

## The influence of anaesthesia on the tensile properties of spider silk

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### Summary

**In this study of the effect of anaesthesia on both the forced silking process and on the properties of the retrieved silk fibres, a monitored forced silking process enables the silking force to be measured during the whole process. Silk samples were tensile-tested and their diameters measured. Force–displacement curves and**

**stress–strain curves were drawn. The evolution of the silking process of anaesthetized spiders is found to be complex, but it sheds light on the details of the spinning mechanism of spider silk.**

Key words: spider silk, anaesthesia, mechanical properties.

### Introduction

The urge to characterize, understand and synthesize spider silk is driven by an exceptional mechanical behaviour in that it has the highest work of fracture in either natural or man-made materials (Kaplan et al., 1991; Elices, 2000; Seidel et al., 1998; Lazaris et al., 2002). In a wider context, spider silk also appears as a fruitful area in which to examine the subtleties and interrelations between composition, processing, microstructure and properties, to ascertain general trends in the emerging field of Biological Materials and Biomimetics.

Spider silk fibres are the result of highly conserved anatomic structures and a basic molecular design (Selden et al., 1991; Gatesy et al., 2001) that allows processing under mild conditions in terms of temperature and pH, and endow the spider with the ability to change the properties of the fibres almost instantaneously to adapt them to its immediate requirements (Madsen et al., 1999; Garrido et al., 2002).

The spinning of spider silk starts with a concentrated aqueous solution of large proteins known as spidroins (Vollrath and Knight, 2001; Xu and Lewis, 1990). These proteins are synthesized in the silk glands and the solution goes through a duct that finally yields the insoluble silk fibre. The duct is thought to promote a liquid crystal phase in the protein solution (Kerkam et al., 1991; Jin and Kaplan, 2003) that fulfils two essential conditions of spinning: decreasing the viscosity of the concentrated solution and pre-aligning the proteins. In turn, pre-alignment of the silk proteins is a necessary step in the formation of the insoluble fibre by matching a number of spidroin motifs in  $\beta$ -microcrystallites (Viney, 2000). This process is prompted by shear stresses (Iizuka, 1985) and probably by the composition of the solution (Vollrath et al., 1998).

Attempts to understand the details of the relationship between the processing and the mechanical performance of the silk fibres

have relied on retrieving silk spun under controlled conditions and trying to establish a correlation between spinning conditions and tensile properties. Forced silking methods, which involve the winding the silk fibre on a rotating mandrel (Work and Emerson, 1982; Guinea et al., 2005) and usually require the previous immobilisation of the spider, have been preferred to silk naturally spun by the spider (Work, 1976; Denny, 1976; Griffiths and Salinatri, 1980; Pérez-Rigueiro et al., 2001) because a number of silking parameters can be easily controlled. However, systematic studies of the influence of the silking speed (Guess and Viney, 1998), silking temperature (Vollrath et al., 2001) or from specimens with varying degrees of starvation (Madsen et al., 1999) did not provide any firm correlation between the silking process and the tensile properties of spider silk. The performance of a monitored silking process in which the silking force is measured (Pérez-Rigueiro et al., 2005), has identified this force as the first measurable parameter with a clear influence on the tensile properties of the fibre. This result has been supported by the identification of a friction brake that allows the spider to control the force exerted on the fibre during spinning (Ortlepp and Gosline, 2004).

In this context, the identification of procedures that modify the silking process in a predictable way is extremely useful in establishing the correlation between processing and properties. Anaesthesia of spiders was introduced to help to manipulate delicate, fast-moving spiders (Work, 1976). Anaesthesia allowed the immobilization of the spider prior to silking, but introduced uncertainties in the silking process that are related to its possible influence on the tensile properties of silk. Consequently, it has been a common practice to allow for complete recovery of the spider before starting the silking process (Work, 1976; Ortlepp and Gosline, 2004).

Anaesthetized spiders have been used to try to unravel some

aspects of the silking process, such as the degree of control that a spider can exert on spinning (Madsen and Vollrath, 2000), since it was thought that disruption of the usual spinning process might modify the properties of the silk fibres. Initial data (Work, 1976) indicated that the morphology of the silk fibres was severely affected by anaesthesia and the tensile strength decreased, as compared with naturally spun fibres. A subsequent report (Madsen and Vollrath, 2000) has shown that the morphology of the silk does not appear to be altered after anaesthesia, but that significant changes are observed in the diameter, breaking strain and elastic modulus.

