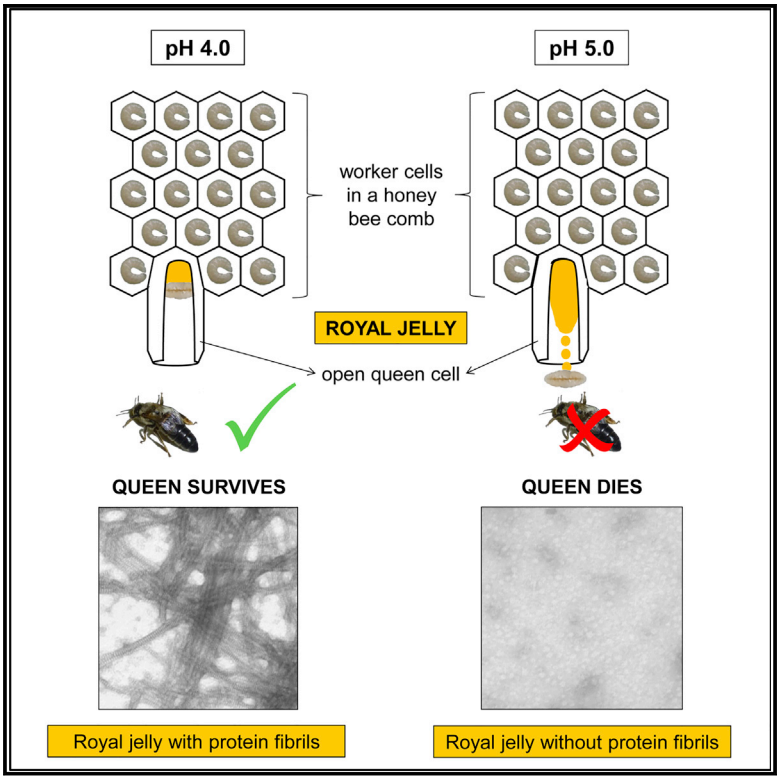
Report

Current Biology

How Honeybees Defy Gravity with Royal Jelly to Raise Queens

Graphical Abstract



Highlights

- Royal jelly ensures queen-bee development also by physical properties
- High-royal-jelly viscosity holds the queen larvae in place
- Nurse bees combine secretions of two head glands to ensure the high viscosity
- A fibril network of two proteins determines royal jelly viscosity

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In Brief

Honeybee queen larvae are raised in specific cells which are vertically oriented, opening downwards. Nurse bees deposit protein rich royal jelly at the cell ceiling as larval food. Buttstedt et al. show that two of these proteins are crucial for queen development. They control royal jelly viscosity, preventing the larva to fall out of its cell.

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How Honeybees Defy Gravity with Royal Jelly to Raise Queens

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SUMMARY

The female sex in honeybees (*Apis spp.*) comprises a reproductive queen and a sterile worker caste. Nurse bees feed all larvae progressively with a caste-specific food jelly until the prepupal stage. Only those larvae that are exclusively fed a large amount of royal jelly (RJ) develop into queens [1]. RJ is a composite secretion of two specialized head glands: the mandibular glands, which produce mainly fatty acids [2], and the hypopharyngeal glands, which contribute proteins, primarily belonging to the major royal jelly protein (MRJP) family [3]. Past research on RJ has focused on its nutritional function and overlooked its central role with regard to the orientation of the larva in the royal brood cell. Whereas workers are reared in the regular horizontal cells of the comb, the queen cells are specifically built outside of the normal comb area to accommodate for the larger queen [4, 5]. These cells hang freely along the bottom of the comb and are vertically oriented, opening downward [6]. Queen larvae are attached by their RJ diet to the cell ceiling. Thus, the physical properties of RJ are central to successful retention of larvae in the cell. Here, we show that the main protein of RJ (MRJP1) polymerizes in complex with another protein, apisimin, into long fibrous structures that build the basis for the high viscosity of RJ to hold queen larvae on the RJ surface.

RESULTS

MRJPs from Gland to Queen Cell

All MRJPs are synthesized into the endoplasmic reticula of the secretory cells in the hypopharyngeal glands at pH 7.0 [7] and subsequently stored in special secretion reservoirs [8]. However, in the larval food in the brood cell, they are subjected to more acid conditions (pH 4.0) as fatty acids from the mandibular glands are added to the secretions of the hypopharyngeal glands [9]. This difference in pH is known to affect the structure of the most abundant protein in RJ, MRJP1, which can be isolated from RJ as a monomer (monoMRJP1) and as an oligomer (oligoMRJP1) [10–13]. At neutral pH, oligoMRJP1 forms a tetramer in complex with four molecules of apisimin, a small 54-amino-acid-comprising protein that is also present in RJ [14, 15]. At pH 4.0, oligoMRJP1 is organized into higher-level oligomers and/or polymers of unknown structure [10]. To verify whether or not apisimin is also bound to MRJP1 at the native pH of RJ, pH 4.0, we purified oligoMRJP1 and monoMRJP1 by ion exchange chromatography from three different RJs (RJ1–RJ3) (Figure 1A, Figure S1, Figure S2, and Data S1). Whereas oligoMRJP1 was recovered in the flowthrough of the ion exchange chromatography resin at pH 4.0, monoMRJP1 bound to the resin and was subsequently eluted with increasing ionic strength. We copurified apisimin with oligoMRJP1 in all three RJs but never with monoMRJP1 (Figure 1B). To ensure that apisimin was not only coincidentally copurified, but actually bound to oligoMRJP1, we further analyzed the band representing oligoMRJP1 in native polyacrylamide gel electrophoresis (PAGE) (Figure 1C, 10% native PA gel). Subsequent sodium dodecyl sulfate (SDS) PAGE of this band revealed—besides the band representing MRJP1 (~60 kDa)—a second band migrating

