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RESEARCH ARTICLE

REVISED A queen's tale: An experimental palaeoproteomic study of a honey bee queen cell specimen from Natural History Museum Denmark

[version 2; peer review: 2 approved, 1 approved with reservations, 1 not approved]

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Abstract

Background

Natural history museum specimens of historical honey bees have been successfully used to explore the genomic past of the honey bee, indicating fast and rapid changes between historical and modern specimens, possibly as a response to current challenges. In our study we explore a potential untapped archive from natural history collections - specimens relating to historical beeswax.

Methods

We examine an *Apis mellifera mellifera* queen cell specimen from the 19th century. The intact and closed cell was analysed by X-ray Computed Tomography (CT) to reveal a perfectly preserved queen bee inside her cell. Subsequently, a micro-destructive approach was used to evaluate the possibility of protein extraction for a palaeoproteomic approach.

Results

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Using proteomics by liquid-chromatography-tandem mass spectrometry, we were able to recover 120 non-contaminant proteins, mostly bee-related, including major royal jelly proteins (MRJPs) crucial for queen nutrition and development, and silk fibroin proteins. Analysis of the protein modifications indicate their endogenous source and show the presence of *in vivo* glycosylation. This probably helped protein preservation and allows for the study of antimicrobial and metabolic functions of bees in the past.

Conclusion

Our exploratory results show that studies on specimens such as these can provide biomolecular information. Such specimens can inform future research on queen development, diet, and hive conditions, providing valuable context for understanding how honey bee populations have responded to changing environments and pressures over time. This highlights further the scientific potential of natural history collections in addressing contemporary challenges in honey bee conservation and health.

Plain language summary

This study investigated a 19th-century queen bee cell from a natural history museum to reveal a perfectly preserved queen bee and successfully extracted proteins from the inside of the cell with information about the development and diet of the bee. These findings offer valuable insights into historical bee rearing practices, which can help us better understand and address current challenges faced by honeybees.

Keywords

Apis mellifera, honeybees, queen bee, beeswax, MRJPs, X-ray Computed Tomography, palaeoproteomics, natural history museum collections

article can be found at the end of the article.

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REVISED **Amendments from Version 1**

This version incorporates updates and clarifications made in response to reviewer comments, as well as additional input and refinements from the authors. In response, we have carefully revised the paper to better reflect our findings, reorganized and expanded the study (e.g. introduction) to more clearly state our research questions and contextual background, and restructured the methods section to better separate experimental and analytical approaches. All reviewer comments are also addressed point-by-point and incorporated to the study to the best of our ability. We are grateful for the constructive feedback, which has helped improve our study.

Any further responses from the reviewers can be found at the end of the article

Introduction

Today, natural history museums play a crucial role in advancing scientific knowledge, offering invaluable resources for research into biodiversity, evolution, and climate change. They serve as educational hubs, fostering public understanding and appreciation of the natural world through exhibits and outreach programs. Additionally, these museums are vital for conservation efforts, providing historical baselines to track environmental changes and inform protection strategies for endangered species and habitats.

Therefore, these museum collections can act as reservoirs of biological knowledge, especially since they contain varieties of natural material collected throughout time and space. One such reservoir is the collections of honey bee material that can be relevant to understand changes that have occurred over time to the species, especially those caused by anthropogenic factors. The western honey bee, *Apis mellifera*, is a eusocial insect vital for its irreparable role in ecology, agriculture and economy (Crane, 1999; Lamei, 2018). Natural history museum collections, with their preserved specimens and historical data, can offer critical insights into the evolution, health, and behaviours of honey bee populations over time. By studying past collections of *Apis mellifera*, researchers can identify genetic diversity, disease patterns, and environmental impacts, aiding in the development of effective conservation strategies in Europe and the rest of the world; this will help to protect and sustain honey bee populations, which are essential for pollination and ecosystem health (Kasso *et al*., 2023b; Parejo *et al*., 2020).

There have been studies successful in extracting DNA from museum specimens of bees (Lozier & Cameron, 2009; Mikheyev *et al*., 2015; Parejo *et al*., 2020; Strange *et al*., 2009), which is especially important for observing the decrease in genetic diversity that has occurred in honey bees over time (Espregueira Themudo *et al*., 2020). Beeswax-containing historical material such as medieval sealing wax, has also been investigated through genomic approaches (Kasso *et al*., 2023a; Szulc *et al*., 2020). As an alternative to ancient DNA, palaeoproteomics explores ancient proteins that could be used to explore questions relating to paleogenomics and microbiology (Hendy *et al*., 2018), while also being more likely to survive than DNA over extended time scales. Proteomics have been applied to modern honey bees successfully for insights into development (Feng *et al*., 2009; Zheng *et al*., 2011), pathogens and disease (Arad *et al*., 2025; Han *et al*., 2013) and major royal jelly, including its impact to the development and resistance of bees (Bíliková *et al*., 2009; Buttstedt *et al*., 2014; Drapeau *et al*., 2006; Mureşan *et al*., 2022; Zhang *et al*., 2014b) However, a purely palaeoproteomic approach has not been attempted before.

While each bee has an important role in the colony, it could be valuable to focus research on queen bees specifically. The queen is the only bee without which the entire hive will not survive; she is the mother of all bees in the colony (Woodward, 2007). Through her pheromones, the colony maintains its order and functions that reflect the requirements of the colony, e.g. the need for foraging (Crane, 1999; Woodward, 2007). Human led queen rearing (i.e. raising and replacing) has been a prominent practice in beekeeping ever since the 19th century, as the quality of the queen significantly affects the whole hive, including its reproductivity and resistance to disease (Crane, 1999; Woodward, 2007). In Denmark, the bees currently used in beekeeping are a developed hybrid species from 3–4 subspecies of *Apis mellifera*, which has replaced the native bee to the area, *Apis mellifera mellifera*, the European Dark bee (i.e. *den brune bi*), that has almost disappeared from Denmark (Nielsdatter *et al*., 2021).

In this study, we used both X-ray Computed Tomography (CT) and palaeoproteomics to investigate a queen cell specimen (Figure 1) from the Natural History Museum Denmark collections, which holds several historical honeycomb specimens. This study was exploratory, to complement the DNA work that has been done on museum bee specimens and to better understand the potential of these techniques applied to historical material. This is especially true of the palaeoproteomics approach, since it has so far not been applied to historical bee specimens. If ancient proteins can be extracted from such specimens, it is feasible that, with more samples, similar work to that applied on modern specimens could be performed on historical specimens to better understand development and disease over time.

An experimental study to extract proteins directly from historical beeswax was also conducted; but due to limited results it is available as Extended Data (ED) (See Data Availability, ED 1).

Methods

The queen cell

The specimen (Figure 1), made accessible by Natural History Museum Denmark, and is estimated to be from the late 19th century, based on the hand-written label (Figure 1b) of the sample, which reads *"Apis mellifica L 2 Dronnningecell Ulvedal 7.88 Borries M 8.4.89"*. "8.4.89" could be interpreted as the date in 1889 when the specimen was collected or recorded by the museum, or it could simply be a catalogue number. Ulvedal could be Ulvedalen in Denmark and Borries



Figure 1. The queen bee cell specimen from all sides. a. Piece of honeycomb with queen cell specimen shown from all sides. Figure 1**b**. Museum label of the specimen. Figure 1**c**. Close-up of top of the cell (far right): the left one is open and was possibly never occupied, the right one is capped.

the name of the beekeeper - yet no historical archival information was found to support this, so we must leave this to speculation. "*Apis mellifica* L" indicates European Dark bee (*Apis mellifera mellifera*), which today is rare in Denmark. It was displaced through the introduction of other subspecies and hybrids, such as the Italian *Apis mellifera ligustica*, which was introduced to Denmark ca. 1860 (Nielsdatter *et al.*, 2021).

The beeswax specimen is formed of two queen cells, with one cell capped (Figure 1c) and the possibility of having an undisclosed queen inside. Therefore, the specimen was studied with X-ray Computed Tomography (CT) to non-destructively image the interior of the closed cell.

X-ray computed tomography

The CT-scanning was performed at the 3D Imaging Centre at DTU using a ZEISS Xradia 410 Versa micro CT, which allows a resolution of a few micrometres and typical sample sizes from millimetres to a few centimetres. The maximum power of the instrument is 10 W, and the energy of the employed X-rays can be varied in the range 40–150 keV to optimise contrast that allows for the separation and configuration of different materials, such as the chitinous insect from the lipidic beeswax. The sample was scanned without a filter using 40 kV and 6.6 W, the large field of view objective and a 2x2 detector binning. 3201 projections were acquired with an exposure time of 2 s per projection, leading to a full scanning time of 3 hours. The reconstructed voxel size was 40.6 µm which results in an approximate resolution of 100 µm. During the scanning process, only 2D X-ray images were captured at different angles as the sample was rotated 360 degrees. However, the queen inside the cell was not visible in these images. However, the reconstruction of the 3D volume and the subsequent visualisation using the ThermoFisher Avizo software (https://www.thermofisher.com/dk/en/home/electron-microscopy/products/software-em-3d-vis/avizo-software.html) allowed the bee to be seen and to segment different components of the image in different colours. Similar analysis and visualisation can be performed with the software tool ITK-SNAP (www.itksnap.org) (Yushkevich *et al.*, 2006).

