Methods to identify silk gland activation patterns in spider spinning behaviours

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Abstract: Spiders possess multiple types of silk glands, producing silk materials with contrasting properties, and which are deployed in distinct behavioural contexts, such as locomotion, prey capture and egg casing. Whereas the diversity of silk glands and spigots across different spider families is relatively well described, their biological functions (i.e., with which behaviour each gland type is activated) are poorly known. Here we provide an overview about available methods and approaches to determine the biological function of spider silk glands, and evaluate their advantages and disadvantages based on our experiences and a test study on *Pholcus phalangioides* (Pholcidae) and *Kukulcania hibernalis* (Filistatidae).

Keywords: Spider silk; silk spinning-behaviour; silk types; multi-method approach

1. Introduction

Unlike other silk-producing animals, spiders deploy silk in multiple ways throughout their life. From predation to reproduction, dispersal, and maternal care, spiders use silk as a

multifunctional tool (Apstein, 1889; Gorb & Barth 1994; Blackledge et al., 2005; Gheysens et al. 2005; Foelix, 2011; Bell et al., 2012; Blackledge, 2012). With the potential for up to eight distinct types of silk glands (excluding the epiandrous glands found in adult males), each presumably serving one or more specific biological functions and specialised to produce fibres and glues with different mechanical properties (Blackledge & Hayashi 2006; Foelix 2011), spiders possess a sophisticated apparatus to spin a variety of fibre-based materials. The silk glands are present in the opisthosoma and open through specialized tube- or nozzle-like cuticular protrusions, the spigots, that are located on modified abdominal appendages, the spinnerets (Foelix, 2011; Eberhard, 2020). The morphological arrangement of spigots on the spinnerets, combined with varied utilization of different silk glands across different behaviours, is distinct between different spider families and has captivated arachnologists for years.

Since the beginning of spider studies, a large variety of silk materials and spinning behaviours has been described, along with the morphological variation of gland types, numbers and spigot arrangements (Apstein, 1889; Coddington, 1989; Eberhard, 2010; Alfaro et al., 2018). However, in only very few cases it was observed which glands are involved in which behavioural contexts. This is a significant caveat for the general understanding of how the morphology of the spinning apparatus, silk material composition and silk property variation have co-evolved with the behavioural ecology in spiders.

A reason for the existence of this knowledge gap is a lack of standardized protocols resulting in a patchwork of multiple methods that are not well documented and have not been evaluated regarding their efficiency. Some studies have relied on studying silk samples collected from spider webs or by intercepting specific spinning behaviours. These samples were then analysed with polarised light microscopy or electron microscopy to distinguish fibres based on diameters or optical properties (Work, 1981; Blackledge, Cardullo & Hayashi 2005; Wolff et al., 2017).

Some approaches, including the paraffin embedding method pioneered by Peters in 1982, have been repeatedly used in behavioural studies (Peters 1990, 1992; Gorb & Barth 1996; Townley & Tillinghast 2003; Grannemann et al. 2019). Other, less explored approaches to

identify the use of specific silk glands in spider behaviours, include snap freezing the spider followed by cryo-scanning electron microscopy, high speed video recording, or the experimental blocking of specific spigots (Barth, Gorb, & Landolfa 1998; Wolff, 2019; Wolff et al. 2015; 2024). Another promising approach is the comparison of the chemical profiles of silk products and gland contents (Lefèvre et al., 2011, Wolff et al, 2024).

Thus far, there is no study that combined these different techniques in the same system. The present study presents an overview of the available set of methodologies to identify gland use in different behavioural contexts or the glandular origin of silk products, with the aim to give a practical guide for experimentalists.

As a test object we studied the behaviourally versatile cosmopolitan cellar spider (Pholcidae: *Pholcus phalangioides*). This species possesses two anterior lateral spinnerets with each one major ampullate gland spigot, and two posterior median spinnerets with each one minor ampullate and one aciniform gland spigot, producing fibres of different sizes. In addition, there are one large piriform and multiple small piriform gland spigots on each anterior lateral spinneret, producing glue-like silk secretions. The posterior lateral spinnerets do not bear any functional spigots in this species. In addition to tests with *P. phalangioides*, paraffin experiments and silk reeling tests were performed on Southern house spiders (Filistatidae: *Kukulcania hibernalis*), which are cribellate spiders possessing a more complex spinning apparatus with seven different types of silk glands.

