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Sexual dimorphism in the spinning apparatus of *Allocosa senex* (Araneae: Lycosidae), a wolf spider with a reversal in typical sex roles

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Abstract. *Allocosa senex* (Mello-Leitão, 1945) is a sex role-reversed wolf spider that inhabits sandy water-margin environments of southern South America. Males are larger than females and dig deeper burrows. Females are the courting sex and they prefer to mate with males that build deep burrows, suggesting high selective pressures on male digging behavior. Our aim was to investigate the external morphology and histological constitution of the spinning apparatus of males, females and juveniles of *A. senex*. Our results showed that *A. senex* adult males possess more piriform glands and spigots than adult females and juveniles. These glands produce silk for attachment discs that are crucial for the stability of the burrows. The differences according to the sex could be related to females' and males' digging strategies and strong selection on male burrow length in this species.

Keywords: spinnerets, spigots, spinning glands, digging behavior

One of the synapomorphies of spiders is the presence of silk glands situated in the ventral part of opisthosoma (Marples 1967). The glands are connected to the outside through thin tubes called the spigots, which are located on the spinnerets (Foelix 2011). The spider spinnerets are thought to arise evolutionarily from biramous opisthosomal appendages, similar to those reported for other arthropods (Shultz 1987). The order Araneae is represented by three clades, Mesothelae, Mygalomorphae, and Araneomorphae (Wheeler et al. 2017). These groups present structural differences in the spinning apparatus (e.g., Glatz 1972, 1973; Haupt & Kovoor 1993; Hajer et al. 2017). The spinnerets in the mesothelids are located at the middle of the opisthosoma, whereas in most mygalomorphs and araneomorphs they are located at the posterior end of the opisthosoma (Marples 1967). The number of spinnerets varies in different groups. The mesothelids present four pairs of spinnerets: anterior median (AMS), anterior lateral (ALS), posterior median (PMS), and posterior lateral (PLS), representing the ancestral state in spiders (Haupt & Kovoor 1993). The araneomorphs, however, possess only three pairs of spinnerets: ALS, PMS and PLS (e.g., Foelix 2011).

In spite of the ecological and evolutionary importance of silk production in spiders, the spinning apparatus has been well studied only in a few taxa, such as orb web spiders and their relatives (Araneoidea) (Kovoor 1977). There are only few studies regarding the spinning apparatus in wolf spiders (Richter 1970; Townley & Tillinghast 2003; Dolejš et al. 2014), which belong to the araneomorph family Lycosidae. Wolf spiders have four types of silk glands: piriform, ampullate, aciniform and tubuliform (Richter 1970). The piriform glands are connected to the ALS and produce attachment discs. Based on histochemical differences, the ampullate glands can be divided into the major ampullate glands (MA) and minor ampullate glands (mA) (Kovoor 1987; Kovoor & Peters 1988). The ducts of the MA glands are connected to spigots located on the ALS, while the ducts of the mA glands are connected to spigots on the PMS. The function of both types of ampullate glands is to produce draglines (silk threads that some spiders release while walking) and attach the egg sac to the spinnerets, as is typical in wolf spiders (Townley & Tillinghast 2003). On the other hand, the aciniform glands are connected to the PMS and PLS (Richter 1970), and one of their possible functions is to produce a scaffold that secures the spider when molting (Dolejš et al. 2014). The tubuliform glands are connected to the PMS and PLS and occur only in adult females; their function is to produce the fibers that constitute egg sacs (Richter 1970; Foelix 2011).