Palaeoproteomics

From the reconstructed 3D image obtained from X-ray CT, a layer of material can be seen beneath the queen bee (see Figure 2B). It was decided to attempt extracting this material for ancient proteins, as it was considered unethical to sample the queen itself, due to the limited sample size of still encapsulated historic bees. Accessing the queen would have required opening the cell and this would risk damaging or destroying the specimen irreparably, posing limitations for future research to study the morphology of the bee and its genetic material.

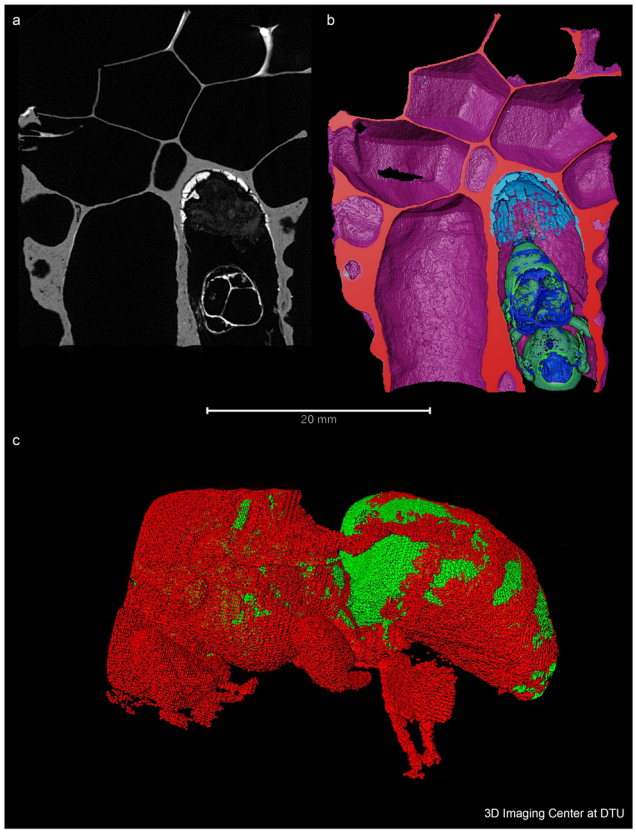

Figure 2. A 2D, 3D volumetric and 3D representation of the bee. a. A 2D slice showing one plane of the 3D volume obtained by X-ray CT. The bee is located in the bottom right cell and the residual layer appears bright on the top of this cell, meaning that it has a higher density or is composed of materials with higher atomic number than the material in the darker areas.. This plane is used to virtually cut the volume which enables the view shown in Figure 2**b**. Figure 2**b**. A 3D volumetric representation, colour coded according to the identity of the different materials. Note the residual layer (illustrated in light blue) at the bottom of the cell. Figure 2**c**. 3D representation of the bee isolated from the wax.

Protein extraction and mass spectrometry

For the micro-destructive extraction of proteins from the queen cell, 100 µL extraction buffer was injected through the wall of the capped cell near the unknown material, using a syringe with a 1mm diameter needle. The extraction buffer was prepared (75:5:4:8:8) with 8M guanidine hydrochloride (Sigma-Aldrich cat. no. G7294), 1M Tris (Invitrogen™ cat. no. 10055704), 0.5M tris(2-carboxyethyl)phosphine) (Sigma-Aldrich cat. no. 646547), 0.5M chloroacetamide (Sigma-Aldrich cat. no. 22790), and ultra-pure H_2O (AccuGENE™ Molecular Biology Water cat. no. 7732-18-5). It was delicately partially aspirated and dispensed in and out of the cell two times. After this careful resuspension, the extraction buffer was fully aspirated and placed into a 1.5 mL Protein LoBind tube (Eppendorf cat. no. 022431081). An immediate observation was a colour change of the buffer from transparent to translucent dark brown, indicating that the extraction was likely successful in retrieving sample material from inside the cell. The following analysis was performed according to best practice for palaeoproteomic samples to limit modern contamination, including the use of a dedicated laboratory space and nitrile gloves (Hendy *et al.*, 2018). A blank extraction was also performed to control for laboratory contamination. The sample and blank were first incubated at 80°C for 1h. Protein quantification was made using bicinchoninic acid (BCA) assay Pierce™ BCA Protein Assay Kits cat. no 23225. This confirmed that a sufficient quantity of protein was extracted and available for downstream processing. The sample was then digested first with 1 µL (0.4 µg/µL) of Lys-C (Promega cat. no. V1671) at 37°C for 1 h, followed by digestion with 1 µL trypsin (0.4 µg/µL) trypsin (Promega cat. no. V5111) overnight at 37°C. The purification of peptides was subsequently performed via StageTips (Rappsilber *et al.*, 2007). The peptides were eluted in 30 µL of 50% acetonitrile (ACN, Thermo Fisher Scientific/Pierce cat. no. 51101) 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich cat. no. T6508).

The samples were analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS) using an EASY nLC 1200 (Proxeon, Odense, Denmark) coupled to a Exploris 480 mass

spectrometer (Thermo Scientific, Bremen, Germany), based on methods already published for historical samples (Mackie *et al.*, 2018). The elutions were vacuum centrifuged at 45°C until approximately 5 µL of sample remained. Samples were then resuspended in 8 µL 5% ACN 0.1% TFA in water. 2 µL of each sample was injected for measurement. The samples were separated on an in-house laser-pulled 15 cm column (75 µm inner diameter, Polymicro Technologies cat. no. TSP075375) and packed with 1.9 µm C18 beads (ReproSil Pur 120 C18-AQ, Dr. Maischcat. no. r119.aq)) over a 77 min gradient with increasing ACN in 0.1% formic acid (FA, Merck/Supelco cat. no. 5330020050). In short, the MS parameters were as follows: MS1- scan range of 350–1400 m/z, 120k resolution, maximum injection time (IT) of 25 ms, and an AGC target of 300% in Top 10 mode. MS2- 60k resolution, maximum IT of 118 ms, minimum intensity 2e5, AGC target 200%, normalised collision energy of 30%, a dynamic exclusion of 20 s, and an isolation window of 1.2 m/z. To hinder cross-contamination, a wash-blank method using 0.1% TFA, and 5% ACN was run in between each sample.

Protein identification and modification data analysis

The raw MS/MS data was analysed in two steps. Firstly, a primary screening was made using MaxQuant (v.1.6.3.4, RRID:SCR_014485) (Cox & Mann, 2008), with a tryptic search of the Swiss-Prot database (downloaded 10/02/22, RRID:SCR_021164) to determine the possible sources of protein in the sample. Search parameters were the defaults for an Orbitrap mass spectrometer. Modifications were as follows: fixed carbamidomethylation of cysteine; variable oxidation of methionine, deamidation of asparagine and glutamine, and pyroglutamic acid of glutamine and glutamic acid. Error tolerances were 10 ppm for the precursor and 0.02 Da for the fragmentions, and the false discovery rate (FDR) was set to 1%. Minimum score cut-off was 60. Identifications from this search led to further searches of the Uniprot (RRID:SCR_002380) honey bee proteome (unspecific digestion search), and a search each for the known Uniprot proteins from *Aspergillus* and *Penicillium* species (trypsin specific), followed by more specific species proteomes with an unspecific digestion search.

All hits from these search paths were then combined into a final database and searched with the same modifications as above but with semi-specific trypsin specificity (max peptide length of 8–25 amino acids), based on the results of the previous searches. The discovered proteins and peptides were then authenticated and identified: proteins with only one peptide detected in the entire dataset were discarded, as well as contaminants clearly deriving from the laboratory process, such as those present in the extraction blank, and from handling, such as keratins. Peptide species specificity was determined by using pBLAST (Altschul *et al.*, 1990) (RRID:SCR_004870). Deamidation was assessed using publicly available code (https://github.com/dblyon/deamidation) (Mackie *et al.*, 2018).

In order to examine other post-translational modifications (PTMs), another search was made with this database using PEAKS (version 7.5, RRID:SCR_022841), to utilise the PEAKS PTM module (Han *et al.*, 2011) to find unspecified modifications. The results of this search identified N-glycosylation sites, specifically N-Acetylhexosamine, which were confirmed with another MaxQuant search with this as a variable modification.

