in *T. serrulatus*, the establishment of who would be the active male and who would be the passive male made the behavior longer. That happens because males take turns on each role several times, until it one of them deposits the spermatophore. Same-sex sexual behavior is often associated with competition between males but also with inaccuracies in choosing a mate (Sales et al. 2018). Several studies suggest that males engage in same-sex sexual interactions due to limitations in sexual partner recognition. In fact, males of different arthropod species have been reported courting other males, heterospecific individuals, or even objects and dead individuals (Scarbrough 1978; Dunning et al. 1979, Wall & Langley 1993, Hassler & Brockmann 2001, Levan et al. 2009, Fedina & Lewis 2008, Scharf & Martin 2013).

If the interaction between two males is due to misidentification, it is probably more difficult to identify the gender of individual when the size is similar, since “dance between males” tends to occur when the males have similar size. An important factor in the *T. serrulatus* male identification error may have been the presence of pheromones. As in all experimental replicates there was a female in the terrarium, the release of possible sexual pheromones may have confused the males. In situations like this, sexual pheromones are a major cause of misidentification in arthropods (Benz 1973; Rasa 1995, Leal et al. 1998; Ruther & Steiner 2008). In the spider *Oedothorax gibbosus* Blackwall 1841, males engage in same-sex sexual behavior more frequently in the presence of females, probably excited by female pheromones (Vanacker et al. 2003**)**. Contact sexual pheromones are important signals in mating of several species of scorpions (Taylor et al. 2012, Pordeus et al. 2019). *T. serrulatus* males show courtship behavior when exposed to surfaces previously occupied by females, indicating the presence of sexual pheromones (Chapter 4). Thus, it would be interesting to evaluate experimentally the role of female chemical signals on the aggressive and same-sex sexual behaviors in *T. serrulatus*.

Same-sex sexual behavior presented by males of *T. serrulatus* may be favored due to the artificial environment in which they found during the observations and may be less frequent in a natural environment. Although same-sex sexual behavior has already been described in the field in some insects, such as beetles and butterflies, some authors suggest that they are favored in an artificial environment (Kuba & Koyama 1985, Shah et al. 1986, Iguchi 1996; Switzer et al. 2004). One reason is that same-sex sexual behavior is favored when individuals are in high density (Livingstone & Ramani 1978), a situation in which *T. serrulatus* specimens met in our observations. To our knowledge this is the first report of same-sex sexual behavior in scorpions. Future field observations may help to understand whether “dance between males” is a common behavior in the species.

Finally, in our observations, the size of the male in relation to the size of the adversary did not influence the success of mating. We believe that this result may be related to the small difference in the size of the males in our study. However, males with significant difference size are found in the nature (personal observation) and different mating tactics can be an alternative for smaller males. As observed in our results, the difference in size between males can influence the tactic used, as occurs in “dance between males”, which usually occurs when males have approximate sizes. In future, individuals with significant differences in size, mainly of the metasoma, which is the structure most used during competition between males, must be investigated in order to understand if the size of the males influences on mating success related to a particular tactic.

