



## Research

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# Adhesion enhancement of cribellate capture threads by epicuticular waxes of the insect prey sheds new light on spider web evolution

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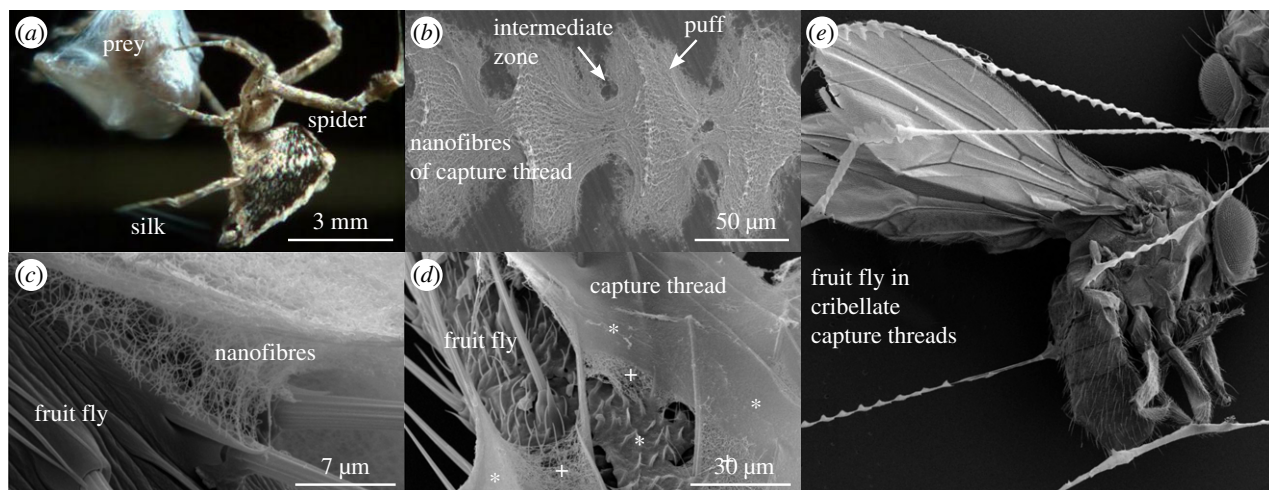
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To survive, web-building spiders rely on their capture threads to restrain prey. Many species use special adhesives for this task, and again the majority of those species cover their threads with viscoelastic glue droplets. Cribellate spiders, by contrast, use a wool of nanofibres as adhesive. Previous studies hypothesized that prey is restrained by van der Waals' forces and entrapment in the nanofibres. A large discrepancy when comparing the adhesive force on artificial surfaces versus prey implied that the real mechanism was still elusive. We observed that insect prey's epicuticular waxes infiltrate the wool of nanofibres, probably induced by capillary forces. The fibre-reinforced composite thus formed led to an adhesion between prey and thread eight times stronger than that between thread and wax-free surfaces. Thus, cribellate spiders employ the originally protective coating of their insect prey as a fatal component of their adhesive and the insect promotes its own capture. We suggest an evolutionary arms race with prey changing the properties of their cuticular waxes to escape the cribellate capture threads that eventually favoured spider threads with viscous glue.

## 1. Introduction

The finding that rather weak van der Waals' forces enable geckos to climb walls provided novel insight into biological mechanisms of adhesion [1]. Since this finding, van der Waals' forces have been proposed to play a role in many biological adhesives, e.g. in insects' or spiders' feet or the capture threads of basal, cribellate spiders (figure 1*a*) [2–5]. In contrast with the reversible adhesion of geckos' or insects' feet, however, for the capture threads of spiders, it is advantageous to produce prolonged adhesion that can help to subdue prey. The capture threads of cribellate spiders consist of nanofibres, arranged in puffs and intermediate zones similar to beads on a string (figure 1*b*). In the intermediate zones, they are intertwined with stabilizing core fibres, the axial fibres, which pervade the complete thread [6]. Compared with viscid silk droplets, the glue of the modern and much more abundant ecribellate spiders, the adhesive forces generated by the nanofibres of cribellate threads are rather weak, at least on artificial surfaces [7–9]. On the other hand, cribellate capture threads can restrain prey for much longer periods of time than ecribellate threads [10]. Thus, ascertaining whether one adhesive is superior to the other (as well as the evolutionary linkage between both) is not simple and has entailed an elaborate debate about the benefits and drawbacks of using cribellate versus ecribellate silk [11–16].