Both types of ampullate glands (MA and mA) can be divided into the primary (1°A) and secondary (2°A) glands (Townley et al. 1993). During the inter-ecdysial period, the ampullate glands that produce the draglines are the primary ampullate glands (1°MA and 1°mA). The primary spigots are not functional during ecdysis and molt *in situ*. The ampullate glands that produce draglines during proecdysis (beginning of molting), are the secondary ampullate glands (2°MA and 2°mA) (Townley et al. 1993). There are two secondary ampullate glands associated with each ALS (two 2°MA) and PMS (two 2°mA), but only one of each pair is functional at a given instar. The other pair of ampullate secondary glands (both 2°MA and 2°mA) is not functional at a given instar, but was functional during the preceding proecdysis. When nonfunctional, these glands are not represented externally by spigots, but only by post-functional tartipores: cuticle openings that allow the glands' ducts to remain connected to spigots on the old exoskeleton during the preceding proecdysis. As a consequence, the two secondary ampullate glands associated with one spinneret take turns functioning from instar to instar (Townley et al. 1993). After the final molt of lycosid males, one of the 2°A spigots turns into a nonfunctional structure called the nubbin (Townley & Tillinghast 2003). Tartipores are associated not only with the secondary ampullate glands but also with piriform and aciniform glands. Tubuliform glands are found only in adult females, therefore are never tartipore-accommodated (e.g., Dolejš et al. 2014).

Allocosa senex (Mello-Leitão, 1945) is a wolf spider that inhabits the sandy coastal areas of northeastern Argentina, southern Brazil and Uruguay (Simó et al. 2017). This species was originally described as *Glieschiella senex* Mello-Leitão, 1945, later it was considered a junior synonym of *Allocosa brasiliensis* (Petrunkevitch, 1910) (Capocasale 2001), and has been recently revalidated (Simó et al. 2017). Individuals construct burrows where they stay during the day, becoming active during the summer nights (Aisenberg 2014). This species is characterized by a reversal in typical sex roles and expected sexual size dimorphism in spiders (Aisenberg et al. 2007, 2011; Aisenberg 2014). Males are larger than females and dig deep tubular burrows, while females construct superficial silk capsules (Aisenberg et al. 2007). Females of *A. senex* prefer to mate with males that present longer burrows (Aisenberg et al. 2007). Copulation occurs within the male burrow; after mating, the males exit and the females remain inside. The male burrows are mating refuges but also breeding nests because the females will lay their egg sac there and will not leave before spiderling dispersal (Aisenberg 2014).

According to previous observations on this species, individuals show longer ALS (anterior lateral spinnerets) compared to other burrowing wolf spider species (Simó et al. 2017). This could be an adaptation for digging in the sand and releasing large quantities of silk. The construction of male burrows in this species begins with the extraction of the sand using chelicerae, pedipalps and occasionally tibiae, metatarsi and tarsi of the first pair of legs (Aisenberg & Peretti 2011). Males combine the extraction of sand with the deposition of multiple layers of silk on the walls and around the entrance of the burrow (Aisenberg & Peretti 2011). The deposition of multiple layers of silk during burrow construction has been described also for other wolf spiders (Gwynne & Watkiss 1975; Henschel 1990) and is probably necessary to maintain a stable burrow in the sandy habitat where *A. senex* is found. Taking into account that males of *A. senex* need to construct long burrows and that this trait is selected under female choice, we could expect differences in spigot morphology according to the sex in this species. Thus, our aim was to investigate the external morphology and histological constitution of the spinning apparatus of males, females and juveniles of *A. senex*, with focus on the sexual strategies in this species.

METHODS

We collected 22 individuals of *Allocosa senex* (7 adult males, 8 adult females, 2 subadult males, 2 subadult females and 3 juveniles) in November 2016 at the coastal area of San José de Carrasco, Canelones, Uruguay (34°51′06.06" S, 55°58′46.71" W). Spiders were captured by hand during the night using headlamps. We housed each individual in Petri dishes 9.5 cm in diameter and 1.5 cm tall, with sand as substrate and cotton soaked in water, under controlled conditions of temperature and humidity. We fed the spiders three times a week with *Tenebrio molitor* larvae (Coleoptera; Tenebrionidae). When individuals molted, we preserved the shed exuviae dry in an Eppendorf tube with their corresponding identification. We deposited voucher specimens in the National Museum, Prague (Czech Republic) under the inventory number P6d-2/2017.

