Invited Review
The Coiled Coil Silk of Bees, Ants, and Hornets

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ABSTRACT:

In this article, we review current knowledge about the silk produced by the larvae of bees, ants, and hornets [Apoidea and Vespoidea: Hymenoptera]. Different species use the silk either alone or in composites for a variety of purposes including mechanical reinforcement, thermal regulation, or humidification. The characteristic molecular structure of this silk is α-helical proteins assembled into tetrameric coiled coils. Gene sequences from seven species are available, and each species possesses a copy of each of four related silk genes that encode proteins predicted to form coiled coils. The proteins are ordered at multiple length scales within the labial gland of the final larval instar before spinning. The insects control the morphology of the silk during spinning to produce either fibers or sheets. The silk proteins are small and non repetitive and have been produced artificially at high levels by fermentation in E. coli. The artificial silk proteins can be fabricated into materials with structural and mechanical properties similar to those of native silks. © 2011 Wiley Periodicals, Inc. Biopolymers 97: 446–454, 2012.

Keywords: *coiled coil; recombinant proteins; hymenoptera; labial gland; silk*

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INTRODUCTION

Hymenoptera is one of the largest insect orders with over 144,000 species. The ability to produce silk has arisen at least six times within the order, primarily as a building material for construction of cocoons, elaborate nests, or webs to cover parasitised hosts.[1] This review focuses on silks produced from the labial gland of the larvae of bees, ants, and hornets—species within the superfamilies Vespoidea and Apoidea. These silks contain proteins with a well-defined coiled coil molecular structure.[2] In contrast, the dominant molecular structures of silk proteins found in other hymenopteran species are extended ß-sheet—akin to that found in spider dragline and lepidopteran cocoon silks—collagen-like or polyglycine-like.[2–5] For the sake of convenience, in this review, we use the term "coiled coil silk" to refer to the larval silks of Vespoidea and Apoidea species.

Coiled coil silk is used for a variety of purposes including the single-use cocoons of bulldog ant pupae, the cocoons constructed by bumblebees that are reused as honey and pollen storage vessels, and the elaborate nests constructed by social species that house multiple generations over entire seasons. Figure 1 shows the approximate evolutionary relationships of the species described in this review with some examples of how their silks are used.

MOLECULAR STRUCTURE

Early X-ray fiber diffraction data demonstrated that silk threads from solitary wasps or fibers drawn from honeybee silk glands contained α-helical proteins assembled into ordered coiled coil