2. Methods and Results

2.1. Direct observation assisted by high-magnification lenses and high-speed camera

Ideally it can be directly observed, from which spigot(s) a given silk product is coming. However, this is possible to observe only in a limited cases of spinning behaviours due to the extremely small size of spigots, thin diameter and translucence of silk fibres (making them hard to see), and the often high speed of movements during silk spinning. Direct observation can best be done in behaviours that only depend on the choreography of the spinnerets and can be performed while the spider is immobilized. One of us (JOW) has previously described

a method that makes it possible to directly observe the spigots that are active during the production of silk anchor points and dragline initiation by letting a spider spin against a glass slide within a confined, highly magnified camera field of view (Wolff, 2020; 2021; Fig. 1.C). High-speed footage was also used to understand the spigot origin and kinematics of spinning of the ribbon-like silk of recluse spiders of the genus *Loxosceles* (Koebley et al. 2017; Magalhaes et al. 2017), and the silk gland usage in the wrap-attacks of Gnaphosidae (Wolff et al., 2017).

Methodology: Here, we tested, how well a high-speed camera (Phantom MIRO LC 320S) equipped with a macro-lens (Canon 100mm) may resolve fibre origination in free moving spiders. For this, videos were recorded while the spiders exhibited behaviours such as dragline spinning, prey wrapping, and bridging. Recordings were done at 2000 frames per second. The camera was placed on a tripod with rolls for enhanced mobility to easily follow the spider and keep it in focus. Recordings were then replayed at low frame rates (slow motion) to discern from which spinnerets fibres were coming (Fig. 1 A,B).

Figure 1. Single frames of high-speed videos of spinning *Pholcus phalangioides*. Arrowheads highlight silk fibre origination. (A). Dragline production, where fibres were drawn from all three glands (major ampullate, minor ampullate and aciniform glands), as indicated by the fibres' number, different thickness and origination from both anterior and poster median spinnerets. (B). Prey immobilisation wrapping behaviour, where fibres are drawn with the help of 4th pair of legs; note that two strands of silk can be distinguished. (C). Filming of dragline initiation with high magnification lens, permitting focus on the spinning apparatus (details on method in Wolff 2021). Here it is visible, that both minor ampullate and aciniform fibres originated from posterior median spinnerets and could be included in the dragline. *Abbreviations*: Ac – aciniform gland silk fibre; ALS – anterior lateral spinneret; MA – major ampullate gland silk fibre; MiA – minor ampullate gland silk fibre; PMS – posterior median spinneret.

111 *Evaluation:* The advantage of this method is that silk emergence can be directly observed. It
112 also allows to infer information on the spinning kinematics, for example with which speed
113 fibres are spun.

114 The disadvantage of this method is that it requires expensive equipment and strong
115 illumination, which might inhibit natural behaviours in many nocturnal species. Further,
116 only in behaviours where most of the spider body stays stationary (e.g., in cribellar combing;
117 Grannemann et al. 2019) it may be possible to focus on the spinning apparatus with a lens
118 that allows sufficient magnification to distinguish between spigots. Otherwise, we found that
119 it may be feasible to observe from which spinnerets fibres are coming, and, in some cases,
120 how many fibres are included into the silk product. In the case of *P. phalangioides*, due to the
121 low number of spigots and comparably high differences between fibre diameters, it was
122 possible to indirectly infer which silk gland was used (i.e., observing from which spinneret
123 silk was coming and then associating diameters of fibres in the final silk products with spigot
124 sizes). However, the necessary magnification to resolve these details came to the cost of a
125 low depth of field, making it impossible to continuously keep the opisthosoma in focus
126 during the rapid spinning, such as during prey wrapping behaviour. Notably, rare
127 behaviours, such as the construction of gumfoot lines or egg sacs, which always happen at
128 night, could not be captured with this method.

129

130 ### *2.2. Paraffin-fixation*

131 Due to the microscopic scale, to enhance clarity in silk fibre emergence from spigots,
132 ideally a scanning electron microscope (SEM) is used. However, as animals (usually) cannot
133 be observed *in vivo* in the SEM, a useful approach is to fix the spinning behaviour. One way
134 to achieve this is to pour hot paraffin onto the spinning apparatus and the spider. The
135 paraffin solidifies and embeds the spinnerets and fibres, fixing their position. The spider is
136 then mounted into a chemical fixative, the paraffin chemically dissolved, and the sample
137 dehydrated, sputter coated and mounted for SEM. This technique was successfully used to
138 infer the origin of airborne ballooning and bridging lines (Peters 1990), draglines (Gorb &

Barth 1996; Townley & Tillinghast 2003), and the fibres composing the cribellar bands (Peters 1982, 1984, 1992).