Following this rationale we applied the monitored forced silking method to anaesthetized spiders to explore its influence on the processing and the tensile properties of silk fibres. The evolution of the silking force was monitored and samples were retrieved; their cross-sections and force–displacement curves were measured, so that the relationship between the tensile properties and the silking force of anaesthetized spider could be established.

### Materials and methods

Silk fibres from the major ampullate gland (MAS fibres) of *Argiope trifasciata* (Forskål) spiders were used in this study. *A. trifasciata* is a mediterranean spider whose size is suited to easy manipulation. The spiders were kept in boxes 70 cm×70 cm×20 cm and fed on a diet of crickets.

The monitored forced silking process has been described in detail elsewhere (Pérez-Rigueiro et al., 2005). Briefly, the spider is immobilised in a self-zipping plastic bag with a small hole so that the spinnerets remain accessible. The bag is fixed with pins on a stereofoam surface, and the stress exerted by the taut bag is enough to immobilise the spider. The initial length of silk is obtained by making contact on the area of the spinnerets with a piece of adhesive tape.

The basic immobilization procedure can be easily modified making it possible to anaesthetize the spider at any time during the silking process. Following previous works (Work, 1976; Madsen and Vollrath, 2000), CO<sub>2</sub> was used as the anaesthetizing gas. CO<sub>2</sub> was produced by slow addition of HCl (35%; Panreac, Barcelona, Spain) to a suspension of CaCO<sub>3</sub> (Merck, Darmstadt, Germany) in water. The flow of gas was controlled by the volume of HCl added per unit time. The CO<sub>2</sub> gas was circulated through a washing flask to remove traces of acid before reaching the spider. The tip of a micropipette perforating the plastic bag was used as the gas inlet.

The immobilized spider was placed upside down on the base of an Instron 4411 testing machine (Canton, MA, USA), and the initial length of silk was attached to a load cell HBM Q-11 (Darmstadt, Germany; resolution ±5 mg) fixed to the crosshead of the machine. Forced silking was done by displacing the crosshead of the silking machine at constant speed. Tensile tests on anaesthetized spiders were performed at 1 mm s<sup>-1</sup>. The experimental set-up is shown in Fig. 1. Four monitored silking processes on two different spiders were performed at 20°C and 35% relative humidity.

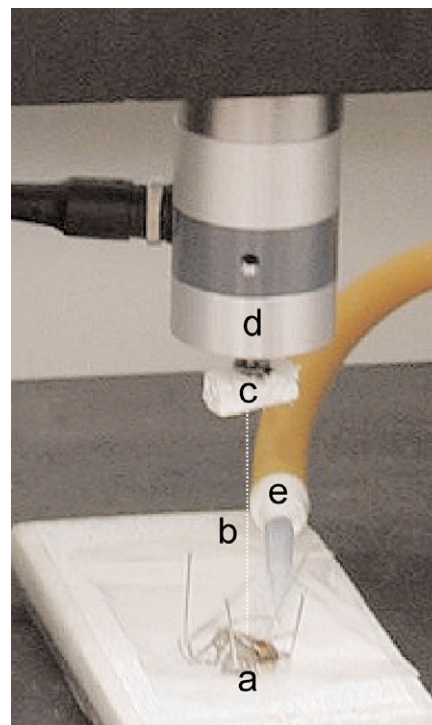


Fig. 1. Experimental set-up of the monitored forced silking process. The immobilized spider (a) is placed upside down, and the tip of fibre (b) is fixed with a magnet (c) to the load cell (d). The tip of a micropipette perforating the plastic bag (e) is used as the gas inlet. The silking process proceeds by displacing the crosshead of the testing machine at constant speed.