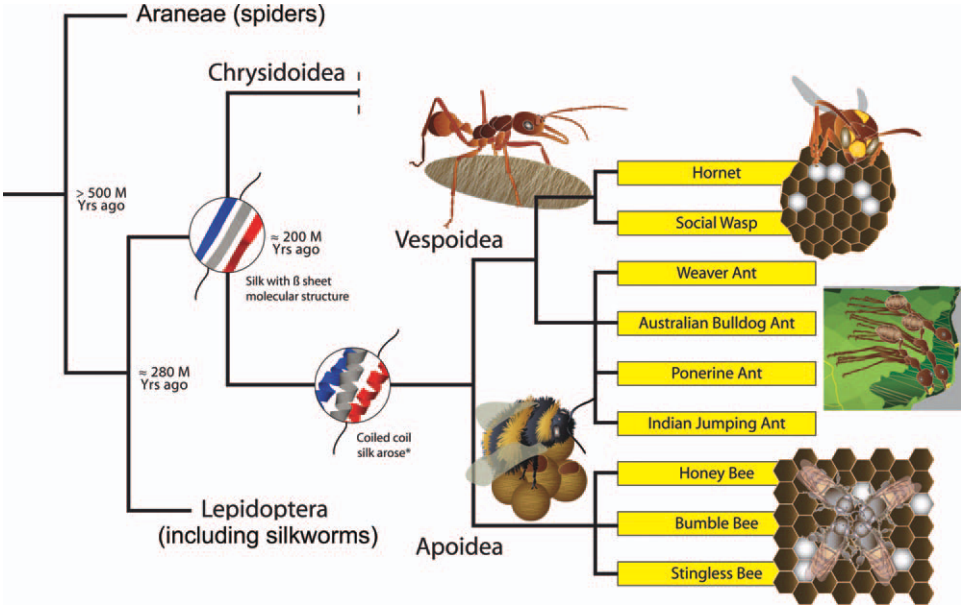

FIGURE 1 Schematic showing approximate evolutionary relationship of spiders, lepidoptera (silkworm), and Hymenopteran species described in this review, including Hornet (*Vespa simillima, V. dybowskii, V. crabo, V. manderina, V. ducalis, V. analis*, and *V. orientalis*); Social Wasps (Paper wasps: *Polistes annularis, P. versicolour, P. simillimus*; European wasp: *Vespula germanica*); Weaver ant (also known as tailor ant, *Oecophylla smargdinia*); Australian Bulldog ant (*Myrmecia forticata*); Ponerine ant (*Pachycondyla villosa*); Indian jumping ant (*Harpegnathos saltator*); Honeybee (*Apis mellifera, A. cerana, A. cerana japonica*); Bumblebee (*Bombus terrestris*); and Stingless bee (*Melipona bicolour; Scaptotrigona postica*). Hymenopteran phylogeny from Ref. 6.

structures.[7,8] Small-angle meridional reflections suggested an axial period of at least 28 nm, with the reflections varying slightly in different species.[8] The patterns from honeybee silk fibers was considered most consistent with a four-strand coiled coil structure with a tighter than expected superhelix radius (0.52 nm).[9] Discovery of the silk protein sequences (see later section describing proteins) provided an explanation of why the coiled coil packing was atypically tight: while usually the core of coiled coils contains large hydrophobic residues such as leucine and isoleucine, in coiled coil silk the most abundant core residue is the small amino acid alanine.[5]

X-ray diffraction patterns from weaver ant silk were reported to contain both the expected pattern attributable to the coiled coil structure and also a ß-sheet pattern,[10] although data were not shown. Our Figure 2 shows a direct comparison of X-ray diffraction data from natural honeybee and weaver ant silk sheets, obtained by synchrotron wide-angle X-ray scattering (WAXS) experiments as described elsewhere.[11] The plots demonstrate that scattering observed from the silk from bees (Apoidea) is dominated by the coiled coil signal, whereas the scattering from silk from the ants (Vespoidea) contains both coiled coil and beta-sheet signals with comparable intensity. Similarly, nuclear magnetic resonance (NMR) analysis identified coexisting α-helix and ß-sheet structures in both honeybee (Apoidea) and hornet (Vespoidea) silk, with the amount of ß-sheet being comparatively larger in the hornet silk.[12,13]

PROCESSING OF SILK PROTEINS

Optical and electron microscopy of silk gland fluids or fixed silk glands from a number of Apoidea and Vespoidea species

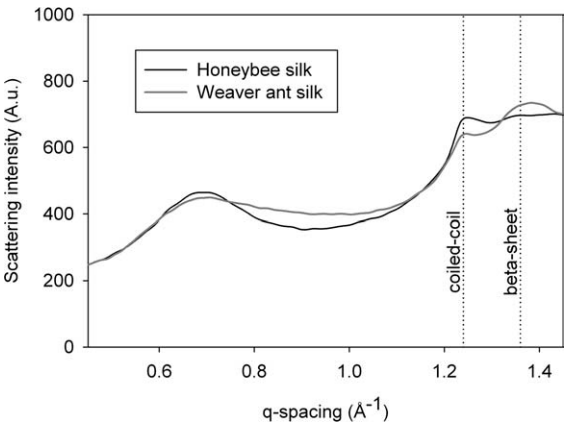

FIGURE 2 Comparison of WAXS plots of native European honeybee silk sheet and of native weaver ant silk sheet, examined under identical experimental conditions.

