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Sexual interactions between adult males and immature females in the subsocial spider *Anelosimus vierae*

Interações sexuais entre machos adultos e fêmeas imaturas na aranha subsocial *Anelosimus vierae*

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Abstract: This study aimed to analyze the consequences of sexual interactions between adult males and penultimate females of the Uruguayan subsocial spider *Anelosimus vierae* Agnarsson, 2012 (Theridiidae). In the laboratory, several experimental groups were formed consisting of unrelated adult males and penultimate females of the species. The courtship and pseudo-copulation behaviors that occur between adult males and penultimate females were described in detail. These sexual behaviors were shown to have positive effects on future sexual encounters.

Keywords: Courtship. Pseudo-copula. Subadult females. Communal spider. Uruguayan spider.

Resumo: Este estudo teve como objetivo analisar as consequências das interações sexuais entre machos adultos e penúltimas fêmeas da aranha subsocial uruguaia *Anelosimus vierae* Agnarsson, 2012 (Theridiidae). No laboratório, foram formados vários grupos experimentais compostos por machos adultos não relacionados e penúltimas fêmeas da espécie. Os comportamentos de cortejo e pseudo-cópula que ocorrem entre machos adultos e penúltimas fêmeas foram descritos em detalhes. Esses comportamentos sexuais mostraram ter efeitos positivos em futuros encontros sexuais.

Palavras-chave: Cortejo. Pseudo-cópula. Fêmeas subadultas. Aranha comunal. Aranha uruguaia.

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INTRODUCTION

The strategies used by males to achieve dominance in the fertilization of females are very varied (Tomkins & Simmons, 2002; Parker, 2006). In both vertebrates and invertebrates, males may guard the females with whom they recently mated to avoid copulation with other males, thus ensuring their paternity (Parker, 1970; Birkhead, 1998; Simmons, 2001). Another male strategy is to guard females before they mature to monopolize the partners until they are receptive to mating (Andersson, 1994; Simmons, 2001). In this sense, in some spiders with sperm priority, males cohabit with subadult females to ensure their paternity by copulating with virgin females that have recently molted (Fernández-Montraveta & Cuadrado, 2003; Viera & Albo, 2008; Uhl et al., 2015; Moura & Gonzaga, 2017).

In many spiders, it is common for adult males to detect subadult females and guard them until they reach adulthood (Jackson, 1986; Bukowski & Christenson, 1997; Fernández-Montraveta & Cuadrado, 2003; Klein et al., 2005; Albo et al., 2007). The males of some spider species even fight among themselves in the webs of penultimate females to monopolize them (Albo et al., 2007; Aisenberg et al., 2015). The attractiveness of subadult females can vary during the penultimate instar, becoming more attractive just before the final molt (Schneider & Lubin, 1998). In cases where there is sperm priority, it is expected that males prefer to associate with subadult females rather than adults, mating with them as soon as they mature to ensure paternity (Eberhard et al., 1993; Elgar, 1998). In extreme cases, males of the theridiids *Latrodectus hasselti* Thorell, 1870 and *L. geometricus* C. L. Koch, 1841 increase their reproductive successes and avoid cannibalization by copulating with penultimate females. Males pierce the exoskeleton of females with their chelicerae to access females 'newly developed spermathecae. Females retain sperm through their final molt and achieve fecundity similar to adult females (Biaggio et al., 2016).

Male guards try to reduce female polyandry, so this behavior has typically been considered as a form of sexual conflict (Parker, 1979; Birkhead, 1998; Jormalainen, 1998; Zeiss et al., 1999; Rodríguez-Muñoz et al., 2011). However, females may obtain direct benefits from mating with guarding males, such as reduced predation (Rodríguez-Muñoz et al., 2011; Cothran et al., 2012) and sexual harassment by other males (Davis, 2002). They might also gain indirect genetic benefits (Prenter et al., 2003), by selecting for costly traits in males that indicate greater heritable variability for their offspring (Williams, 1966). Females who mate with males who guard efficiently would pass that ability on to their offspring.

Finally, cohabiting with a male during the pre-copulatory guard allows the female to evaluate the partner as a potential mate for the future (Hebets, 2003; Johnson, 2005; Hebets & Vink, 2007; Rutledge et al., 2010; Kasumovic & Seebacher, 2013). For example, Hebets (2003) conducted laboratory experiments with the wolf spider *Schizocosa uetzi* Stratton, 1997 (Lycosidae). He formed two groups of males, one with tibia I and patella I brown painted and the other with tibia I and patella I black painted, and exposed each group to juvenile females. When females matured, they were exposed again to these two groups of males. It was observed that females mated more frequently with males with familiar traits and cannibalized males with unfamiliar traits (Hebets, 2003).

Anelosimus vierae Agnarsson, 2012 is a Uruguayan subsocial spider. Individuals live in communal nests in evergreen trees. The mother cares for the young, and the juveniles show a lack of conspecific aggression. Males mature earlier and consistently disperse, resulting in inbreeding avoidance (Viera et al., 2007). Females can display one of two reproductive tactics depending on the availability of resource: they may remain in the nest with the mother and share the web with other sisters or they may disperse as subadults and build individual nests (Viera et al., 2007). Therefore, when males disperse from their natal nest, they may encounter one of two scenarios: a nest with many subadult females and one or a few adult females, or a nest made up of a single subadult female. These two scenarios will determine the reproductive tactics and mate preferences of males (Viera & Albo, 2008). Viera and Albo (2008) performed an experimental design simulating a nest made up of several females of *A. vierae*, which gave the males the possibility of choosing between females of different ages and reproductive status. These nests were made up of two adult females of different ages and a penultimate subadult female. All males courted at least one adult female, but no courtship was observed towards subadult females. On the other hand, Albo et al. (2007) simulated a nest made up of a single subadult female. In this scenario, the subadult female was exposed to two males. Subadult females were attractive to males, who courted and even fought among themselves. They also observed that the subadult females courted the males and adopted a receptive posture. In this position, males faced their pedipalps towards the epigynum area of the females and made several insertion attempts by drumming the epigynum of subadult females. They called this behavior 'pseudo-copula' because males were could not insert their copulatory organs due to a mechanical impediment, linked to the sexual immaturity of the female (Albo et al., 2007; Viera & Albo, 2008; Rojas-Buffet & Viera, 2015). Pseudo-copula has also been reported in the subsocial species *A. studiosus*, where authors have referred to these behaviors as "nonconceptive sexual behaviors" (Pruitt & Riechert, 2011, p. 790).