Sample preparation: One of us (MJR) has applied the paraffin method to the Southern house spider *Kukulcania hibernalis* (see Grannemann et al. 2019). The melted paraffin was stored in a 50 ml Falcon vial immersed on a thermos with hot water, then transferred to a smaller vial to drop on the spinning spiders. The spiders were transferred to 95% ethanol for a week, the paraffin was afterwards dissolved with xylol, then through a graded series to 100% ethanol, critical point dried and sputter coated with Au/Pd, and finally observed standard (FEI-XL30TMP) or field emission electron scanning microscope (Zeiss Supra). The rate of success was moderate and disparate for different fibre types, ca. 1 successful fixation per 3-5 trials for cribellar or minor ampullate fibres, but 1 out of 10 for the paracribellar and major ampullate fibres.

Results: The technique allowed to image short stretches of fibres coming out of the spigots (Fig. 2.A-C). The subsequent matching of those fibres to the natural web structures was done indirectly by comparing fibre morphology and diameter. The matching of ampullate and cribellar fibres was clear, because of their disparate diameter and characteristic morphology: the cribellar fibres were ultra-thin, major ampullate fibres were thick, and minor ampullate fibres were undulating, very thick and oval in section. Paracribellar fibres, on the other hand, were tentatively matched by diameter alone. Finally, in addition to individual fibre morphology and diameter, the number of spigots observed in SEM preparations of the spinning organs corresponded with the fibres found in the cribellate band and its supporting thread: two minor ampullate gland spigots corresponded to the thick undulating fibres, six paracribellar gland spigots with the thinner undulating fibres, many cribellar gland spigots with the nano-fibrils, and six major ampullate gland spigots with the fibres of the supporting thread.

Evaluation: The advantage of this method is that fibre origin can be directly observed. The technique can be applied in the field by transporting the molten paraffin in a thermos. The disadvantages are the time-consuming sample preparation and high error rate, i.e., it

requires the killing of many spiders to obtain suitable results. It is therefore not suitable for species that occur in low abundance nor for rarely observed behaviours.

