Supporting Information

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SI Text

SI Materials and Methods. All honeycombs used in this study were collected from the Bee Research Institute of Chinese Academy of Agricultural Sciences. The macrostructure of the honeycombs was observed by an optical microscope. The thickness of the uniform part of the cell wall was measured after cutting off the top 1 mm of the cell, which is bulbous (typical of the two-day-old (i.e., fresh), five-month-old and one-year-old combs) or tapered (typical of the two-year-old comb) (Fig. 1). To improve the accuracy of measurements, the thickness of the walls was also measured on the uniform part of sections cut along the axial direction of the cells (Fig. 1). We cut flat slabs from the cell walls and embedded them in epoxy. Then, we cut samples by an ultratome (LKB-2088) during which they were cooled by liquid nitrogen. These samples had smooth cross sections. We used them to examine the microstructure of the honeycomb by an environmental scanning electron microscope (ESEM, Quanta 200 FEG). For nonmetallic specimens, the use of an environmental SEM helps to avoid the accumulation of electric charge on the surfaces. The pressure of the gas in the chamber of ESEM however needs to be adjusted according to the conductive properties of the specimen. To obtain high quality images, the pressure of 0.8 Torr was chosen in our study. The temperature of the microscope stage was kept at 22 °C. To characterise the microstructure further, we carefully peeled thin layers (2–3 μm) off the old honeycomb wall using tweezers and examined them in a transmission light optical microscope (Olympus IX71).

We also weighed a rectangular slab (27×7 cells in size) of a one-year-old comb before soaking it first in hot water (100 °C), followed by alcohol, and finally acetone to dissolve the beeswax. We then weighed the dried residue to obtain the mass fraction of the silk cocoons.

The indentation modulus was measured using a TriboIndenter (Hysitron) with a standard 50 nm Berkovich diamond tip or a sapphire spherical (400 μm radius) tip. We used the multicycle testing method, in which a sequence of multiple loading-unloading cycles was applied at the same position. This method can determine the properties with varying indentation depths. However, to confirm that the multicycle testing method has no influence on the resulting elastic modulus, we also used a single load-unload cycle force control testing method with the same load parameters (see below). The two test methods gave almost identical results. The indentation loads, together with the corresponding displacement data, were used to determine the indentation modulus. In our experiment, 10 loading-unloading cycles were performed at each indentation point. The longitudinal indentation moduli of silks and the walls of the combs were measured on samples prepared in the same way as above for microstructural examination by ESEM. The transverse indentation moduli of silks and the walls of the combs were however measured on flat cut slabs of comb walls bonded to a metallic substrate. Optical and in situ images of the nanoindentations were taken to ensure that the local area of the indented surface was sufficiently flat. The local surface roughness for the transverse cross section of the silks and walls was about 40 nm, whereas that for the longitudinal surface was 90 nm. All indentation depths used in our study are larger than the corresponding surface roughness.

The nanoindentation test with the 50 nm Berkovich tip was carried out to determine the properties of the silk and the walls of the honeycomb at different ages. Before the nanoindentation test, we first checked the possible influence of the loading rate on the measurements on the fresh and one-year-old walls. We carried out indentation tests by using the same load and different loading rates 200 μN/s, 375 μN/s, 500 μN/s, 750 μN/s, 1000 μN/s, and found that they virtually had no influence on the results. As mentioned above, both the multicycle testing method and the force control mode were used. The maximum loads were set to 1000 μN for the fresh comb and 4000 μN for the one-year-old comb, respectively, resulting in the maximum indentation depth of about 3 μm for both combs.

According to the rule that the indentation depth should be limited to <10% of the film thickness (1), the load functions were characterized by the following: a constant loading/unloading rate of 50 μN/s, and a constant hold time at the peak load of 0.5 s prior to unloading to 40% of the peak load. The peak load was 300 μN for the silk (200 μN for the fresh comb wall), and only the readings with the maximum depth of indentation for the silk under 150 nm (100 nm for fresh comb wall), which is well below 10% of the diameter of the indented silk specimens (or the thickness of the fresh comb wall), were retained. At least 10 indentations at different locations were made. A different loading function, in which the peak load was 800 μN, with the loading/unloading rate of 200 μN/s and the hold time of 0.5 s, was used to determine the variation of the indentation elastic modulus across the wall thickness of the one-year-old honeycomb (maximum indentation depth of 1 μm). Five indentations were made in each layer. Samples from four different one-year-old combs were tested. The nanoindentation measurements with the 50 nm Berkovich diamond tip were carried out in an ambient condition at 28 ± 2 °C and $50 \pm 5\%$ relative humidity.