Results and discussion

X-ray CT

The CT investigation successfully resulted in locating the queen and 3D images were generated of the bee that looks well developed (Figure 2). The formation stages of the queen bee from a *larvae* to a queen are well known (Woodward, 2007), and the adult queen usually emerges from the cell around the 16th day. As both the development and hatching of the bee needs a temperature of 35°C (Woodward, 2007) maintained by the worker bees, removing this cell from the beehive and keeping it at a lower temperature likely terminated the development and caused the death of the queen. However, pathogens may also have caused disease and impacted the queen.

From the reconstructed 3D image obtained, a layer of material can be seen beneath the queen bee (see Figure 2B). In order to better characterise this layer, a palaeoproteomic workflow was performed on the material, using a minimally destructive method that avoided opening the cell completely.

Palaeoproteomics

We successfully identified 120 proteins (ED 2) from the material retrieved from the encapsulated cell sample, with the most relevant groups being bee-related proteins (Figure 3). The most abundant proteins are mostly represented by major royal jelly proteins (MRJPs) and silk fibroin proteins. In addition, proteins from *Penicillium* and *Aspergillus* species (ED 2) were also recovered. It is clear that the unknown material seen in the X-ray CT scan is mostly composed of MRJPs and silk fibroins, left behind from the development of the queen. During this development, queens are fed MRJPs and the queen larvae makes a silk cocoon for itself, covering the cells (Hepburn *et al.*, 2013; Micas *et al.*, 2016). There are four different types of bee silk proteins (Micas *et al.*, 2016), and we identified all of them in our data. Due to the presence of other proteins, such as enzymes, it is likely that some of this material also represents faecal or other depositional material from the bee, such as the meconium (i.e. the gut contents that are voided as the bee emerges from the pupa).

Many of the royal jelly proteins were recovered. There is no inherent difference between the eggs which develop into workers and queens. The only difference is the nutrition provided, with the prospective queens fed royal jelly (Buttstedt *et al.*, 2014; Woodward, 2007), which is more nutritious than the standard worker fare. Royal jelly (or 'bee milk') is high in both protein and carbohydrates and triggers the queen's development; its quality is critical for this event, despite the actual biological mechanism behind it not being completely understood (Buttstedt *et al.*, 2014; Crane, 1999; Woodward, 2007). MRJPs form up to 15% of the royal jelly that is fed

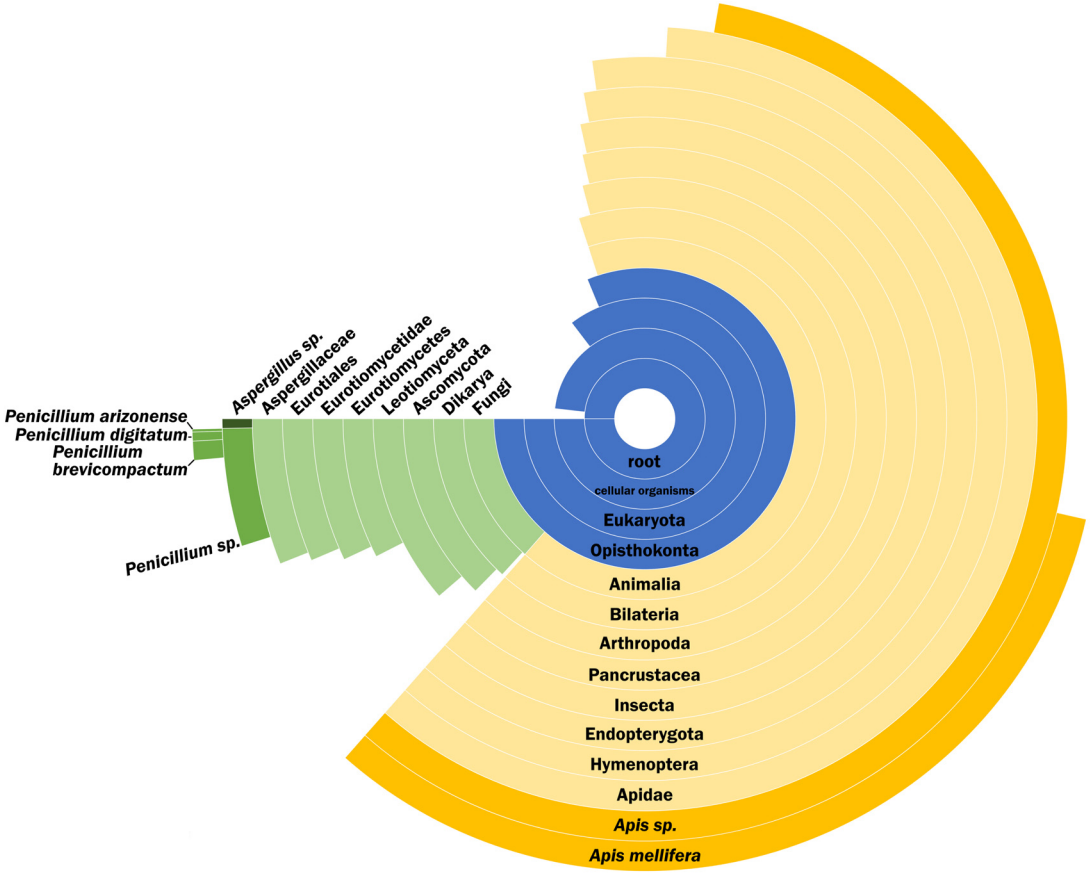

Figure 3. Peptide Species Specificity distribution of the sample. Peptide Species Specificity distribution of the sample after removal of lab reagent contamination and proteins reported with only one peptide. Percentage of the circle of each group indicates the percentage of peptides specific to that group.

to the queen during its development and its life (Buttstedt *et al.*, 2014; Woodward, 2007). We were able to identify five of the nine MRJPs known (MRJP 1, 2, 3, 5 and 7), and these are also the most abundant ones in royal jelly (Buttstedt *et al.*, 2014), explaining the better coverage of them in our sample.

In addition to its nutritional value, royal jelly is considered to be both antifungal and antimicrobial (Shen *et al.*, 2012). Some *Apis* specific enzymes found were Defensin-1 and glucose oxidase. Defensin-1 is found in royal jelly (Bucekova *et al.*, 2017), and in low concentrations it acts as a mechanism against gram-positive bacteria, e.g. *Paenibacillus larvae*, the cause of American Foulbrood disease (Ryba *et al.*, 2009; Shen *et al.*, 2012). Related to antimicrobial properties, we were also able to detect N-Acetylhexosamine glycosylation at several sites in MRJPs 1 and 2 (ED 3) three of which have been predicted to occur from sequence analysis (see UniProt Knowledgebase, https://www.uniprot.org, for these proteins). In addition, there was spectral evidence for a previously unrecorded hexose modification site on MRJP3. Some other sites were detected with varying confidence (ED 3), and more research needs to be done to confirm their presence. Their presence is important here because this modification has shown to give these proteins antimicrobial effects (Bíliková *et al.*, 2009; Mureşan *et al.*, 2022). In MRJPs, it is also related to metabolic activities important for the high metabolic fuel demands of an egg-laying queen (Zhang *et al.*, 2014a). This modification also may increase the stability of the proteins (Lis & Sharon, 1993), which may explain why it has been found in modern and ancient bones, as well as ancient eggshell (Cleland, 2018; Demarchi *et al.*, 2016; Schroeter, 2024).

While the modern bee proteome contains thousands of proteins, we have recovered comparatively few. This is probably due to both the nature of the sample and its age. Over time, the degradation of proteins from environmental conditions (and possibly enzymes present from the bee itself) likely limited the recovery to those that were more abundant or more stable. The sample is also likely not representative of the bee proteome anyway, as the sample was only taken from around the bee. Finally, it is possible that wax or some other substance was extracted along with the proteins that caused interference with

the trypsin digestion and/or the mass spectrometry, and limited the peptides recovered. However, there were still several proteins extracted that can give some information about the development of the bee and the environment of the queen cell. It is also of note that this project was much more successful than a similar approach applied to beeswax itself, which only provided minimal proteomic evidence of royal jelly proteins and only from wax from queen cell specimens (See ED 1).

Other non-bee related proteins were also recovered, specifically to *Penicillium* and *Aspergillus* species. The microbial environment of the hive is a delicate balance that protects the bees against pathogens, including fungi. The fungi *Penicillium* and *Aspergillus* are present in the natural environment, therefore also present in the hive as well. *Penicillium* and *Aspergillus* species also thrive indoors and are associated with dust and/or biodeterioration of museum pieces, therefore, they could originate from the sample being from a (natural) museum environment. In fact, some of the specific species detected have been detected in indoor Danish dust (Andersen *et al.*, 2021; Frisvad & Gravesen, 1994), although it is obviously not specific to Denmark. However, dust contamination is somewhat unlikely as the cell itself was closed, and the area outside cleaned prior to perforating the wall of the specimen for extraction. Also, no contaminant keratin proteins were recovered from the sample, only the extraction blank, indicating limited dust presence in the sample. It is possible that the fungi proteins are endogenous to the sample, as honeybees also collect fungal spores for nutrition (Parish *et al.*, 2020). Therefore, another advantage of using closed cells in future biomolecular studies of historical bees is the isolated environment that may reflect a pristine proxy for the original developmental environment of the queen bee.