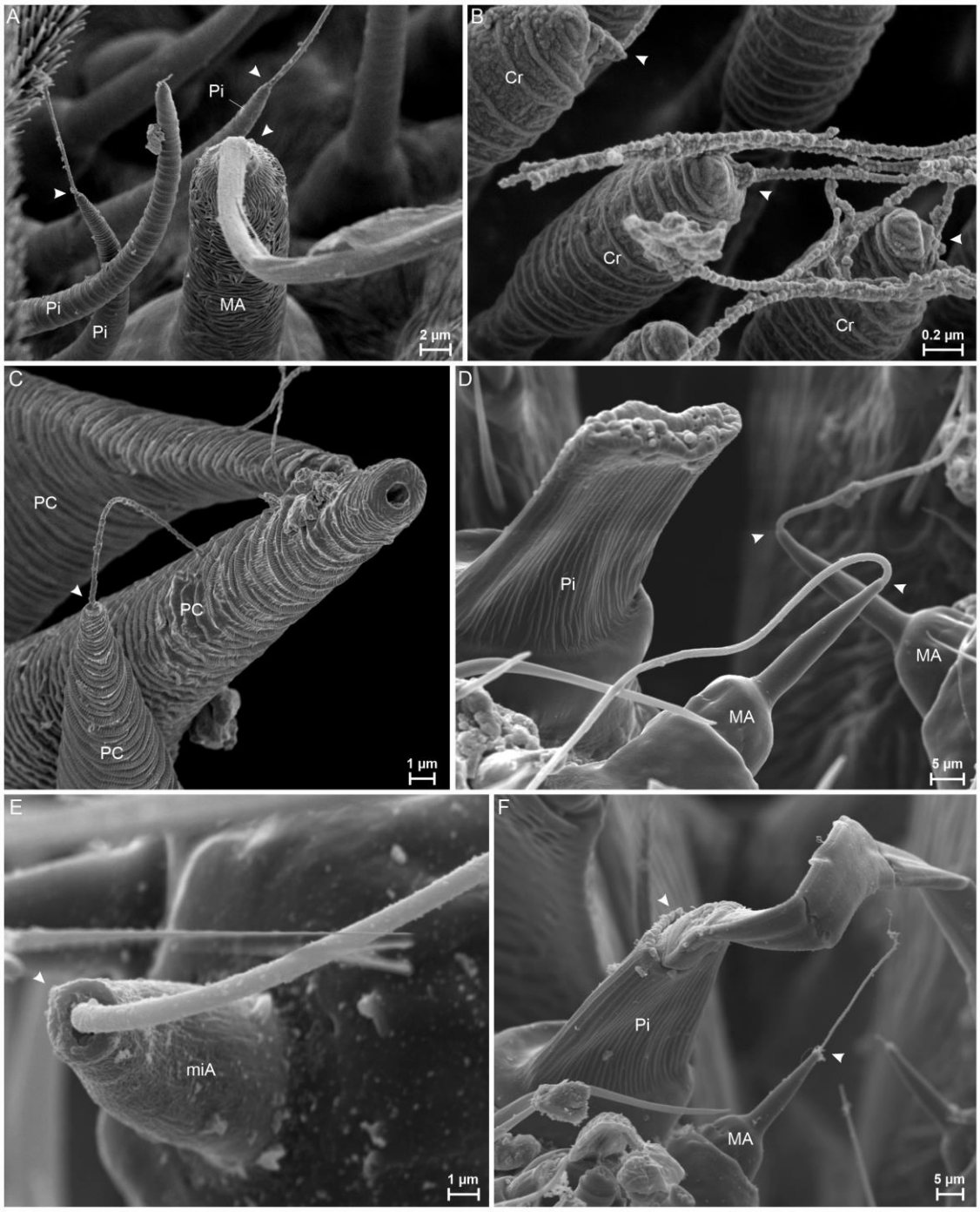

Figure 2. SEM images from paraffin and cryo fixation methods. Arrowheads highlight silk fibre origination. (A-C). Paraffin fixation of *Kukulcania hibernalis*. (A) Fixation of anchor spinning behaviour, showing fibres emerging from

piriform and major ampullate glands. (B) Fixation of cribellar combing behaviour, showing fibres originating from cribellum spigots and (C) fibres from paracribellar spigots. (D-F). Cryo fixation images of *Pholcus phalangioides* while producing (D) dragline, where major ampullate glands were active, (E) bridging line, where minor ampullate glands were active, and (F) dragline anchorage, where both the large piriform gland (emitting glue to attach fibres to the substrate) and major ampullate glands were active. *Abbreviations*: Cr – cribellar spigot; MA – major ampullate spigot; MiA – minor ampullate spigot; PC – paracribellar spigot; Pi –piriform spigot.

2.3. Cryo-fixation

The idea of this approach is the same as with the paraffin method: the instant fixation of a given behaviour to enable the microscopic observation of from which spigots silks are emitted. For the fixation, liquid nitrogen is used, that freezes the spider in an instant. These frozen specimens can than directly be transferred to a Cryo-scanning electron microscope (Wolff et al., 2015), or fixed in chilled ethanol and investigated with classical SEM as described below.

Experimental design: Here, two different experimental setups were employed to study the dragline formation. For draglines produced while walking, the individuals were allowed to traverse a stand lined with tissue paper. When the spider started walking with a dragline trail, the tissue paper, along with the spider, was swiftly submerged in a liquid nitrogen container.

The dragline spun during abseiling behaviour was studied by letting the spider descend from an elevated surface (rod or vial) and directly plunge it in a liquid nitrogen container.

Dispersal behaviour, characterized by the use of an airborne bridging line (Huber, 2023), was investigated by confining individuals to a small stand, coating the surrounding area with Vaseline to restrict movement, and submerging the stand in liquid nitrogen when spiders started to show bridging behaviour.

To study the prey wrapping behaviour, each individual was housed in 15 × 10 × 10 cm plastic boxes for 1 to 2 weeks during which a web was constructed. The housing container was opened on the front and placed in a larger polystyrene box to prevent spillage during liquid nitrogen application. Then one blowfly was presented to the spider with forceps, which was then attacked and wrapped. Once prey wrapping was initiated, liquid nitrogen was immediately poured onto the spider to snap-freeze it.

Sample preservation, preparation and investigation: Spiders should be immediately preserved in chilled ethanol, or there is a chance that the abdomen bursts and leaks, rendering any further investigation impossible.

After cryo-fixation, spiders were immediately transferred to chilled (from -80°C storage) 80% ethanol and stored at -80°C or -40°C (no difference in the outcome was observed between both storage temperatures). Prior sample preparation, the ethanol concentration was gradually increased to 96% before transferring the spiders to increasing temperatures (-40°C, -10°C), and finally to room temperature. The spiders were then subjected to critical point drying and affixed to the aluminium stubs with conductive silver glue. With scanning electron microscopy (SEM), spigots were examined for the presence of silk fibres coming from the spigot pores, indicative of gland activity during observed behaviours.