Samples were retrieved and glued on perforated cardboard frames (Pérez-Rigueiro et al., 1998) 20 mm long. Tensile tests were performed in an Instron 4411 testing machine at a constant crosshead speed and an average strain rate of  $2 \times 10^{-4}$  s<sup>-1</sup>. The load applied to the sample was measured with a balance (AND 1200 G, A&D Instruments, Oxford, UK, resolution ±10 mg) attached to the lower end of the sample. The crosshead displacement was taken as a direct measurement of the sample deformation, since the compliance of silk has been estimated as 1000 times that of the equipment. The tests were performed in air under nominal conditions of 20°C and 35% relative humidity.

Both ends adjacent to the length of the fibre to be tested were secured and retrieved before tensile testing to determine the fibre's cross-sectional area. Samples were sputtered with gold, and examined in a JEOL 6300 scanning electron microscope (observation conditions  $V=10$  kV,  $I=0.06$  nA) (Guinea et al., 2005). Brin diameters were measured from each micrograph, and the mean value of the diameters of both ends of a given fibre were used to compute the cross-sectional area of the fibre, assuming a circular cross-section (Pérez-Rigueiro et al., 2001).

### Results

Fig. 2A shows the typical features of the silking force vs time. Since the silking process proceeds at a constant speed of

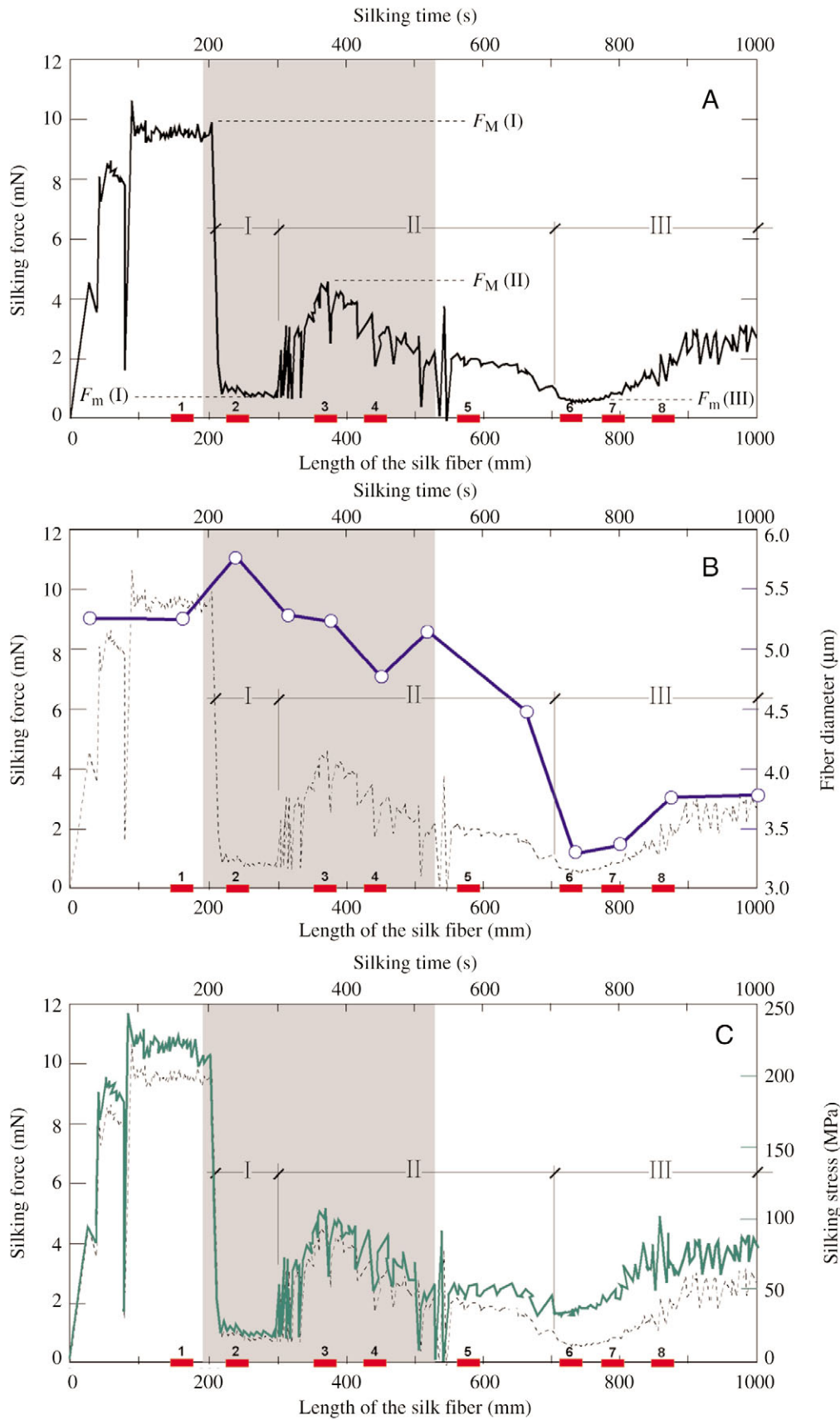


Fig. 2. Variation of the silking force (A), fibre diameter (B) and silking stress (C) during the silking process at  $1 \text{ mm s}^{-1}$  of an anaesthetized spider. The shaded zone spans the period during which the spider was exposed to the  $\text{CO}_2$  flow. The three regions I, II and III, referred to in the text are indicated. The samples whose tensile properties were measured are numbered from 1 to 8 and their positions along the silking process are indicated at the bottom of each panel.  $F_M(I)$ , maximum force in region I;  $F_m(I)$ , minimum force in region I;  $F_M(II)$ , maximum force in region II;  $F_m(III)$ , minimum force in region III.

$1 \text{ mm s}^{-1}$ , the distance from the origin is calculated as the product of the silking speed and time, and gives the silking force at which any given sample is spun. The samples whose tensile properties were measured are numbered from 1 to 8 and their positions along the silking process are indicated at the bottom of the figure. Three regions I, II and III, are marked to clarify subsequent discussion, and the period during which the spider was exposed to the  $\text{CO}_2$  flow (190 s–530 s) is shaded. The labelled forces correspond to maximum force in region I [ $F_M(\text{I})$ ], minimum force in region I [ $F_m(\text{I})$ ], maximum force in region II [ $F_M(\text{II})$ ] and minimum force in region III [ $F_m(\text{III})$ ].

