Queens, but not their stored sperm, are resilient to temperature stress

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Abstract

Abiotic stressors such as extreme temperatures can reduce stored sperm viability within queen honey bees. However, little is known about how thermal stress may directly impact queen performance or other maternal quality metrics, such as queen mass, egg laying rate, and development of embryos within eggs. Here, in a blind field trial, we recorded laying pattern, queen mass, and average callow worker mass before and after exposing queens to a cold temperature (4°C, 2 h), hot temperature (42°C, 2 h), and hive temperature (33°C) to serve as a handling control. We then used proteomics to investigate potential vertical effects of maternal temperature stress on embryos, as well as to measure the abundance of previously determined protein markers for temperature stress in the spermathecal fluid. We found no significant effect of abiotic stress on any of the metrics we recorded. These data suggest that there are likely no lasting maternal effects of temperature stress on honey bee queens, and that the queens themselves are highly stress tolerant, but not their stored sperm.

Introduction

The queen is normally the sole reproductive female within a honey bee colony and can live up to eight years, though normally not more than three [1]. Therefore, any impairment to her fecundity has a large impact on a colony's productivity and ability to withstand environmental challenges. Concerningly, poor queen quality is a frequently reported problem in beekeeping operations [2, 3]. Indeed, survey results show that over half of colonies in some operations are requeened within the first six months of being established [4], but surprisingly little is known about why queens are failing so frequently [5].

When they are about one week old, queens embark on one or more nuptial flights and store a proportion of each mate's sperm, which must last for their lifetime since they are unable to mate again once they deplete their sperm stores. As such, a queen's lifetime reproductive capacity is ultimately sperm-limited, and any decrease in the viability of her stored sperm results in a permanent change to her fecundity and longevity [1, 6]. Previous research has shown that extreme temperatures (both hot and cold) can reduce viability of stored sperm, and temperature stress has been proposed as potential causal factor underlying queen failure in apiculture operations [7–9].

Queens are vulnerable to temperature stress during shipping and potentially inside small colonies during extreme weather events (i.e., heatwaves) [7–9]. Queen honey bees are routinely shipped both domestically and internationally to satisfy the seasonal needs of beekeepers. For example, Canada imports over 200,000 queens annually, mainly from warmer geographic regions (predominantly the US, New Zealand, Australia, and Chile) to satisfy early season demand before locally produced queens become available [10]. In the United States, queens are mainly produced by a relatively small number of large-scale queen production operations in Hawaii, California, and the southeastern states for domestic distribution. Queens are typically shipped in small cages with poorly thermoregulated environments, leaving them vulnerable to perturbations in ambient temperatures [7–9].
We previously determined that queens are safe between 15 and 38°C; outside this range, stored sperm viability tends to decrease [8]. Interestingly, Withrow et al. [11] found that queens from honey bee packages experiencing deviant temperatures during transport were more likely to fail, despite those temperatures being well within the safe temperature zone. However, this effect was apparently not linked to sperm viability, suggesting that temperature may impact other aspects of queen physiology. Worryingly, temperature stress events have been documented repeatedly during routine queen shipping [7–9]. While this is known to directly impact queen fertility via reductions in sperm viability, neither maternal effects on egg laying nor vertical impacts on her progeny have been investigated.

Transgenerational effects of temperature stress have been described in other animals, including insects [12–15]. For example, eggs fertilized with sperm from heat-stressed males develop into adults with shorter lifespans and reduced reproductive potential in the flour beetle, Tribolium castaneum [12]. In a parasitoid wasp (Aphidus ervi), which is in the same Order (Hymenoptera) as honey bees, maternal heat stress increases the developmental time and decreases the survivorship of her progeny [13]. However, little is known about the physiological impacts of temperature stress on honey bee queens, including their performance within colonies and potential altered physiology of their progeny. Heat stress could therefore not only reduce the fertility of the queen, but also change the physiology of the eggs she lays and the resulting adults. As such, it is conceivable for there to be consequences of thermal stress on queens above and beyond that of her stored sperm.