Evaluation: The advantage of this approach is that it permits the direct visualization of silk gland activity during behaviours. We could observe not only the emergence of fibres (Fig. 2.D-F), but also the addition of sticky silk from the large piriform glands in silk anchor spinning (Fig. 2.F). The sample preparation for this method is less time-consuming than for the paraffin method. In the case a direct transfer to a Cryo-SEM is performed, it should be even possible to observe the emergence of viscid silk products, such as aggregate silk, that are soluble in polar liquids and would get lost during chemical fixation procedures.

The disadvantages are that it is a destructive method (spider dies and can only be observed once). Usually a high number of spiders is needed, as the silk emergence is visible only in a subset of the samples. The technique requires lab facilities to store and handle liquid nitrogen. Therefore, in contrast to the paraffin technique, it cannot be used in the field. In comparison to other approaches, the cryo-fixation method followed by SEM is comparably time-consuming and labour-intensive. Furthermore, we found that the freezing sometimes leads to squeezing out of excess liquid silk in spigots, forming a tiny blob or fibre piece coming out of spigots. While regularly spun fibres usually show a smooth and even shape, sometimes it is difficult to interpret if material seen coming from the spigot was an artifact due to the method.

2.4. Experimental spigot blocking

This follows the classical approach in functional morphology to identify the role of a certain structure in a certain behavioural context, by modifying or removing the structure and investigating how the behavioural performance changes. By blocking (sealing) or destroying specified spigots, the production of this silk by the spider is inhibited. By comparing with control spiders with unblocked spigots or observations made on the same spiders before the experimental manipulation, it is then determined, which silk materials change their composition or which silk-based behaviours are altered or not displayed any longer.

Anaesthesia and preparation: Live individuals of *P. phalangioides* were anesthetized with CO_2. Subsequently, the spiders were immobilized with the ventral side upwards on a soft Styrofoam platform using masking tape, allowing for clear observation of the spinnerets under a stereomicroscope. To avoid that the spiders are completely unable to spin silk structures, care was taken not to block the large piriform and major ampullate spigots, which are required for dragline production and anchorage and are the prerequisite to construct silk scaffolds. Instead we tried to target the spigots of the posterior median spinnerets or the small piriform spigots.

Spigot blocking procedure: Various materials, including nail polish, eyelash glue, Cyanoacrylate super glue, and water-based (Elmer's) glue were tested to block specific spigots. The suitability of each material in targeting individual spigots, durability of adhesion and likelihood of contaminating other spigots due to fluid properties was assessed.

Nail polish: The advantage is the bright colour making it easy to clearly see even small amounts, but due to its comparably high viscosity, nail polish proved challenging to be applied in small amounts to target specific spigots. Small amounts tended to stick not very well on the cuticle, and could only be secured by further spreading the nail polish along the lateral edge of the spinneret. On the other hand, the viscosity prevented the contamination of other spigots. In many cases, the spiders were able to remove the nail polish using their hind legs within the two days after treatment. This could be mitigated by applying larger

amounts of nail polish, which, however, may cause uneven weight distribution, affecting spider mobility.

Eyelash glue: Despite being sufficiently sticky, the eyelash glue exhibited a slow drying time. This caused problems when the anaesthesia wore off and spiders started to move their spinnerets, leading to cross-contamination.

Super glue: Due to its initially low viscosity, even tiny amounts of super glue were spreading across all spinnerets, causing the accidental blockage of multiple spigots.

Water-based glue: This adhesive had a comparably high viscosity and extended drying period, and was therefore ineffective for applying it to specific spigots.

Evaluation: It was found that it was barely possible to block specific spigots or spigot fields. If small enough amounts were used, the spiders were able to remove the block. The spider's ability to remove adhesive blocks may be taxon-specific. *Pholcus phalangioides* is known to exhibit a thick coating of fluid hydrocarbon on their cuticle, which might act as a repellent to adhesives (Frutiger & Kropf 2019). If higher amounts of glue were used, it contaminated other spigots. Overall, we found that spiders were highly stressed by the experimental treatment and their behaviour was largely affected, even if only single spigots were blocked. Treated spiders usually stopped to spin webs and draglines altogether, making it impossible to determine changes in gland-specific behaviours. This caveat might be species-specific, and it remains unclear, if the experimental approach may be effective for other spider taxa.

2.5. Correlating fibre and spigot pore diameters

This approach is based on the observation that the spigots of different gland types often differ in the diameter of their terminal pore opening. Assuming that the spigot cuticle is relatively stiff, small pores would not be able to emit thick fibres, and hence fibres of thicker diameters originate from spigot with larger diameters. As the spigot pores are in the micron to sub-micron scale their investigation in the SEM is required. Fibres may either be visualised with an SEM or polarised light microscopy (PLM) (Blackledge, Cardullo & Hayashi, 2005).