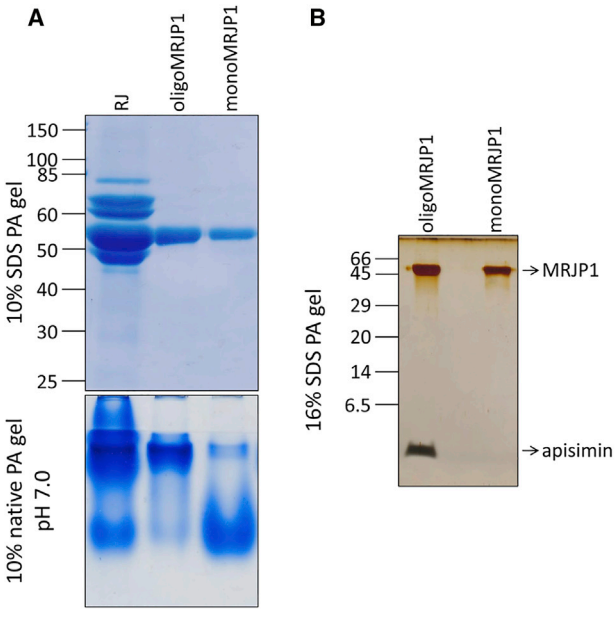

Figure 1. Protein Purification and Analysis

Protein marker bands are depicted as lines, and molecular weights are given in kDa.

(A) Purification of oligo and monoMRJP1 exemplarily shown for RJ3. The lane RJ shows the protein extract before purification. Protein identities were confirmed via mass spectrometry.

(B) Apisimin was only found co-purified with oligoMRJP1, but not with monoMRJP1.

(C) OligoMRJP1 of RJ1-3 was analyzed with native PAGE. The band representing oligoMRJP1 was cut and loaded on a 16% SDS PA gel.

See also Figures S1 and S2 and Data S1.

below 6.5 kDa, which has been identified by mass spectrometry as apisimin (Figure 1C, 16% SDS PA gel, Figure S2C, and Data S1).

To further elucidate the fate of the oligoMRJP1/apisimin complex from hypopharyngeal glands (pH 7.0) to RJ in the queen cell (pH 4.0), we studied the complex with analytical ultracentrifugation. Therefore, oligoMRJP1/apisimin isolated from RJ at pH 4.0 was dialysed against citrate-phosphate buffer with different pH values. At pH 7.0, 6.0, and 5.0, oligoMRJP1/apisimin sedimented as homogeneous species with sedimentation coefficients of 8.5 ± 0.2, 8.7 ± 0.1, and 9.3 ± 0.2 S, respectively (means ± standard errors [SE]) (Figure 2A squares and Table S1). At lower pH (4.75 and 4.5), sedimentation coefficients increased, confirming a pH-dependent change in size. However, instead of one homogeneous species, we found two different species: a slower- (pH 4.75: 10.7 ± 1.2 S; pH 4.5: 18.3 ± 6.5 S) (Figure 2A black circles) and a faster-sedimenting one (pH 4.75: 18.3 ± 2.5 S; pH 4.5: 35.0 ± 6.1 S) (Figure 2A red circles) (Table S1). Finally, at pH 4.0, the native pH of RJ, oligoMRJP1/apisimin exhibited sedimentation coefficients higher than 50 S, and homogeneous species could no longer be observed suggesting high level polymerization of the complex.

To visualize the different levels of polymerization, we used electron microscopy to show that oligoMRJP1/apisimin forms a protein fiber network at pH 4.0 (Figures 2B and 2C). These fibrous structures dissociated into smaller oligomers at pH 5.0. The process was completely reversible, and even after dissociation of the fibrils into smaller oligomers at pH 7.0, the fibrous structures reassembled again after decreasing the pH (Figure 2B, pH 4.0 → pH 7.0 → pH 4.0). These reassembled fibrils had the same structure as those directly isolated at pH 4.0 and were composed of disk-like subunits with a mean disk height of 3.40 ± 0.07 nm (mean ± SE) and a fibril width of 9.39 ± 0.14 nm (n = 60) (Figure 2B). Although fibril formation of MRJP1 has already been observed [12], its pH dependence and biological effects had not been addressed.

The Importance of pH for RJ Viscosity

We evaluated the consequences of pH-dependent oligoMRJP1/apisimin fibril formation for RJ viscosity via rheological measurements. 350 μL sodium hydroxide with different molarities were added to 5 g RJ to adjust the pH from 4.0 to 5.6. RJ viscosity was then characterized by determining the shear-rate-dependent flow behavior (Figure S3A). The viscosity at a shear rate of 500 s^{-1} was plotted against the pH, and Figure 3A illustrates the decrease in viscosity with increasing pH. Whereas below pH 4.3 viscosity was above 170 mPa × s (174.8 ± 6.6 to 177.1 ± 11.8 mPa × s), it decreased to below 70 mPa × s above pH 5.0 (67.7 ± 6.3 to 64.2 ± 2.1 mPa × s) (Figure 3A). The data were fitted to an equation describing a sigmoidal curve with a transition midpoint at a pH of 4.57 ± 0.03 (χ^2 = 115.00). This pH-dependent decrease in RJ viscosity was well in line with the dissociation of the oligoMRJP1/apisimin fibrils into smaller oligomers with increasing pH from 4.0 to 5.0.