In addition, the deamidation rate tells us about the preservation and the relative age of the proteins from the samples. This modification occurs to the amino acids asparagine (N) and glutamine (Q) over time. Older, and therefore more likely to be endogenous proteins, should have higher deamidation. The contamination present (mostly human keratins in the laboratory blank) has much lower rates of deamidation than those relating to the actual sample (mean of 3.6% for N and 4.3% for Q in the contamination; compared to 14.5% and 23.9%, respectively, for the queen sample), supporting that the proteins from the actual sample are endogenous to the queen cell (ED 4). Since this specimen is from the 19th century, this level of damage is consistent with the historical age, compared to archaeological samples (Ramsøe *et al.*, 2021; Schroeter & Cleland, 2016). However, it really only shows that they are similarly damaged, as deamidation cannot really be used as an age indicator (Ramsøe *et al.*, 2021; Schroeter & Cleland, 2016). It is also of note that the fungi and bee samples seem equally damaged (deamidation mean of 13.6% for N and 29.9% for Q for the fungi), despite the bee samples appearing to have more unspecific hydrolytic cleavage (61.4% semi-tryptically cleaved peptides versus 32.0%, ED 4). This cleavage also occurs over time with the breakdown of proteins due to hydrolysis, and is associated with older samples (Cappellini *et al.*, 2018; Schroeter & Cleland, 2016). The similar deamidation levels could indicate that the fungal and bee proteins are relatively the same age, therefore the fungi proteins could be from an earlier source than dust contamination. The higher rate of unspecific hydrolytic cleavage in the bee proteins might then be from enzymes from the digestive tract of the bee itself.

Conclusions

In this study, we used both X-ray Computed Tomography (CT) and palaeoproteomics to investigate a queen cell specimen from the Natural History Museum Denmark in order to better understand the specimen and evaluate the possibility of biomolecular (protein) extraction to gain biological insight to past bee rearing.

With the help of X-ray CT, the bee and the residue of its developmental environment were identified in a closed cell. This allowed us to carefully identify and place the optimum sampling location for maximum information with as little intervention to the bee as possible through micro-destructive sampling. This allows maximal information retrieved with limited damage to the queen.

We were successful in extracting proteins from the residual material inside the queen cell, identifying several bee related proteins, including major royal jelly proteins (MRJPs). (MRJPs). The MRJPs and defensive enzymes are interesting from the perspective of the development and nutrition of the queen, in addition to the protective microbiome of the hive against pathogens. The detection of glycosylation sites informs on antimicrobial properties of MRJPs in the past. Discovering and imaging the fully preserved queen in conjunction with the proteomics data will allow comparisons with modern samples. These studies, in conjunction with further work on other historic or ancient samples, could inform on changes in queen bee development over time.

This experimental study of a queen cell from the Natural History Museum Denmark brings forward the profound potential these collections hold. This small study not only shows that proteinaceous material can be recovered from such a specimen, but that it can be a source of information to better understand bees in the historic past. It can illuminate aspects of the queen bee's diet (MRJPs, potentially fungal spores) and antimicrobial protection of the cell environment, but it also demonstrates how such preserved specimens in general can serve as time capsules for future scientific research. In contrast to historical beeswax specimens, which we attempted unsatisfactorily (ED 1), closed cells are an excellent source of proteins and potentially other biomolecules. Their preservation and isolation from the environment renders these samples the most promising for future studies on historical bees and could be a future direction or application for this type of targeted sampling. Future research integrating both DNA and proteomic analyses of more samples could provide a more comprehensive understanding of historical honey bee populations, including potential subspecies identification and their adaptive responses to past environmental conditions.

Ethics and consent

Ethical approval and consent were not required.

Data availability

Underlying data

EMBL-EBI :PRIDE database: Assessing the hidden potential of beeswax specimens in Natural History Museum collections.

The underlying data has been deposited in the ProteomeXchange Consortium via the PRIDE partner repository, accession number PXD034106: https://www.ebi.ac.uk/pride/archive/projects/PXD034106 (Mackie, 2024).

Data are available under the terms of the Creative Commons Public Domain license (CC0).

Extended data

EMBL-EBI :PRIDE database: Assessing the hidden potential of beeswax specimens in Natural History Museum collections. https://www.ebi.ac.uk/pride/archive/projects/PXD034106 (Mackie, 2024).

This project contains the following extended data:

- Extended_Data_2_Queen_Cell_protein_tables.xlsx
- Extended_Data_1-3-4.pdf

Data are available under the terms of the Creative Commons Public Domain license (CC0).

References

Altschul SF, Gish W, Miller W, *et al.*: **Basic Local Alignment Search Tool.** *J Mol Biol.* 1990; **215**(3): 403–410.
PubMed Abstract | Publisher Full Text

Andersen B, Frisvad JC, Dunn RR, *et al.*: **A pilot study on baseline fungi and moisture indicator fungi in Danish homes.** *J Fungi (Basel).* 2021; **7**(2): 71.
PubMed Abstract | Publisher Full Text | Free Full Text

Arad M, Ku K, Frey C, *et al.*: **What proteomics has taught us about honey bee (*Apis mellifera*) health and disease.** *Proteomics.* 2025; **25**(1–2): e2400075.
PubMed Abstract | Publisher Full Text | Free Full Text

Bíliková K, Mirgorodskaya E, Bukovská G, *et al.*: **Towards functional proteomics of minority component of honeybee royal jelly: the effect of post-translational modifications on the antimicrobial activity of apalbumin2.** *Proteomics.* 2009; **9**(8): 2131–2138.
PubMed Abstract | Publisher Full Text

Bucekova M, Sojka M, Valachova I, *et al.*: **Bee-derived antibacterial peptide, defensin-1, promotes wound re-epithelialisation *in vitro* and *in vivo*.** *Sci Rep.* 2017; **7**(1): 7340.
PubMed Abstract | Publisher Full Text | Free Full Text

Buttstedt A, Moritz RFA, Erler S: **Origin and function of the Major Royal Jelly Proteins of the honeybee (*Apis mellifera*) as members of the *yellow* gene family.** *Biol Rev Camb Philos Soc.* 2014; **89**(2): 255–69.
PubMed Abstract | Publisher Full Text

Cappellini E, Prohaska A, Racimo F, *et al.*: **Ancient biomolecules and evolutionary inference.** *Annu Rev Biochem.* 2018; **87**(1): 1029–1060.
PubMed Abstract | Publisher Full Text

Cleland TP: **Solid digestion of demineralized bone as a method to access potentially insoluble proteins and post-translational modifications.** *J Proteome Res.* 2018; **17**(1): 536–542.
PubMed Abstract | Publisher Full Text

Crane E: **The world history of beekeeping and honey hunting**. Routledge, Inc. Abingdon, Oxford, 1999.
Reference Source

Cox J, Mann M: **MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification.** *Nat Biotechnol.* 2008; **26**(12): 1367–1372.
PubMed Abstract | Publisher Full Text

Demarchi B, Hall S, Roncal-Herrero T, *et al.*: **Protein sequences bound to mineral surfaces persist into deep time.** *eLife.* 2016; **5**: e17092.
PubMed Abstract | Publisher Full Text | Free Full Text

Drapeau MD, Albert S, Kucharski R, *et al.*: **Evolution of the yellow/Major Royal Jelly Protein family and the emergence of social behavior in honey bees.** *Genome Res.* 2006; **16**(11): 1385–94.
PubMed Abstract | Publisher Full Text | Free Full Text

Espregueira Themudo G, Rey-Iglesia A, Tascón LR, *et al.*: **Declining genetic diversity of European honeybees along the twentieth century.** *Sci Rep.* 2020; **10**(1): 10520.
PubMed Abstract | Publisher Full Text | Free Full Text

Feng M, Fang Y, Li J: **Proteomic analysis of honeybee worker (*Apis mellifera*) hypopharyngeal gland development.** *BMC Genomics.* 2009; **10**(1): 645.
PubMed Abstract | Publisher Full Text | Free Full Text

Frisvad JC, Gravesen S: ***Penicillium* and *Aspergillus* from Danish homes and working places with indoor air problems. Identification and mycotoxin determination.** In: *Health Implications of Fungi in Indoor Environments: Air Quality Monographs*. Pergamon Press, London, England, 1994; **2**: 281–290.
Reference Source