To explain this prolonged retention of prey in capture threads of cribellate spiders compared with prey in capture threads of ecribellate spiders, it was suggested that the nanofibres' adhesion relies not only on van der Waals' forces, but also on



**Figure 1.** Cribellate spiders capturing prey. (a) *U. plumipes* (hanging upright) wrapping prey after capturing it in its horizontal orb web. (b) Cribellate capture thread of *U. plumipes* adhered to a metal surface. The adhesive nanofibres are organized in regularly spaced puffs and intermediate zones. (c) Single nanofibres adhering to prey (*D. melanogaster*). (d) When in contact with *D. melanogaster*, cribellate threads show areas where single nanofibres (+) are no longer discernible individually (\*). (e) Overview of *D. melanogaster* covered in capture threads of *U. plumipes*. (b–e) SEM images. (c–e) Provided by H. Adamova. (Online version in colour.)

hygroscopic forces and, finally, entrapment of insects' surface structures in a Velcro-like manner [2,17]. But the discrepancy between the adhesive force on artificial surfaces and prey cannot be explained solely by entrapment: the highest adhesive force ever determined for cribellate threads was measured on the elytra (hardened forewings) of a lady beetle (*Hippodamia convergens*), which lacks any surface irregularities [17,18]. van der Waals' forces and hygroscopic forces can explain the adhesive forces of cribellate threads on smooth artificial surfaces, but the 10-fold increase in adhesive force between smooth prey surfaces and cribellate threads may suggest an additional, unrecognized adhesive mechanism [17,18].

The aim of this study is to determine what forces are responsible for restraining prey in cribellate threads for elongated periods of time. This information could also help to address other long-standing questions, such as the uncertain evolutionary history of the modern viscoelastic glue of the cribellate threads, and the question of how cribellate spiders persisted as a lineage for more than 145 million years, despite using adhesives that had been presumed ineffective [11,12,19,20].

## 2. Material and methods

### (a) Spiders and insects

*Uloborus plumipes* (Lucas, 1846) and *Kukulcania hibernalis* (Hentz, 1842) were raised at room temperature (approx. 21°C), ambient humidity (approx. 30%), and northern European diurnal rhythm. *U. plumipes* were kept in large terraria shared by several spiders as a laboratory colony. Each spider was able to build a web of its own. *K. hibernalis* were kept separately in 1 l containers covered with gauze. Once a week, spiders were fed with *Drosophila melanogaster*, juvenile *Acheta domestica*, or *Callosobruchus maculatus*. Water was provided once to twice per month by sprinkling the web. In the case of *K. hibernalis*, water was provided by applying droplets near the burrow. Wetted threads were not used for further research.

Thread samples of *Amaurobius ferox* (Blackwall, 1861) were collected in the Roonstraße in Mainz (Germany) using two parallel metal wires (zinc paper clips) with a fixed distance of approximately 0.7 cm. Thread samples of *Badumna longinqua* (L. Koch, 1867) were provided by the Queensland Museum (Brisbane, Australia). Samples collected in the field were

stored in the dark and protected against dust in a box at room temperature and room humidity until use.

Most insects (true bugs: *Eurydema oleraceum*, *Peribalus strictus*, *Pyrhocoris apterus*, *Cercopis vulnerata*; beetles: *Donacia marginata*, *Hemicrepidius niger*, *Ampedus sanguineus*, *Harpalus distinguendus*, *Cantharis fusca*, *Harmonia axyridis*, *Psyllobora vigintiduopunctata*; lepidopterans: *Xanthorhoe fluctuata*, *Colostygia pectinataria*; true flies: *Sarcophaga* sp., *Lucilia* sp., *Xanthogramma citrofasciatum*; for more information, refer to electronic supplementary material, table S1) were collected in May 2016 in the vicinity of the department of the RWTH Aachen University. The true fly *D. melanogaster* (fruit fly), the cricket *A. domestica*, and the beetle *C. maculatus* (cowpea weevil) were taken from our laboratory colonies. *D. melanogaster* was used to determine the retention time of living prey in cribellate threads, and the elytra of *C. maculatus* were used to characterize the cribellate adhesive. All insects were used to validate the generality of the experiments performed only with *C. maculatus*.