The methodology follows Dolejš et al. (2014). We examined the spinning glands of males, females, and juveniles histologically (light microscopy), and the exterior of their spinnerets through scanning electron microscopy (SEM). For both types of microscopy spiders were fixed in modified Bouin-Dubosque-Brasil fluid (Smrž 1989) for 5 days, rinsed in 80% ethanol that was changed every 12 hours for 5 days, and stored in propanol until further processing. For histological evaluation, the specimens were embedded in Paraplast Plus (Fluka) and sectioned by sledge microtome (Reichert-Jung 407) at 7 μm. We stained sections in two triple-stains: Masson-Goldner's (Masson 1929; Foot 1933; Goldner 1938) and Gomori's (Gomori 1950) trichrome to discriminate between the gland types based on their color, to observe their position in the spider's opisthosoma and to detect carboxyl groups by hematoxylin present in both triple-stains. We also applied the ferric ferricyanide reaction – FFR (Adams 1956) to test for reducing groups of secreted proteins. We inspected the preparations under a light microscope (Olympus BX50) and photographed selected sections using a 3CCD color video camera (Olympus DP70).

For SEM examination, we transferred fixed specimens into acetone, air-dried, and inspected them using a scanning electron microscope (HITACHI S-3700N). Terminology of silk glands, spigots, tartipores, and nubbins follows Townley & Tillinghast (2003). We obtained additional images of juvenile spinning apparatus from exuviae. The exuviae were treated in a glycine SDS buffer (Novex, Invitrogen) for two weeks. Subsequently, we detached distal parts of the opisthosomal exuviae containing spinneret cuticle. Each spinneret was re-expanded by using watchmaker pincers to pull the spinneret down onto the pointed end of an appropriately sized pin embedded in and protruding from dark wax in a Petri dish (Townley & Tillinghast 2009). The cuticles of re-expanded spinnerets were rinsed in distilled water, dehydrated through an ascending ethanol series to propanol, and prepared for SEM examination as described above. We identified the spigots generally according to the spinnerets on which they appeared, and based on their shape, size and number. To distinguish between the aciniform and tubuliform spigots that are similar and present on the same spinnerets of adult females, we compared the spinning fields of adult females, subadult females and adult males.

RESULTS

The silk glands in *Allocosa senex* opened in three pairs of spinnerets (Fig. 1A) through four types of spigots. Anterior lateral spinnerets (ALS) were the largest, notably prolonged spinnerets (Fig. 1A). We observed the spigots corresponding to the major ampullate glands, 1°MA and 2°MA in juveniles, females and subadult males. The bases of both types of MA spigots were relatively small (Fig. 1B) and difficult to locate at the spinning field. However, the base of the 2°MA spigot is larger than that of the 1°MA spigot. Beside the spigots, the spinning field was densely covered by setae. The presence of the 2°MA tartipore was recorded. The 2°MA glands and tartipores persisted in adult females. In adult males, however, only 1°MA spigot was present and the nubbin appeared