The microindentation with a sapphire sphere (400 μm radius) was carried out to measure the microindentation moduli of the honeycomb wall. The data obtained by nanoindentation (50 nm indenter) reflect an individual wax grain property rather than the bulk mechanical property of the wall, whereas microindentation (400 μm indenter) gives an average property of an ensemble of nearly two thousand wax grains, including the weak interfaces and pores. Before microindenting the fresh and old walls, we had measured the indentation moduli of the fresh wall by nanoindentation on the cross sections and longitudinal surfaces, and found them to be nearly the same, thus confirming the isotropy of the wax structures. It is well known that for isotropic materials, the indentation modulus E_r is related to other elastic constants by (1) $1/E_r = (1-\nu_s^2)/E_s + (1-\nu_i^2)/E_i$, where E_s, E_i, ν_s and ν_i are the elastic moduli and Poisson's ratios of the indented specimen and of the indenter, respectively. As the elastic modulus of the indenter is much larger than that of the honeybee comb specimen, the preceding relation may be approximated by $E_r \approx E_s/(1-\nu_s^2)$. As the wall of the fresh comb is isotropic, this relation was used to estimate its Poisson's ratio by substituting the microindentation modulus E_r and the elastic modulus E_s obtained from the tensile test. The Poisson ratio for the fresh comb is 0.313. The wall of the old comb is an anisotropic medium due the silks. Nanoindentation technique has also been used to measure the indentation moduli of anisotropic biomaterials (2–6), although for anisotropic media, the indentation modulus is a combination of the moduli in all directions (2, 3, 6), which does not follow the above simple relation between the elastic moduli and the indentation moduli for the isotropic media. Therefore, we also estimated the Poisson ratio of the old comb walls using another theoretical method independent of the measurements of the old comb walls. By regarding the old comb walls as fiber-reinforced composites, we can estimate the Poisson ratio of the old comb walls theoretically using the Mori–Tanaka scheme, which is known to be accurate for stiff fiber-reinforced composites. In this way, we got the theoretical estimates as 0.319 (five-month wall), 0.326 (one-year wall), 0.327 (two-year wall).

Microindentations were made on the longitudinal surfaces of the walls to ensure larger contact areas. A load profile with the peak load of 1000 μN, the loading/unloading rate of 200 μN/s, a hold period of 0.5 s, was applied to the fresh honeycomb. For the five-month-old honeycomb, the load profiles had the peak load of 4000 μN with the loading/unloading rate of 400 μN/s and hold time of 0.5 s, and the load profile for the one-year-old and two-year-old combs had the peak load of 6000 μN with the loading/unloading rate of 600 μN/s and hold time of 0.5 s. The maximum indentation depth for the walls of the fresh and old honeycombs was about 2.4 μm, which is well below 10% of the thickness of the indented wall specimens. Ten microindentations each were made on the fresh and old honeycomb walls. The microindentation measurements with the 400 μm sapphire spherical tip were carried out in an ambient condition at 23 ± 2°C and 30% relative humidity.

Tensile tests of slabs cut from the comb walls were conducted in a MicroTester (Instron 5848) at a displacement rate of 0.1 mm/ min, and at least 10 specimens each were tested from the fresh and old honeycombs. The slabs from the fresh comb were approximately 6.4 mm × 3.1 mm in size, and those from the old combs 5.9 mm × 2.8 mm. The macroscopic shear properties of the combs were measured on rectangular specimens approximately 8 × 15 cells in size, cut from the fresh and old combs. The specimens were bonded between two aluminum plates and the simple shear loading was realised by clamping and pulling the offset ends of the plates in the MicroTester. The nominal shear strength is the ratio of the maximum load to the entire surface area of the comb and not just that of the solid material. At least five specimens each were tested in simple shear from the fresh and old honeycombs at a displacement rate of 0.1 mm/ min. The tensile and shear tests were performed at 23 ± 2°C and a relative humidity 11 ± 1%.