### (b) The retention time of *Drosophila melanogaster*

To determine the retention time, fruit flies eclosed from pupae that very day were taken by picking them up with a feather-weight forceps. They were placed into the capture threads of a web of *U. plumipes*, from which the spider had been removed. Subsequently, the time it took the fruit flies to escape the capture thread was measured. Flies were not cooled down or narcotized to eliminate any delay in reaction.

### (c) Microscopy

To analyse the interaction between spider species and insect prey, all insects were killed by freezing them at  $-20^{\circ}\text{C}$ . Elytra or wings were separated from the body and dried at room temperature for at least 1 week previous to any further use. The dried prey was placed onto conductive foil (Plano GmbH) and covered with cribellate capture threads by pulling the complete sample through a web of *U. plumipes*. Elytra and threads were left for 30 min to provide time for interactions. After this time, the samples were sputtered with gold (Hummer; Technics Ins.) and examined with a scanning electron microscope (REM 525 M; Philips AG). Elytra of *C. maculatus* were also used to observe interactions with capture threads of *K. hibernalis*, *A. ferox*, and *B. longinqua*.

Capture thread samples of *U. plumipes* were also observed with a high-speed light microscope (500 fps; Keyence VW-600C) or a compound microscope with epifluorescence (DAPI-filter

combination; Axioplan 2; Carl Zeiss) after or during contact with an elytron of *C. maculatus*.

After observing structural changes of the capture threads after contact with the prey's cuticle with both light and scanning electron microscopy, we also studied the modified threads with the transmission electron microscope (TEM 10; Carl Zeiss). Finder-grid (400er mesh, 3.5 mm; Plano GmbH) were covered with parts of the structurally altered capture threads of *U. plumipes* and analysed without any further treatment.

#### (d) Influence of cuticular wax concentration

To evaluate the effects of cuticular wax concentrations, 20 elytra of *C. maculatus* were washed for 1 min in 100  $\mu\text{l}$  *n*-hexane (95%; AppliChem). The supernatant was transferred to a tube containing a metal wire, and the tube was left open for at least 4 h to dry before the wires (now coated with wax) were used. We defined 20 elytra per 100  $\mu\text{l}$  *n*-hexane as our stock solution (100%). To reduce the amount of wax used for coating the metal wire, this stock solution was diluted further to yield concentrations of 50, 25, 12.5, and 6.25%.

Next, cribellate capture threads of *U. plumipes* were picked up with one coated and one uncoated metal wire, placed in a box for protection, and stored at room temperature (22°C). After 10 and 60 min, the changes in the appearance of the thread were documented by using a camera (Canon EOS 550D) mounted to a binocular dissection microscope. The distance between the front of the matrix and the starting point (i.e. contact of the thread to the metal wire) was measured, and the velocity ( $\mu\text{m s}^{-1}$ ) was determined. Because there was no structural change of the capture thread on uncoated metal wires, there are no quantitative data for the negative control.

To obtain wax-free elytra, single elytra were washed four times for 1 min with *n*-hexane and dried afterwards for at least 4 h. These samples were also used to examine the interaction with threads of *U. plumipes* via scanning electron microscopy.

#### (e) Influence of temperature

To determine whether the interactions observed were temperature-sensitive, metal wires were coated with 100% cuticular wax solution (see above). These samples were exposed to different temperatures (4, 22, 40, 50, and 80°C) and analysed as described above (see 'Influence of cuticular wax concentration').

#### (f) Removing elytra (as prey surrogate) from capture threads

To determine whether something propagating from the elytra was migrating through the capture thread, we separated the elytra from the capture thread. Therefore, threads were picked up with two parallel metal wires. One elytron of *C. maculatus* was adhered at the margin of each thread. After 2 days, allowing the propagation of the matrix to seize half of the thread's length, an additional wire was included, separating the elytra from the rest of the thread (electronic supplementary material, figure S1). Every second thread was cut between the elytron and the additional wire to eliminate continuous contact. The other threads served as a control. The propagation of structural changes was documented every day for at least 5 days.