When comparing the diameters it is important to keep in mind that silks, and potentially cuticle, too, might slightly shrink in the SEM (Greco et al., 2023).

Methodology: In our case study, seven different silk products (drop-down dragline, walking dragline, bridging line, gumfoot line, web tangle, prey wrap, and egg sac) were collected from multiple individuals on sample holders as described by Ramírez et al. (2013). The opisthosomata of the same individuals were prepared for SEM (sample preparation was performed as mentioned above, except for the temperature gradient steps).
High-resolution images of the spigots were captured, and subsequently, each spigot pore diameter was measured using the ImageJ software (Schneider, Rasband & Eliceiri, 2012). Spigot pore dimensions were correlated with fibre diameters to establish relationships between spigots and their respective silk products.

Results: In *P. phalangioides*, we found that each spigot type emits a distinct fibre type. The fibres from different silk samples were visually distinguished as thin, thick, and medium sized using the PLM (Fig. 3.E-G). In the SEM, the aciniform spigot pore showed as the smallest, the major ampullate as medium sized (Fig. 3.A) and the minor ampullate as the largest (Fig. 3.B). However, we found that the minor ampullate spigot had a flexible cuticle surrounding the pore and in the SEM samples the pore opening was often collapsed, making it hard to quantify the effective diameter (in these cases the average of the long and the short diameter across the oval or slit-like pore was taken). By comparison with the results from the Cryo-method, we found that this spigot ejects fibres with a range of diameters, some of which may be indistinguishable from the diameters of major ampullate and aciniform silk fibres. These characteristics may be more specific to Pholcidae, and less so of a problem for spiders of other families.

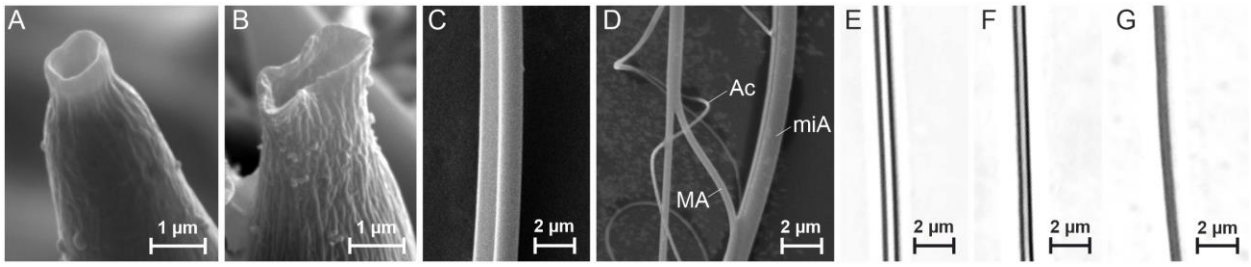

Figure 3. SEM (A-D) and PLM (E-G) images for spigot pore size measurements and fibre diameter measurements of *Pholcus phalangioides*. (A) Major ampullate spigot pore. (B) Minor ampullate spigot pore. (C) Dragline showing two fibres. (D) Detail of gumfoot line (tangle capture line from web), showing all three silk types, distinguishable by fibre width. (E-G) Fibres of different widths observed in egg sac and prey wrap samples. Abbreviations: Ac – aciniform silk fibre; MA – major ampullate silk fibre; MiA – minor ampullate silk fibre.

Evaluation: The advantage of the approach to correlate fibres with spigots based on diameters is that with PLM a large number of silk samples can be analysed in a comparably short time.

The limitation is that the matching of fibres and spigots based on diameters must be regarded as a hypothesis, as it cannot be fully excluded that large pores emit thin fibres. Furthermore, the approach is only applicable if spigots of different silk types are clearly distinct in their diameter, and exhibit a stable pore shape. Especially in families where more than four silk gland types are present, it might be increasingly difficult to distinguish fibre types based on diameters alone, and additional characteristics might have to be taken into account (e.g., birefringence, Raman spectra, etc., see below).