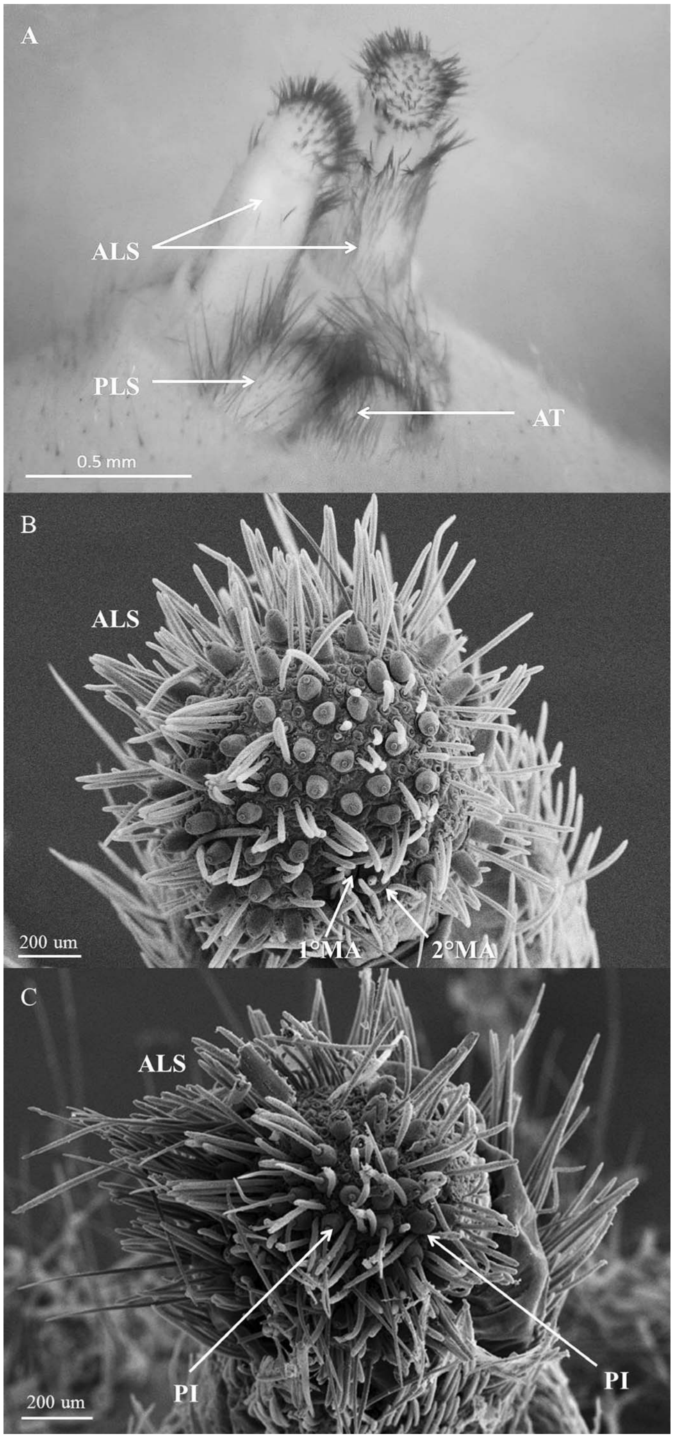

Figure 1.—*Allocosa senex*: A. Spinnerets, note extremely prolonged anterior lateral spinnerets. B. Anterior lateral spinneret of a female, with relatively small major ampullate spigots. C. Anterior lateral spinneret of a male, with higher number of piriform spigots; 1°MA = primary major ampullate spigot, 2°MA = secondary major ampullate spigot, ALS = anterior lateral spinnerets, AT = anal tubercle, PI = piriform spigots, PLS = posterior lateral spinnerets.

instead of the 2°MA spigot. We counted the spigots belonging to the piriform glands (Table 1). Adult males possessed a greater number of piriform glands (Fig. 1C), compared to juveniles and adult females (Table 1). Histologically, the piriform glands appeared as oval or bean-shape structures with a clearly bipartite epithelium (Fig. 2A, B). The proximal part stained dark purple via hematoxylin, revealing carboxyl groups present in the secretory cells. The basophilic distal part of the gland stained grey or greenish. The FFR revealed reducing groups of medium intensity present in the proximal part, as well as in the lumen of the gland. The piriform glands were located not only at the ventral area of the spinning apparatus, but also on the dorsal area, together with the aciniform glands (Fig. 2A). Relative diameter of piriform glands was larger than that of aciniform glands, being apparent already in subadult males (Fig. 2B).