#### (g) Analysis of cuticular waxes

Because our experiments hinted at the possibility that the epicuticular waxes of the prey insects might be absorbed by the cribellate capture thread, we analysed whether the waxes of the cuticle are indeed found in structurally changed capture threads. Cuticular waxes were removed and transferred to a metal wire as described before. Capture threads were picked up with coated metal wires

and stored dust-protected until the complete threads were structurally changed (about a week at room temperature; for more information about the time dependency of this process, refer to electronic supplementary material, figure S3). Afterwards, the now structurally changed capture threads were separated from the coated metal wires by using a new uncoated metal wire. As a negative control, capture threads without any previous treatments were used. All threads were extracted with *n*-hexane for 10 min and subsequently analysed using gas chromatography–mass spectrometry (GC–MS). For comparison, we extracted the cuticular hydrocarbon profile of 20 freshly killed *C. maculatus* individuals by immersing them in *n*-hexane for 10 min.

Each extract was concentrated under a gentle nitrogen stream to approximately 20  $\mu\text{l}$  and injected into a GC (7890A; Agilent Technologies, Santa Clara, CA, USA) at a temperature of 250°C in splitless mode (injection volume: 2  $\mu\text{l}$ ). The GC was equipped with a Zebron Inferno DB5-MS capillary column (Phenomenex; length 30 m,  $\varnothing$  0.25 mm, 0.25  $\mu\text{m}$  coating), and helium was used as the carrier gas with a flow rate of 1.2 ml min<sup>-1</sup>. After 2 min at 60°C, the oven heated at a rate of 60°C min<sup>-1</sup> up to 200°C and afterwards at 4°C min<sup>-1</sup> up to 320°C. The mass-selective detector (5975C; Agilent Technologies) used electron ionization at 70 eV and scanned for molecular fragments in a range of 40–550 *m/z*. Data were acquired using the software MSD ChemStation (E.02.02.1431; Agilent Technologies). Hydrocarbons were identified based on retention index and diagnostic ions. We included all hydrocarbons longer than C20 and with a relative abundance of at least 0.01% (electronic supplementary material, table S2).

#### (h) Measurements of adhesive forces

To determine whether the observed phenomenon is a new adhesive mechanism or perhaps a defensive reaction of the prey, the adhesion between capture threads of *U. plumipes* and native, as well as wax-free elytra of *C. maculatus*, was measured. Single threads were taken from the web by picking them up gently with a sample holder, consisting of two parallel metal wires (distance 0.7 cm). Special care was taken not to stretch the sample during this procedure. The sample holder was placed onto a microbalance (JB1603/C-FACT; Mettler Toledo AG). Native as well as wax-free elytra were fixed to toothpicks using superglue (Uhu Sekundenkleber blitzschnell Supergel; Uhu GmbH & Co. KG). From above, the elytra were brought into contact with the threads for 30 s and afterwards slowly and steadily pulled upward and perpendicular to the threads using a micromanipulator (MM 33; Märzhäuser Wetzlar GmbH & Co. KG). The maximal value recorded with the microbalance was taken as maximal adhesion force. Data were normally distributed (Kolmogorov–Smirnov test; calculated by SPSS Statistics (Version 21)). Data from both samples were compared with a two-tailed *t*-test and  $\alpha = 0.05$ . For monitoring the threads, single trials were recorded with a digital reflex camera (50 fps; Canon EOS 550D).

### 3. Results

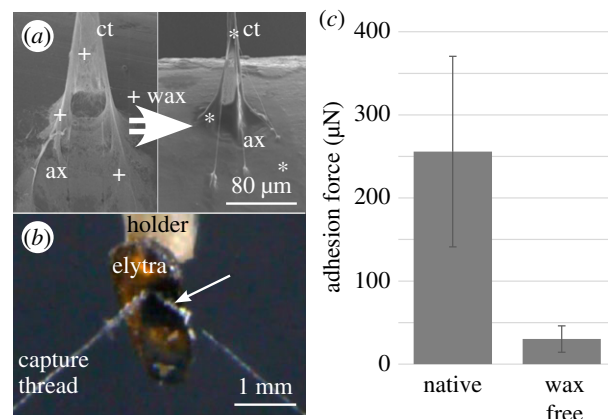
In contrast with the data generated with artificial surfaces, cribellate capture threads can restrain prey for longer periods of time than ecribellate threads can [10]. We even observed prey such as fruit flies and winged ants dying in abandoned cribellate webs, apparently unable to struggle free. Measuring the retention time of fruit flies (*D. melanogaster*) in abandoned cribellate orb-webs of *U. plumipes*, only 8% were able to struggle free within 5 min ( $N = 13$ ). Because spiders capture prey typically within seconds and prey not captured within the first 3 min is assumed to be ignored by the spider, this retention time enables even very slow spiders to subdue the prey [21,22].