Region I corresponds to the initial period of anaesthesia and is characterized by a sudden drop of the silking force shortly after the spider is exposed to the gas flow. Region II starts with an increase in the silking force that reaches a maximum force and descends smoothly, even after the gas flow has stopped. Finally, the beginning of region III is indicated by a new drop in the silking force. This pattern has been systematically observed in all the silking processes as illustrated in Table 1, which shows the values of the selected silking forces as defined above for the four silking processes performed.

The three regions can be assigned to different stages during the process of anaesthesia. Region I corresponds to the initial effect of anaesthesia when the spider is probably not fully anaesthetized. Its main feature is the sudden drop – more than one order of magnitude – of the silking force. The first part of region II probably corresponds to the fully anaesthetized spider and is characterized by a significant initial increase of the silking force, which reaches a maximum and is followed by a smooth decay. Although anaesthesia is removed in approximately the middle of region II, the first spontaneous movement of the spider was observed within region III at 850 s, so the initial decrease in silking force can correspond to the beginning of the spider's recovery from anaesthesia.

The evolution of the diameters of the fibres during silking is shown in Fig. 2B. A slight increase in the diameter in region I as compared with the diameter measured before anaesthesia was observed. The diameter decreases along region II, although it is not completely regular and some fluctuations are observed, and it reaches a minimum at the beginning of region III. The overall morphology of the silk fibres is presented in Fig. 3. Fig. 3A shows sample 1 (before anaesthesia), Fig. 3B

sample 4 during anaesthesia (region II, in the notation of Fig. 2A), and Fig. 3C sample 8 (region III) after the removal of anaesthesia and the observation of spontaneous movement of the spider. It is apparent that the morphology of the fibres is not affected by anaesthesia.