Previously, we conducted short-term (2 d) queen stress experiments to evaluate changes in protein abundance in the spermathecal fluid, from which we proposed a panel of six candidate queen stress biomarkers linked to cold-stress, heat-stress, and pesticide exposure [16]. Here, we conducted a blind field trial to investigate the effects of temperature stress and direct pesticide exposure on queen performance and productivity metrics. Reproductive potential (quality) can vary substantially among individual queens [17], so we recorded non-destructive quality metrics before and after exposure to thermal stress in order to account for changes relative to an individual queen's baseline. We also performed proteomic analysis on eggs laid before and after the queen was stressed to investigate potential vertical impacts of temperature stress (e.g., through differential egg provisioning or embryo development). At the end of the experiment, we sacrificed queens to measure their stored sperm viabilities and abundance of candidate stress biomarkers to evaluate their utility for field-realistic stress diagnostics.

**Results And Discussion**

**No direct effects of temperature stress on non-destructive queen quality metrics**

To determine how queen performance is impacted by temperature stress, we performed a blind field trial in which we compared queen laying pattern and queen mass before and after exposing the queen to extreme cold (4°C, 2 h, n = 9), extreme heat (42°C, 2 h, n = 10), and a handling control (33°C, 2 h, n = 9). This experimental design enabled us to account for phenotypic differences between individual queens,
which had different baseline body weights and laying patterns. We found no effect of temperature stress on these phenotypes (Fig. 1a & b; see Table 1 for statistical parameters, indicating the queens themselves are apparently resilient to temperature stress. Queen mass has been previously shown to be poorly correlated with ovary mass [18, 19], but because the queens’ laying patterns are also not affected it is unlikely that their ovaries were significantly impacted.

We and others have previously found that exposure to extreme temperatures significantly reduces viability of stored sperm [7–9], as well as drone ejaculates [8, 20], but here we detected no effect of temperature on sperm viability (Fig. 1c). However, upon inspection of proteins quantified within the spermathecal fluid, we found that one of the six proteins significantly correlating with sperm viability (5% false discovery rate) was Mellitin, which is the main protein component of honey bee venom (Table 2), and which we have not observed in our previous spermatheca proteomics dataset [21]. Here, abundance of Mellitin is inversely linked to sperm viability, strongly suggesting that venom contamination during the dissection process impaired our ability to detect differences in sperm viability between experimental groups.

Table 1
Statistical parameters for queen quality metrics*

| Independent variable | Test                                      | df  | Contrast    | |t/z|    | P    |
|----------------------|-------------------------------------------|-----|-------------|-----|-----|-----|
| Sperm viability      | Generalized linear mixed model (glmer)    | 23* | Hot:Control | 0.421 | 0.677 |
|                      |                                           |     | Cold:Control| 0.419 | 0.679 |
| Pattern ratio        | Linear mixed model (lme)                  | 27  | Hot:Control | 1.03  | 0.314 |
|                      |                                           |     | Cold:Control| 0.001 | 0.99  |
| Mass ratio           | Linear mixed model (lme)                  | 26  | Hot:Control | 1.13  | 0.271 |
|                      |                                           |     | Cold:Control| 1.37  | 0.184 |
| Average worker mass  | Linear mixed model (lme)                  | 26* | Hot:Control | 0.452 | 0.655 |
| ratio                |                                           |     | Cold:Control| 0.666 | 0.512 |

*In all models, temperature was a fixed effect and queen mating location was a random effect
No vertical effects of queen stress on callow worker mass nor egg proteins

Transgenerational effects of abiotic stressors have been documented in other insects, including hymenoptera [12, 13, 15], but they have not been investigated in honey bees. In a previous study, we identified proteomic changes in the ovaries of queens shortly (2 d) after being temperature stressed [8], indicating a potential route for a maternal effect of temperature stress. To test the hypothesis that abiotic stress on the queen could affect her progeny, we sampled callow workers (n = 9–12 per colony) that developed from eggs the queen laid before and after being stressed and recorded their emergence mass. We found no differences in worker mass ratios (post-stress relative to pre-stress) between treatment groups (Fig. 1d; see Table 1 for statistical parameters).