In this study, we described in details the courtship of *A. vierae* males towards penultimate females and their pseudo-copulatory behavior. We also evaluated the consequences and possible benefits of pre-copulatory sexual interactions between males and penultimate females. The following specific objectives were proposed: (1) to determine when, after the penultimate molt, females are receptive and/or pseudo-copulate with adult males; (2) to describe courtship and pseudo-copula in details; (3) to investigate whether penultimate females can modulate their development rates until reach adulthood, according to the occurrence of previous sexual experience; (4) to determine if there are differences in copulation latency between couples with previous sexual experience and sexually naïve couples; and (5) to determine if males prefer females that had sexual experience during their penultimate stage or sexually naïve females.

MATERIAL AND METHODS

COLLECTION AND MAINTENANCE

Nests of *A. vierae* were collected from several evergreen trees located in Parque Rodó, Montevideo, Uruguay (34° 54' 48" S, 56° 10' 2" W). The nests were transported in nylon bags to the laboratory of the Sección Entomología, Facultad de Ciencias, Montevideo, Uruguay. In the laboratory, individuals were sexed and raised separately in Petri dishes (4 cm in diameter and 1.2 cm high). All spiders were fed *ad libitum* with *Drosophila* spp. (Diptera) and were supplied with moisture, using cotton wool soaked in water.

EXPERIMENTAL STAGE

Receptivity, courtship, and pseudo-copula of penultimate females (specific objectives 1 and 2)

Penultimate females were used to form four groups (n = 30 per group), according to their age (i.e., number of days) since they molted to the penultimate stage:

- Group 1: three days after molting to penultimate.
- Group 2: ten days after molting to penultimate.
- Group 3: 20 days after molting to penultimate.
- Group 4: 25 days after molting to penultimate.

Each female was placed individually in a Petri dish (4 cm diameter and 1.2 cm high) she was exposed to an adult virgin male of a different nest (non-siblings) 24 h later. Each individual was used only once. The interactions between both individuals were recorded for 1 h, using a video camera (Sony DCRA-C151, 2.7" Wide Hybrid LCD, Handycam Station Supplied, 12x optical zoom, 800x digital zoom). The occurrence of courtship by males and females and the occurrence and number of instances of pseudo-copulations were recorded for each group. Courtship behavior was considered from the first courtship behavioral unit to the first pseudo-copula behavioral unit. The behavioral units involved in courtship and pseudo-copula were defined and described. Unit transition matrices were constructed for each sex in each couple and then the overall transition matrix was calculated for each sex (by consolidating all matrices). A flow chart was constructed to visualize the degree of association between the units. Z-value was calculated to determine which transitions occur more frequently than expected by chance. Z = values > 1.96 occur significantly more or less frequently than expected by chance. Based on the generally observed matrix and the expected matrix, Z is calculated as $Z = [(\text{observed frequency } B|A \pm 1/2) - \text{expected frequency } B|A)]/\text{standard deviation}$ (Blumstein & Daniel, 2007). The durations and frequencies of behaviors were recorded. The videos were analyzed with the behavioral analysis program JWatcher (Blumstein et al., 2000).

After each trial, cephalothorax width of all individuals was measured to relate it to the characteristics of courtship and pseudo-copula. After the experiences where pseudo-copula occurred, the epigynous area of ten females were observed under a stereoscopic magnifying glass to rule out traumatic inseminations that the males could have carried out on the integument during pseudo-copula. After the experiments, the same females were raised individually in Petri dishes, until they naturally died, to record the day of molting to adulthood and the possibility of laying oothecae during the adult stage.

Modulation of the development rate until adulthood according to the occurrence of previous sexual experience (specific objective 3)

Females and males were reared individually in Petri dishes from juveniles. In the beginning, cotton was supplied with water and ENSURE supplement, because they cannot capture prey on their own during the first stages (Ghione et al., 2004). From the third instar, each individual was fed twice a week with the same amount of fruit flies (*Drosophila* spp.).

Two experimental groups were formed (n = 30 each):

- Group 1: a penultimate female 25 days after molting and an adult male.
- Group 2: a penultimate female 25 days after molting and a penultimate female.

Penultimate females were placed individually in Petri dishes (4 cm in diameter and 1.2 cm high), and after 24h, an adult male (group 1) or another penultimate female (group 2) was placed with her. In group 2, it was decided to place another penultimate female instead of an adult one to avoid their territorialism and the risk of cannibalism. Furthermore, it is common for penultimate females to coexist in nature (Viera et al., 2007).

The occurrences of receptive postures of penultimate females were recorded: ventral area up, and body axis inclined approximately 45° (Albo et al., 2007) and the occurrence of pseudo-copula. After 1 h, focus females were separated and returned to their Petri individual dishes. The development time of sexually experienced penultimate females (i.e., those that engaged in pseudo-copula or at least were receptive to male courtship) was monitored between (1) molting to the penultimate instar to adulthood and (2) from the day of the mating experience to adulthood. Both periods were compared with those of the females of the group 2. Cephalothorax width was measured and the weight of all focus females were measured to rule out that the latency to reach adulthood was related to the size and/or weight of the females.

Copulation latency as a consequence of previous sexual experience (specific objective 4)

The following five experimental groups were formed (n = 20 each):

- Group 1: Adult female and male with sexual experience (original dyad).
- Group 2: Adult female and male with sexual experience (new dyad).
- Group 3: Adult female with sexual experience and naive adult male.
- Group 4: Naive adult female and adult male with sexual experience.

- Group 5: Naive adult female and male.

For females, 'sexually experience' means that during their penultimate stage, they were exposed to adult males, and the 'naive' are those who, as penultimate, were never exposed to adult males. For males, 'sexually experience' means that during their adult stage, they were exposed to penultimate females, and the 'naive' are those who, as adults, were never exposed to penultimate females. All used individuals were virgins. For each experimental group, the female was placed in a Petri dish (4 cm in diameter and 1.2 cm high). After 24 h, the adult male was placed. Copulation latency was compared between the five experimental groups. Copulation latency was measured from the moment the male was placed in the Petri dish until the first palp was inserted into the female's genital opening.