suggest that the silk proteins are highly organized in the gland lumen before spinning (Ref. 14: honeybee, bumblebee, and colonial wasps; Ref. 15; Ref. 16: honeybee; ponarine ants; Ref. 17: stingless bees, ponerine ants, and paper wasps). In honeybee glands, the proteins are ordered into cigar-shaped tactoids of up to 40 μm length, with cross banding along the tactoids of high-density, anisotropic bands 320–370 nm wide and low-density, isotropic bands 160–170 nm wide.[14,15] The majority of the silk proteins are organized into the tactoids, which are suspended in an isotropic solution with a low protein concentration.[15] Tactoids similar to those observed in honeybee silk glands are also observed in the silk glands of ants.[16] In bumblebees and colonial wasps, the macromolecular organization is slightly different: although some tactoids are evident the majority of the proteins are arranged into birefringent fibrous rods $\sim$1 μm long that are assembled laterally into large "fibrous bars." Electron microscopy of silk gland contents diluted into KCl solution shows that both the tactoids and the fibrous bars break up into long, fairly rigid fibrils (250–500 nm long for honeybee, over 1 μm long for bumblebees and wasps) that are about 4–5 nm in width.[14] High magnification analysis suggests that the fibrils are composed of pairs of thin filaments 2–2.5 nm wide, comparable to the expected diameter of a single tetrameric coiled coil. The organization of the coiled coil proteins in the silk gland has been proposed to (1) prevent coagulation of the proteins within the silk gland[2] and (2) reduce the flow viscosity of the protein solution in order to allow the concentrated silk dope to pass through the spinneret.[1]

There are several reports of chemical or physical changes that occur in coiled coil silks after formation of the silk fibers. The silk produced by larvae of the hornet is initially white but gradually darkens to a deep gray in a process described as tanning and postulated to involve the crosslinking of proteins.[18] Similarly, ant cocoon silks show evidence of tanning.[10] The nest silk of ants does not appear to tan but does change after spinning, with mats fabricated by weaver ants being hydrophilic when freshly made and hydrophobic when matured.[19]

SILK COMPOSITION

Coiled coil silk is primarily protein. Our quantitative amino acid analysis of clean silk from the end caps of European wasp nests recovered more than 75% of sample weight as amino acids, with some losses expected by this technique (unpublished result). As well as organic elements, significant levels of P, S, Cl, K, and Ca have been found in the silk of the hornet[18] and Mg, Al, Si, S, and K (0.04–0.13% weight) have been reported in weaver ant silk.[19] In honeybee silk, material from the Malpighian tubules and faeces are interposed between silken layers,[20] although the role of these materials is not clear.

The protein composition of the silks from a number of hornet species have been investigated after the silks were solubilized in concentrated lithium bromide and then analyzed by SDS-poly acrylamide gel electrophoresis.[12,21] The gels showed four major protein bands of comparable intensity with molecular weights of 35–60 kDa and one or two minor bands. The protein composition in other silks has been inferred from analysis of silk gland cDNA libraries and gene sequences. Coiled coil silk sequences have been identified from silk gland cDNA libraries from European honeybees,[22] hornets,[23] weaver ants, Australian bulldog ants, and bumblebees.[5] Silk genes have been isolated from Asian honeybee genomic DNA using oligonucleotides designed from European honeybee silk cDNA sequences.[24] Further genomic silk gene sequences can be identified by sequence homology in the recently published Indian jumping ant genome[25] (NCBI accession numbers: EFN81669, EFN81671, EFN81672, and EFN81674).

All species contain a copy of each of four paralagous genes that encode proteins of 29–45 kDa with a central block of $\sim$20 kDa predicted to form the characteristic coiled coil molecular structure (Figure 3). An additional gene, encoding $\sim$42 kDa protein, is found at high levels in the silk and silk gland of honeybees and bumblebees.[5,22] Approximately 35% of the weight of mature honeybee silk and the majority of the 42 kDa protein could be removed by boiling in sodium carbonate solution, suggesting that the protein is nonfibrous and may have a cementing or gluing role analogous to the role of sericin in silkworm cocoons. Although no homolog of the 42 kDa protein has been identified in ants or hornets, a nonrelated silk gene that encodes the minor proteins present in the hornet silk has been identified.[26]

CHARACTERISTICS OF SILK GENES AND PROTEINS

Four genes encoding fibrous coiled coil silk proteins are present in each Apoidea and Vespoidea species investigated. The amino acid composition of the proteins are similar, with each containing very high levels of alanine and high levels of serine, glutamic acid, leucine, lysine, glutamine, and threonine. The architecture of all the proteins includes a central region of $\sim$210 residues predicted in silico to form coiled coil structure, flanked by N- and C-termini regions of varying length (Figure 3). The Vespoidea species in general have longer terminal regions than the Apoidea species. NMR studies of hornet silk (Vespoidea) suggested that the alanine-rich central regions preferentially form alpha-helices, whereas the serine-rich terminal regions form beta-sheet structure.[12,23]