Posterior middle spinnerets (PMS) were short and extremely reduced. In juveniles, females and subadult males of *A. senex*, we observed the spigots corresponding to the minor ampullate glands: 1°mA and 2°mA, and the 2°mA tartipore. The bases of both types of mA spigots were relatively large; the base of the 2°mA spigot was larger than that of the 1°mA (Fig. 3A). Intensive dark blue color of the FFR revealed that silk present in the ampullate glands (both MA and mA) consisted of proteins containing strong reducing groups (Fig. 2C) enabling clear identification of the ampullate glands in the histological slides (cf. Fig. 2D). In adult males, the 2°mA glands were reduced, and the nubbin could also be observed (Fig. 3B). The number of spigots corresponding to the aciniform and tubuliform (the latter only in adult females) glands was relatively low compared to other wolf spider species (Table 1; Dolejš et al. 2014). The aciniform glands appeared histologically as small roundish structures with a bipartite epithelium situated at the dorsal area of the spinning apparatus, close to the PMS, PLS and the anal tubercle. The proximal area stained darker red than the distal area, differing in a degree of acidity. The lumen stained pinkish orange (Fig. 2A, B) and the result of the FFR was negative. The tubuliform spigots appeared at the periphery of the spinning field and their shafts were thicker than those of the aciniform spigots (Fig. 3A).

At the posterior lateral spinnerets (PLS), we observed the spigots corresponding to aciniform and tubuliform glands (the latter in adult females only). In adult females, aciniform and tubuliform spigots could not be accurately discriminated due to its poor visualization and/or breakage of the shafts (Table 1). Beside the spigots, the spinning field was covered densely by setae (Fig. 3C). Histologically, however, the tubuliform glands were easily distinguishable as a cluster of glands with a thin epithelium, filled with a green substance. These glands were situated ventral to the ovaries.

DISCUSSION

We found differences in the spinning apparatus, namely in number of piriform glands, between males and females of *A. senex*. The spinning apparatus of *A. senex* differs in comparison to other wolf spider species regarding the size of ALS, PMS and number of spigots occurring on those spinnerets (Dolejš et al. 2014). Based on our results, *A. senex* adult males possess more piriform glands and spigots than

Table 1.—Number of spigots per each spinneret of *Allocosa senex*; ac, aciniform spigot; pi, piriform spigot; tu, tubuliform spigot. The juveniles examined were on their 5th to 9th instar.

Spinnerets	Juvenile (n = 8)	Sub-adult ♂ (n = 4)	Adult ♂ (n = 8)	Sub-adult ♀ (n = 4)	Adult ♀ (n = 4)
ALS	21–45 pi	36–38 pi	64–86 pi	43–50 pi	49–52 pi
PMS	5–9 ac	7–9 ac	5–9 ac	13–14 ac	7–9 ac, 11–12 tu
PLS	20–27 ac	22–27 ac	23–28 ac	31–46 ac	33–47 ac+tu

adult females. Probably the higher quantity of piriform glands in males compared to females of *A. senex* is closely related to the construction of the burrow, i.e., the need for deposition of several silk layers and the function of these glands (producing attachment discs). This result is contrary to that reported by Dolejš et al. (2014) for four species of lycosids from Europe with traditional sex roles, in which adult females had a greater number of piriform glands. In their study, one of the species, *Tricca lutetiana* (Simon, 1876), is a burrowing wolf spider also possessing large piriform glands (Dolejš et al. 2014), which, however, present a smaller number of piriform, aciniform and tubuliform glands compared to *A. senex*. One of the probable reasons why *T. lutetiana* has a smaller number of piriform glands could be that its burrow is not covered with silk (Dolejš et al. 2008, 2014). Likewise, possibly the increase in the number of piriform glands in the adult males of *A. senex* could be determined by its habitat: sandy substrate instead of soil as it occurs in *T. lutetiana* typical habitat (Dolejš et al. 2008). Also, the spider's life history can increase the number of piriform glands as it was observed in gnaphosids that use piriform silk for prey capture (Wolff et al. 2017). In general, the number of glands increases from sub-adult to adult wolf spider stages (Dolejš et al. 2014), but in *A. senex*, we did not find differences in the number of spigots between subadult and adult spiders (with the exception of newly formed tubuliform glands in adult females and increased number of piriform glands in adult males).