The simultaneous measurement of the silking force and the cross section of the fibres allows a calculation of the stress exerted on the fibres during silking. The silking stress is shown in Fig. 2C. The evolution of the silking stress is seen not to coincide with that of the silking force. Thus, silking stress at the beginning of region III is comparable to silking stress in region II, whereas it differs from that in region I. In contrast, the silking forces in region I and at the beginning of region III almost coincide and are significantly lower than those in region II. This pattern of the silking stress has been observed in the four silking processes as shown in Table 2, where the values

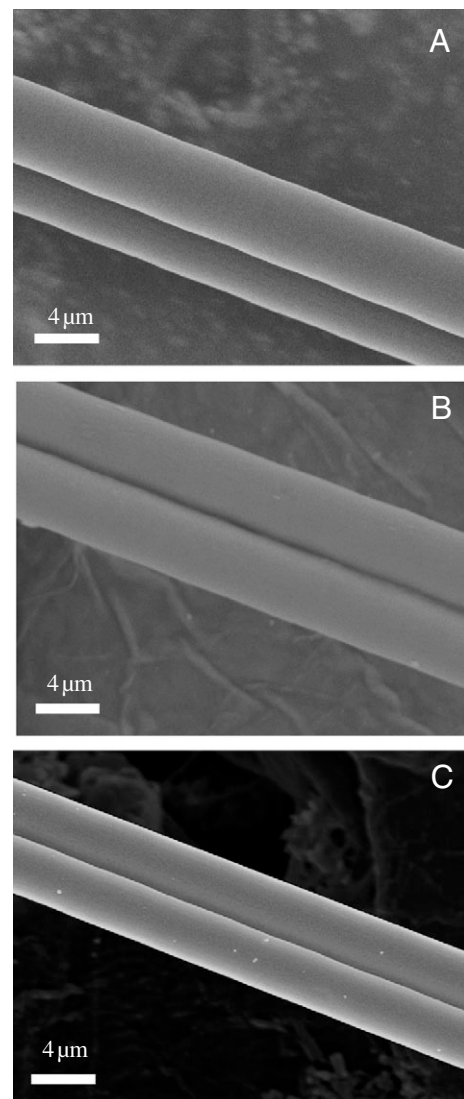


Fig. 3. Comparison of the morphology of silk fibres spun before anaesthesia (A), during anaesthesia (B) and after the removal of anaesthesia (C).

Table 1. Selected silking forces from four different monitored forced silking processes on anaesthetized spiders

Silking process	$F_M(\text{I})/F_m(\text{I})$	$F_M(\text{II})/F_m(\text{I})$	$F_m(\text{III})/F_m(\text{I})$
(i)	13.8	6.7	0.9
(ii)	13.8	5.2	0.7
(iii)	11.2	6.5	0.7
(iii)	14.2	7.1	1.3
Mean $\pm$ s.d.	$13.3 \pm 0.8$	$6.4 \pm 0.4$	$0.9 \pm 0.1$

$F_M(\text{I})$ , maximum force in region I;  $F_m(\text{I})$ , minimum force in region I;  $F_M(\text{II})$ , maximum force in region II;  $F_m(\text{III})$ , minimum force in region III. The values are normalized by dividing them by  $F_m(\text{I})$ .



Table 2. Selected silking stresses from four different monitored forced silking processes on anaesthetized spiders

Silking process	$\sigma_M(I)$ (MPa)	$\sigma_m(I)$ (MPa)	$\sigma_M(II)$ (MPa)	$\sigma_m(III)$ (MPa)
(i)	216	15	107	32
(ii)	272	17	119	17
(iii)	289	26	138	39
(iv)	300	21	205	29
Mean $\pm$ s.d.	270 $\pm$ 20	20 $\pm$ 2	140 $\pm$ 20	29 $\pm$ 3

$\sigma_M(I)$ , maximum stress in region I;  $\sigma_m(I)$ , minimum stress in region I;  $\sigma_M(II)$ , maximum stress in region II;  $\sigma_m(III)$ , minimum stress in region III.

of selected silking stresses corresponding to the three regions are indicated.

The stress–strain curves of the silk samples of the silking process shown in Fig. 2 are presented in Fig. 4. The number adjacent to each curve indicates each sample according to the numbering in Fig. 2. The range of tensile properties spanned by naturally spun (NS) fibres (Pérez-Rigueiro et al., 2003) is shaded, and an example of a characteristic fibre retrieved from a forced silking process of an unanaesthetized spider is also shown (FS fibre). Almost all the fibres spun during anaesthesia are close to the stiffest NS fibres and more compliant than a typical FS fibre. The only exception is sample 2, which was spun in region I and its stress–strain curve coincides with the most compliant NS fibre.