In order to show that the pH-dependent viscosity change of RJ is indeed caused by proteins, we incubated water-diluted RJ (1:1) at pH 4.0 with Proteinase K at 34°C for up to 72 hr and compared the viscosity with water-diluted RJ controls (Figure 4A and Figure S3B) (Kruskal-Wallis ANOVA, df = 10, H = 40.34, n = 44, $p < 0.001$). The viscosity in the controls was the same within the first 2 hr (16.6 ± 1.2 mPa × s to 17.9 ± 1.7 mPa × s) and did slightly increase after 7 hr (19.6 ± 0.8 mPa × s) and further increased after 72 hr (23.7 ± 1.7 mPa × s), albeit not significantly. In strong contrast, protease treatment reduced viscosity slightly already after 30 min (13.4 ± 0.2 mPa × s), and viscosity decreased even further after 7 and 72 hr of protease treatment (11.7 ± 0.3 mPa × s and 10.8 ± 0.1 mPa × s) being significantly different from the control samples (7 hr, $p = 0.032$, $z' = 3.44$; 72 hr, $p < 0.001$, $z' = 4.40$). Thus, the degradation of proteins causes a lower viscosity of RJ (Figure 4A).

To further narrow down the role of the oligoMRJP1/apisimin fibril network for RJ viscosity, we tested the protein complex purified from RJ between pH 4.0 and 6.0 (Figure 4B and Figure S3C). Again, we found a clear impact of pH on viscosity (Kruskal-Wallis ANOVA, df = 7, H = 31.91, n = 36, $p < 0.001$). Although the protein complex in solution at pH 6.0, 5.0, and 4.5 did not show significantly increased viscosity compared

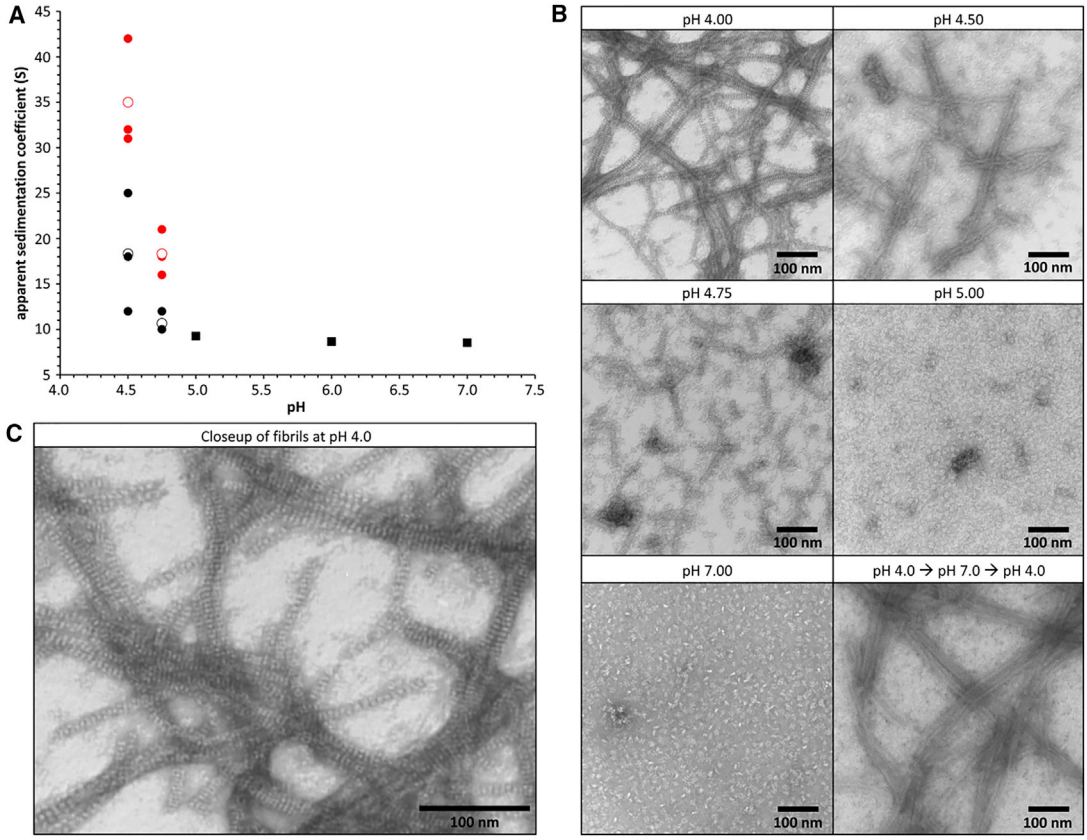

Figure 2. Polymerization of OligoMRJP1/Apisimin Depending on pH

(A) Analytical ultracentrifugation. From pH 7.0 to pH 5.0, a stable sedimentation velocity was measured for all three replicates (black squares). At pH 4.75 and 4.5, two species were observed: one exhibiting a lower (black circles) and a second one with a higher sedimentation velocity (red circles). Closed circles show individual measurements for oligoMRJP1/apisimin purified from RJ1-3 and open circles show means. See also Table S1.