Male preference (specific objective 5)

Three experimental groups were formed (n = 15 each):
- Group 1: Adult female and male with sexual experience (original dyad) and a sexually naive female.
- Group 2: Adult female and male with sexual experience (new dyad) and a sexually naive female.
- Group 3: Adult female with sexual experience and sexually naïve male and female.

The two females of each experimental group were placed at the same time in a Petri dish (5 cm in diameter and 1.2 cm high). After 24 h, the adult male was placed, as equidistant as possible from the females in all trails. We evaluated (1) male choice through the occurrence of copulation in the presence of both females and (2) competition between females through the occurrence of agonistic behavioral units such as chasing, chelicerae confrontation or biting that have been reported for this species (Santana et al., 2017). Both male choice and competition between females were evaluated during 2 h of observation. After each experience, cephalothorax widths of the experienced and non-experienced females of each group were compared to rule out that the male copulation with one or another female was affected by this variable.

STATISTICAL ANALYSIS

The results were analyzed with the PAST v program. 4.09 (Hammer et al., 2003). The normal distribution of the data was examined using the Shapiro-Wilk test and the homogeneity of variances with the Levene test. In cases with normality and homogeneity of variances, the parametric Student's t test was applied; otherwise, the Mann-Whitney U test was applied. To compare frequencies, Fisher's Exact test (used when expected frequencies in any cell of a contingency table are less than 5) or Chi-square test (used when all expected cell frequencies in the contingency table are greater than 5) were used.

RESULTS

RECEPTIVITY, COURTSHIP, AND PSEUDO-COPULA OF PENULTIMATE FEMALES

All the males courted all groups of females. But females of groups 1, 2, and 3 did not accept the courtship of the males, they did not court and there was no pseudo-copula. In the group 4, 80% of the penultimate females were receptive to the males' courtship and 54% of the receptive females pseudo-copulated. Courtship latency was 3.33 ± 4.72 min (mean and standard deviation). In all cases, courtship was initiated by the males. Courtship behavior had a total duration of 12.96 ± 9.68 min (range = 1.10 - 33.37 min; minimum and maximum).

Table 1 details the behavioral units involved in courtship. In summary, males approached the potential partners, while females approached or moved away from males. Males remained motionless in front of both females and then they began to tighten silk threads with their front legs. Receptive females responded to the males' courtship by approaching them and stretching threads in their direction. Following this, males and females extended their legs and palps, and females adopted the receptive posture, with the ventral area up, and body axis inclined approximately 45°, similar to that of adult females. In this position, males touched females with their front legs until pseudo-copula occurs (Figure 1).

The most frequent behavioral units of courtship in both sexes were: stillness (♂ = 18.61 ± 14.11 times/individual; ♀ = 25.77 ± 24.69 times/individual), silk thread tension (♂ = 16.92 ± 15.14 times/individual; ♀ = 15.54 ± 19.94 times/individual) and walk (♂ = 8.46 ± 8.71 times/individual; ♀ = 6.61 ± 10.36 times/individual).

Table 1. Description of the courtship behavioral units of males and penultimate of *A. vierae* females. Who performs each unit is indicated, whether male (♂), female (♀), or both sexes (♂, ♀).

Behavioral unit	Description	Sex
Abdominal vibration	Vertical oscillations of the abdomen	♂
Insertion attempt	Repeatedly touching the female's epigynum with one of the copulatory organs	♂
One leg extension	While still, extends and rotation one of the legs	♀
Silk thread tension	Tension and release of silk threads with leg I, II and III	♂, ♀
Touch	Touches the other sex with front legs	♂, ♀
Palps and legs extension	Extension of palps and anterior legs towards the other sex	♂, ♀
Stillness	Absence of movement	♂, ♀
Remoteness	Walks in the opposite direction from the couple	♂, ♀
Approach	Walks towards the couple	♂, ♀
Walk	Movement from one place to another sometimes also moving palps alternately	♂, ♀
Place silk threads	Walks and leaves silk threads	♂, ♀
Clean of legs and/or palps	Carries some of its legs or palps towards the chelicerae	♂, ♀

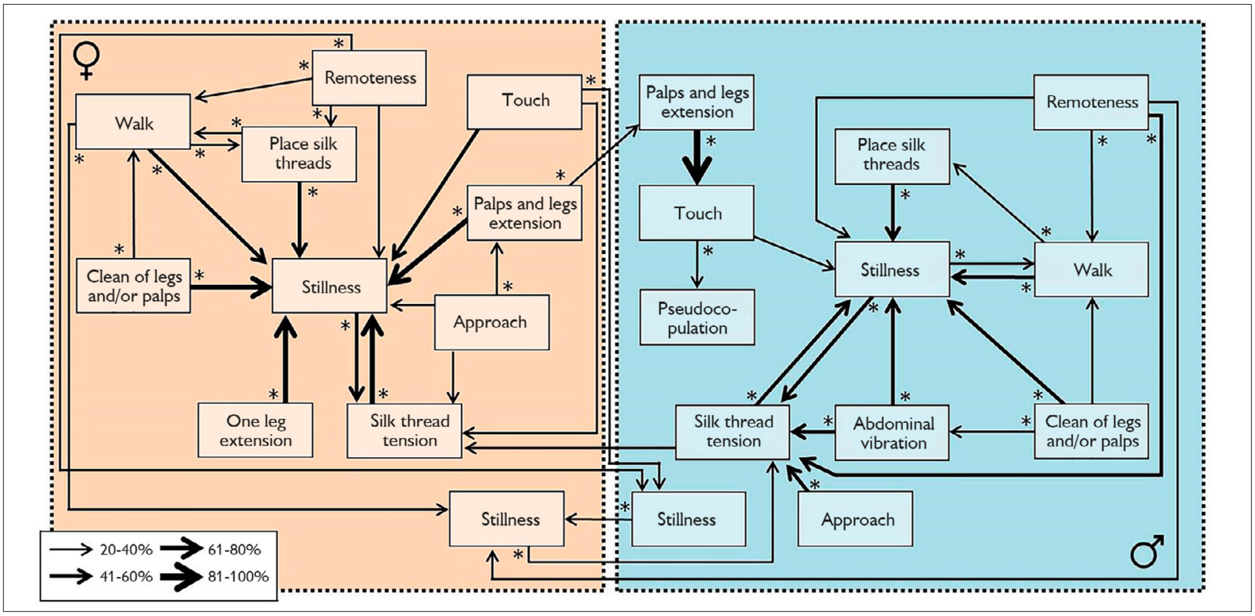

Figure 1. Flow diagram of courtship in *A. vierae* in pairs of an adult male and a penultimate female of 25 days after molting. The orange box represents the units performed by the females and the light blue box by the males. The thickness of the arrows indicates the probability of transition from one unit to the next. Only probabilities greater than or equal to 20% are represented for intra-sexual transitions, and those greater than or equal to 25% for inter-sexual transitions are represented. Asterisks indicate transitions that occur more frequently than expected by chance (with Z values > 1.96).