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Figures Chapter 5

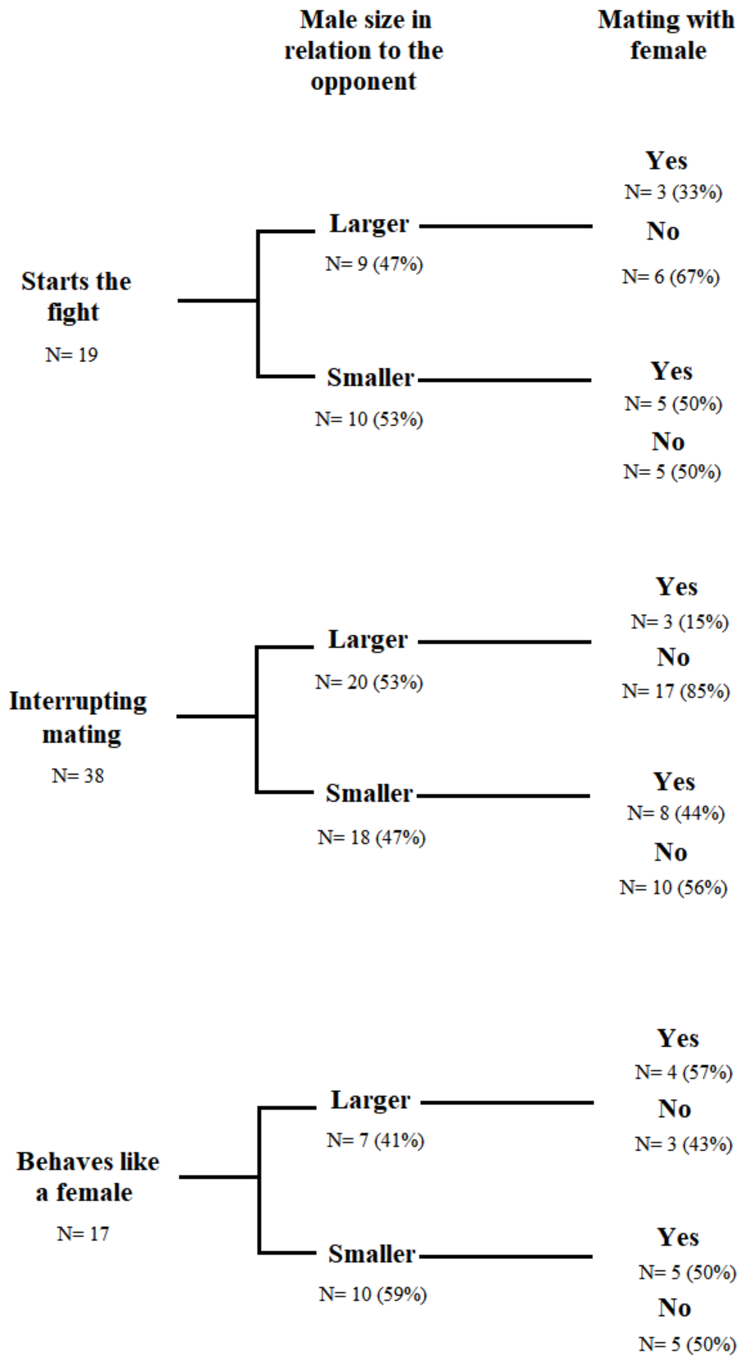


Figure 1. Male competition tactics used by male *Tityus serrulatus*, showing use frequency and outcomes of each tactic when employed by smaller and larger males.