Despite there being no effect on adult mass at emergence, it is possible that there may have been more subtle developmental changes early in life; for example, differences in egg provisioning could lead to altered embryo development. To determine if temperature stress impacts the proteins expressed in or provisioned to eggs, we sampled 48–72 h old eggs (n = ~ 30 eggs per sample) laid by queens before and
after treatment and quantified proteins by label-free quantitative proteomics. We identified 4,604 unique protein groups at a 1% false discovery rate, which is the richest honey bee proteomics dataset yet reported. There is one other report with higher numbers (8,609 proteins) [22], but the authors’ definition of proteins was non-parsimonious (i.e., all proteins within a protein group were counted as unique identifications) thereby inflating the results in a way that is not the accepted standard in the field. Despite these rich data, we still did not identify any protein expression differences among treatment groups even at a relatively loose FDR (multiple samples test, Benjamini-Hochberg correction, 10% FDR), indicating that there are no vertical effects of queen temperature stress on embryo protein expression (Fig. 2a).

These data show no obvious effect of maternal temperature stress on the eggs (embryos) nor adult phenotypes, which does not support the hypothesis that maternal temperature stress adversely impacts the queen’s progeny. We acknowledge, however, that we did not record an exhaustive list of all potential phenotypes (e.g., behavioral ontogeny), and it is possible that there is some impact on progeny that is as yet unrealized. Regardless, the data that we acquired also shows a lack of apparent impact on the queen’s phenotype (body mass) and productivity (laying pattern), which point to a surprisingly robust stress-tolerance system. Indeed, queens are highly tolerant of heat stress, in terms of survival, compared to drones [8]. Since similar reductions in sperm viability are observed when both ejaculates and queens are heat-shocked [8], even the negative effects of heat on sperm stored within the queen can be explained by direct impacts on the sperm cells rather than indirectly through heat harming the queen’s ability to keep the sperm alive.

Evidence consistently points to queens having a high tolerance to temperature stress, which is puzzling because queens do not often naturally experience extreme temperatures; therefore, there should be little opportunity for selective pressure to drive the evolution of extreme temperature tolerance. However, drones are mortally sensitive to temperature extremes [8, 20], among other stressors [23–26]. This is at least in part due to their haploid genome—without the potential for compensatory alleles, individuals with recessive deleterious or “susceptible” genotypes are more likely to manifest and are thus purged from the population [27, 28].

It has been previously proposed that this “haploid susceptibility,” where haploid males more sensitive to stressors than their diploid counterparts, has an adaptive advantage: culling harmful mutations or unfavourable recessive alleles from the gene pool [27–29]. Although it is possible that queens’ exposure to temperature fluctuations during mating flights, swarming, and inside the hive is sufficient to select for a wide range of tolerance, we think that is an unlikely scenario. Rather, we speculate that selection for queen temperature tolerance is at least in part achieved indirectly through the drones, which share 100% of their genetic material with their mother queen, contribute to 50% of the genetic material to their daughters (which may become queens), exhibit well-documented susceptibility to temperature and other stressors [8, 20, 23–26], and are also more likely to be exposed to extreme temperatures since they themselves engage in more mating flights and occupy the periphery of the nest, which is relatively poorly thermoregulated [30].
As such, drones can be thought of as physical extensions of the queen, providing bodies more exposed to selective pressure, ultimately helping to drive selection for robust stress tolerance in queens and females in general. We admit, however, that this does not entirely explain queens’ resilience to extreme temperatures. Given that 50–77% of drones die after several hours of heat exposure, and that drones share 100% of their genes with their queen, we would expect at least some queens to have the unfortunate genetic combinations leading to heat sensitivity, and therefore other factors must also contribute.

**Longevity of queen stress biomarkers is less than two weeks**

Previously, we developed a panel of candidate stress protein biomarkers to aid with queen failure diagnostics. This panel consists of six proteins that are uniquely upregulated in spermathecal fluid of queens exposed to heat-stress, cold-stress, or pesticides relative to controls (two per stressor) [16]. The length of time that these markers remain abundant in the spermathecal fluid directly impacts their utility for queen failure diagnostics because symptoms of queen failure may not manifest or be noticed until long after a queen stress event has occurred. Our initial experiments identifying the candidate stress markers evaluated queens two days after applying the stress [16], but after a longer period of time marker abundance may fade or the stress response may evolve to a different protein fingerprint. Here, we evaluated global changes in protein abundances, as well as the candidate stress markers specifically, in queens two weeks after temperature stress exposure to identify potential longer-term candidate markers and to evaluate the temporal stability of the ones we already proposed. We found that no global changes in protein expression remained two weeks after the queens were exposed to stressors (Fig. 2b; limma, Benjamini-Hochberg false discovery correction to 10% FDR). Theoretically, the venom contamination that we noted above could have skewed the protein normalization procedure conducted by MaxQuant, but this is highly unlikely because the amount of venom contamination was very small in proportion to the amount of spermathecal fluid: We identified twelve protein groups containing proteins annotated as venom proteins, which on average make up less than 1% of the cumulative ion intensity per sample.