The tensile parameters measured from the fibres retrieved in each of the three regions for the four silking processes are

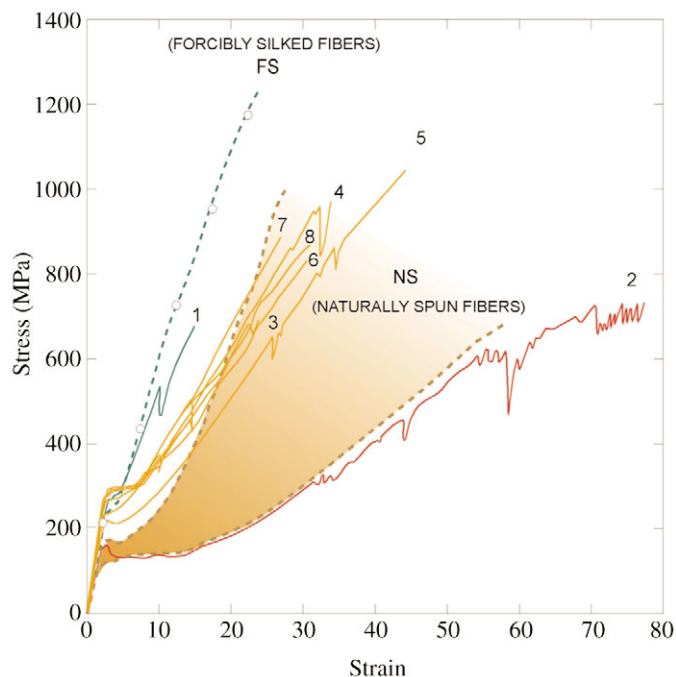


Fig. 4. Stress–strain curves of the silk samples as labelled in Fig. 2. The number adjacent to each curve identifies each sample. The range of tensile properties spanned by naturally spun (NS) fibres is shaded, and an example of a characteristic forcibly silked fibre retrieved from an unanaesthetized spider is also shown (FS fibre).

presented in Table 3. These results show that regions I, II and III are not only consistently defined in all the silking processes in terms of both silking force and silking stress, but also that silk fibres produced in each region share some characteristic features that differ from those produced in the other regions.

### Discussion

Silking of anaesthetized spiders is an involved process, in which silking force and fibre diameter change during the procedure. Also, it appears that the processes that control the fibre diameter and silking force are independent. This assertion is supported by the fact that the diameter of fibres silked with similar forces (samples 2 and 6) differ by almost a factor of two.

Observation of Fig. 2A–C indicates that (1) in region I the silking force drops and the diameter increases slightly, leading to a sudden drop of the silking stress. (2) At the beginning of region II the silking force increases and subsequently decays smoothly. In contrast, the average diameter decreases steadily, leading to a moderate decrease in the values of silking stress that does not reach the low limit of region I. The decrease in the diameter supports previous findings (Madsen and Vollrath, 2000) of a decrease in the diameter of the fibre after exposure of spiders to CO<sub>2</sub> for more than 400 s. (3) In region III, when the spider is awake, both silking force and silking diameter increase. As a result, the silking stress increases moderately.

It has been proposed (Madsen and Vollrath, 2000) that the valve, present in the distal part of the silk gland, is responsible for controlling the diameter of the fibre through muscular control exerted by the duct levator muscle. The valve has also been credited with the biological function of grasping the silk within the duct so that the spider can control its descent

Table 3. Tensile parameters of the fibres retrieved in regions I, II and III of the four silking processes

	$E$ (GPa)	$\sigma_u$ (MPa)	$\epsilon_u$
Region I	7.2 $\pm$ 0.4	800 $\pm$ 70	60 $\pm$ 20
Region II	9.9 $\pm$ 0.4	1000 $\pm$ 50	34 $\pm$ 2
Region III	9.2 $\pm$ 0.6	880 $\pm$ 20	30 $\pm$ 3

Values are means  $\pm$  s.d.  
 $E$ , elastic modulus;  $\sigma_u$ , tensile strength;  $\epsilon_u$ , strain at breaking.