Standard histochemical characteristics (e.g., Kovoor & Peters 1988; Haupt & Kovoor 1993) of all four types of silk glands observed in *A. senex* resemble those of other wolf spiders (Richter 1970; Dolejš et al. 2014 and references therein). A region containing carboxyl groups seems to be

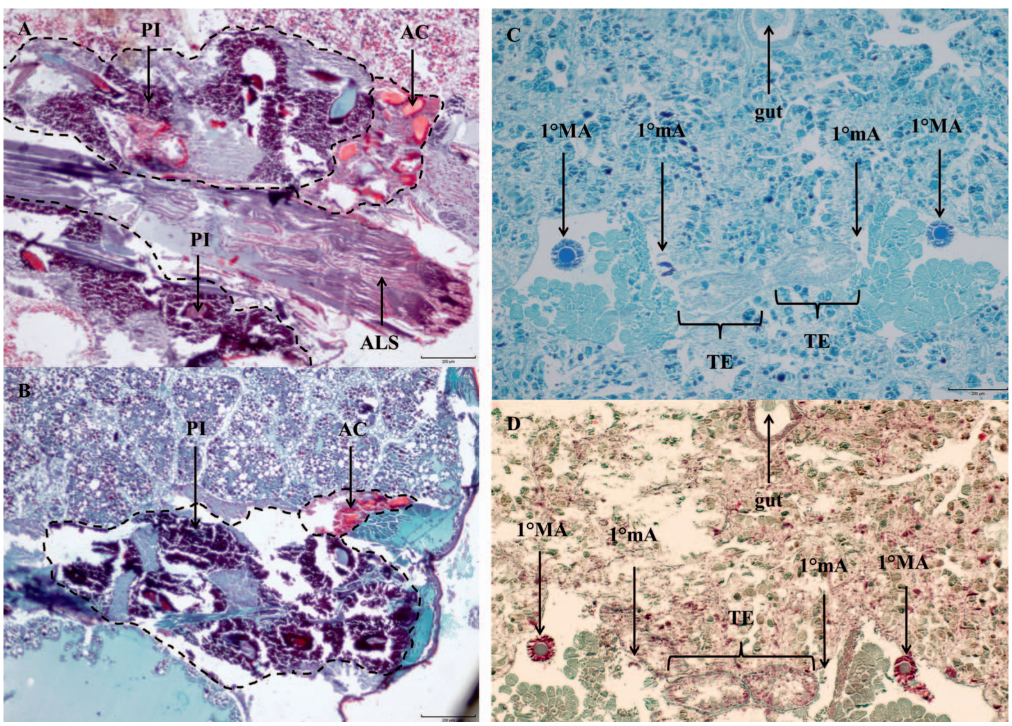

Figure 2.—Spinning glands of *Allocosa senex*: A. Adult male (sagittal section, Gomori trichrome), note large piriform glands (PI) located also very close to the aciniform glands (AC); dashed line = approximate borders of piriform and aciniform glands; ALS = retracted anterior lateral spinneret. B. Subadult male (sagittal section, Gomori trichrome), note the difference in size between piriform (PI) and aciniform (AC) glands; dashed line = approximate borders of piriform and aciniform glands. C. Transversal section of an adult male (Ferric-ferricyanide reaction) showing reducing content in dark blue, of primary ampullate glands. D. Corresponding (more anterior) image as in C providing general histological overview (Masson-Goldner trichrome). Despite comprehensible depiction, the minor ampullate glands are hardly visible; 1°MA = primary major ampullate gland, 1°mA = primary minor ampullate gland, TE = testis.