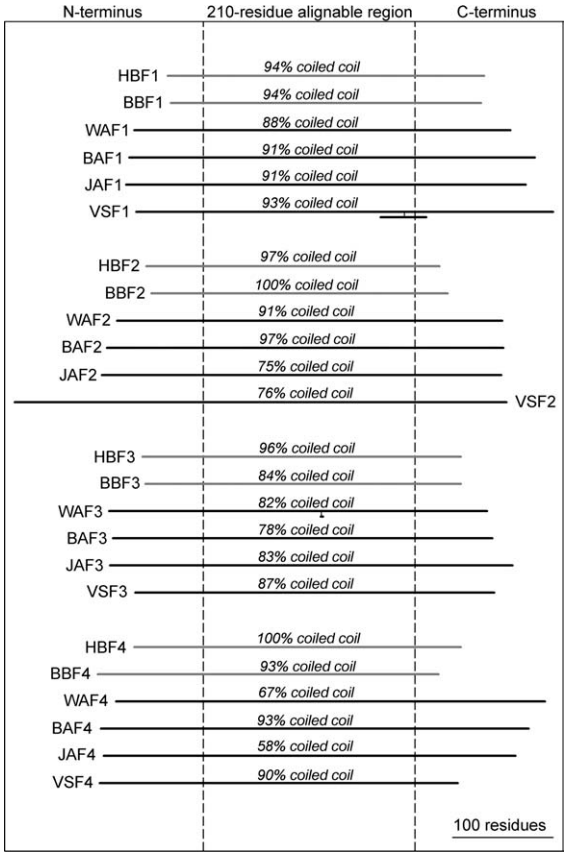

FIGURE 3 Schematic architecture of the four coiled coil silk proteins (F1–F4) of honeybees (HB, both European and Asiatic), bumblebees (BB), weaver ants (WA), and bulldog ants (BA), Indian jumping ants (JA) and hornets (VS). Apoidea species proteins are shown as gray lines and Vespoidea as black. Predicted levels of coiled coil structure within the conserved 210-residue central region are obtained from MARCOIL.

Sequence comparisons indicate that different regions of the silk proteins have different levels of sequence constraint. For example, pairwise alignment of the closely related silk proteins from European honeybees and Asiatic honeybees had on average 3% amino acid changes in predicted coiled coil core positions, 8% amino acid changes in predicted coiled coil noncore positions, and 14% amino acid changes in the N- and C-termini regions. Between proteins from the two superfamilies, the N- and C- termini have diverged beyond sequence recognition, whilst the residues in the coiled coil regions retain around 30% identity. The average amino acid composition in different coiled coil positions (Figure 4) is of interest since it is atypical of coiled coils, with the entire coiled coil and particularly the hydrophobic a and d core positions exceptionally rich in alanine, rather than the expected large hydrophobic residues. This would usually be expected to reduce coiled coil stability in solution. Reduced solution stability and specificity of the coiled coil silk proteins is likely compensated for by the high concentrations of the proteins in the silk glands.

The genetic organization of the fibrous silk genes in the superfamilies Apoidea and Vespoidea is not conserved, which is unsurprising since their genomes diverged over 150 million years ago.[27] The fibrous silk genes of honeybees (Apoidea) occur sequentially and in a single direction on the genome.[22] In comparison, the silk genes of the Indian jumping ant (Vespoidea) occur in a different order, are more widely spaced, are located on both strands of the DNA, and are interrupted by two intervening nonsilk genes (Figure 5). Despite the obvious genetic rearrangements that have occurred between the two genomes, each species has retained a single copy of each of the four homologous silk genes.

The retention of the genes encoding the four fibrous proteins in all seven Apoidea and Vespoidea species studied implies a critical role for each of these proteins in coiled coil silk. If the proteins were functionally redundant then gene duplication and/or deletion events would be expected in some lineages. The four silk genes are expressed at equivalent levels,[5] the proteins are produced in approximately equimolar quantities,[21] and the molecular structure of the silk is a tetrameric coiled coil.[9] The simplest explanation of these findings is that the proteins assemble into a hetrotetramer and that the hetrotetrameric structure is critical to either silk synthesis or silk function.