(B) Electron micrographs of RJ3 oligoMRJP1/apisimin forming fibrillary structures depending on pH.

(C) Fibrils at pH 4.0 are composed of disk-like subunits.

to buffer controls ($p > 0.1$, $z' < 2.90$), the viscosity of oligoMRJP1/apisimin solutions gradually increased with decreasing pH (pH 6.0 = 2.00 ± 0.13 mPa × s; pH 5.0 = 2.23 ± 0.06 mPa × s; pH 4.5 = 2.40 ± 0.18 mPa × s), whereas buffer viscosity did not (Figure 4B) (1.78 ± 0.02 to 1.81 ± 0.05 mPa × s). Finally, at pH 4.0, the viscosity of oligoMRJP1/apisimin solutions (3.16 ± 0.07 mPa × s) was significantly higher compared to the buffer control (1.82 ± 0.04 mPa × s) ($p = 0.03$, $z' = 3.29$). Thus, the pH-dependent fibrillary structures resulting from the oligoMRJP1/apisimin complex are indeed involved in the pH-dependent viscosity change of RJ.

We established a bioassay to show the biological relevance of this pH-dependent viscosity change of RJ by testing the pH-dependent ability of RJ to hold larvae in queen cells. We used standard queen-rearing equipment where a bee larva is placed with a grafting tool in an artificial queen cell cup filled with RJ (Figure S4). The cups were then turned into a vertical position, and larvae that adhered to the RJ were counted (Figure 3B, Movie S1) ($n = 10$ per pH). At pH 4.0, all larvae remained in the queen cell cups even overnight; four out of ten larvae fell out of the cups immediately at pH 4.8. The drop rate of larvae increased to 90% at pH 5.3, and all larvae fell out of the cells at pH 5.9 ($\chi^2 = 26.50$, $p < 0.00001$). Thus, RJ pH needs to be at 4.0, which is indeed the pH naturally occurring in the biological system, to ensure that the royal larvae do not fall out of their brood cells.

DISCUSSION

We have shown that oligoMRJP1 in complex with apisimin acts as a structural protein, ensuring that the viscosity of RJ is such that the queen larvae are retained in position in their vertically oriented cells to complete their development. This is similar to other structural proteins, e.g., collagen, keratin, and actin [16], that confer a greater degree of viscosity to the otherwise more fluid biological components. In case of oligoMRJP1/apisimin, this fibril formation is strongly pH-dependent and proceeds from smaller oligomers between pH 7.0–5.0 to long fibrous structures at pH 4.0, the pH of native RJ in the brood cell. Similar pH-dependent mechanisms for protein fibril formation can be found in a number of other biological systems; the repeat domain of the human premelanosome protein (Pmel17), where fibrils serve as structural scaffolding required for melanin synthesis, only forms fibrils below pH 6.0 in acidic melanosomes that dissolve at neutral pH [17]. Silk proteins produced by spiders and silkworms are extremely soluble at high pH (~8.0) in their secreting glands and change confirmation to fibers in response to ionic strength,

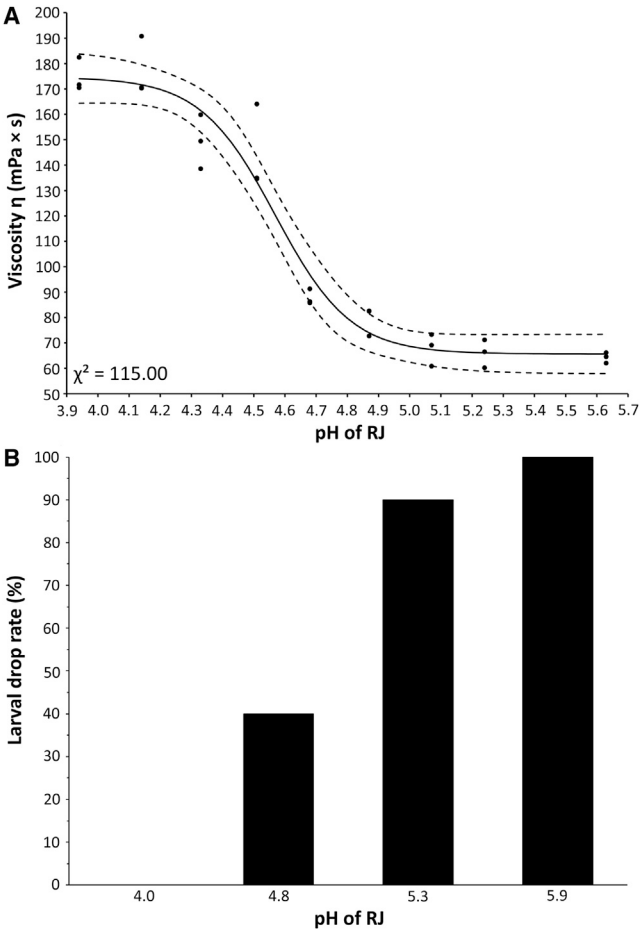

Figure 3. pH Dependency of RJ Viscosity

(A) For viscosity analysis of pH-changed RJ, 350 μL NaOH with different molarities were added to 5 g RJ to achieve a pH range between 3.96 and 5.63. Data measured at a shear rate of 500 s^{-1} were plot in dependence of pH and fit to an equation describing a sigmoidal curve (solid line), including the 95% confidence interval (dashed lines). As reference, viscosity of pure RJ was also measured and determined to be 253.4 ± 9.7 mPa × s. See also Figure S3A.