They are followed by abdominal vibration (6.31 ± 6.18 times/individual) in males; palps and legs extension (6.08 ± 7.26 times/individual) in females and one leg extension (5.77 ± 7.06 times/individual), also in females. The longest behavioral unit was stillness for both sexes (♂ = 6.79 ± 7.69 min; ♀ = 8.22 ± 6.31 min), followed by silk thread tension (2.13 ± 1.97 min) in males, and walk (1.44 ± 3.05 min) in females (Figure 2).

When investigating the influence of cephalothorax width of males and females on the duration of courtship, no significant relationship was found neither for males (F = 0.11; DFn = 1, DFd = 11; p = 0.74), nor for females (F = 0.51; DFn = 1, DFd = 11; p = 0.49). Only considering group 4, thirteen of the 30 dyads pseudo-copulated, and the latency was 16.00 ± 12.90 min from the beginning of the experience. Strictly, pseudo-copula involved three behavioral units. In males: attempt to insert the left palp and attempt to insert the right palp onto the epigynum; in females: extension of legs and palps in a receptive position. During each encounter, pseudo-copulations occurred in an average of 2.25 ± 0.87 instances. The duration of pseudo-copula (the sum of the durations of the pseudo-copulations instances) was 18.41 ± 11.64 s (range = 6.88 - 50.14 s) (Figure 3).

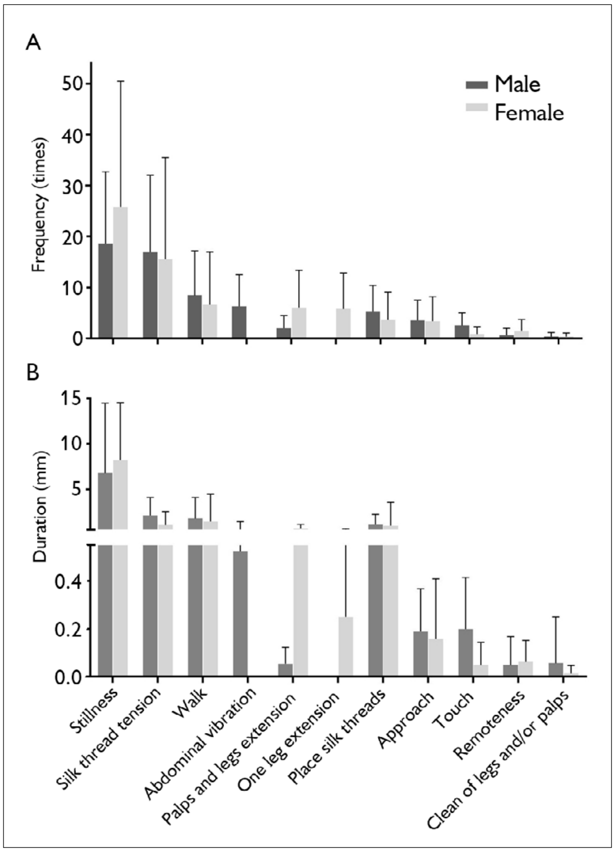

Figure 2. (A) Frequency and (B) duration of each behavioral unit involved in the courtship of adult males and penultimate females in *A. vierae*. The mean and standard deviation of the values are plotted.

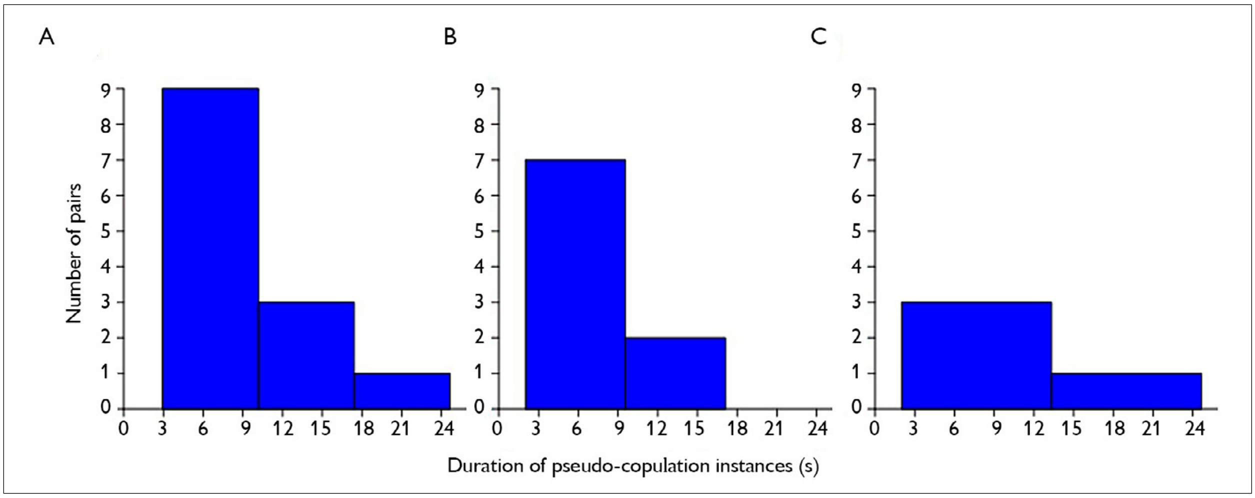

Figure 3. Histogram of pseudo-copula duration, during the first (A) (n = 13), second (B) (n = 9), and third instance (C) (n = 4). The 4th instance of pseudo-copulation is not depicted because it occurred in only one pair.

For the description of pseudo-copula, we considered all that occurred between the first unit involved during the first instance of pseudo-copulation to the last unit of the last instance of pseudo-copulation. Table 2 describes the units involved throughout the process and details of the units performed by both sexes. In summary, once the penultimate female accepted the male's courtship, she adopted the typical receptive posture of an adult female. In this position, the male made several insertion attempts, drumming the female's epigynum with one or both copulatory organs until one of the sexes moved away from the other. Pseudo-copulations may occur more than once during the same encounter, and in that case, the male continued with a new cycle of drumming until they moved away from each other and remained still. The units left palp insertion attempt and right palp insertion attempt had a frequency of 1.61 ± 1.12 times/individual for the left palp and 1.46 ± 1.26 times/individual for the right one, and a duration of 9.66 ± 7.46 s and 10.95 ± 9.30 s, respectively.