Recently, we demonstrated that a solution of a single recombinant honeybee silk protein could form coiled coils in solution and in solids[28] and therefore all four proteins are not required for formation of the coiled coil structure. The mechanical properties of silken fibers from single-protein or four-protein solutions were equivalent suggesting that multiple proteins do not improve the performance of mature silk. By deduction, it is likely that the critical role for all four of the silk fiber proteins are in the supramolecular assembly of the proteins in the silk gland before spinning.

SILK SPINNING BEHAVIOR

Spinning behavior has been studied in honeybee larvae, which build cocoons that both protect the pupae and reinforce the structure of the communal hive. Silk is generated from the labial gland as the larvae perform random head movements in all directions whilst moving in slow somersaults within the cell.[20] The colorless silk is produced through a slit-like spinneret located at the tip of the combined labium-hypopharynx. After spinning the larvae smear a small amount of material from the Malpighian tubules onto the hardened silk layers and faeces are also excreted between silk

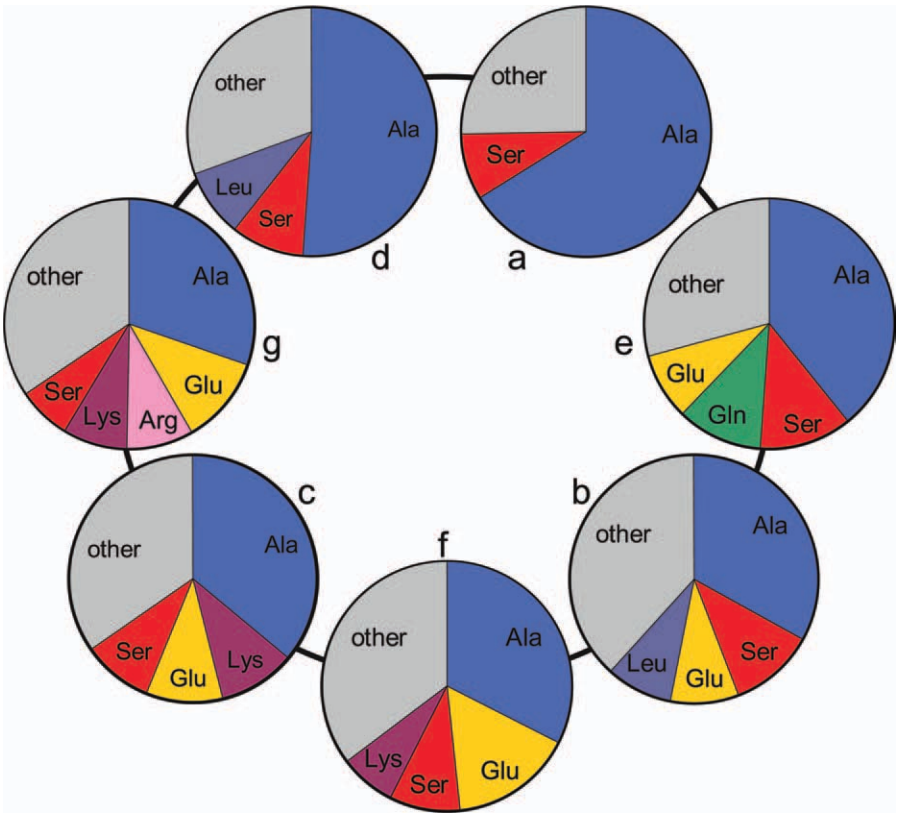

FIGURE 4 Schematic top–down view of one strand of a coiled coil generated from coiled coil silk proteins. Formation of coiled coils occurs when two strands of protein containing repeats of amino acids in the pattern HPPHPPP (where H are generally hydrophobic residues and P are generally polar residues) come together to shield the hydrophobic residues from the solvent. The heptad repeat is commonly denoted as a–g with the a and d positions corresponding to the core residues. Abundance of different amino acids in each position, averaged over all silk proteins in seven species, is shown in pie chart form.

layers. The entire spinning process of worker bee larvae takes 24–48 h.[20]

Hornets similarly produce cocoons as part of a larger nest structure. Hornets fabricate silk more rapidly than honeybees, completing the cocoon in 5 h.[29] Initially, the larvae fabricate a cap from fibers and small films that extends over the brood comb cell. The larvae proceed to spin the remaining covering from the end cap up to the opposite end and then back down to the end cap.[29] There is no evidence for deposition of additional material onto the silk.