2.6. Distinguishing gland products by chemical analysis (Raman spectroscopy)

Different silk gland products differ in their chemical composition and base protein (spidroin) structure. By comparing signatures in silk chemistry between the contents of the identified glands and that of the silk product it is possible to determine, which gland was involved in the production of which material. There is a large range of techniques available, such as mass spectroscopy, nuclear magneto-resonance spectroscopy or X-ray diffraction (Blamires et al., 2023; Wolff et al., 2023). However, most of these techniques usually require high amounts of material. Raman spectroscopy is especially promising as it allows the recording of chemical profiles directly in the native sample with high spatial resolution. By obtaining Raman profiles on both the silk products and the content of dissected glands (silk dope), it is, in principle, possible to identify the glandular origin of the silk product. However, it is important to note that the Raman profiles differ between pre- and post-spinning silks due to structural changes occur during the spinning process (Lefèvre et al. 2010). The

efficiency of this approach highly depends on how much the Raman profiles of the gland products differ. We have previously used the technique to determine, which of diverse adhesive silks produced by *Pholcus phalangioides* likely are the same gland products (Wolff et al., 2024). In this case, the approach prooved very useful in distinguishing the silks. However, we could not obtain the corresponding profiles of gland contents as it was not possible to clearly separate the silk dope from the surrounding epithelia, which probably is only feasible for large species.

2.7. Distinguishing gland products by mechanical properties

Different gland products are known to differ in their mechanical properties (Blackledge and Hayashi, 2006). Especially ultimate strain (indicator of how much a material can be stretched before it breaks) and Young's modulus (an indicator of material stiffness) differ between major ampullate, minor ampullate, aciniform and flagelliform silks (Blackledge and Hayashi, 2006). These characteristics may be used to distinguish whether sampled silk fibres are made from the same material or not. While the estimation of silk strength and Young's modulus requires a sophisticated setup (such as a universal testing machine with high resolution load cells), silk strain can be relatively easily quantified by attaching the silk sample to the forks of callipers and stretching out the silk until it breaks.

However, there are strong limitations to the approach of determining the glandular origin of silk materials based on mechanical properties. First, silk properties are almost exclusively known for major ampullate silk, and knowledge on the tensile properties of other silks is extremely limited. In order to create a reference database, it is necessary to do tensile test of isolated silk fibres whose glandular origin is known. This can be done by forcibly silking spiders, where in a restrained spider, specific spigots are brushed with a fine probe that may lead to silk release that is slowly pulled into a strand that is then transferred to the sample holder (Work & Emerson, 1982). Our experience with this technique is mixed. In *P. phalangioides*, distinct silk samples can be obtained that way, but is often difficult to identify from which spigot the silk is coming, even if using a stereo microscope with high magnification. We were able to forcibly pull single aciniform and major ampullate fibres, but the minor ampullate fibres tended to stick to aciniform fibres and it was not possible to obtain isolated minor ampullate silk samples with this method. In *Kukulkania hibernalis* we

found silking was almost impossible, as the spinning field was inaccessible due to the specific spinneret morphology and setal field covering the spigots in rest.

Second, tensile properties are affected by the way they are spun (Greco et al. 2023) and it is likely that behaviours, in which spiders rapidly pull fibres from the glands (such as during prey wrapping) result in different mechanical properties than behaviours in which spiders slowly and steadily extend the silk (such as in web building), even if the used silk material is the same. Spinning effects on tensile properties seem to be more expressed in some spider taxa than in others, and were previously found insignificant for ampullate silks of *Pholcus phalangioides* (Boutry et al., 2011). However, we have found that bridging lines, that are pulled by *P. phalangioides* at high speed, have different and more variable tensile properties than draglines, even if used materials are the same (Jani et al, *in prep.*).

And third, spiders often combine multiple silks in their threads and sheets, and such material mixture creates complex mechanical behaviour, in which the properties of the single components may be masked (Liprandi et al. 2024).

Finally, tensile testing is a destructive technique, and therefore cannot be used on rare and valuable samples.

3. Discussion: Comparative evaluation of the methodology

We found that each method offered their own advantages, challenges and limitations (summary in Table 1, at the end of this preprint). None of the methods is applicable for all behaviours, silk products or species, therefore we propose a combination of methods as the best approach for a more comprehensive understanding of spider silk production and gland functionality. While SEM and PLM remain essential tools for the detailed structural analysis of silk products and spigots, our observation suggests that relying solely on fibre diameter measurements may not provide a complete understanding on which silk types (gland products) are included in a given silk material. Flexible spigot openings and variation in fibre production rates can lead to variation in fibre diameters (Madsen, Zheng & Vollrath 1999, Vollrath & Köhler 1996). This may lead to discrepancies and false assumptions about thin fibre origins. Therefore, it is recommended that gland uses are further studied via direct

observation methods, like high-speed videography and cryo-fixation, where possible. Even if silk emergence cannot be observed directly, often the movement of the animals or their body parts can be informative to logically infer fibre origins in conjunction with the number and diameters of the fibres in the spun silk material, and the number, sizes and arrangement of spigots (Eberhard, 2010).