Despite the lack of globally differentially expressed proteins, we still checked our original candidate temperature stress biomarkers specifically, since evaluating four specific proteins does not suffer from substantially reduced statistical power owing to reduced multiple hypothesis testing. We also did not identify differences in expression of these *a priori* defined proteins of interest (XP_026296654.1, XP_395122.1, XP_001120006.2, and XP_395659.1) (Fig. 3), indicating that either (a) two weeks is sufficient time for them to revert to constitutive expression levels and that they are mainly an acute stress response mechanism, or (b) the stress treatments did not elevate these biomarkers in the first place.

However, we note that candidate heat-stress biomarker expression is a reproducible response in queens from different genetic sources (Californian and multi-generational local queens) [8, 16]. Indeed, the candidate markers XP_001120006.2, and XP_395659.1 are also conserved heat-shock proteins (HSPs); therefore, it is highly unlikely that these proteins simply failed to become upregulated at all after heat-
shock. Replication of the cold-shock markers has not yet been attempted, but regardless of whether this stressor induces different proteins in different queens or if the proteins were induced but regressed back to baseline expression, these data show that protein markers expressed shortly after queen stress, as previously determined, do not have sufficient stability for diagnostic purposes.

While it is unfortunate that no proteins were significantly different in queens as a result of temperature stress—especially the candidate biomarkers which had the potential to be used for queen failure diagnostic tests—it is not entirely surprising. Evidently, two weeks is a sufficient recovery period for queens, at which time the acute stress response is no longer apparent. Coupled with a lack of observable effects on other queen performance metrics and no apparent vertical impacts of queen stress on workers, these results are an encouraging tribute to queens’ potential for resilience against temperature stress.

We caution that although no significant effects were identified in these experiments, this does not mean that all queens are universally resilient. Indeed, an abundance of previous work clearly shows that temperature stress can seriously impact queen quality by damaging their sperm [7–9], and it is possible that our field observations did not extend long enough to observe potential negative effects of queen stress. Although there was no molecular evidence of queen stress at the end of the experiment, we only analyzed the queen's spermatheca and her eggs, not other tissues. In addition, even a dramatic reduction in stored sperm viability may not manifest immediately, since queens normally use a set volume of spermathecal contents for each fertilization event, corresponding to multiple sperm cells [6, 31]. For example, if queens normally use an average of two sperm cells per egg fertilization, as has been previously suggested [6], a queen could theoretically experience a drop in stored sperm viability from 100–50% and still be able to lay predominantly fertilized (diploid) worker eggs. Therefore, it makes sense for failed fertilization events to only manifest later in a queen's life, when she uses up more sperm (but the volume of fluid used for fertilizations remains the same) and thus exhibits a higher chance of releasing no viable sperm per egg. Future research should focus on longer-term experiments identifying how long after temperature stress events a queen is expected to last before exhibiting the anticipated deterioration of worker laying into drone laying, which has not yet been demonstrated.

**Conclusion**

Queen failure is a common problem in beekeeping operations, but surprisingly little is known about the underlying factors. Here, we investigated short-term (two weeks post-stress) impacts of heat-stress and cold-stress on queen quality metrics. We found no significant effects on any of the phenotypes we recorded (laying pattern, queen mass, and average callow worker mass) nor protein expression differences in eggs laid before and after stress exposure. We also identified no molecular evidence of the stress event in the queens’ spermathecal fluid, indicating that two weeks post-stress is sufficient time for previously identified candidate stress biomarkers to revert back to baseline expression. Cumulatively, the data acquired consistently demonstrate resilience of the queen herself to temperature stress, even if it may decrease the viability of the sperm she stores.
Methods