Han X, He L, Xin L, *et al.*: **PeaksPTM: Mass spectrometry-based identification of peptides with unspecified modifications.** *J Proteome Res.* 2011; **10**(7): 2930–2936.
PubMed Abstract | Publisher Full Text

Han B, Zhang L, Feng M, *et al.*: **An integrated proteomics reveals pathological mechanism of honeybee (*Apis cerena*) sacbrood disease.** *J Proteome Res.* 2013; **12**(4): 1881–97.
PubMed Abstract | Publisher Full Text

Hendy J, Welker F, Demarchi B, *et al.*: **A guide to ancient protein studies.** *Nat Ecol Evol.* 2018; **2**(5): 791–799.
PubMed Abstract | Publisher Full Text

Hepburn HR, Duangphakdee O, Pirk CWW: **Physical properties of honeybee silk: a review.** *Apidologie.* 2013; **44**: 600–610.
Publisher Full Text

Kasso TM, Bruun Jensen A, Johns S, *et al.*: **Beescapes – extracting pollen from historical Danish beeswax to explore honeybee foraging.** *Palynology.* 2023b; **48**(2): 2288220.
Publisher Full Text

Kasso TM, Enevold R, Johns S, *et al.*: **ArcHives—combined palynological, genomic and lipid analysis of medieval wax seals.** *Herit Sci.* 2023a; **11**: 11
Publisher Full Text

Lamei S: **The effect of honeybee-specific Lactic Acid Bacteria on American foulbrood disease of honeybees**. Doctoral dissertation. Uppsala: Sveriges Lantbruksuniv. Acta Universitatis Agriculturae Sueciae. 1652-6880; 2018; 23.
Reference Source

Lis H, Sharon N: **Protein glycosylation. Structural and functional aspects.** *Eur J Biochem.* 1993; **218**(1): 1–27.
PubMed Abstract | Publisher Full Text

Lozier JD, Cameron SA: **Comparative genetic analyses of historical and contemporary collections highlight contrasting demographic histories for the bumble bees *Bombus pensylvanicus* and *B. impatiens* in Illinois.** *Mol Ecol.* 2009; **18**(9): 1875–1886.
PubMed Abstract | Publisher Full Text

Mackie M: **Assessing the hidden potential of beeswax specimens in natural history museum collections.** *PRoteomics IDEntifications Database.* 2024.
https://www.ebi.ac.uk/pride/archive/projects/PXD034106

Mackie M, Rüther P, Samodova D, *et al.*: **Palaeoproteomic profiling of conservation layers on a 14th century Italian wall painting.** *Angew Chem Int Ed Engl.* 2018; **57**(25): 7369–7374.
PubMed Abstract | Publisher Full Text | Free Full Text

Micas AFD, Ferreira GA, Laure HJ, *et al.*: **Proteins of the integumentary system**

of the honeybee, *Apis mellifera*. *Arch Insect Biochem Physiol.* 2016; **93**(1): 3–24.
PubMed Abstract | **Publisher Full Text**

Mikheyev AS, Tin MMY, Arora J, *et al.*: **Museum samples reveal rapid evolution by wild honey bees exposed to a novel parasite.** *Nat Commun.* 2015; **6**: 7991.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Mureşan CI, Dezmirean DS, Marc BD, *et al.*: **Biological properties and activities of Major Royal Jelly Proteins and their derived peptides.** *J Funct Foods.* 2022; **98**: 105286.
Publisher Full Text

Nielsdatter MG, Larsen M, Godthaab Nielsen L, *et al.*: **History of the displacement of the European dark bee (*Apis mellifera mellifera*) in Denmark.** *J Apic Res.* 2021; **60**(1): 13–18.
Publisher Full Text

Parish JB, Scott ES, Hogendoorn K: **Nutritional benefit of fungal spores for honey bee workers.** *Sci Rep.* 2020; **10**(1): 15671.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Parejo M, Wragg D, Henriques D, *et al.*: **Digging into the genomic past of swiss honey bees by whole-genome sequencing museum specimens.** *Genome Biol Evol.* 2020; **12**(12): 2535–2551.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Ramsøe A, Crispin M, Mackie M, *et al.*: **Assessing the degradation of ancient milk proteins through site-specific deamidation patterns.** *Sci Rep.* 2021; **11**(1): 7795.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Rappsilber J, Mann M, Ishihama Y: **Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips.** *Nat Protoc.* 2007; **2**(8): 1896–906.
PubMed Abstract | **Publisher Full Text**

Ryba S, Titera D, Haklova S, *et al.*: **A PCR method of detecting American Foulbrood (*Paenibacillus larvae*) in winter beehive wax debris.** *Vet Microbiol.* 2009; **139**(1–2): 193–196.
PubMed Abstract | **Publisher Full Text**

Schroeter ER: **Characterization of diagenetiforms in an expanded proteome of the extinct moa (dinornithidae): identifying biological, diagenetic, experimental artifact, and mislabeled modifications in degraded tissues.** *Minerals.* 2024; **14**(2): 137.
Publisher Full Text

Schroeter ER, Cleland TP: **Glutamine deamidation: an indicator of antiquity, or preservational quality?** *Rapid Commun Mass Spectrom.* 2016; **30**(2): 251–255.
PubMed Abstract | **Publisher Full Text**

Shen L, Liu D, Li M, *et al.*: **Mechanism of action of recombinant acc-royalisin from royal jelly of Asian honeybee against gram-positive bacteria.** *PLoS One.* 2012; **7**(10): e47194.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Strange JP, Knoblett J, Griswold T: **DNA amplification from pin-mounted bumble bees (*Bombus*) in a museum collection: effects of fragment size and specimen age on successful PCR.** *Apidologie.* 2009; **40**: 134–139.
Publisher Full Text

Szulc J, Jablonskaja I, Jabłońska E, *et al.*: **Metabolomics and metagenomics characteristic of historic beeswax seals.** *International Biodeterioration & Biodegradation.* 2020; **152**: 105012.
Publisher Full Text

Woodward DR: **Queen bee: biology, rearing and breeding**. Balclutha, New Zealand, 2007.
Reference Source

Yushkevich PA, Piven J, Cody Hazlett H, *et al.*: **User-guided 3D active contour segmentation of anatomical structures: significantly improved efficiency and reliability.** *Neuroimage.* 2006; **31**(3): 1116–28.
PubMed Abstract | **Publisher Full Text**

Zhang L, Han B, Li R, *et al.*: **Comprehensive identification of novel proteins and N-glycosylation sites in royal jelly.** *BMC Genomics.* 2014a; **15**(1): 135.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Zhang Y, Zhang G, Huang X, *et al.*: **Proteomic analysis of *Apis cerana* and *Apis mellifera* larvae fed with heterospecific royal jelly and by CSBV challenge.** *PLoS One.* 2014b; **9**(8): e102663.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Zheng A, Li J, Begna D, *et al.*: **Proteomic analysis of honeybee (*Apis mellifera* L.) pupae head development.** *PLoS One.* 2011; **6**(5): e20428.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

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Version 2

Reviewer Report 29 August 2025

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✔ **Richard Bernstein**
Institute for Bee Research Hohen Neuendorf, Hohen Neuendorf, Germany

The suggestions from my earlier report were incorporated into the manuscript or reasons were provided why this is not possible.
The authors added more background on Proteomics in honeybees and other studies of ancient materials from honeybees in the Introduction. In the Results & Discussion, the manuscript now mentinons the limitations of working with ancient material for proteomics more clearly.
The authors explained in their response that a comparison of their results to studies of current bees would not be fruitful, as changes over time in their samples and the bees are probably large, and their results are of limited amount. A larger sample number or optimisation of the retrieval method could change this situation.
Work on museum samples is challenging, and the study successfully retrieved proteomic data from museum samples.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: population genetics of honeybees based on DNA data

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 12 August 2025

https://doi.org/10.21956/openreseurope.21939.r56539

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✔

Nelson Enrique Arenas
Universidad de Cartagena, Cartagena, Bolívar, Colombia

The authors addressed accurately all comments.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 24 December 2024

https://doi.org/10.21956/openreseurope.20042.r47994

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?

Richard Bernstein
Institute for Bee Research Hohen Neuendorf, Hohen Neuendorf, Germany

The authors studied a single queen from the 19th century collected in Denmark. The analysis of museum samples is very challenging, but could yield very interesting information. The authors focus on palaeoproteomics which is a rather new method compared to DNA studies. The sequences of the identified proteins are accessible in the extended data, which is beneficial for future research.

The main success of the study probably lies in the identification of some proteins from the challenging sample material. At least to my knowledge, proteomics on ancient bee samples have not been done yet, although genomic studies of bee samples from the 19th century have been done. Compared to the number of proteins in a honeybee, only a small amount could be identified, namely 120 proteins. Results and Discussion focus on specific proteins, and explain their role in honeybees in general, or why some finds might be due to dust contamination.