When investigating the influence of the cephalothorax widths of males and females on the duration of pseudo-copula, no significant relationship was found for males (F = 1.43; DFn = 1, DFd = 11; p = 0.26), or for females (F = 0.89; DFn = 1, DFd = 11; p = 0.36). No significant differences were found in the cephalothorax width of females that pseudo-copulated (0.99 ± 0.07 mm) and those that did not pseudo-copulate (1.01 ± 0.06 mm) (U = 89; p = 0.38). Likewise, no differences were found in the cephalothorax width of males that pseudo-copulated (1.04 ± 0.04 mm) and those that did not (1.03 ± 0.06 mm) (U = 108.5; p = 0.95).

No evidence of traumatic inseminations was observed in the 13 females analyzed and none of them laid egg sacs after reaching the adult stage. During the experiments, two females began the molting process into adults 27.82 ± 6.36 min after being exposed to the adult males (Figure 4A). Previously, both had been receptive to the males' courtship, but pseudo-copula did not occur. Females molted during the filming and males remained most of the time, immobile, although on some occasions both males carried out courtship units but not copulation attempts. Molting process lasted 28.10 ± 0.16 min. At the end, males approached females and courted them. Females were receptive and copulated (Figure 4B).

Table 2. Description of the pseudo-copula behavioral units of *A. vierae*. Who performs each unit is indicated, whether male (♂), female (♀), or both sexes (♂, ♀). RP: right palp; LP: left palp.

Behavioral unit	Description	Sex
LP insertion attempt	Makes several insertion attempts on the female's epigynum with the left palp	♂
RP insertion attempt	Makes several insertion attempts on the female's epigynum with the right palp	♂
Abdominal vibration	Vertical oscillations of the abdomen	♂
One leg extension	While still, extends and rotates one of the legs	♀
Touch	Touches the other sex with front legs	♂, ♀
Palps and legs extension	Extension of palps and anterior legs towards the other sex	♂, ♀
Silk thread tension	Tension and release of silk threads with leg I, II and III	♂, ♀
Clean of legs and/or palps	Carries some of its legs or palps towards the chelicerae	♂, ♀
Stillness	Absence of movement	♂, ♀
Remoteness	Walks in the opposite direction from the couple	♂, ♀
Approach	Walks towards the couple	♂, ♀
Walk	Walks and moves palps alternately	♂, ♀
Place silk threads	Walks and places silk threads	♂, ♀

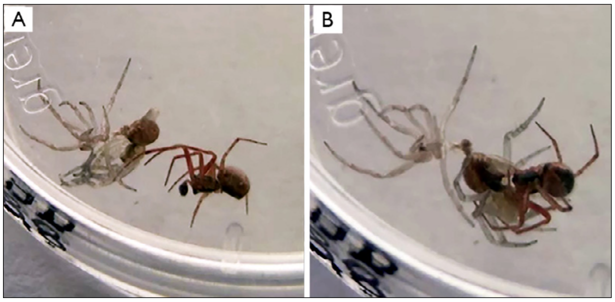

Figure 4. (A) Penultimate female molting into adult and adult male close to her. (B) Copulation of recently molted female. Photos taken from the filming.

The latency of copulation after molting differed greatly between both pairs, one of the pairs copulated after 1.67 min and the other after 55.13 min. The male of this last pair made numerous insertion attempts, and the difficulty was evident in inserting any of his copulatory organs into the still soft epigynum of the female. The total duration of copulation for one couple was 84.96 min and for the other couple was 93.78 min. Only one of the females laid an egg sac, and it was the female of the couple with the shortest copulation. The female laid the egg sac after 23 days of copulation, from which 23 viable offspring were born.

Modulation of the development rate until adulthood according to the occurrence of previous sexual experience (specific objective 3)

In the group 1, 90% of penultimate females were receptive and 10 of them performed pseudo-copula, so the development times of these 27 females were compared with those of the 30 females of group 2. Female maturation time from the penultimate instar in group 1 was 30.67 ± 5.06 days, and in group 2, was 31.10 ± 2.47 days (Figure 5). Besides, the time from the day of experience to adulthood was 5.67 ± 5.06 days for females of group 1 and 6.10 ± 2.47 days for females of group 2.

No significant differences were found between the maturation times since the females' penultimate molting in both groups (U = 319; p = 0.17). There were also no differences in the times from the day of the experiences to adulthood (U = 319; p = 0.17). Therefore, the latency towards the last molt in penultimate females with and without sexual experience was similar.

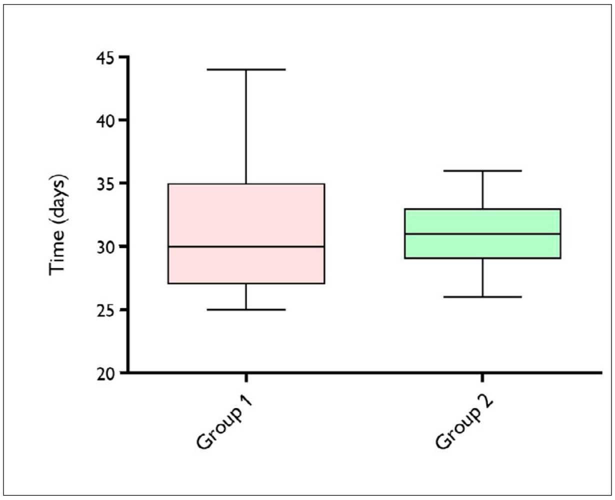

Figure 5. Box plot showing the time it took for the penultimate females of *A. vierae* to reach adulthood in both experimental groups.

When investigating the existence of significant differences in maturation times from the penultimate molting, separating within group 1 the females that pseudo-copulated (n = 10) from those that were receptive but did not pseudo-copulate (n = 17), no significant differences were found (U = 74; p = 0.42). No differences were found either between receptive females of group 1 and females of group 2 (U = 149.5; p = 0.13), or between pseudo-copulated females of group 1 and females of group 2 (U = 169.5; p = 0.51).