Weaver ants make elaborate nests by binding together living leaves with silken curtains, and some species also make silken shelters for honey-dew producing aphids.[30] Final instar larvae produce the silk, with the adults controlling the process by holding the larvae between their mandibles. After being manipulated into position, the larvae touch their spinnerets to a surface and draw out a fiber. The adults then move them across the gap to be filled to the surface on the other side. Up to 10 adult-larva pairs may participate in a weaving session that lasts 5–10 min and results in a flat curtain of silk binding the leaves together.[30]

MORPHOLOGY OF SILK MATERIALS

The diameter of coiled coil silk fibers is related to the size of the insect, with nanoscale fibers produced by the minute weaver ant larvae (0.76 ± 0.32 μm diameter),[19] larger fibers produced by honeybee larvae (5.4 ± 1.5 μm diameter),[31] and larger yet fibers produced by hornet larvae (4–15 μm diameter).[18]

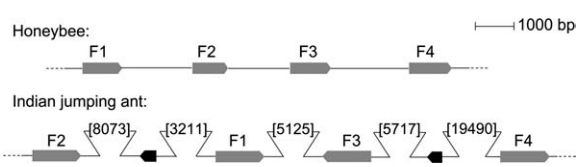

FIGURE 5 Comparative arrangement of coiled coil silk genes on the genomes of European honeybee (Apoidea) and Indian jumping ant (Vespoidea).

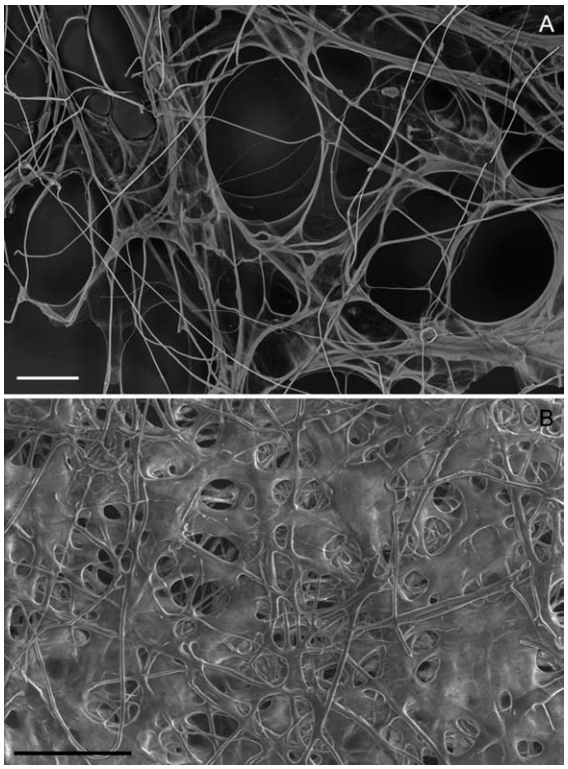

FIGURE 6 Scanning electron microscopy images of silk fibers and sheets produced by European honeybee larvae (A) or European wasp larvae (B). Scale bars are 100 μm. Late final instar honeybee larvae were induced to spin silk within plastic tubes, which were maintained at 30°C and 60% humidity to mimic their natural environment, and the clean silk was removed before the larvae added any further material. European wasp silk was harvested from freshly spun silk end-caps covering nest brood cells.

In close-up, hornet silk fibers appear grooved and comprised of multiple nanoscale fibrils.[18] Transmission electron microscopy of fiber cross sections suggests that each fibril has a fibrous core surrounded by an envelope of a different density.[29] The cross section of fibers produced by honeybee larvae transferred from hive cells to glass slides was smooth with no obvious internal nanostructure.[31]

In addition to fibers, Apoidea and Vespoidea species produce sheets from the same silk dope (Figure 6).[18,32] The spinneret of Apoidea and Vespoidea species including bumblebees (Figure 7), honeybees,[20] ants,[30,33] vespid wasps,[34] chrysidid wasps,[35] and sphecid wasps[36–38] is a long, thin slit ideally suited to the production of either fibers or films. The behavior of thin sheets of fluid is primarily driven by surface tension. Deformities in thin films will rupture into cylindrical fibers that are expected to have approximately the same diameter as the film's thickness. Consistent with this, Jay[20] observed that fibers were formed when the honeybee spinneret was drawn away from the cell wall. In contrast, films were formed when the spinneret was dragged over the cell wall,[20] presumably because the substrate stabilized the thin film. Mature coiled coil silk material consists of overlapping fibers joined together with thin sheets (Figure 6).