(B) Capability of RJ to hold a larva in a queen cell. Larvae were placed on top of queen cell cups filled with RJ (n = 10 larvae per pH). After turning the queen cell cups downward, larvae still adhered to the RJ were counted.

See also Figure S4 and Movie S1.

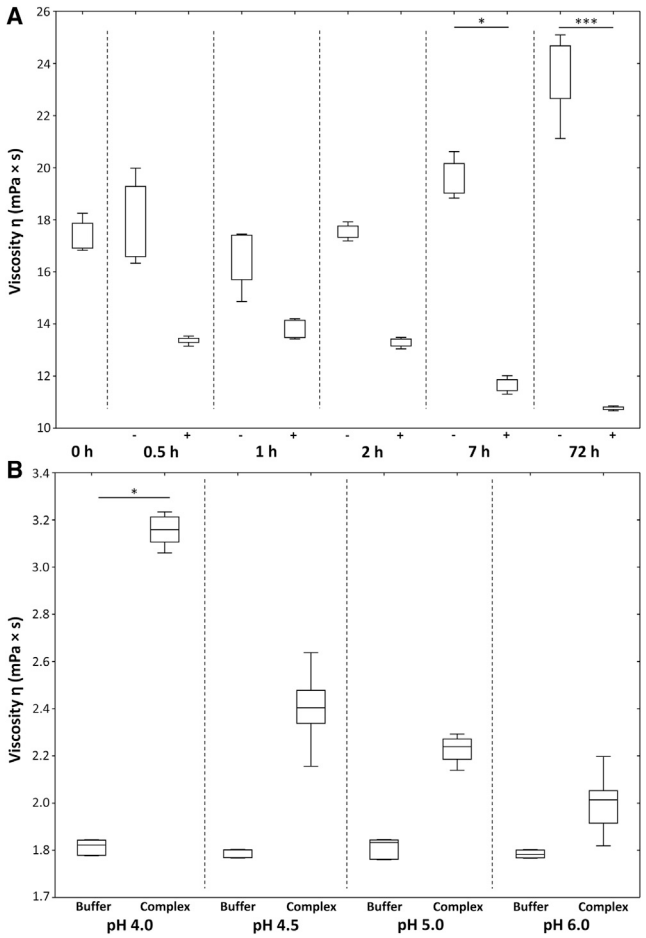

Figure 4. Protein-Dependent Viscosity at 34°C

Boxes show medians ± 25% percentiles and whiskers show the non-outlier range. Significant differences (Kruskal-Wallis ANOVAs) are marked by asterisks, *p < 0.05; ***p < 0.001.

(A) Viscosity of RJ incubated with proteinase K (+) compared to controls without proteinase K (−).

(B) Viscosity of the oligoMRJP1/apisimin complex at different pHs compared to the buffer controls.

See also Figures S3B and S3C.

shear forces, and also decreasing pH (~6.0) when they are transported through the gland to the opening pore [18]. In both cases, the resulting fibrils are amyloids characterized by a typical β sheet structure in cross-β conformation known to bind the dye Thioflavin T [19]. However, both the disk-like structure of the oligoMRJP1/apisimin fibrils and the inability to bind Thioflavin T (data not shown) make an amyloid structure of these fibrils unlikely.

Along the secretion pathway of MRJPs from within the secretory cells of the hypopharyngeal glands to the lumen of the secretory duct, the pH becomes more acidic the closer the secretion comes to the plasma membrane (endoplasmic reticulum, pH ~7.0; Golgi network, pH ~6.3; secretory vesicles, pH ~5.5) [7]. However, these values are still high enough to maintain a liquid phase, which, at this stage, allows for a smooth passage through the glandular ducts. Viscosity only needs to be high in the RJ in the queen cell so as to hold the larva in place. The highly concentrated proteinaceous hypopharyngeal gland secretion is released into the collecting duct and reaches the mouth cavity of the honeybee with a pH of 5.1 ± 0.1 [9], still at low viscosity as the oligoMRJP1/apisimin complexes form smaller oligomers at this pH (Figure 2). Indeed, it is the fatty acids from the mandibular glands that increase the acidity of RJ to pH 4.0. It is not exactly known when and where the hypopharyngeal gland secretion is mixed with the mandibular gland secretion, which is rather acidic (pH 3.9 ± 0.1) [9]. As the mandibular glands open through ducts on the inner margin of the mandibles [1] and as the nurse bees deposit two differently colored secretions (clear and white) into the brood cell [9, 20], a mixing of both secretions outside of the bees' body is most likely. This is when the oligoMRJP1/apisimin complexes form the fibril networks and increase viscosity to the levels required to attach to the cell

bottom and hold the larvae. Thus, MRJP1 is indeed crucial for queen rearing, but not as erroneously claimed in its monomeric form as physiological caste determinator [21], which has been clearly rebutted [10]. The current evidence suggests that quantity, rather than the quality, of the larval food is the trigger for queen determination [22–24]. In any case, it is obvious that the correct viscosity of RJ is vital for queen rearing in honeybees; if the larva falls out of the cell, food quality becomes irrelevant. These physical requirements are achieved by oligomeric MRJP1 in complex with apisimin, which is of particular importance in the entire genus *Apis*, where the workers construct upside-down-oriented queen cells [25].