No significant differences were found in the groups between the cephalothorax width (Group 1 = 1.01 ± 0.06 mm; Group 2 = 0.99 ± 0.05 mm; U = 378; p = 0.67) and between the weight of females (Group 1 = 3.67 ± 0.63 mg; Group 2 = 3.39 ± 0.56 mg; t = 1.65; df = 55; p = 0.10).

Copulation latency as a consequence of previous sexual experience

Copulation occurred in all trials, and the copulation latencies for each experimental group were: group 1 (known experienced female and male) = 23.90 ± 14.48 min;

group 2 (unknown experienced female and male) = 24.07 ± 12.28 min; group 3 (experienced female and naïve male) = 37.36 ± 20.97 min; group 4 (naïve female and experienced male) = 47.37 ± 31.96 min; and group 5 (naïve female and male) = 44.12 ± 26.78 min.

When comparing copulation latency between the five experimental groups, significant differences were found (H = 13.03; p = 0.011). The copulation latency of group 1 was significantly lower than that of group 3 (U = 112; p = 0.018), group 4 (U = 113; p = 0.019), and group 5 (U = 111; p = 0.017). The copulation latency of group 2 was significantly lower than that of group 3 (U = 125.5; p = 0.045), group 4 (U = 118; p = 0.027), and group 5 (U = 114; p = 0.021). Figure 6 shows the copulation latency of the different experimental groups.

When evaluating differences in copulation latency between pairs in which at least one of the sexes previously pseudo-copulated (n = 36) and couples that were receptive but did not reach pseudo-copula (n = 44), no significant differences were found (U = 758.5; p = 0.75).

Male preference

All males in the three experimental groups courted at least one of the females. The males courted one of the two females more frequently, both in total, taking into account the three groups ($\chi^2 = 54.44$, n = 45, $p < 0.0001$), and by group (Group 1: Fisher's Exact Test, n = 15, p = 0.0001; Group 2: Fisher's Exact Test, n = 15, $p > 0.0001$; Group 3: Fisher's Exact Test, n = 15, p = 0.0009). Likewise, males courted experienced females more frequently than sexually naive females, both in total (taking into account the three groups) ($\chi^2 = 35.28$, n = 45, $p > 0.0001$), and by group (Group 1: Fisher's Exact Test, n = 45, p = 0.0001; Group 2: Fisher's Exact Test, n = 45, p = 0.0092; Group 3: Fisher's Exact Test, n = 45, p = 0.0025).

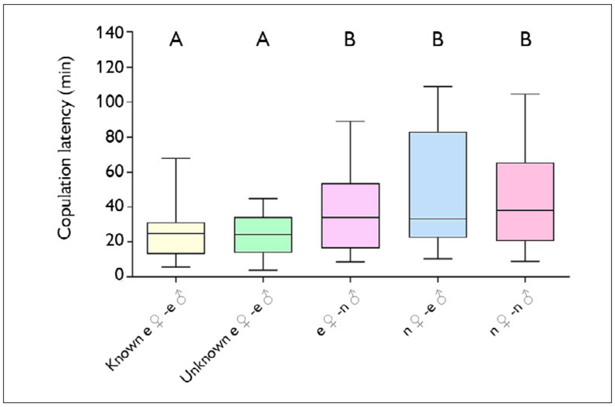

Figure 6. Box plot comparing copulation latency among the five experimental groups (test U for Mann Whitney). ♀: female; ♂: male; e: sexually experienced; n: sexually naive. Different Letters above the boxes denote significant differences between groups ($p < 0.05$).

In 29 experiences out of 45, it was exclusively the experienced females who courted the males (Group 1: n = 10; Group 2: n = 11; Group 3: n = 8), and in eight experiences it was exclusively the naive females (Group 1: n = 2; Group 2: n = 3; Group 3: n = 3). Only in two experiences of group 1 both females courted. In six trials, none of the females courted (Group 1: n = 1; Group 2: n = 1; Group 3: n = 4). The experienced females courted more frequently than the sexually naive ones in the total, taking into account the three groups ($\chi^2 = 20.84$, n = 45, $p < 0.0001$), in Group 1 (Fisher's Exact Test, n = 15, p = 0.0025) and in Group 2 (Fisher's Exact Test, n = 15, p = 0.0092). In Group 3, there were no differences between the courtship frequency of both females (Fisher's Exact Test, n = 15, p = 0.128).

Copulation occurred in 93.3% of the pairs in group 1, in 86.7% of the pairs in group 2, and in 73.3% of the pairs in group 3. In cases where copulation did not occur, all males courted but none of the females were receptive. All the males that copulated did so with a single female. When analyzing the total (taking into account the three groups), it was observed that copulations were more frequent between sexually experienced males and females ($\chi^2 = 19.95$, n = 45, $p < 0.0001$). Similarly, in groups 1 and 2, copulations occurred more often between sexually experienced males and females (Group 1: Fisher's test, n = 15, p = 0.0007; Group 2: Fisher's test, n = 15, p = 0.025). In all experiences in which experienced males and females copulated, both individuals courted. In eight experiments the experienced females were the ones that actively began courtship (Group 1: n = 5; Group 2: n = 3). In two experiments in Group 2, naive females initiated courtship, but males copulated with experienced females. In all cases of copulations between males and naive females, only the naive females courted. In Group 3, no differences were found in the frequency of copulations with experienced and naive females (Fisher's Exact Test, n = 15, p = 0.128) (Figure 7). In this group, in all cases of copulations between males and experienced females, only the experienced females courted, and in all cases of copulations with naïve females, only the naïve females courted. So females courted males in all copulation cases. The duration of courtship and copulation (in minutes) of the three experimental groups are shown in Table 3.

No significant differences were found between the body sizes of the experienced females and the naive ones in each group (Table 4). During the experiments, no disturbances of the copulating pair by the other female were recorded, as well as no agonistic behavioral units such as chasing, chelicerae confrontation or biting between the adult females.