When dead and air-dried *D. melanogaster* were brought into contact with cribellate threads (figure 1e) of the *U. plumipes*, we observed single cribellate nanofibres attached to the fly surface, probably due to van der Waals' forces as described previously (figure 1c). However, in other parts of the thread, single fibres were not distinguishable anymore but seemed to have fused into one layer (figure 1d). This 'fusion' of the cribellate fibres was observed after contact with almost 60% of the insect species we tested (electronic supplementary material, table S1). Furthermore, threads of all cribellate species tested (*U. plumipes*, *K. hibernalis*, *B. longinqua*, and *A. ferox*) showed this 'fusion' of nanofibres (electronic supplementary material, figure S2). Such 'fusion' was never observed on any artificial surface (figure 1b).

To further characterize this interaction between cribellate nanofibres and the prey surface, we performed further experiments using the elytra of cowpea weevils, *C. maculatus*, and capture threads of *U. plumipes*, as here the 'fusion' effect was most prominent. An effect was already visible in the light microscope. After contact with the *C. maculatus*' elytra, the light reflection of the thread of *U. plumipes* increased and a blue autofluorescence developed under UV illumination. We could observe a steady migration of a front of 'fusing' fibres, seizing the complete capture thread when given enough time (electronic supplementary material, movie S1). Hence, the interaction is a time-dependent process, which can lead to a structural change of the thread even beyond the area where thread and elytra are in contact. The velocity of this structural change was temperature-sensitive, increasing with temperature (electronic supplementary material, figure S3b). Cooling to 4°C did not completely stop the movement of the front of 'fusing' fibres. The process could only be stopped by removing the elytra from the thread. These results show that the nanofibres of the thread are not changing their structure after initial contact by some kind of domino effect.

Closer inspection with a transmission electron microscope of any such 'fused' areas revealed that the nanofibres are still recognizable individually, but are embedded in a fluid matrix with a low vapour pressure (electronic supplementary material, figure S3a). A chemical analysis of the nanofibres embedded in the matrix revealed that the hydrocarbon composition of the embedded nanofibres is similar to that on the cuticle of *C. maculatus* (electronic supplementary material, table S2). No hydrocarbons could be detected in the capture threads alone. Correspondingly, reducing the amount of waxes on a surface (by dilution) also reduced the velocity of matrix propagation (electronic supplementary material, figure S3c). When removing all cuticular waxes from the elytra, structural changes of the capture thread were no longer induced. Moreover, the transfer of these waxes to an artificial surface immediately led to structural changes of the capture thread after contact (figure 2a).

A comparison between native, untreated elytra and the wax-free elytra (width of elytra:  $0.77 \pm 0.04$  mm;  $N = 21$ ) revealed that the adhesion force on native elytra was eight times stronger than that on wax-free elytra ( $260 \pm 117$   $\mu$ N ( $n = 15$ ),  $31 \pm 16$   $\mu$ N ( $n = 11$ ), figure 2c). This is significantly different according to a two-tailed *t*-test ( $p = 1.2 \times 10^{-6}$ ). Hence, the observed structural change of the capture threads must be the previously undetected adhesive. During these recordings, 87% of the threads tore before coming free of the elytra. Indeed, the stability of the axial fibres seemed to be the limiting factor for this adhesive, because they have a