It is highly adaptive that RJ secretion in honeybees is handled by two separate specialized head glands. It is the mixture of the hypopharyngeal and mandibular gland secretions that reduces pH levels such that the oligoMRJP1/apisimin complexes form fibril networks to achieve the required RJ viscosity. The nurse bees eventually control the viscosity of RJ when filling a queen cell by simply combining the secretions of two independent glands, an extraordinary adaptation that is even more sophisticated than the two-component adhesives used in human technology since it also serves as excellent food.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, one movie, and one data file and can be found with this article online at https://doi.org/10.1016/j.cub.2018.02.022.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.B. and R.F.A.M.; Methodology, A.B., G.H., H.L., C.H.I., and S.-H.S.; Validation, A.B., G.H., H.L., and C.H.I.; Formal Analysis, A.B.; Investigation, A.B., C.I.M, G.H., H.L., and C.H.I.; Resources, C.I.M., G.H., H.L., C.H.I., M.P., S.-H.S., and R.F.A.M.; Writing – Original draft, A.B. and R.F.A.M.; Writing – Review & Editing, A.B., C.I.M., G.H., H.L., C.H.I., M.P., S.-H.S., and R.F.A.M.; Visualization, A.B.; Supervision A.B. and R.F.A.M.; Project Administration, A.B.; Funding Acquisition, C.I.M., M.P., and R.F.A.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Royal jelly 1	Cluj-Napoca, Romania – July 2015	N/A
Royal jelly 2	Bratca, Romania – August 2015	N/A
Royal jelly 3	Cluj-Napoca, Romania – June 2015	N/A
Royal jelly for viscosity analysis	Naturprodukte Lembcke GbR	Cat#1395
Chemicals, Peptides, and Recombinant Proteins		
Sulfopropyl (SP) Sepharose Fast Flow	GE Healthcare	Cat# 17-0729-10
Proteinase K	Carl Roth	Cat# 7528
Software and Algorithms		
HAAKE RheoWin	Thermo Fisher	http://www.rheowin.com/rheowin43.htm
Origin 5.0	OriginLab	https://www.originlab.com/
Sedfit	[26]	http://www.analyticalultracentrifugation.com/download.htm
Statistica 8.0	Statsoft	http://www.statsoft.de/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Anja Buttstedt (anja.buttstedt@gmail.com).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Royal jelly samples

Fresh *Apis mellifera* royal jelly (RJ) samples used for protein purification were acquired in 2015 from three different beekeepers in Romania (RJ1 - Cluj-Napoca, Cluj County - July / RJ2 - Bratca, Bihor County - August / RJ3 - Cluj-Napoca, Cluj County - June). Due to limited quantities of fresh RJ, experiments regarding the viscosity of RJ (see viscosity analysis of royal jelly) were performed with RJ purchased from Naturprodukte Lembcke GbR (Faulenrost, Germany). All RJ samples were stored at −20°C.

METHOD DETAILS

Protein purification from royal jelly

RJ protein extract was prepared in 20 mM sodium citrate/citric acid, pH 4.0 (buffer A) and oligoMRJP1 and monoMRJP1 were purified via cation exchange chromatography according to Buttstedt et al. [10]. Briefly, 1 g of RJ was dissolved to a total of 10 mL in buffer A. After centrifugation of the extract (8,500 × g, 10 min, 4°C) and dialysis of the supernatant against the same buffer (Spectra/Por 6 Dialysis Membrane MWCO: 25 kDa, Spectrum Laboratories, Rancho Dominguez, CA, USA), the dialysate was loaded onto 1.5 mL Sulphopropyl (SP) Sepharose Fast Flow (GE Healthcare, Little Chalfont, UK). OligoMRJP1/apisimin was recovered in the flow through and found to be during the experiments presented in this study in the fibrillary state. Thus, this method is suitable to directly purify fibrillary oligoMRJP1/apisimin from RJ. The column was then washed with 10 mL buffer A, followed by 10 mL of buffer A containing 200 mM NaCl. Monomeric MRJP1 was eluted with 10 mL of buffer A containing 300 mM NaCl. Throughout the purification, 1 mL fractions were collected and protein content in the fractions was determined by following the absorption at 280 nm on a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) (Figure S1A). The purity of protein containing fractions was verified with sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and pure fractions were combined. For further experiments, buffer exchange was performed with PD-10 desalting columns (GE Healthcare, Little Chalfont, UK). The flow through containing oligoMRJP1/apisimin showed a normal protein absorption spectrum with a higher absorption at 280 nm than at 260 nm only after buffer exchange (Figure S1C). Protein concentrations were determined after buffer exchange via UV spectroscopy. The molar extinction coefficient was calculated with ProtParam [27]) as 56,185 M-1 cm-1 for MRJP1. Apisimin does not contain any Tryptophan or Tyrosine and does thus not show any absorption at 280 nm. The molecular weight of glycosylated monoMRJP1 was determined with mass spectrometry as 51.3 ± 2.0 kDa (Figure S2B). Apisimin has not been shown in the literature to be posttranslationally modified and has a molecular weight of 5.5 kDa (Protparam, ExPASy Bioinformatics Resource Portal).