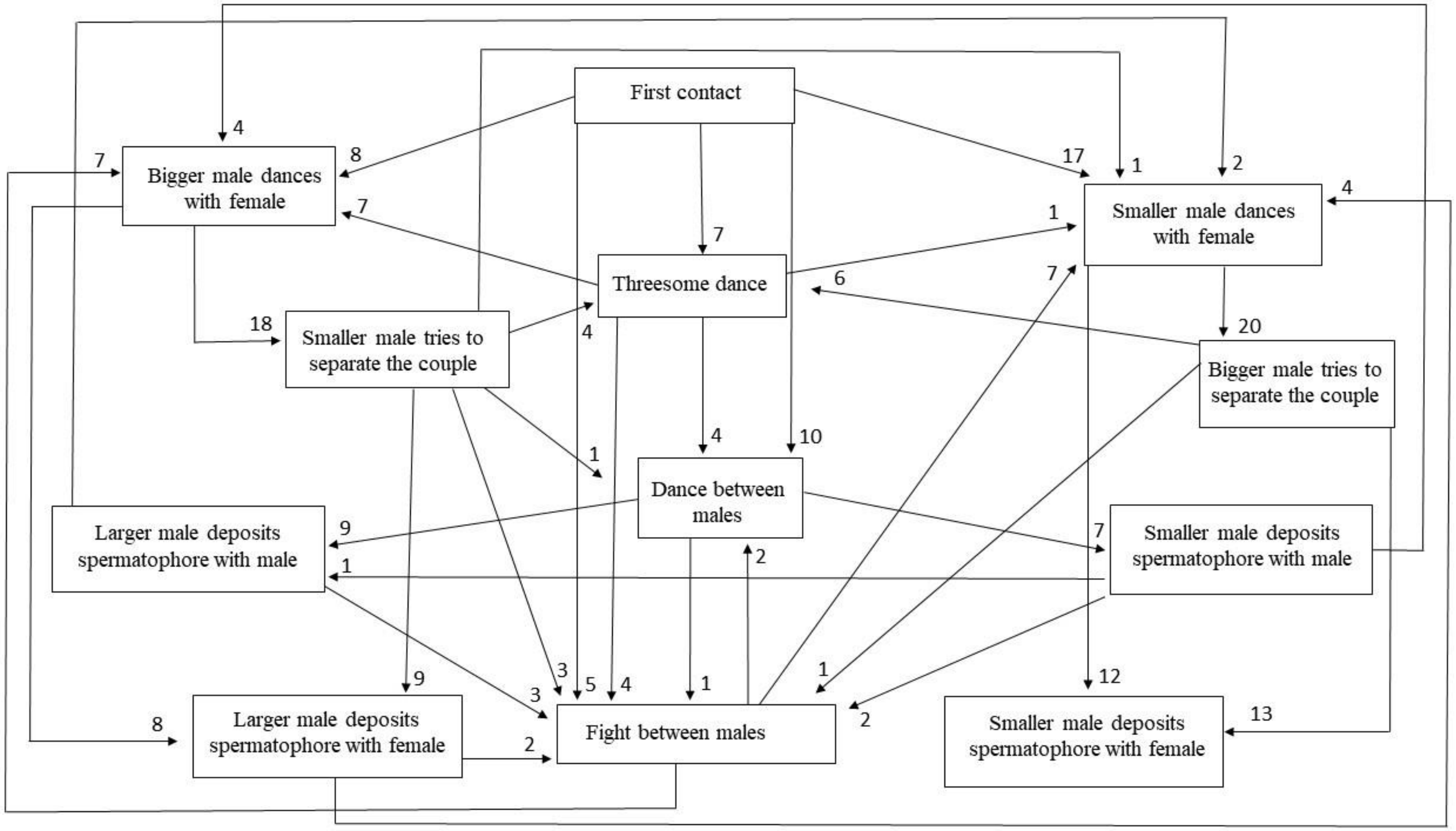


Figure 2. Flow diagram of the behaviors observed during the competition between two males of *Tityus serrulatus* for a female. The values next to the arrows represent the frequency of behavioral transition.