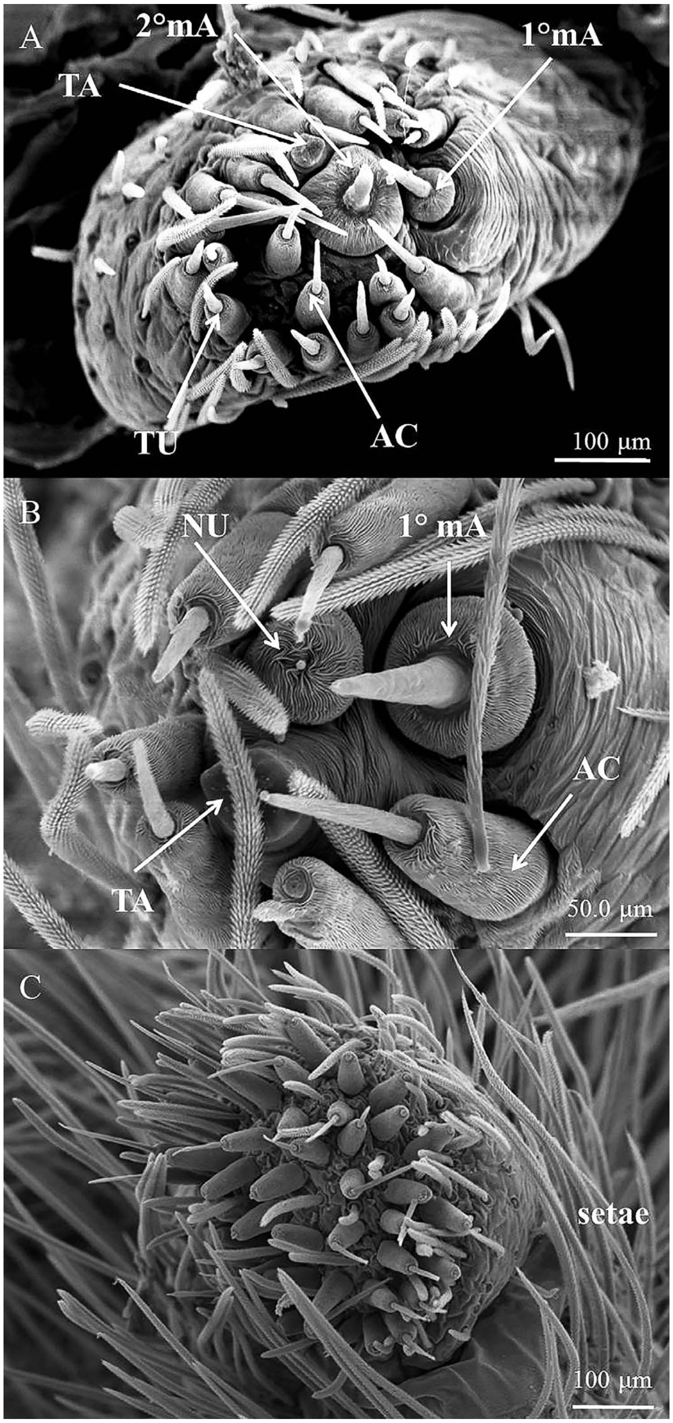

Figure 3.—*Allocosa senex*: A. Posterior median spinneret of a female, note large base of secondary minor ampullate spigot and position of tubuliform spigots. B. Posterior median spinneret of a male, with secondary minor ampullate nubbin. C. Posterior lateral spinneret of a female, see the spinning field densely covered by setae. 1°mA = primary minor ampullate spigot, 2°mA = secondary minor ampullate spigot, AC = aciniform spigot, NU = nubbin, TA = tartipore, TU = tubuliform spigot.

typical for piriform glands, as well as acidophilic reaction of aciniform glands (Kovoor 1987). Content of proteins rich on reducing groups increase from proximal to distal of any prolonged (e.g., ampullate) glands (e.g., Kovoor 1990; Haupt & Kovoor 1993). Thus, the FFR can be used for visualization of ampullate glands (not only) on transversal sections.

Simó et al. (2017) reported that *A. senex* possesses anterior lateral spinnerets twice as long as the rest. Studies under the electron microscope revealed that burrow silk in this species is composed by hundreds of thin silk lines that cover each sand grain (Foelix et al. 2017). The enlarged ALS and reduced PMS seem to be a common feature among sand-dwelling spiders across families as described by Peters (1992) for an eresid of the genus *Seothyra* (Purcell, 1903) and the sparassid *Leucorchestris arenicola* (Lawrence, 1962).