For honeybee biology in general, only the identified sequences are interesting, but a comparison of honeybees in the 19th century to current ones is not presented in this study. The authors merely suggest that such an comparison may be possible in the future. Such comparisons are possible with DNA analyses of museum samples [1]. With DNA data from the queen, we could e.g. check whether the sample was from *Apis mellifera mellifera* or another subspecies of *Apis mellifera*.

I criticize that the results are about methods in paleoproteomics, and not highly relevant to the biology of honeybees. E.g. the history of honeybees in Denmark is mentioned in the Introduction, but not relevant for the Results and Discussion. I suggest that the effectivity of the method should

be discussed, and a comparison with other methods to investigate historic honeybee populations should be included. In particular, the current study does not cite any previous paleoproteomic work in honeybees. The authors do provide literature on paleoproteomics, but this is not sufficient. There are certainly studies which speculate about the history of honeybee proteins [2]. If it is the first study of this kind, then the authors should mention it. Another important question would be, how well the proteomic analysis of museum samples performed compared to proteomic analysis of recent samples.

References

1. Parejo M, Wragg D, Henriques D, Charrière JD, et al.: Digging into the Genomic Past of Swiss Honey Bees by Whole-Genome Sequencing Museum Specimens.*Genome Biol Evol*. 2020; **12** (12): 2535-2551 PubMed Abstract | Publisher Full Text
2. Drapeau MD, Albert S, Kucharski R, Prusko C, et al.: Evolution of the Yellow/Major Royal Jelly Protein family and the emergence of social behavior in honey bees.*Genome Res*. 2006; **16** (11): 1385-94 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: population genetics of honeybees based on DNA data

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 24 Apr 2025

Tuuli Kasso

We kindly thank the reviewer for the detailed comments and helpful suggestions that have

significantly improved our paper. Following is our responses in a point-by-point manner (in bold): The authors studied a single queen from the 19th century collected in Denmark. The analysis of museum samples is very challenging, but could yield very interesting information. The authors focus on palaeoproteomics which is a rather new method compared to DNA studies. The sequences of the identified proteins are accessible in the extended data, which is beneficial for future research. The main success of the study probably lies in the identification of some proteins from the challenging sample material. At least to my knowledge, proteomics on ancient bee samples have not been done yet, although genomic studies of bee samples from the 19th century have been done. Compared to the number of proteins in a honey bee, only a small amount could be identified, namely 120 proteins. Results and Discussion focus on specific proteins, and explain their role in honey bees in general, or why some finds might be due to dust contamination. Response:. We have rephrased and revised the paper on Results and Discussion, especially in adding a part that talks about the limited protein retrieval. We have not gone too in depth on the proteins present, as we do not want to overinterpret at this point in time, but more indicate the retrieval of protein from this kind of specimen is possible. For honey bee biology in general, only the identified sequences are interesting, but a comparison of honey bees in the 19th century to current ones is not presented in this study. The authors merely suggest that such an comparison may be possible in the future. Such comparisons are possible with DNA analyses of museum samples [1]. With DNA data from the queen, we could e.g. check whether the sample was from Apis mellifera mellifera or another subspecies of Apis mellifera. Response: We acknowledge that our study does not directly compare historical and modern honey bee proteomes. As mentioned in response to another reviewer, there is no real comparable data to use at the moment. Any abundance patterns when compared to even the same kind of sample from modern times would be fraught with uncertainty since each protein would degrade differently based on its composition/structure and the conditions in which it was kept. Understanding changes in abundances over time would certainly be interesting, but it would need many more samples and is out of the scope of this exploratory study.
Our primary aim was to explore whether proteins could be retrieved from a 19th-century queen cell and what insights this might offer about historical bee biology. While DNA studies have provided direct comparisons of genetic variation over time, our work demonstrates that palaeoproteomics could complement these studies by revealing protein expression patterns. In general, more samples will need to be taken to make any useful comparisons. We started with one sample, to limit the destruction of irreplaceable samples in case it was not successful. Method development could also be possible to optimise the retrieval of proteins. We have revised the discussion to more explicitly state that future research could integrate DNA and proteomic data to gain a deeper understanding of historical honey bee populations, including subspecies identification. I criticize that the results are about methods in paleoproteomics, and not highly relevant to the biology of honeybees. E.g. the history of honeybees in Denmark is mentioned in the Introduction, but not relevant for the Results and Discussion. I suggest that the effectivity of the method should be discussed, and a comparison with other methods to investigate historic honeybee populations should be included. In particular, the current study does not cite any previous paleoproteomic work in honeybees. The authors do provide literature on paleoproteomics, but this is not sufficient. There are certainly studies which speculate about the history of honeybee proteins [2]. If it is the first study of this kind, then the authors should mention it.

Another important question would be, how well the proteomic analysis of museum samples performed compared to proteomic analysis of recent samples. Response: We appreciate the reviewer's feedback and agree that a more detailed discussion of method effectiveness and comparative approaches would strengthen the manuscript. We expanded our discussion to address the advantages and limitations of palaeoproteomics, and have revised our literature on proteomics to honey bees, albeit there has not been previous studies on applied palaeoproteomics on honey bees. Additionally, our data is from the inside materials of the queen cell, therefore we acknowledge that our data is not as representative of the bee, more more so of her environment. We have refined this in the paper, to better align with the study's findings.

Competing Interests: No competing interests.

Reviewer Report 14 December 2024

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Simon Hammann
University of Hohenheim, Stuttgart, Germany

In their article "A queen's tale: Assessing the hidden potential of beeswax specimens in Natural History Museum collections" the authors describe the analysis of a queen bee beeswax cell specimen from the 19th century from a museum collection. The authors emphasise how museum collections can be a valuable source of information. They used µCT to visualise the position of the queen bee and extracted a part of additional material found in the cell. Using LC-MS/MS – based proteomics they identified the material to be consistent with royal jelly, but they also found fungal proteins. Deamidation patterns suggested the royal jelly proteins and the fungal proteins to be contemporary, suggesting it was not a modern contamination. They paper also mentions the extraction of proteins from beeswax itself, but these data are not presented in the main paper.
I like the general premise of the paper to tap into these rich resources in Natural History Museums. However, I feel the paper is lacking a clear hypothesis or research question. The paper is also written a bit vague in the introduction section ("If the queen was present, proteomics would..."). Furthermore, the first and second paragraph feel unconnected, as there is a very sudden jump from museums to bees. Please try to make this a bit more cohesive. Also, I suggest to emphasise the specific research questions for proteomics part a bit more (third paragraph): What information could be gathered through proteomics potentially? How does this refer to paleogenomics? What questions exactly could be answered here.
The introduction ends with a reference to the extraction of proteins from beeswax itself and that this is not presented in the main manuscript. Looking at the supplementary material it seems that this part is in length and data richness almost comparable to the main manuscript. I do not really understand why the authors mention this, but then hide it away in the Extended Data (which is

generally not reviewed). In my opinion this is not extended data for the main manuscript but a whole different paper/dataset (with its own introduction and Experimental section!). If the authors want to publish this, it should be included into the main manuscript and subject to proper review. If not, I suggest the authors delete this completely from this submission.

Specific comments:
Method section, line 11: Should this be 40 keV?
“During the scanning…”: This sentence is quite complicated. Please consider rephrasing.
Results and Discussion: When talking about the deamidation damage, it would be nice to have the numbers in the main text and not only a reference to the ED
Since I am not an expert on proteomics I can’t fully evaluate the quality of the proteomics results. But with the reported sequence coverage of the MRJP species the identification of the material as royal jelly looks very reasonable to me.
Generally, the results and discussion section feels short compared to Introduction and Methods. Figure 2, currently cited in the introduction, should be moved to the results and discussion. Figure 2 also shows the intact bee, which also doesn’t work with the speculation about the presence of the queen in the penultimate paragraph of the introduction.
In summary, I believe the authors have a nice little case study highlighting the potential to access these specimens on a larger scale. I believe the manuscript could be improved by emphasising more what exactly could be learned from doing so. I also believe, the publication of the beeswax part of the paper as unreviewed extended data is inappropriate and should be reconsidered.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Food chemistry, Lipid analysis, Organic Residue Analysis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 24 Apr 2025

Tuuli Kasso

We kindly thank the reviewer for the detailed comments and helpful suggestions that have significantly improved our paper. Following is our responses in a point-by-point manner, in bold: In their article “A queen’s tale: Assessing the hidden potential of beeswax specimens in Natural History Museum collections” the authors describe the analysis of a queen bee beeswax cell specimen from the 19th century from a museum collection. The authors emphasise how museum collections can be a valuable source of information. They used µCT to visualise the position of the queen bee and extracted a part of additional material found in the cell. Using LC-MS/MS – based proteomics they identified the material to be consistent with royal jelly, but they also found fungal proteins. Deamidation patterns suggested the royal jelly proteins and the fungal proteins to be contemporary, suggesting it was not a modern contamination. They paper also mentions the extraction of proteins from beeswax itself, but these data are not presented in the main paper.
I like the general premise of the paper to tap into these rich resources in Natural History Museums. However, I feel the paper is lacking a clear hypothesis or research question. The paper is also written a bit vague in the introduction section (“If the queen was present, proteomics would…”). Furthermore, the first and second paragraph feel unconnected, as there is a very sudden jump from museums to bees. Please try to make this a bit more cohesive. Also, I suggest to emphasise the specific research questions for proteomics part a bit more (third paragraph): What information could be gathered through proteomics potentially? How does this refer to paleogenomics? What questions exactly could be answered here.