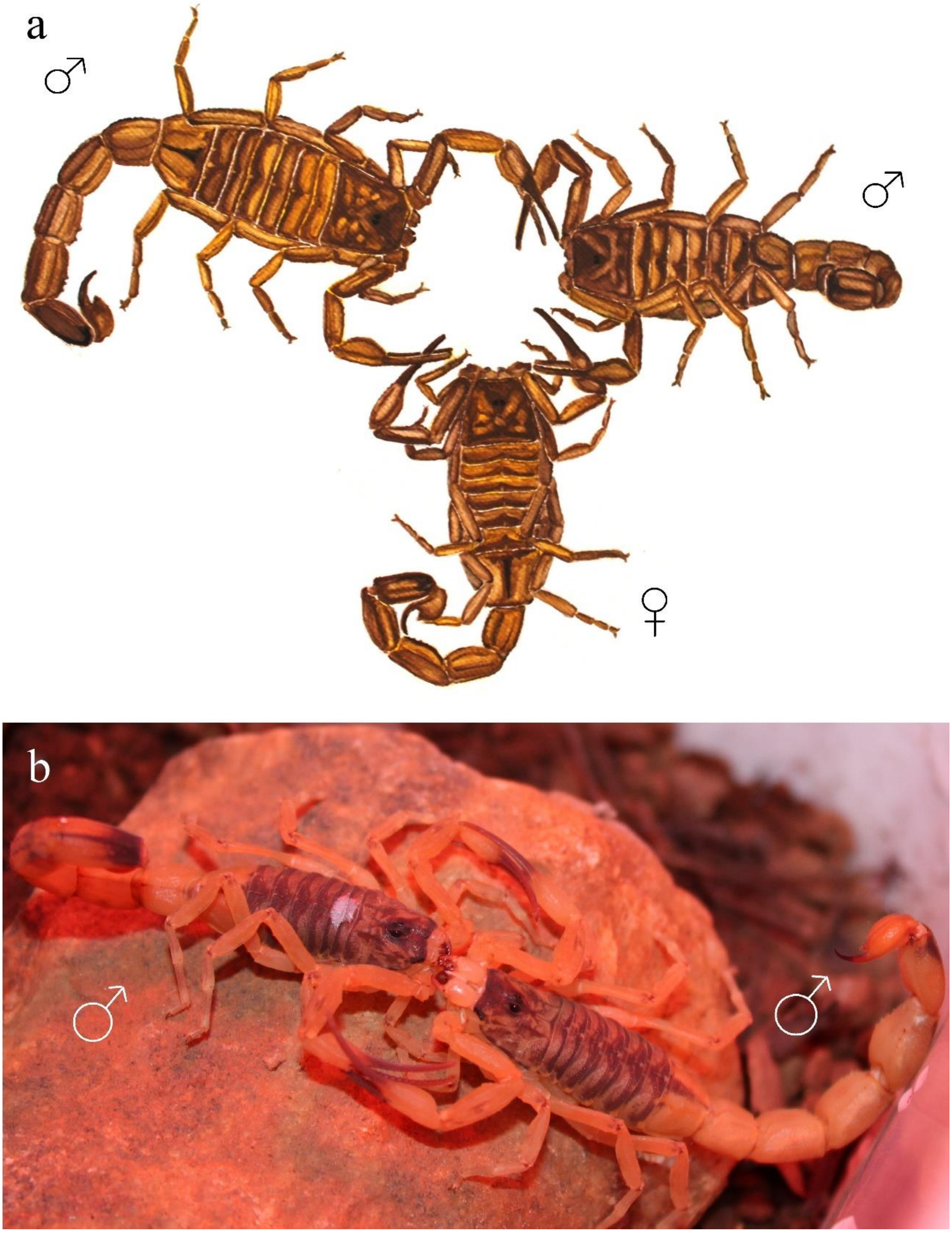


Figure 3. a) Threesome dance between two males and one female of *Tityus serrulatus*, a behavioral event observed during our male competition observations. **b)** Dance between two males of *Tityus serrulatus*, a possible alternative tactic used by individuals to avoid physical combat.