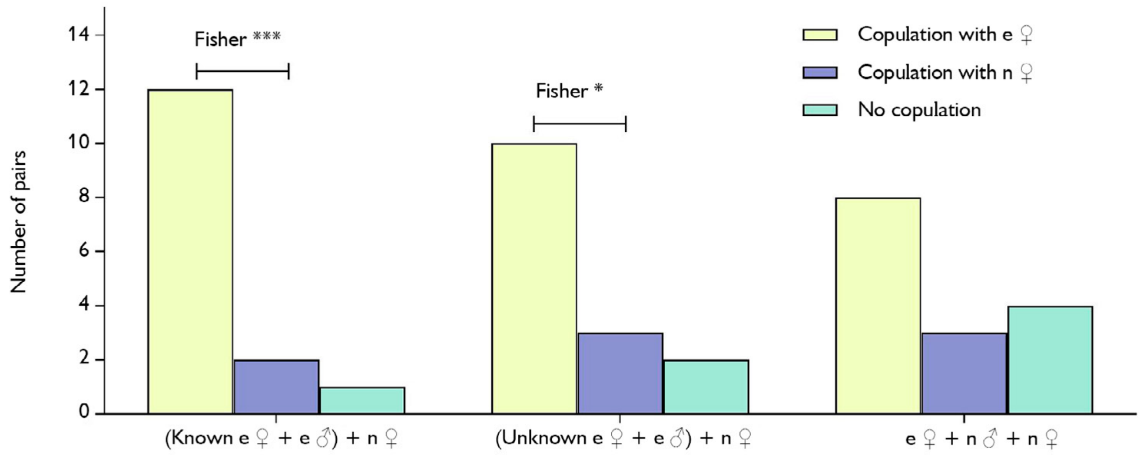

Figure 7. Number of copulations for each experimental group, between males and experienced females (e♀) and naïve females (n♀). Significant differences are shown with asterisks: * = $p < 0.05$; *** = $p < 0.005$.

Table 3. Courtship and copulation durations (in minutes) in males with experienced females (e♀) and with naïve females (n♀) for each experimental group. Values are shown as the mean ± standard deviation.

	Group 1		Group 2		Group 3	
	e♀	n♀	e♀	n♀	e♀	n♀
Courtship latency	4.34 ± 11.09	11.10 ± 8.88	3.29 ± 3.88.09	0.75 ± 0.62	1.73 ± 1.70	9.49 ± 12.73
Courtship duration	27.73 ± 17.95	17.00 ± 6.20	32.86 ± 19.12	10.65 ± 6.41	13.14 ± 8.83	12.93 ± 7.60
	n = 14	n = 3	n = 12	n = 4	n = 13	n = 4
Copulation latency	27.23 ± 21.22	21.62 ± 8.84	36.20 ± 21.47	13.81 ± 5.40	11.22 ± 6.96	23.85 ± 13.53
Copulation duration	106.86 ± 54.07	95.49 ± 4.62	69.39 ± 21.32	115.17 ± 88. 13	77.79 ± 10.55	76.89 ± 14.21
	n = 12	n = 2	n = 10	n = 3	n = 8	n = 3

Table 4. Comparison (Mann Whitney U test) of body size (cephalothorax width) between experienced females (e♀) and naïve females (n♀) from the three experimental groups. Values are shown as the mean ± standard deviation.

	Group 1		Group 2		Group 3	
	e♀	n♀	e♀	n♀	e♀	n♀
Cephalothorax width (mm)	1.04 ± 0.05	1.04 ± 0.06	0.99 ± 0.25	0.98 ± 0.25	0.97 ± 0.24	0.99 ± 0.25
Statistic	U = 103.5; p = 0.72		U = 108.5; p = 0.88		U = 96; p = 0.51	

DISCUSSION

All penultimate females of *A. vierae* at 3, 10, 20, and 25 days were attractive to males. In nature, it is common to observe males of this species cohabiting nests made up of penultimate females (Viera et al., 2007). This is because, although the primary sex ratio of *A. vierae* is 1:2, biased towards females, females mature asynchronously and later than males. Consequently, at the beginning of the reproductive season, the operational sex ratio is biased towards males, with second-to-last females being common (Viera et al., 2007). For this reason, penultimate females can be attractive to males. The attractiveness of penultimate females varies between species. For example, in the subsocial species of the same genus, *A. arizona*, penultimate females are unattractive to males, and male guardings are not very common in nature (Klein et al., 2005).

Although males courted penultimate females of all four ages, 3, 10, and 20-day-old, females were not receptive and rejected courtship from adult males. Coexistence between adult males and penultimate females in nature could be costly for young penultimate females, since males do not cooperate in communal activities (Viera et al., 2007), therefore, there would be costs associated with time and/or energy by rejecting them (Albo et al., 2007). There are studies on spiders that show that, although some females are attractive to males, they are not necessarily receptive. For example, *Schizocosa malitiosa* (Tullgren, 1905) males are highly attracted to chemical trails of virgin females, but they are also attracted to non-receptive females carrying oothecae (Baruffaldi & Costa, 2010).

Only the penultimate females close to maturity (i.e., 25 days after molting to penultimate) accepted the courtship of the males and were receptive to mating attempts. Males began the courtship and carried out the same courtship units as in the presence of adult females (Viera & Albo, 2008). As in adult pairs, the behavioral unit most used during the courtship of males exposed to penultimate females was the tension of the silk threads with front legs. Penultimate females were active during the encounters, courted and indicated their receptivity by approaching and extending their legs and palps towards males. They also exhibit the receptive posture characteristic of the adult female.

Although many of the penultimate females close to the next molt were receptive to the males' courtship and adopted the copulatory posture typical of adult females, pseudo-copula behavior did not turn out to be as frequent as expected. Pseudo-copula occurred only in cases in which males got close enough to females and made multiple insertion attempts. Therefore, males would be the ones promoting pseudo-copula behavior. No behaviors of males that seemed to be forcing females to pseudo-copulate were observed. Thus, sexual conflict (Arnqvist & Rowe, 2005; Chapman, 2006) in relation to this behavior would be ruled out in this species). It would be interesting to evaluate the circumstances that cause this behavior to occur in the future. Considering that no significant differences were found between the size of the individuals involved in pseudo-copulations and those who did not, this variable would be ruled out as influential in this behavior occurrence.

The pseudo-copula duration in *A. vierae* was, comparatively, of very short duration as none of the couples reached a minute. This differs from what was found by Albo et al. (2007) for the same species, in which pseudo-copula had a duration of 13.8 ± 17.7 min. Some of the causes of this inequality could be differences in the age of the penultimate females used (in Albo et al., 2007, they were penultimate in general, while they were penultimate after 25 days of molting in this study); and in the duration of the observations (in Albo et al., 2007, it was 4 h, while it was 1 h in this study). In the species *Anelosimus studiosus* (Henz, 1850), Pruitt & Riechert (2011) also found a longer duration of pseudo-copula (more than half an hour). In this work the observations also had the same duration as in Albo et al. (2007).