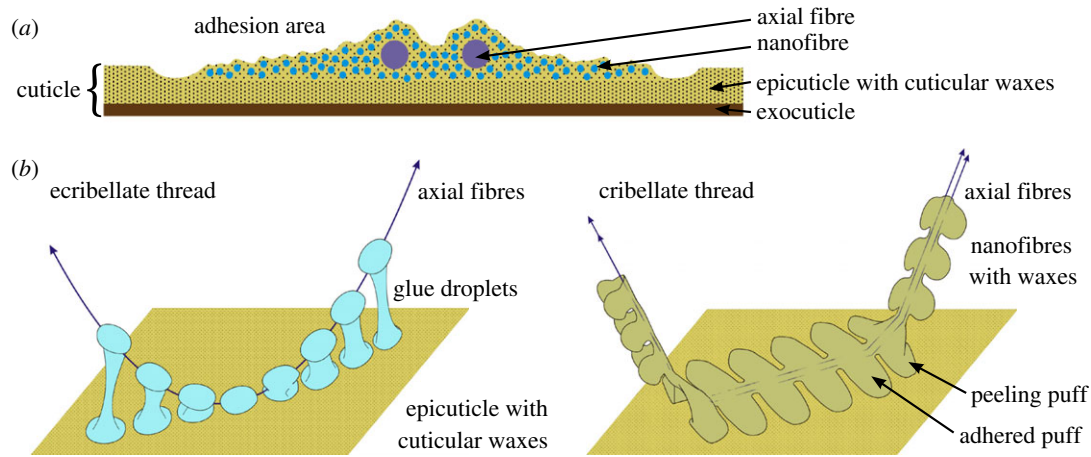
**Figure 2.** Epicutular waxes enhance adhesion between cribellate capture thread and prey. (a) A cribellate thread (ct) adhered to an untreated metal surface (left panel) shows individual nanofibres (+). Previous coating with epicutular waxes of cowpea weevils, *C. maculatus*, however, led to a 'fusion' of nanofibres into a homogeneous sheet-like structure (\*) (right). Note that in both cases, the axial fibres (ax) are partly separated from the cribellate fibres. SEM image. (b) When pulling a native elytron out of a cribellate capture thread, the separation proceeds by single puffs peeling off individually (arrow; see electronic supplementary material, movie S2). (c) Removal of waxes from the elytra led to a significant decrease in the adhesive force (two-tailed *t*-test:  $\alpha = 0.05$ ;  $p = 1.2 \times 10^{-6}$ ). (Online version in colour.)

similar breaking strength to our measured adhesive force [23]. In the other cases, the adhering puffs peeled off the elytra one after another (figure 2b, electronic supplementary material, movie S2).

## 4. Discussion

In this study, we were able to determine the adhesive mechanism that enables cribellate spiders to restrain prey for longer periods of time than would be predicted by the measured adhesive force of their capture threads on artificial surfaces. In fact, the predominant adhesive component, occurring in threads of all tested cribellate species, deploys the epicuticular waxes of insects, leading to an eightfold increase in adhesion force compared with wax-free surfaces.

The prey or rather its cuticular waxes are necessary for attachment and propagate a structural change of the capture thread. This structural change of the thread is in fact an advancing fluid matrix embedding the nanofibres. Because the propagation of the matrix stops as soon as one removes the elytra of *C. maculatus* (as prey surrogate), the elytra acts as a source for this matrix. In addition, metal surfaces coated with the epicuticular waxes could elicit similar effects. Combining these results, we conclude that the epicuticular waxes of the prey are the material of the matrix. This hypothesis is supported by the chemical analysis that revealed the presence of the cuticular waxes of *C. maculatus* within structurally changed capture threads. The propagation of the epicuticular waxes may be induced by capillary forces. Such forces were described previously in the context of water collection by cribellate threads, although the biological function of this collection remained controversial [24,25]. Epicuticular waxes outside the hardened exocuticle are actually a viscous fluid, which could be absorbed by the thread through capillary forces [26]. Because their viscosity is sensitive to temperature, it is not surprising that the velocity of the



**Figure 3.** Model of adhesive mechanism of cribellate capture threads. (a) Cross-section of an adhered cribellate thread, showing the nanofibres and the axial fibres embedded in the epicuticular waxes of the prey's cuticle. (b) Comparison of the adhesion mechanism of ecribellate and cribellate threads illustrates differences in their peeling behaviour due to employment of different adhesives. For detaching from a surface, single droplets of viscous glue (ecribellate thread) stretch between prey and capture thread, distributing the applied traction more equally between the points of attachment. By contrast, the single puffs of the cribellate capture thread peel off individually one after another. (Online version in colour.)

matrix propagation within cribellate threads increased after increases in ambient temperature.