Response: We thank the reviewer for constructive feedback on the introduction to the manuscript/ project. In order to connect paragraph 1 and 2, we have added a new section to highlight the importance of bee collections in natural history museums.
We have also strived to clarify more of the research questions. In the third paragraph we have added examples of how proteomics have been applied to modern honey bee specimens, and what questions those solve. We have also specified in the 5th paragraph the exploratory nature of this work, to see if palaeoproteomics is a viable route in the future to better understand bee development and disease overtime.

The introduction ends with a reference to the extraction of proteins from beeswax itself and that this is not presented in the main manuscript. Looking at the supplementary material it seems that this part is in length and data richness almost comparable to the main manuscript. I do not really understand why the authors mention this, but then hide it away in the Extended Data (which is generally not reviewed). In my opinion this is not extended data for the main manuscript but a whole different paper/dataset (with its own introduction and Experimental section!). If the authors want to publish this, it should be included into the main manuscript and subject to proper review. If not, I suggest the authors delete this completely from this submission.
Response: Thank you for your feedback. We appreciate your perspective on the beeswax analysis report and the role of supplementary materials. Our intent in mentioning the beeswax analysis was to acknowledge that we attempted protein extraction from this material, rather than to present it as a central part of the study. Unfortunately, it did not

work, and it is important that this information is available to other researchers so they do not waste precious, irreplaceable, historic material on a similar method. We recognize the importance of ensuring that supplementary materials are scientifically and properly reviewed, therefore we also cite it in our paper and have provided it as an open source (since Open Research Europe does not allow a supplementary), so that it can be reviewed along with the manuscript and other supplementary material. We strongly believe that supplementary data can contribute valuable context to a study, and we believe it also brings transparency.
We do not want to delete ED1 for these reasons, and in order to remind the reader of its existence and to quickly summarise the results and their relevance of to this project, we have added the following in the main manuscript: "It is also of note that this project was much more successful than a similar approach applied to beeswax itself, which only provided minimal proteomic evidence of royal jelly proteins and only from wax from queen cell specimens (See Data Availability, ED 1)."

Specific comments: Method section, line 11: Should this be 40 keV? "During the scanning…": This sentence is quite complicated. Please consider rephrasing.
Response: We have corrected the typo and rephrased the text.

Results and Discussion: When talking about the deamidation damage, it would be nice to have the numbers in the main text and not only a reference to the ED
Response: We have added the % of deamidation and semi-tryptically cleaved peptides to the main text to better illustrate the damage without looking at the ED.

Since I am not an expert on proteomics I can't fully evaluate the quality of the proteomics results. But with the reported sequence coverage of the MRJP species the identification of the material as royal jelly looks very reasonable to me. Generally, the results and discussion section feels short compared to Introduction and Methods. Figure 2, currently cited in the introduction, should be moved to the results and discussion. Figure 2 also shows the intact bee, which also doesn't work with the speculation about the presence of the queen in the penultimate paragraph of the introduction.
Response: We appreciate your assessment of our proteomics results, particularly regarding the identification of royal jelly proteins. We acknowledge that the Results and Discussion section was relatively concise compared to the Introduction and Methods, and we have revised it to ensure a more balanced presentation of our findings. Regarding Figure 2, we agree that it would be more appropriately placed within the Results and Discussion section, where it directly supports our analysis, and it has been moved. Additionally, the introduction was refined to ensure consistency in our description of the queen's presence, aligning it more clearly with the evidence presented in the results.

In summary, I believe the authors have a nice little case study highlighting the potential to access these specimens on a larger scale. I believe the manuscript could be improved by emphasising more what exactly could be learned from doing so. I also believe, the publication of the beeswax part of the paper as unreviewed extended data is inappropriate and should be reconsidered.
Response: Thank you for your thoughtful and encouraging feedback. We appreciate your recognition of our study's potential to inspire broader research on historical specimens. We

have worked to strengthen our discussion to better highlight the insights that could be gained from scaling up this approach. Regarding the report on beeswax analysis, we have decided not to include it in the main paper, as we feel it would dilute the focus of our core findings. Our intent was simply to acknowledge that we attempted protein extraction from beeswax, as our results were not useful. We do not wish to pursue the report as a full scientific paper, but still provide all the necessary data, in case it is of use to future study, and would expect it to also be reviewed for content

Competing Interests: No competing interests.

Reviewer Report 14 December 2024

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Luca Fontanesi
University of Bologna, Bologna, Italy

This study investigated a queen cell specimen approx. 150 years old collected from a natural museum in Denmark. The study is simple as just one specimen was analysed using X-ray CT and paleoproteomics.
A few corrections are needed:
1) the title is misleading, it should be changed indication what was done and the specific objective/results of the study.
2) Introduction: "...while also being more likely to preserve than DNA.". This is not completely true.
3) Methods: the following sentence is not clear and should be re-phased: "*Apis mellifica* L indicates European dark bees, which today as a honeybee subspecies is rare in Denmark due to its displacement by other subspecies and hybrids, such as the Italian *Apis mellifera ligustica* which was introduced to Denmark ca. 1860 (Nielsdatter *et al.*, 2021)."
4) Methods: It is not clear the meaning of "... It was decided to experiment with extracting this material for ancient proteins to learn about this specimen, as it was considered unethical to sample the queen itself." Ethical issues in this context are not relevant.
5) It not completely clear how the queen cell was accessed.
6) Methods: please refine the way in which numbers and subsequent metrics are written - numbers should be separated from metric acronyms - and then be consistent in writing L derived measures using the same acronym: uL and not ul
7) Results: it is not clear if from the results or the information acquired on the specimen it is possible to obtain an indication on how old is the analysed queen cell material.
8) Results: no relevant results were obtained - it seems that the proteomics analyses did not identify anything unexpected. It would be good to list some of the most relevant proteins detected and add a table for that.
9) Conclusions. this sentence is not correct or is too bold: "These studies could inform on changes

in queen be development over time, such as possible changes and developments in major royal jelly proteins." it is actually one study and I doubt that there could be any information useful to evaluate changes of development and the possibility to estimate differences of composition of the royal jelly compared to what we have now.
10) It is not clear how "This small study not only illuminates aspects of the queen bee's diet and hive conditions ..." This study could not detect any relevant results for this.
11) I wonder how many capped queen cells could be studied from museum specimens to have such a high expectation
12) DNA analyses of this specimens should be mentioned as one of the most important net step

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Apiculture, animal genetics, eDNA, animal food research and authentication

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 24 Apr 2025

Tuuli Kasso

We kindly thank the reviewer for the detailed comments and helpful suggestions that have significantly improved our paper. Following is our responses in a point-by-point manner, in bold: This study investigated a queen cell specimen approx. 150 years old collected from a natural museum in Denmark. The study is simple as just one specimen was analysed using X-ray CT and paleoproteomics. A few corrections are needed:

1) the title is misleading, it should be changed indication what was done and the specific

objective/results of the study. Response: We have reformulated the title.

2) Introduction: "...while also being more likely to preserve than DNA.". This is not completely true. Response: While there could be cases where DNA preserved better than proteins, it is well established in the ancient biomolecule community that proteins are more stable over longer time scales than ancient DNA due to their chemical and physical properties (see Hendy, J. et al. (2018) 'A guide to ancient protein studies', Nature Ecology & Evolution, 2(5), pp. 791–799., and Demarchi, B. et al. (2016) 'Protein sequences bound to mineral surfaces persist into deep time', eLife, 5, p. e17092. And also studies where proteins have been recovered where DNA has not, for example : Welker, F. et al. (2015) 'Ancient proteins resolve the evolutionary history of Darwin's South American ungulates', Nature, 522(7554), pp. 81–84.; Cappellini, E. et al. (2019) 'Early Pleistocene enamel proteome from Dmanisi resolves Stephanorhinus phylogeny', Nature, 574(7776), pp. 103–107. However, we have reformulated the sentence to make this more clear.