In araneoids, contrary to the lycosids, the 2° ampullate glands produce silk only during proecdysis (Townley et al. 1993) and, therefore, these glands are not necessary and non-functional in adults of both sexes. In lycosids (and also in pisaurids, agelenids, amaurobiids, thomisids and some dionychans), the 2°MA and 2°mA are transformed into non-functional nubbins in males only, whereas females' glands and spigots are maintained as they produce silk to attach the egg-sacs to the spinnerets (Townley & Tillinghast 2003). Our results indicate that the bases of the MA spigots in males, females and juveniles of *A. senex* are relatively small and difficult to locate in the spinning field even with SEM, due the high density of hairs. It could be expected that their size is limited by spatial constrictions and as a way to generate more space for the piriform spigots. However, the MA spigots, while relatively small, differ in size with the 2°MA spigot base which is clearly wider than the 1°MA spigot base. Next to the spigots, the spinning field was densely covered by setae, and it can be inferred that the large number of setae would have the function of protecting the spigots. Small MA spigots as well as ALS densely covered with setae were also recorded in other sand-dwelling spiders (Peters 1992).

Simó et al. (2017) reported the presence of the 2°mA spigots at the PMS of *A. senex* (therein see 'MAP' in Figure 7E). The bases of both types of mA spigots are relatively large and the base of the 2°mA spigot is greater than that of the 1°mA. Generally, 2°A (both 2°MA and 2°mA) spigots with wider bases have been observed to coincide with larger diameter silk fibers produced by these 2°A as compared with fibers from the 1°A (Townley & Tillinghast 2003). Thus, as in some other lycosids, the 2°A likely play an important role compared to the 1°A in attaching the egg sac to the spinnerets in *A. senex*. However, the 2°A spigot bases can be notably larger (genus *Pardosa* C.L. Koch, 1847) or only slightly larger (or even of similar size) compared to 1°A spigots (other genera) (Townley & Tillinghast 2003; Dolejš et al. 2014).

The small size of the PMS and a small number of aciniform glands on them were observed. The function of the small PMS in *A. senex* is probably limited to carrying the egg sac due to enlarged bases of mA spigots, whereas the anchor threads that support the body of the spider during the molting process, as was suggested by Dolejš et al. (2014) for other wolf spider species, could be supplied mostly by aciniform glands on the PLS.

Finally, Alfaro et al. (2018) reported that the number of piriform gland spigots of females in several spider families increased with adulthood. Also these authors observed sexual dimorphism in this type of spigots in *Dolomedes tenebrosus* Hentz, 1884 (Pisauridae), in which males have lower number of spigots than females. In our study, we observed the opposite: males with larger number of spigots compared to females. We could infer that the large number of piriform glands in males of this species is responsible for producing and depositing silk to keep the burrow stable in fine sand, the typical substrate of *A. senex* (Aisenberg & Peretti 2011). It can be assumed that digging activity would bear an important associated cost in the production of large amounts of protein, similarly to what has been reported for other burrowing spider species (Prestwich 1977; Henschel & Lubin 1992; Peters 1992). For example, it has been reported that the burrow of *C. rechenbergi* lasts approximately one month before being replaced, and if it is damaged the spider repairs it instead of constructing a new one (Foelix et al. 2017). The burrowing spider *Geolycosa missouriensis* (Banks, 1895) stays practically all its life in the same burrow and only maintains and enlarges the tube (Wallace 1942). Unfortunately, the total excavation time-period and the associated costs of burrow construction in *A. senex* are still unknown and remain to be determined. Future studies will focus on determining whether there are differences in spinneret size and the number of spigots in *A. senex* spiders that live on sandy substrates with different grain size or in Allocosinae species inhabiting different substrates.

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