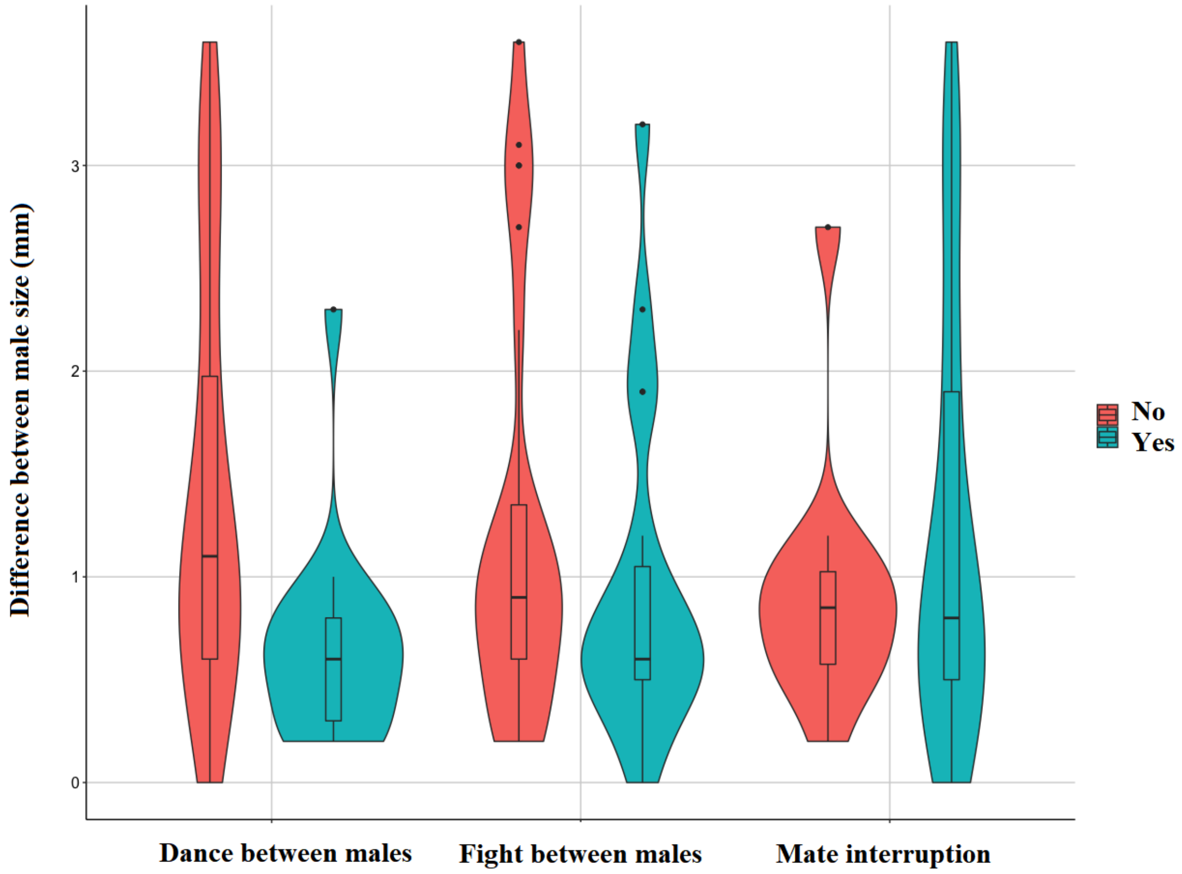


Figure 4. Frequency of male competition behaviors, as related to difference in male size. Box plots show median, quartiles, total range, and outliers in male size difference in each experiment. The violin plot shows the distribution of male size difference in each experiment.