Honey bee colonies

We purchased twelve 1.5 kg honey bee packages from Tasmania in order to ensure that our experimental honey bees were all free from mites at the beginning of the experiment. Packages were installed in standard 10 frame deep hive bodies and supplied with pollen and syrup to encourage brood rearing and population growth. After 1.5 months (two brood cycles), we split the colonies into thirty 3-frame nucleus colonies (nucs; 1 frame of honey, 1 frame with open brood, 1 frame with capped brood) and supplied each nuc with a frame feeder for light syrup (~35% sucrose) and a ½ lb pollen patty (15% protein), which we fed continuously throughout the duration of the field trials except for during a period of nectar flow and population growth when we replaced the frame feeders each with two frames of drawn comb. All nucs were kept in a single apiary in Richmond, Canada.

Temperature stress field trial

To eliminate a potential confounding effect of extreme temperature exposure during shipping, we produced queens used for the temperature stress trial locally. We produced queen cells using standard queen rearing techniques (1–2 d old larvae were grafted from a single colony into queen cups and inserted to a queenless cell builder) [32]. Twelve days after grafting, we supplied each experimental nuc with a capped queen cell and the queens were allowed to emerge and naturally mate. Two weeks after queen cell introduction, we checked the nucs to see if the queens were laying eggs. Five queens were either laying poorly (few eggs or multiple eggs per cell) or were not laying, and these were replaced with queens which had been concurrently reared and mated in a second nearby apiary. One colony became queenless during the course of the experiment (in the control group), leaving 29 queens at the time of experiment termination.

Once each queen had been laying eggs for at least two weeks, we evaluated their laying patterns by locating a patch of approximately 100 eggs and recording how many cells within that patch were apparently missed (i.e., the cell was not otherwise occupied but lacked an egg). We avoided patches at the edge of the brood area. If a patch included an occasional cell with a newly eclosed larva, it was counted as ‘laid’ since it was likely that the eggs were on the verge of hatching. This method does not distinguish between eggs that were laid and then cannibalized by workers; however, since all colonies were fed supplemental protein, egg cannibalization should be linked to developmental deficiencies rather than nutritional stress, which is a desirable feature to which our method should be sensitive. We repeated this procedure two weeks after the queens were experimentally stressed in order to calculate a change in laying pattern (the ratio of the fraction of cells laid post-stress relative to pre-stress).

On the day that laying pattern was evaluated, queens were caged with five attendants and candy, then transported to the laboratory where a colleague not otherwise involved in the study briefly anesthetized the queens with carbon dioxide, weighed them on an analytical balance, and randomized them into three treatment groups (cold-stress at 4°C for 2 h, heat-stress at 42°C for 2 h, and control at 33°C for 2 h),
keeping the experimenter blind to their assignments. Queens were then transported back to the apiary and re-introduced to their respective colonies, but remained caged. After two days, we sampled approximately 30 pooled eggs per colony (at which time all eggs were >2 and <3 days old), froze them on dry ice, and released the queens. Two weeks post-stress, laying patterns were again evaluated, the queens were transported to the laboratory, anesthetized, weighed, and sacrificed for sperm viability analysis. Spermathecae were removed from the abdomen with forceps and blotted dry on a kimwipe, then clean forceps were used to gently remove the tracheal net surrounding the spermatheca. The spermatheca was then lysed in an Eppendorf tube containing Buffer D (17 mM D-glucose, 54 mM KCl, 25 mM NaHCO$_3$, 83 mM Na$_3$C$_6$H$_5$O$_7$) and sperm viability was measured using dual fluorescent staining exactly as previously described [21]. Two days after queens were sacrificed, we returned to the nucs to sample remaining eggs as already described, destroy any queen cells that had been built, normalize colony populations, and introduce new adult queens for the next round of the queen stress trial.

We collected newly emerged (callow) workers from each colony four weeks after the beginning of the experiment and four weeks after the queens were stressed. It takes 21 days for worker eggs to develop into adults; therefore, callow workers collected four weeks after the beginning of the experiment developed from eggs laid one week after the experiment began (i.e., before the queens were stressed). Likewise, callow workers collected four weeks after the queens were stressed developed from eggs laid one week post-stress. Callow workers are easily recognizable due to their light grey color, soft bodies, and inability to fly. We collected 9–12 workers per colony per time point in order to calculate a change in average mass at emergence.