(Wilson, 1962; Wilson, 1969). The ability of the valve to grasp the fibre suggests that it can act as a friction brake, a structure hypothesised to explain the ability of the spider to modify the silking force (Ortlepp and Gosline, 2004), but which had not been assigned to any anatomical feature.

The double function of the valve does not necessarily imply a contradiction, even if it has been proved that both mechanisms can be controlled independently. The silking force is the result of the friction exerted on the fibre during spinning, so the silking force can be varied by modifying the friction coefficient through variations of the solution in which the fibre is processed (Vollrath et al., 1998), and not by changing the inner diameter of the silking duct. Changes in the inner diameter in the duct would control the fibre's diameter.

From Figs 2 and 4 it can be deduced that the tensile properties of spider silk are strongly correlated with the silking stress. This result completes previous findings (Pérez-Rigueiro et al., 2005), that showed how the tensile properties of spider silk can be traced back to the silking force. That study considered only processes that rendered samples with similar diameters so the independence of the mechanisms that control diameter and silking stress could not be assessed. Besides, determination of the tensile properties of a given sample could only be made after tensile testing and by comparing yield force and silking force. From the present data a relation between the value of the silking stress and the tensile properties of the fibre can be established directly.

The silking stress correlates well with the tensile properties regardless of the anaesthetizing conditions. It must be noted, however, that strain at breaking tends to be greater in samples spun under the effect of anaesthesia (i.e. samples 2, 4 and 5, Fig. 4) than in samples reeled from unanaesthetized spiders (Guinea et al., 2005). A similar increase in the strain at breaking has been found in anaesthetized *Nephila edulis* spiders (Madsen and Vollrath, 2000). Fig. 4 also shows that the yield stress of forcibly silked fibres in regions II and III tends to be larger than that of naturally spun (NS) fibres.

From Fig. 2C the range of silking stresses that yield fibres whose tensile properties correspond to naturally spun fibres (NS), extends from ~16 MPa (region I, sample 2) to ~80 MPa (average) for sample 3 in region II. The stress-strain curve of sample 2 is identical to that of the most compliant fibres spun naturally by the spider (Garrido et al., 2002) and it has been silked at ~1 mN from a spider with a body weight of 10 mN (1 gf). This is consistent with the estimation that the minimum silking force is roughly 0.1 of body weight (Ortlepp and Gosline, 2004). Silking forces below this limit occur in region II, but, it appears that the spider lacks the ability to sustain such low forces for longer than a few seconds. Samples 3–8 (regions II and III) were silked with comparable average stresses (from 40 MPa to 80 MPa) and their stress-strain curves show a similar behaviour, close to the upper limit of NS fibres (Fig. 4). In contrast, sample 1 spun at ~230 MPa before anaesthesia shows FS tensile properties, in accordance with previous findings by the authors.

Spinning of silk fibres under anaesthesia yields information

on the control mechanisms of the silking process. Thus, the high silking stress observed during forced silking under anaesthesia is the result of the performance of the spinning system in its passive state. The tensile properties of silk retrieved from anaesthetized spiders are characteristic of FS fibres, so that the spinning system in its passive state leads to fibres that are stiffer than the naturally spun (NS) fibres. Spinning of NS fibres would be the result of adjusting the spinning system by actively reducing its silking stress.

### Conclusions

The forced silking process of anaesthetized spiders is an intricate process that prevents its use as a source of spider silk with predictable properties but offers the opportunity to analyse some significant details of the spinning process.

It has been found that during spinning, the diameter of the fibres and their intrinsic properties, in terms of stress-strain curves, appear to be controlled independently. Samples with the same diameter can be spun that render very different stress-strain curves, and samples with similar tensile properties can be spun in a range of different diameters.

Silking stress appears to be the key parameter that controls the properties of the fibre. A correlation can be established between silking stress and the stress-strain curves of the samples. This correlation is valid for both anaesthetized and unanaesthetized spiders.

Forcibly silked (FS) fibres are spun at significantly higher silking stresses than NS fibres. An effective lower limit of the silking stress was found, and samples spun at this silking stress correspond to the most compliant fibres naturally spun by the spider.

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