Finally, the fresh and one-year-old combs fully laden with honey and bees were analyzed by the finite element method (FEM) using the commercial package ANSYS with the Solid Element 185. Beeswax behaves like a viscoelastic material whose mechanical properties vary with temperature and time. Our nanoindentation tests on the walls of the fresh and one-year-old combs show no obvious influence of the loading rate on the mechanical properties of the comb walls at 28 ± 2°C (see above). Moreover, the honeybee silk does not exhibit noticeable viscoelasticity at 23 ± 2°C (7). These results suggest that at 25 °C the fresh and one-year-old combs can be considered to behave elastically. Furthermore, the typical stress-strain curves obtained from tensile tests on the comb walls exhibit a distinct linear elastic behavior. Thus, for all the FEM analyses at 25 °C, we have calculated the stress and strain fields in the fresh and old combs using the linear elastic finite element model, along with the measured elastic constants of the walls.

It is known (2) however that fresh wax wall of the African honeybee comb softens when the temperature rises from 25 to 45 °C, losing its elastic modulus by a factor of 3.5 and its tensile strength by an order of magnitude, whereas those of an old comb wall that contains 34% silk cocoons by mass are considerably less sensitive to an increase in temperature. Given the fact, that the mass fraction of silk cocoons in the walls of Italian honeybee combs is practically equal to that in the African honeybee comb, it can be assumed that a similar temperature dependence prevails in the Italian honeybee combs. Thus, the elastic tensile and shear moduli of the fresh comb wall are assumed to reduce from 156 MPa and 59.41 MPa at 25 °C to 44.6 MPa and 16.98 MPa at 45 °C, respectively, while the moduli of the old comb wall remain unchanged. However, the viscoelastic behavior will determine the properties of the fresh comb at 45 °C for which a viscoelastic model is needed. It has been reported that mixtures of paraffin and the beeswax have the same time dependence of the elastic properties (8). Moreover, it is widely accepted (9) that viscous effects are observed only in the shear modulus, but not in the volumetric deformation of semicrystalline polymers. For the preceding two reasons, we have used the Prony model to obtain the shear relaxation modulus of beeswax $G(t)$ as a function of time t on the basis of the data from the dynamic shear test on the Cerita™ wax (paraffin wax) (9). The normalized shear relaxation modulus for the paraffin wax at 45 °C is $G(t)/G(0) = 0.134168 + 0.29698\,e^{-0.0017366t} + 0.3219\,e^{-0.027677t} + 0.1142\,e^{-0.40471t} + 0.049368\,e^{-4.4212t} + 0.083384\,e^{-36.853t}$ where the unit of the time t is second and $G(0)$ is the initial (elastic) shear modulus at time $t = 0$ when the load is first applied, which for the fresh comb wax is 16.98 MPa. We calculated the stress and strain fields in the wall of the fresh comb at 45 °C by the FEM during a period of 10 minutes under a constant load resulting from the weight of the honey and the worker bees.

The FEM computation was performed for a comb area with 8(horizontal) × 15(vertical) cells. The comb is filled with honey (density = 1400 kg/m^3) and covered by 26 worker bees (mass of each bee about 0.1 g) such that the total load is approximately 0.301 N. The load on each cell is 2.51 mN and is distributed along the cell span. The comb is modeled as a short cantilever (span of cell = 10 mm) with the back faces of the cells clamped, as shown in Fig. S1*A*. In view of potential stress concentration at the clamped end, the finite element mesh on the back part of each cell was finer than the rest of the cell (the smallest size of element was 10 × 40 × 125 μm), as shown in Fig. S1*B*. To avoid any free surface effects, we use the maximum stresses and strains of the innermost cell unit (1 × 15). At 45 °C, the creep deformation increases with time for about 10 minutes and remains almost constant thereafter. Because both the maximum displacement and shear strain occur at the bottom of the comb, the displacement along the direction of the load and the out-of-plane shear strain distribution in the bottom central cell of the fresh comb at 45 °C are shown in Fig. S1 *C* and *D*. The out-of-plane shear strain in the bottom central cell of the one-year-old comb is shown in Fig. S1*E*.

We also simulated the shear tests of the fresh and one-year-old combs using the FEM and the displacements at the maximum load measured in the tests (0.22 mm for the fresh comb and 0.34 mm for the one-year-old comb). The maximum shear strain of the fresh comb is found to be 4.3% at 25 °C and 1.5% at 45 °C. The maximum shear strain of the one-year-old comb is always 7% irrespective of the temperature. The corresponding out-of-plane shear strain distributions in the innermost cells (1 × 15) of the fresh and one-year-old comb at 25 °C are shown in Fig. S2 *A* and *B*. Note that apart from the corners near the clamped end where there is a strain concentration, the maximum shear strain away from these corners is roughly one half the values reported above (see Fig. S2 *A* and *B*). In other words, the strain concentration factor near the clamped corners is around 2.