**Proteomics analysis**

Proteins were extracted, digested, and purified from spermathecal fluid exactly as previously described [16]. Briefly, sperm cells were spun down from the Buffer D-diluted spermathecal fluid solution (Buffer D) and soluble proteins in the supernatant were precipitated with four volumes of ice-cold acetone. The pellets were washed and resuspended in urea digestion buffer (6 M urea, 2 M thiourea, in 100 mM Tris, pH 8). Preliminary tests showed that each spermatheca yields approximately 5–10 µg of protein. The proteins were reduced, alkylated, then digested with 0.2 µg of Lys-C (3 h, room temperature) followed by 0.2 µg of trypsin (overnight, room temperature). Peptides were desalted using in-house made C18 STAGE-tips, dried, suspended in Buffer A (0.1% formic acid, 2% acetonitrile), and quantified using a Nanodrop (280 nm absorbance). One µg of peptides were injected on a Thermo easy-nLC 1000 liquid chromatography system coupled to a Bruker Impact II mass spectrometer. Sample orders were randomized prior to loading, and instrument parameters were set exactly as previously described.

We followed the same procedure for analysis of the egg proteins, except that protein was extracted into 6 M guanidinium chloride (in 100 mM Tris, pH 8) using a Precellys homogenizer with ceramic beads. We digested approximately 25 µg of protein per sample using 0.5 µg of Lys-C and trypsin.

Raw mass spectrometry data were searched using MaxQuant (v 1.6.1.0) exactly as previously described [16]. We used the most recent honey bee canonical protein database available on NCBI (HAV3.1,
downloaded November 18th, 2019) with honey bee pathogen sequences added. Protein and peptide identifications were filtered to 1% FDR based on the reverse hits approach. All specific search parameters are available within the mqpar.xml file included in our data repository (see Data Availability).

**Statistical analysis**

We analyzed sperm viability, laying pattern ratio, queen mass ratio, and worker mass ratio data using R (v3.5.1). For sperm viability analysis, the data was non-normal; therefore, we used a generalized linear mixed model (glmer(), family = binomial) with live sperm counts as ‘successes’ and dead sperm counts as ‘failures,’ as has been conducted previously [33]. Ratio data were analyzed using a linear mixed model. For all models, temperature and pesticide trial data were analyzed separately, queen treatment was included as a fixed effect, and queen source was included as a random effect (queens for the temperature stress trial came from two different genetic lineages and mating locations, and queens for the pesticide stress trial came from either New Zealand or California).

For spermathecal fluid data, protein intensities ('LFQ intensity' columns from the MaxQuant output) were first log2 transformed, then reverse hits, contaminants, protein groups only identified by site, and protein groups without at least three defined values per treatment group were removed, leaving 1,899 and 1,794 proteins quantified for the temperature stress and pesticide stress experiments, respectively. Differential expression analysis was performed using limma() (example code is provided, see Data Availability) and a Benjamini-Hochberg multiple hypothesis testing correction. We analyzed expression of individual candidate biomarkers (six proteins) using a Dunnett’s test (hive temperature = control for the temperature trial, and solvent = control for the pesticide trial). For egg proteomics data, we calculated the ratio of each protein's expression post-stress relative to pre-stress prior to log2 transformation; otherwise, analysis was conducted using limma() following the same procedure as spermathecal fluid data. After filtering, 4,184 egg proteins were quantified. Heatmaps were generated using Perseus v1.6.1.1 (clustered via Euclidian distance, 300 clusters, 10 iterations).

**Data availability**

Raw mass spectrometry data for the spermathecal fluid and egg analyses are available on the MassIVE proteomics archive (massive.ucsd.edu; accession MSV000086862). We have also made the protein group tables and experimental design tables more easily accessible as supplementary information (see Table S1 for spermatheca proteomics data and Table S2 for egg proteomics data). Data for all colony and queen measurements is available in Table S3. R scripts underlying data analysis and figure generation are available as Files S1 (limma analysis) and S2 (biomarker expression comparison).

**Declarations**

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