ROLE OF SILK IN COMPOSITES

The coiled coil silks are used as structural materials alone, as in weaver ant silk, or in composites with other materials. Honeybee nests are composites of wax and silk. Initially, the waxen comb cells are fabricated by adult bees, with some designed to store honey or pollen and others as brood comb to raise the young. The larvae add a layer of silk to the walls of the brood comb cells before pupation, and as successive generations of brood apply more silk to the walls, the proportion of silk can reach over 40% of the total nest mass.[39] Mechanical tests have found that older combs that are reinforced with more silk are stronger, stiffer, and more distensible than fresh waxen comb.[31,39] At temperatures >40°C, the silk-wax composite material is an order of magnitude stronger than the wax alone, suggesting that the silk provides important thermal stability.[31,39] Furthermore, the silk-wax composite can also absorb over 10% of its mass in water in comparison to only 3% water absorption in wax-only nests, suggesting that the silk is instrumental in maintaining the highly humid (~60%) environment found in brood comb.[40]

Wasps and hornets use silk combined with material collected from the environment, including tree bark and mineral matter.[18,41] Hornet pupae are extremely sensitive to temperature, with developmental damage occurring outside a 4°C temperature range,[42] but hornets are frequently found in deserts with up to 30°C daily temperature swings. Survival in these temperature extremes is made possible because the hornet nest maintains an almost steady temperature. The thermal regulation is attributed primarily to the larval silk: empty silk-free brood comb fluctuates in temperature according to the environment, whereas silk-containing cells do not.[42,29]

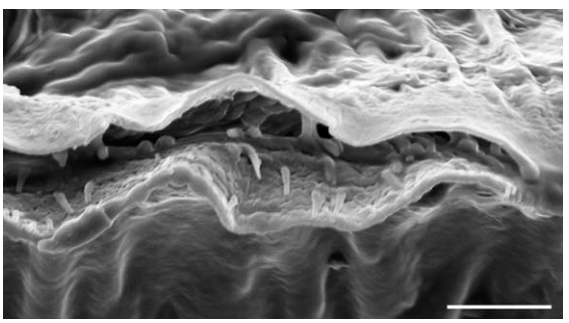

FIGURE 7 Scanning electron microscopy image of part of bumblebee spinneret. Scale bar is 10 μm. Total length of spinneret is ~200 μm.

Studies suggest that hornet silk functions as a thermostat, acting as an organic thermoconductor that can store energy from heat to be used later as required for thermal regulation.[43–49]

MECHANICAL PROPERTIES OF SILK FIBERS

Little mechanical data is available for naturally produced individual fibers of coiled coil silk, primarily due to the difficulty in obtaining lengths of material suitable for mechanical testing. Individual fibers from weaver ants are extensible by ~109% before breaking.[10] The elastic properties of coiled coil silk fibers are desirable for tough materials that can withstand movements of the nest under normal environmental stresses.

Mechanical properties have been obtained from silk threads that were hand-drawn from living honeybee larvae. At 65% relative humidity, the stress–strain curves of these fibers indicate a breaking stress of 132 MPa and breaking strain of 204%.[50] When submerged in water the fibers were less stiff, with breaking stress of 118 MPa and breaking strain of 235%. The curves are characterized by an initial linear region until 10% (wet) or 30% (dry) strain, followed by a decrease in the slope of the curve and then a return to linearity at around 100% extension.[50] Mechanical testing data were also collected from fibers spun by honeybee larvae removed from their wax cells and induced to spin on glass slides. Stress–strain curves obtained at 30% relative humidity indicated that these fibers were brittle, with breaking strength of 164 MPa and breaking strain of 4%.[31]