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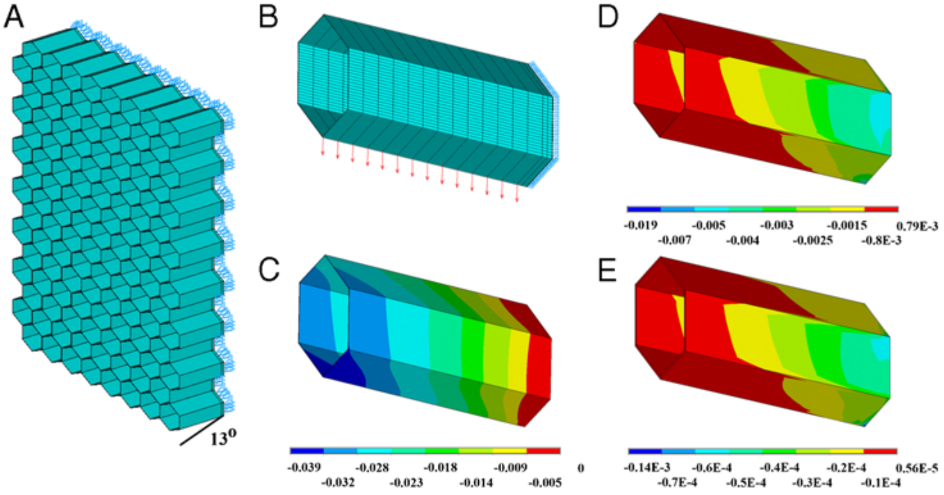

Fig. S1. Fresh and one-year-old combs fully laden with honey and bees were analyzed by the FEM. (*A*) A comb with 8 × 15 cells is modeled. The back of the comb is clamped. (*B*) One cell of the comb with the load distributed along its span (10 mm). (*C*) Displacement distribution in the direction of load in the bottom cell of the innermost cell unit (1 × 15) of fresh comb at 45 °C. Unit is mm. The maximum displacement is 39.4 µm when the temperature is increased to 45 °C and held constant for ten minutes. (*D*) Out-of-plane shear strain distribution for the bottom cell in the innermost cell unit (1 × 15) of fresh comb at 45 °C. The maximum out-of-plane shear strain reaches 1.9% after ten minutes and remains almost constant thereafter. (*E*) Out-of-plane shear strain distribution for the bottom cell in the innermost cell unit (1 × 15) of one-year-old comb at 25 °C. The maximum out-of-plane shear strain reaches 0.014%.

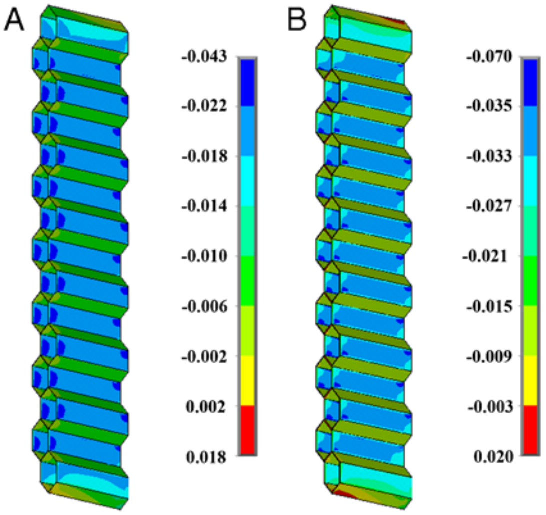

Fig. S2. Shear tests of the fresh and one-year-old combs at 25 °C were simulated by using the FEM. (*A*) Out-of-plane shear strain distribution in the innermost cell unit (1 × 15) of fresh comb under the displacement at the maximum load (0.22 mm). The maximum shear strain of the fresh comb is 4.3% near the corners, but otherwise 2.2%. (*B*) Out-of-plane shear strain distribution in the innermost cell unit (1 × 15) of one-year-old comb under the displacement at the maximum load (0.34 mm). The maximum shear strain of one-year-old comb is 7% near the corners, but otherwise 3.4%.