3) Methods: the following sentence is not clear and should be re-phased: "Apis mellifica L indicates European dark bees, which today as a honey bee subspecies is rare in Denmark due to its displacement by other subspecies and hybrids, such as the Italian Apis mellifera ligustica which was introduced to Denmark ca. 1860 (Nielsdatter et al., 2021)." Response: This sentence has been amended.

4) Methods: It is not clear the meaning of "... It was decided to experiment with extracting this material for ancient proteins to learn about this specimen, as it was considered unethical to sample the queen itself." Ethical issues in this context are not relevant. Response: We would argue that the sampling of historical material always has an element of ethical issues. Since these samples are irreplaceable, the amount of damage done must be worth the information gained. In this situation, the fully formed queen still in a closed cell was determined to be too rare to cause that much damage, but the material in the cell was considered okay to sample from. In order to make this more clear, we have added ", due to the limited sample size of still encapsulated historic bees." at the end of the sentence.

5) It not completely clear how the queen cell was accessed. Response: We have clarified accessing the specimen in the collections and also added a note to the acknowledgements.

6) Methods: please refine the way in which numbers and subsequent metrics are written - numbers should be separated from metric acronyms - and then be consistent in writing L derived measures using the same acronym: uL and not ul Response: The inconsistencies in the metric reporting have been fixed.

7) Results: it is not clear if from the results or the information acquired on the specimen it is possible to obtain an indication on how old is the analysed queen cell material. Response: Unfortunately, protein damage does not give a precise indication on the age of a sample, since this is influenced by many factors. We know the specimen is from the 19th century, and the damage is consistent with this. Finding the exact age of the sample was not a research question, however, we did attempt radiocarbon dating of the beeswax of the specimen, with no better resolution for the date (data not shown).

8) Results: no relevant results were obtained - it seems that the proteomics analyses did not identify anything unexpected. It would be good to list some of the most relevant proteins detected and add a table for that. Response: For future research, there is a full list of the recovered proteins in the extended data (ED 2), accessible on the PRIDE upload https://www.ebi.ac.uk/pride/archive/projects/PXD034106. We mention major royal jelly proteins (MRJPs) and silk fibroin proteins, and have since reworded this to show that most of the abundant proteins fall into either of these groups. The material seen in the X-ray CT scan was unknown and we were able to identify it as material left behind from the development of the queen, and we have added a sentence to make this more clear. The Penicillium and Aspergillus proteins and the glycosylation of the MRJPs were unexpected findings that have also contributed to our interpretation of the material, as mentioned in the discussion.

9) Conclusions. this sentence is not correct or is too bold: "These studies could inform on changes in queen be development over time, such as possible changes and developments in major royal jelly proteins." it is actually one study and I doubt that there could be any information useful to evaluate changes of development and the possibility to estimate differences of composition of the royal jelly compared to what we have now. Response: This sentence has been amended.

10) It is not clear how "This small study not only illuminates aspects of the queen bee's diet and hive conditions ..." This study could not detect any relevant results for this. Response: This sentence has been amended to make it more clear.

11) I wonder how many capped queen cells could be studied from museum specimens to have such a high expectation Response: Indeed, and we understand that in our case, the sample may be a one-off study. However, we hope that our investigation may give new ideas for different lines of research on Natural History Museum collections.

12) DNA analyses of this specimens should be mentioned as one of the most important net step Response: Unfortunately, ancient DNA (aDNA) analysis on leftover extraction buffer was attempted, but with no success. We have not included it in this study in order to streamline the story. We have also attempted aDNA analysis from beeswax (Kasso, T.M., Enevold, R., Johns, S. et al. ArcHives—combined palynological, genomic and lipid analysis of medieval wax seals. Herit Sci 11, 11 (2023). https://doi.org/10.1186/s40494-022-00848-6), and it similarly did not provide sufficient information.

Competing Interests: No competing interests.

Reviewer Report 08 November 2024

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Nelson Enrique Arenas
Universidad de Cartagena, Cartagena, Bolívar, Colombia

The study presented in the natural history of *A. mellifera* specimens from the Natural History Museum collection is quite relevant to understanding the biology of pollinators through ancient times.

Abstract: This section must be strengthened since the findings are interesting, but its abstract is the weakest. Methods and results are not described in detail.

Introduction: Authors should connect paragraphs to present the background of the study sequentially. For example, one paragraph describes honeybees, and the next Natural History Museum collections are without any context. Perhaps check how to order the whole story more connected and add the study aims at the end of this section. Authors should describe better if there is any other study in the field of palaeoproteomics with a similar approach.
The fifth paragraph must be moved to the "Results and Discussion" section including Figures 1 and 2.

Methods: In the section "Palaeoproteomics" separate the data analysis or bioinformatic methods from the experimental settings. I also recommend to define a little longer subtitle for each section.

Results and discussion: The CT´s results appeared in the introduction section and should be moved here. I suggest including a list of the proteins identified as supplementary material despite that no differences were found. Authors must include a major information regarding the fold change of MRJPs and perhaps comparing with other honeybees' species or consider to review if abundances have any explanation through ancient times.
Since the *A. mellifera*´s proteome has thousands of proteins, authors must explain why this low number of proteins, if any technical problems at obtaining clean protein fractions which is difficult due to exoskeleton or complex polysaccharides, wax or lipid content, or some specific inhibitors. The last part of the technical issues in sample processing was not clear.

Conclusions: The authors must summarize this section.

The manuscript must be proofread by a native speaker.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biochemistry, molecular biology, bioinformatics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 24 Apr 2025

Tuuli Kasso

We kindly thank the reviewer for the detailed comments and helpful suggestions that have significantly improved our paper.
Following is our responses in a point-by-point manner, in bold: The study presented in the natural history of A. mellifera specimens from the Natural History Museum collection is quite relevant to understanding the biology of pollinators through ancient times.
Abstract: This section must be strengthened since the findings are interesting, but its abstract is the weakest. Methods and results are not described in detail.
Response: We have added to the abstract to provide more detail in response to this and the other reviewers.

Introduction: Authors should connect paragraphs to present the background of the study sequentially. For example, one paragraph describes honey bees, and the next Natural History Museum collections are without any context. Perhaps check how to order the whole story more connected and add the study aims at the end of this section. Authors should describe better if there is any other study in the field of palaeoproteomics with a similar approach. Response: The introduction has been restructured according to all the comments from reviewers.

The fifth paragraph must be moved to the “Results and Discussion” section including Figures 1 and 2.
Response: Most of the fifth paragraph has been removed or moved to the more appropriate sections, as suggested. We have kept Figure 1 in the materials section, as it is not a result of the methodology, but Figure 2 is still in the results section.

Methods: In the section “Palaeoproteomics” separate the data analysis or bioinformatic methods from the experimental settings. I also recommend to define a little longer subtitle for each section.
Response: We have added the sections “Protein Extraction and Mass Spectrometry” and “Protein identification and Modification Data Analysis”.

Results and discussion: The CT´s results appeared in the introduction section and should be moved here.
Response: We have removed any results from the introduction.

I suggest including a list of the proteins identified as supplementary material despite that no differences were found. Authors must include a major information regarding the fold change of MRJPs and perhaps comparing with other honey bees' species or consider to review if abundances have any explanation through ancient times.
Response: We have already provided a list of the proteins identified in the extended data on the PRIDE upload (ED2), since Open Research Europe does not allow supplementary files to be uploaded. We are not sure what the reviewer is referring to when mentioning differences or fold change, since we are not comparing our recovered proteins to anything else. There is no real comparable data to use. Any abundance patterns when compared to even the same kind of sample from modern times would be fraught with uncertainty since each protein would degrade differently based on its composition/structure and the conditions in which it was kept. Understanding changes in abundances over time would certainly be interesting, but it would need many more samples and is out of the scope of this exploratory study.

Since the A. mellifera´s proteome has thousands of proteins, authors must explain why this low number of proteins, if any technical problems at obtaining clean protein fractions which is difficult due to exoskeleton or complex polysaccharides, wax or lipid content, or some specific inhibitors.
Response: We have added a paragraph to the discussion about the limits of our protein recovery.

The last part of the technical issues in sample processing was not clear.
Response: We are unsure what the reviewer is referencing here, as we do not discuss sample processing issues in the results and discussion. We discuss the possibility of proteins coming from contaminant sources, but clarify that the Penicillium and Aspergillus peptides are possibly endogenous and the bee proteins we recover are likely also endogenous due their level of protein damage (deamidation).

Conclusions: The authors must summarize this section.
Response: This section has been condensed to better conclude the manuscript.

The manuscript must be proofread by a native speaker.
Response: We have had the manuscript proofread by a native speaker, including some of the authors.

Competing Interests: No competing interests.