These results indicate that a previously undescribed adhesive mechanism is deployed by the cribellate capture threads: the epicuticular waxes of the prey itself engulf the cribellate nanofibres. Thus, the prey itself actually contributes to establishing a fibre-reinforced composite-like bond with the capture thread (figure 3a). This embedding of the nanofibres in the prey's epicuticular waxes leads to an increased adhesion force between prey and cribellate thread, limited (in the case of *U. plumipes*) by the strength of the stabilizing axial fibres. We know of no other case where prey actually facilitates its own capture (excluding erroneous behaviour). Because the adhesive force we measured on wax-free elytra resembled the data generated on artificial surfaces, van der Waals' and hygroscopic forces still account for some attachment in the absence of epicuticular waxes [18]. Nevertheless, the predominant adhesive component, occurring in threads of probably all cribellate species, has to be the interaction with the epicuticular waxes of prey insects.

The adhesive forces generated by cribellate capture threads after absorbing the prey's cuticular waxes seem superior to those of ecribellate capture threads: adhesive forces similar to our data were measured for capture threads of the ecribellate *Argiope trifasciata*, a spider 35 times heavier than the cribellate *U. plumipes* examined here (*U. plumipes* is approximately 14 mg ( $n = 8$ )) [27]. A higher adhesive strength could explain why a cribellate spider can capture prey larger than itself and restrain it for longer periods of time [10,28]. The prolonged retention could also be favoured by an adhesive force independent of the length of the thread (figure 3b) [2,11]: to break the adhesion, each puff of nanofibres has to be peeled off individually and, hence, struggling prey has to keep up a constant opposing force for a long time. This lowered chance of the prey escaping might also account for the degeneration of the venom glands in Uloboridae, the only spider family in which these are absent [29]. Future studies are needed to evaluate these hypotheses.

If the capture technique of cribellate spiders is indeed superior, why do the great majority of recent species belong to the ecribellate spiders? In an evolutionary arms race scenario, selection pressure on insects living in a world

dominated by the basal cribellate spiders was high. The adhesive mechanism of cribellate threads, though, relies on the epicuticular waxes of the prey, the composition of which is most likely easy to change during an adaptive process. In fact, the diversity of epicuticular waxes even within one species is very high [26,30]. The waxes cannot be eliminated completely, because they are needed to minimize water loss and for communication [31,32]. The development of a shielding layer of different compositions (like the cement layer covering the waxes in some insects [33,34]) could prevent direct contact between capture thread and epicuticular waxes. Alternatively, waxes with very high viscosity should be inert to capillary forces generated between the nanofibres. Such adaptation could have promoted the development of alternative adhesives such as the viscous glue of ecribellate spider's threads. A viscous epicuticular wax layer on insects, however, is supposed to be detrimental for the glue of ecribellate capture threads [35]. Any adaptations to counteract threads with viscous glue would in turn favour the cribellate spiders and might be the reason for their long evolutionary history.

## 5. Conclusion

Capture threads of cribellate spiders absorb the epicuticular waxes of most insects to establish a fibre-reinforced composite, which increases the adhesive force by approximately eight times compared with wax-free surfaces. The discovery of this adhesive mechanism enables a new interpretation of the evolution of spider webs: the cribellate adhesive depends on the cuticular waxes of the insect prey. In an adaptive process, insects could have changed their cuticular composition, by shielding their epicuticular waxes (e.g. with the cement layer) or increasing the viscosity of their waxes. The entailing evolutionary arms race might have favoured the development of an inherent adhesive such as the viscoelastic glue of ecribellate spiders. To corroborate these hypotheses, future studies would have to determine the benefits and drawbacks of the cribellate silk adhesion in comparison to viscid silk. Knowledge of the influence of cuticular wax composition on the adhesion of insects immune to cribellate threads to viscid silk could help shed light on the evolution of spider adhesives.

**Ethics.** All species used in the experiments are not endangered or protected species. Special permits were not required. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

**Data accessibility.** Data supporting this article have been uploaded as electronic supplementary material.

**Authors' contributions.** R.A.B., W.B., P.B., and A.-C.J. designed the experiments. F.M. performed the cuticular wax analysis. All other experiments were performed by R.A.B. and A.-C.J. All authors

contributed to the writing of the manuscript and gave final approval for publication.

**Competing interests.** We declare we have no competing interests.

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