Protein analysis via polyacrylamide gel electrophoresis

Proteins were analyzed with SDS-PAGE in 10 or 16% acrylamide gels in 25 mM Tris, 187 mM Glycin, 0.1% (*w/v*) SDS [28] at 175 V for 70 min or with native PAGE (10% acrylamide) in 50 mM NaH_2PO_4 / NaOH, pH 7.0 according to Gallagher [29] at 20 mA for 6 h. Gels were stained either with 0.08% (*w/v*) Coomassie Brilliant Blue G250 in 1.6% (*w/v*) phosphoric acid, 8% (*w/v*) ammonium sulfate, 20% methanol (*v/v*) [30] or with silver according to the short silver nitrate staining protocol in Chevallet et al. [31]. For the silver staining, gels were fixed twice for 30 min in 30% (*v/v*) ethanol, 10% (*v/v*) acetic acid, followed by two incubations for 10 min in 20% (*v/v*) ethanol and two additional incubations for 10 min in bidestilled water. Subsequently, gels were soaked for 1 min in 0.8 mM sodium thiosulfate followed by two washing steps (1 min each) in bidestilled water. Gels were stained for 20 min in 12 mM silver nitrate, rinsed for 10 s in bidestilled water and developed in 3% (*w/v*) sodium carbonate, 25 μl formalin and 12.5 μl 10% (*w/v*) sodium thiosulfate per 100 mL bidestilled water. Color development was stopped with 4% (*w/v*) Tris, 2% (*v/v*) acetic acid and gels were washed with water before scanning.

Analytical ultracentrifugation

Analytical ultracentrifugation was conducted in a Beckman XL-A centrifuge (Beckman Coulter, Palo Alto, CA, USA) with an An50Ti rotor. Measurements were performed at 40,000 rpm, 20°C with 10 μM oligoMRJP1/apisimin in 50 mM Na_2HPO_4/citric acid, 150 mM NaCl, pH 4.0-7.0. Protein sedimentation was recorded every 10 min at 280 nm during centrifugation. Data analyses and calculation of the sedimentation coefficient were done with the software Sedfit [26].

Electron microscopy

For electron microscopy analyses, 2 μl protein solutions (approximately 2 mg/mL) were applied on formvar coated copper grids. After 1 min of adsorption, excess liquid was blotted off with filter paper. Subsequently the grids were air-dried for 30 s, washed with water (3 times for 1 min), placed on a droplet of 2% aqueous uranyl acetate and drained off after 1 min. The dried specimens were examined with an EM 900 transmission electron microscope (Carl Zeiss Microscopy, Jena, Germany) at an acceleration voltage of 80 kV. Electron micrographs were taken with a Variospeed SSCCD camera SM-1k-120 (TRS, Germany).

Mass spectrometric analysis

For mass spectrometric identification, the proteins were prepared and analyzed as described in Buttstedt et al. [10]. Therefore, a fourfold volume of ice-cold acetone was added and the solutions incubated overnight at −20°C to precipitate the proteins. Proteins were centrifuged at 14,000 x g for 15 min and the pellets resuspended in 0.4 M ammonium bicarbonate, 8 M urea. Protein disulfides were reduced with DTT and cysteines were alkylated with iodoacetamide. Trypsin was added after dilution of the protein solution to a final concentration of 1 M urea and incubated overnight at 37°C. Peptides were analyzed by LC/MS/MS on an Ultimate 3000 RSLC nano-HPLC system (Thermo Fisher Scientific) coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). Samples were loaded onto a trapping column (Acclaim PepMap C8, 300 μm x 5 mm, 5 μm, 100Å, Thermo Fisher Scientific), and washed for 15 min with 0.1% TFA at a flow rate of 30 μL/min. Peptides were eluted and separated at a flow rate of 300 nL/min using a 90 min gradient from 1 to 40% solvent B (separation column: Acclaim PepMap C18, 75 μm x 250 mm, 2 μm, 100Å, solvent A: 0.1% formic acid in water, solvent B: 0.08% formic acid in acetonitrile). During gradient elution, online MS data were acquired in data-dependent MS/MS mode: full scans were acquired over the m/z range 300-1500 every 5 s (R = 120,000 at m/z 200, AGC (automated gain control) target value 4x105, max. injection time 50 ms). Within these 5 s, the most abundant signals of the full scans were selected for MS/MS experiments: HCD product ion spectra were acquired in the orbitrap analyzer at a resolution of R = 15,000 at m/z 200 (quadrupole isolation window 2 Th, 27% normalized collision energy, AGC target 5x104, max. injection time 200 ms). Exclusion time was set to 60 s, dynamic exclusion was enabled. Data analysis was performed using the Proteome Discoverer 2.0 (Thermo Fisher Scientific). MS/MS data of precursor ions in the mass range 700-5000 were searched against the Swissprot Database (version 5/2013, 256,924 entries) using Sequest HT. Mass accuracy was set to 5 ppm and 0.02 Da for precursor and fragment ions, respectively, oxidation of methionine and protein N-terminal acetylation was set as possible modifications, carbamidomethylation of cysteines as fixed modification and two missed cleavages of trypsin were allowed.