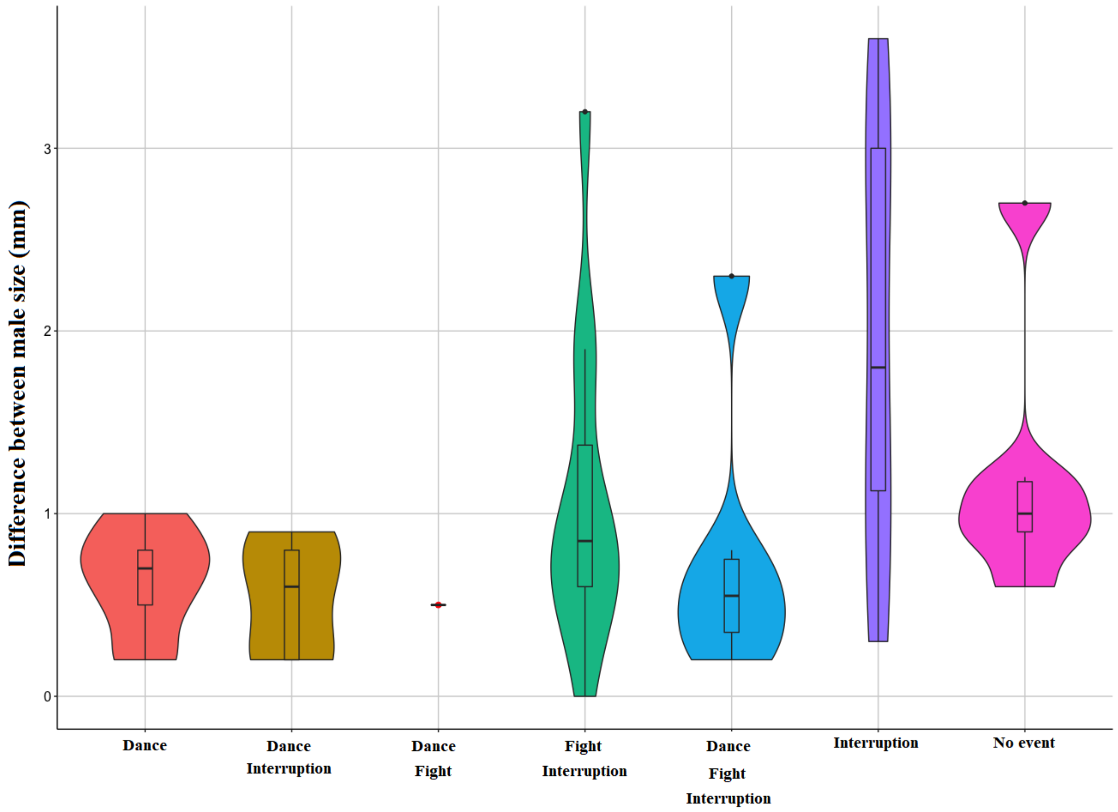
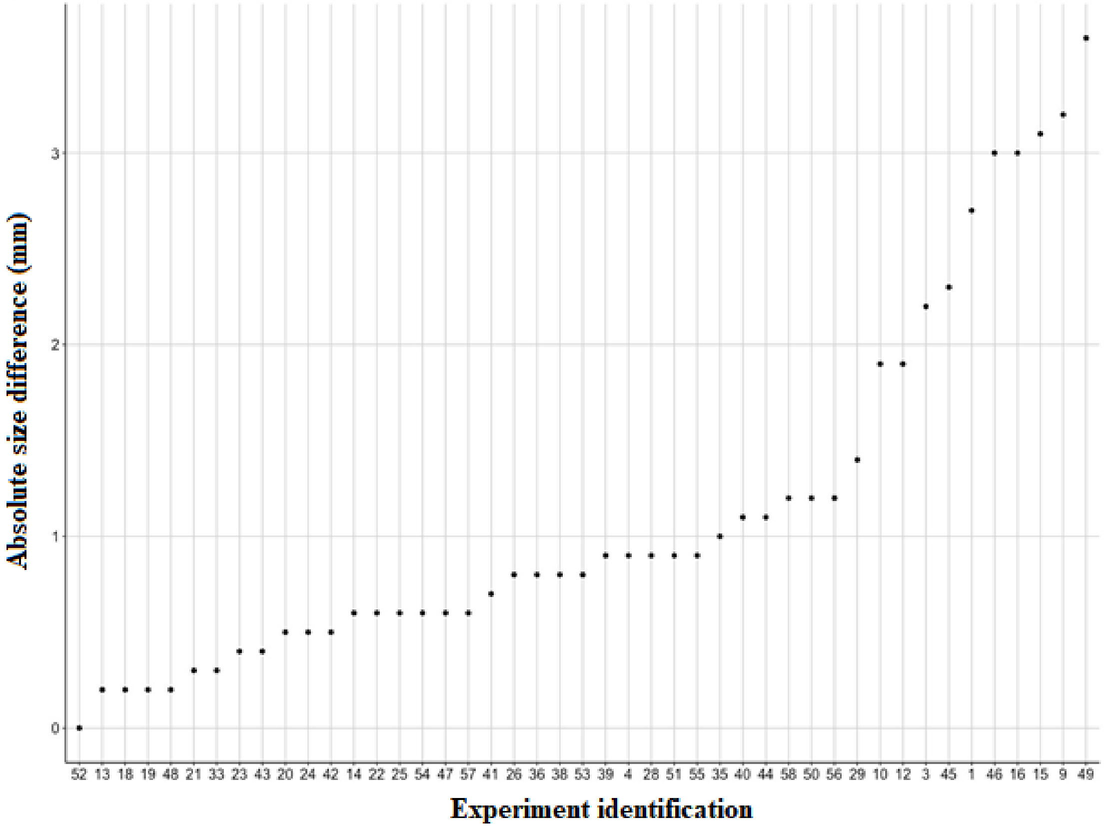


Figure 5. Frequency of combinations of male competition behaviors in *Tityus serrulatus* (Buthidade) as related to the difference contending male size. Box plots show median, quartiles, total range, and outliers in male size difference in each experiment. The violin plot width shows the Kernel density in relation to male size distribution.

Supplementary Materials Chapter 5

Supplementary material 1: Behavior tactics of *Tityus serrulatus* (Buthidae) males for competition for a female: “fight between males”, “mate interruption” and “dance between males”. Link: https://youtu.be/_dAdnq4OSOo



Supplementary material 2: Difference, in millimeters, in the size of the carapace of males of *Tityus serrulatus* (Buthidae) used in this study.