As we progressively learn from direct observations such as silking, video recording, paraffin- and cryo-fixation, it becomes possible to extrapolate such knowledge to related species, so that fibre origin can be in certain cases safely inferred from indirect observations, such as fibre diameter and morphology, and their disposition in compound structures such as cribellar bands, viscid threads and silk anchors.

In conclusion, our study underscores the importance of adopting a multi-method approach to study spider behaviour and silk gland utilization effectively. By combing direct observation methods with the microscopy of silk products, researchers can gain deeper insights into the complex dynamics of spider silk production, behavioural ecology and gland functionality.

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Method	Advantages	Disadvantages
Behavioural observation aided by high-speed camera / high-magnification lenses	• Direct observation of silk emergence. • May also provide insight into spinning kinematics, including speed of fibre spinning. • Can infer information on silk gland usage and fibre inclusion.	• Requires expensive equipment and strong illumination. • May inhibit natural behaviours, especially in nocturnal species. • Applicability to only a limited set of behaviours. • Low depth of field may make continuous focus challenging during rapid spinning.
Paraffin fixation	• Direct observation of fibre origin. • Field applicability with molten paraffin in portable thermos container.	• Time-consuming sample preparation. • High error rate and moderate success, requiring multiple trials. • Requires killing of spiders, unsuitable for low abundance species or rare behaviours.
Cryo-fixation	• Direct visualization of silk gland activity during behaviours. • Less time-consuming sample preparation compared to paraffin method. • Permits observation of both solid and viscid silks.	• Destructive method, spiders die and can only be observed once. • Requires a high number of spiders due to high error rate. • Requires lab facilities for liquid nitrogen handling. • Not suitable for field use. • Time-consuming and labour-intensive compared to other methods. • Freezing may lead to squeezing out of excess liquid silk, potentially creating artifacts.
Spigot blocking	• Experimental approach to infer the role of specific glands in different behaviours, including such that cannot be directly observed.	• High stress on spiders during experimental treatment. • Limited success in blocking specific spigots or spigot fields. • Spiders may remove adhesive blocks, especially smaller amounts (potential taxon-specific differences in ability to remove blocks). • Contamination of other spigots if higher amounts of adhesive used. • Applicability may vary among spider species.
Correlating fibre and spigot pore diameters	• Allows correlation of spigots to fibres even when not functional at the moment of fixation. • Allows the inference of gland origin for silk products, whose construction is difficult to observe (such as egg cases or nocturnal web construction). • Large number of silk samples can be analysed comparatively quickly with polarized light microscopy (PLM).	• Matching of fibres and spigots based on diameters is a hypothesis and may not always be accurate. • Possibility that large pores emit thin fibres may challenge correlation. • Applicability limited to cases where spigots of different silk types exhibit clearly distinct diameters and stable pore shapes. • Difficult for species with many silk gland types. • Additional characteristics such as birefringence or Raman spectra may be necessary for distinguishing silk types.

Distinguishing silk types by chemical analysis (Raman spectroscopy)	• Allows the inference of gland origin for silk products, whose construction is difficult to observe. • May also inform about differences in silk composition and/or protein structure. • Raman spectroscopy provides high spatial resolution chemical profiles directly in native samples. • Can be applied to both solid and viscid silks.	• Most techniques (except Raman spectroscopy) require high amounts of material, which may be unfeasible to collect. • Raman profiles differ between pre- and post-spinning silks due to structural changes, which may challenge correlations. • Efficiency depends on the degree of difference in Raman profiles of different gland products. • Difficulty in obtaining profiles of gland contents due to challenges in separating silk dope from surrounding epithelia, particularly in smaller species.
Distinguishing silk types by mechanical properties	• Allows the inference of gland origin for silk products, whose construction is difficult to observe. • Also informs about the mechanical properties of silk lines, with ecological implications. • Silk strain can be relatively quantified without expensive equipment.	• Limited knowledge on tensile properties of non-major ampullate silks. • Requires a reference database with silks whose glandular origin is known (e.g., collection by forcibly silking, which can be challenging in some species and/or gland types). • Tensile properties may be affected by spinning speed and behaviours (such effects may vary between silk types and taxa). • Spiders often combine multiple silks, masking the properties of individual gland products. • Destructive technique, unsuitable for rare or valuable samples.

Table 1. Overview on approaches to identify spider silk gland use in behavioural contexts.