Due to only two trypsin cleavage sites in apisimin at position 1 and 7, apisimin was digested with chymotrypsin instead of trypsin before mass spectrometric analysis. However, in all three bands representing apisimin (Figure 1C) only one apisimin peptide (KTSISVKGESNVD) was found representing the N terminus of the protein (Uniprot ID: Q8ISL8; KTSISVKGESNVDVVSQINSLVSSIVS GANVSAVLLAQTLVNILQILIDANVFA) (Figure S2C). The chymotrypsin cleavage might have generated too small peptides that are in addition very hydrophobic and do thus not elute from the used C18 column. This difficulty to detect apisimin with mass spectrometry due to the high hydrophobicity of resulting peptides has already been described by Tamura et al. [13].

Molecular weight of monoMRJP1 was determined as 51.3 ± 2.0 kDa (Figure S2B) using matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry. Briefly, purified proteins were desalted by solid phase extraction (ZipTip C4, Merck Millipore, Darmstadt, Germany).and co-crystallized with a saturated sinapinic acid solution in 50% acetonitril, 0.1% TFA on a MALDI target plate. Spectra were acquired using an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) in linear positive mode.

Viscosity analysis of royal jelly

Viscosity of all samples was measured at 34°C with a rotational rheometer (HAAKE MARS 2, Thermo Fisher Scientific, Waltham, MA, USA) using plate and cone (60 mm / 1°) geometry with a titanium layer. All experiments were conducted at a constant plate gap of 0.051 mm and a sample volume of 1 mL. First, constant rotation at a shear rate of 100 s^{-1} was applied for 30 s to adjust temperature. Viscosity η was further measured applying a ramp in shear rate ranging from 0.1 to 1000 s^{-1} within 120 s. For all measurements, the viscosity η was plotted against the shear rate γ (Figure S3).

For viscosity analysis of pH-changed RJ, 350 µl NaOH with different molarities were added in triplicates to 5 g RJ to achieve a pH range between 3.96 and 5.63. The pH was measured after adding 5 mL of distilled water (pH 7.0) to the remaining pH-modified RJ (~4 g) using a digital pH meter (FiveEasy FE20, Mettler Toledo AG, Schwerzenbach, Switzerland). The viscosity over pH at 500 s^{-1} was fitted to the sigmoidal Boltzman equation with Origin 5.0 (OriginLab, Northhampton, MA, USA) with η_{MIN} = minimal viscosity and η_{MAX} = maximal viscosity:

$$\eta = \eta_{MAX} + \frac{(\eta_{MIN} - \eta_{MAX})}{1 + \exp\left(\frac{pHm - pH}{dpH}\right)}$$

In addition to the finally used fit described beforehand, data were also fitted to an equation describing a two-state titration:

$$\eta = \frac{\eta_{MIN} + \eta_{MAX} \times 10^{(pH-pK)}}{1 + 10^{(pH-pK)}}$$

However, this fit (χ^2 = 220.86) was not as good as the fit according to the Boltzman equation (χ^2 = 115.0) and thus the Boltzman equation was used to fit the data and it can be excluded that a single titratable group is responsible for the viscosity change.

To evaluate the dependency of RJ viscosity on intact proteins the following protease experiment was performed in duplicate: 5 g RJ were diluted with 5 g bidestilled water and 1:200 proteinase K was added (179 µl of 20 mg/mL stock solution in bidestilled water) based on a RJ protein concentration of 14.3% (Intertek Food Services GmbH, Bremen, Germany). The samples were incubated at 34°C for 0.5, 1, 2, 7 and 72 hr and PMSF was added to a final concentration of 2 mM (100 mM stock solution in isopropanol) to inactivate poteinase K. Subsequently, the samples were stored at −20°C and viscosity was measured immediately after thawing. Negative controls were processed in the same way but by adding bidestilled water instead of proteinase K. The viscosity recorded in duplicate per sample was used at a shear rate of 500 s^{-1} for statistical analysis with STATISTICA 8.0 (StatSoft, Tulsa, OK, USA). Log-transformed data fulfilled the criteria for normality (Shapiro-Wilk test, $p = 0.11$) but not for homoscedasticity (Levene's test, $p < 0.001$) and thus original data were further analyzed using a Kruskal-Wallis analysis of variance (ANOVA) followed by Bonferroni adjusted Dunn's post hoc test using z test statistics.

For measuring viscosity of the purified oligoMRJP1/apisimin, the complex was purified from two different RJs and 4 mL each three times dialysed for 2 hr against 2 l of 50 mM Na_2HPO_4/citric acid, 150 mM NaCl, pH 4.0, 4.5, 5.0 and 6.0. Protein concentration was set to 8.5 mg/mL in all samples. Again viscosity (triplicate measurements per pH and purified complex) recorded at a shear rate of 500 s^{-1} was used for statistical analysis. Data were analyzed as above using a Kruskal-Wallis ANOVA as they did not fulfill criteria for normality and homoscedasticity (Shapiro-Wilk and Levene's test, $p < 0.001$).

To determine the effect of the pH on the capability of RJ to hold larvae in a vertical cell, commercial queen cell cups facing upward were filled with RJ adjusted to pH 4.0, 4.8, 5.3 and 5.8. Ten four days old worker larvae taken from brood combs were placed on top of the RJ droplet in each cell (Figure S4). After turning the queen cell cups downward, all larvae still adhering to the RJ were counted (Movie S1). Differences in drop numbers were analyzed with a chi-square test.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analysis were performed with STATISTICA 8.0 (Statsoft) and details regarding the analyses were for clarity reasons explained directly after the described experiments in the method details section. Significance was defined in any case as $p < 0.05$