Pseudo-copula in *A. vierae*, in addition to being a short-term behavior, was an intermittent behavior and occurred in several instances throughout the observations. This is expected due to the simple contact that the male's copulatory bulb has with the female's epigynum, which is not introduced because the genital openings are not fully developed. Pseudo-copulation frequencies of *A. vierae* also varied in relation to those found by Pruitt and Riechert (2011) in *A. studiosus.* Instances of pseudo-copulation in *A. vierae* averaged two per pair, while in *A. studiosus* there were eight.

Albo et al. (2007) suggested that pseudo-copula could have benefits for both sexes. The penultimate females that have this physical stimulus would accelerate their maturation and synchronize the couple's mating. However, the results of this study show that females that pseudo-copulate, as well as those that have sexual experience without pseudo-copulating and those that are sexually naive, had similar maturation rate. Thus, in this species, there would be no effect of early sexual experience on the rate of female development.

In two cases, mating occurred after the maturation molt process. Mating just after the final molt of females is a common strategy in male spiders to avoid sexual cannibalism. Males copulate with these females while their exoskeleton is still soft, and their ability to move and attack them is diminished (Robinson & Robinson, 1980; Foelix, 2011; Uhl et al., 2015). However, this does not seem to be the case in *A. vierae*. In the first instance, females began the molting process sometime after we placed the males in their containers. The most surprising thing was that, after molting, they were receptive and copulated easily, without having their exoskeleton completely sclerotized. Despite not having found that penultimate females accelerate their maturation molt by having sexual contact with adult males, these two cases of maturation in the presence of males could be a sexual strategy that females adopt to copulate quickly. Males would also gain benefits from staying close to these females waiting for them to molt into adults. On the one hand, males would ensure that they were the first to copulate with these females, and on the other hand, they would avoid the risks of predation when moving from one nest to another. In addition, males could avoid the high probability of encountering other male(s) by dispersing to another nest and having to compete for access to the female (Viera & Albo, 2008).

Copulation latency in couples with sexual experience, both known and unknown, is lower than in couples consisting of at least one of the sexes had no previous sexual experience. In *A. vierae*, it is not enough for either sex to have had previous sexual experience for copulation latency to decrease, but both individuals must have had it. If pseudo-copula were a sexual rehearsal, it was expected that in pairs made up of at least a single experienced individual, copulation latency would also decrease (Burghardt, 2005).

Early sexual experience in *A. vierae* could confer benefits for both sexes. Mating earlier would be a benefit for males due to their behavioral strategies. Satellite males usually try to supplant dominant males and/or disturb their copulations (Foellmer & Fairbairn, 2005; Albo et al., 2007; Rojas-Buffet & Viera, 2015; Stoffer et al., 2016). Therefore, males that mate more quickly with females are less likely to be supplanted before mating. On the other hand, Pruitt and Riechert (2009) found that high mating rates in *A. studiosus* are associated with a reduction in the risk of sexual cannibalism. Males that are faster at copulating are more likely to survive and can mate with more females. This would not be happening with *A. vierae* males since no cases of sexual cannibalism have been observed in this species.

A. vierae females would also gain benefits from mating earlier. Because they are polyandrous (Albo et al., 2007; Viera et al., 2007; Rojas-Buffet & Viera, 2015), mating more quickly would allow them to copulate with several males during their reproductive period. Although polyandry is a common phenomenon in a wide range of arthropod taxa (Arnqvist & Nilsson, 2000; Jennions & Petrie, 2000), the benefits for females are not so clear. The costs of having multiple copulations, for both males and females, are well documented in nature. Increased risk of physical injury, predation, and disease transmission are some of the known costs (Daly, 1978; Parker, 1979; Hurst et al., 1995; Blanckenhorn et al., 2002). Therefore, the benefits that females obtain from mating with multiple males must outweigh these costs. Some of these benefits may be to compensate for possible errors in mate choice and/or poor quality of previous copulations (Petrie & Kempenaers, 1998), to promote sperm competition processes and/or cryptic female choice, increasing the probability of fertilization by sperm (males) of high quality (Madsen et al., 1992; Birkhead, 1998) and to increase the genetic variability of the offspring, protecting the progeny against environmental uncertainties (Loman et al., 1988).

Sexually experienced males of *A. vierae* prefer to copulate with experienced females, regardless of whether they are known or unknown. This preference may be mediated by differences in female behavior. Experienced females courted more than naive ones perhaps also used different pheromones. In contrast, naive males do not show a preference in copulating with experienced or naive females. Apparently, there would be recognition between individuals who had sexual experience, but naive males would not distinguish females with and without experience. These results agree with those obtained in copulation latency experiences. Couples made up of both sexually experienced individuals obtain benefits in reproductive terms.

In conclusion, the sexual behavior of mature males and immature females of *A. vierae* has positive implications for future sexual encounters. Firstly, pairs consisting of individuals with prior sexual experience copulated more quickly than pairs in which at least one individual lacked such experience. Secondly, individuals with prior sexual experience showed a preference for mating with others who also had previous sexual experience.

In the future, it would be interesting to evaluate whether pseudo-copulated females invest more energy in the offsprings of the males with whom they pseudo-copulated. This was demonstrated in the species *A. studiosus*, where pseudo-copulated females invest more energy in the ootheca cover, prioritizing the offspring of males that pseudo-copulated with them, suggesting a sexual preference for these males (Pruitt & Riechert, 2011). Two groups of copulations could be carried out: females with males that pseudo-copulated and females with males that did not pseudo-copulate, and compare the number of clutches, eggs, spiderlings, weight of the oothecae and weight of the offspring. Furthermore, it would be interesting to assess the consequences of courtship between penultimate females and adult males, as well as pseudo-copulation, by evaluating not only the presence or absence of these behaviors but also their duration and intensity, as these factors could provide valuable insights into their outcomes, including potential influences on reproductive success or other fitness-related aspects, and serve as important elements for understanding their ecological and evolutionary significance.

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AUTHOR'S CONTRIBUTION

C. Rojas-Buffet contributed to formal analysis, funding acquisition, data curation, writing (original draft), investigation and methodology; C. Viera contributed to project administration, funding acquisition, conceptualization, writing (review and editing), methodology and supervision.