RECONSTITUTED SILK MATERIAL

Artificial silken materials are frequently fabricated from reconstituted silkworm silk proteins. When silk from hornets was reconstituted in a similar manner to silkworm silks by solubilization in saturated lithium bromide followed by extensive dialysis and concentration, it formed a hydrogel at comparatively low concentrations (1% weight/volume).[51] The hornet silk hydrogels could be pressed and dried to form transparent films with a protein structure mimicking the native silk, with coexisting α-helices and β-sheet.[51] Drawing dramatically improved the tensile strength of the dried films, with breaking strengths of 170 MPa reported for films drawn to twice their original length. Solid-state NMR and WAXS spectra showed that drawing induced a moderate transformation in the molecular structure of the films from α-helix to β-sheet in the predicted coiled coil region but no structural change in the termini regions. Drawn films showed an increase in birefringence, indicating a significantly higher level of molecular orientation in the drawn material and suggesting that the strength gains are primarily the result of an increase in intermolecular interactions between aligned proteins.[51]

RECOMBINANT SILK MATERIAL

The large size and highly repetitive nature of silkworm and spider silk proteins has prevented recombinant synthesis of full-length silk proteins from these species. In contrast, the coiled coil fibrous silk proteins are small (30–45 kDa) and only moderately repetitive and therefore can be produced by fermentation in *E. coli*. The four fibrous silk genes from the Asiatic honeybee were engineered to encode N-terminal tags of six histidines, expressed in *E. coli* at yields of 10–60 mg L^{-1} ferment then purified by nickel agarose affinity chromatography and FPLC.[24] NMR and near-UV circular dichroism (CD) in the presence and absence of denaturant indicated that 10 μM solutions of tagged proteins had no tight tertiary packing. Similarly, CD on 10–20 μM protein solutions showed mainly random coil structure and dynamic light scattering on 50 μM recombinant protein solutions showed particles of the size expected for monomers or very slight assembly.

Much higher expression levels were obtained when untagged European honeybee silk proteins were expressed into the inclusion bodies of *E. coli* (rather than the soluble fraction) with yields of 200–2500 mg L^{-1} ferment.[52] CD studies indicated that single honeybee silk proteins refolded from inclusion bodies formed predominantly coiled coil structure in solution and dynamic light scattering of protein solutions diluted in 0.1M NaCl measured particles with diameters similar to those predicted for coiled coils.[28] FTIR of the four fibrous proteins dried into films predicted that ~59% of the material had a coiled coil molecular structure, when compared with ~65% coiled coil in the native silk.[52] Fibers could be hand-drawn from concentrated (15% w/v) solutions of all four proteins, but solutions of single proteins precipitated before they could be concentrated to this level, supporting a role for the four different proteins in stabilizing the proteins in solution.[52]

Recombinant honeybee silk fibers of 40–60 μm diameter have been produced by extruding 3% w/v solutions of silk proteins into an aqueous methanol bath. Subsequent drawing of these fibers to two to four times their original length led to alignment of the proteins and gave the fibers mechanical strength comparable to native honeybee silk.[52] Fibers that were fabricated from solutions of a single protein using this method had essentially the same mechanical properties as fibers fabricated from mixtures of all four proteins, suggest-

ing that the role of the four different proteins is not connected to increasing mechanical strength.[28] Mats of much smaller fibers (around 200 nm diameter) have been produced by electrospinning concentrated (10–12%) solutions of one of the recombinant honeybee silk proteins mixed with 1% polyethylene oxide.[53] The proteins within the electrospun mats had predominantly coiled coil structure and were soluble in water before aqueous methanol treatment. After methanol treatment, the fibers promoted the attachment and proliferation of cells in cell culture assays.[53] The initial studies of recombinant coiled coil proteins suggest that the silk has potential applications as a biomaterial.

CONCLUSIONS

Coiled coil silks composed of a family of four fibrous proteins are conserved in all studied bee, ant, and hornet species [Apoidea and Vespoidea: Hymenoptera]. The silk is used for a wide variety of purposes either alone or adding functionality to composite materials. The availability of this versatile building material may have contributed to the immense evolutionary diversification and success of ants, which comprise 15–20% of terrestrial animal biomass.[54] The coiled coil silk proteins are small compared with the fibrous silk proteins of spiders and silkworms, and therefore can be produced as full length proteins by fermentation in *E. coli*. The native coiled coil silk self assembles within the silk gland before spinning, and key elements of this self assembly are replicated in reconstituted or recombinant silk, potentially allowing straightforward capture of native silk functionality in a biomaterial.

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