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# Queen honey bees combat heat stress-induced loss of stored sperm viability with ATP-independent heat shock proteins

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**Abstract**

All species need to reproduce to maintain viable populations, but heat stress kills sperm cells across the animal kingdom and rising frequencies of heatwaves are a threat to biodiversity. Honey bees (*Apis mellifera*) are managed micro-livestock with globally viable populations; therefore, they could serve as environmental biomonitors for heat-induced reductions in fertility. Here, we exposed queens to a range of temperatures (5 to 42 ˚C) and measured the impact on viability of their stored sperm. Fitting a non-linear regression model shows that temperatures of 13 to 39 ˚C are safe for queens at a tolerance threshold of 20% loss of sperm viability. We found that exposing queens to 42 ˚C for 4 h reduced sperm viability by 56% and induced expression of ATP-independent heat-shock proteins (HSPs) in their spermathecae, but not in their ovaries. The strict ATP-independent heat-shock response in the spermatheca points to a tightly controlled ATP economy for stored sperm. Drones (males) are mortally heat-sensitive, with only 42% of drones surviving 6 h at 42 ˚C. Conversely, 100% of queens survived. This sex-biased heat tolerance may be a quality control mechanism to ensure that queens are inseminated only with high-quality sperm. Finally, we quantified HSP expression in queens that failed in the field and found that 45% possessed a heat-shock signature similar to the laboratory heat-shocked queens. These signatures could serve as biomarkers for heat stress to enable post-failure diagnostics for the beekeeping industry, as well as surveying the prevalence of heat-induced loss of sperm viability in diverse landscapes.

**Introduction**

Climate change is threatening biodiversity around the globe[1-3], and one potential driver is through heat-induced reductions in fertility[4-6]. The impact of heat on fertility is far-reaching in the animal kingdom, affecting mammals[7-11], birds[12], fish[13], nematodes[14], and insects[4,15-21]. At temperatures of 40-43 ˚C, spermatogenesis is compromised[22], sperm viability drops[4,18,19], sperm are less competitive[4], and motility is compromised[15,23,24]. Extreme weather events such as heatwaves are increasing in frequency and severity[25-27], which could have widespread effects on populations via reduced reproductive output[4-6].

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Insects and other ectothermic animals are especially vulnerable to changes in local temperatures because, unlike mammals, they are less able to thermoregulate. Insects have an extremely large collective biomass and are critical components of many ecosystems: they provide a major food source for predators, they aid in biomass decomposition through consumption and burial, and they enable plant reproduction through pollination[28]. In the latter example, economic estimates place the global value of insect pollination at approximately €153 billion annually[29]. Declines in insect populations[30] and pollinating species[31-35] have already been reported, with habitat destruction, range compression, pesticide residues, diseases, and their interactions commonly reported as potential drivers[29,36,37]. However, there has been relatively little research on the effects of heat on pollinating insect reproduction[18,19].

Honey bees (*Apis mellifera*) are the most commonly managed insect pollinators and are found on every continent except Antarctica. They are employed in the majority of agricultural operations that require active pollination (*i.e.*, operations that rent pollinating units to improve crop quality, production, and seed set)[38], with bumble bees (most commonly *Bombus terrestris* and *B. impatiens*)[39], leaf-cutting bees (*Megachile rotundata*)[40], and alkali bees (*Nomia melanderi*)[41] playing important roles for specific crops. Honey bees and their role in agricultural productivity are estimated to contribute up to $20 billion annually to the North American economy alone[42-45]—an enormous contribution for a single pollinating species.

Like other insects, individual honey bees are ectothermic, but their colonies (which are mainly composed of tens of thousands of sterile workers) are able to thermoregulate remarkably well[46]. However, female and male reproductive individuals (queens and drones, respectively) could be vulnerable to temperature extremes during mating flights or in poorly ventilated colonies, and queens are especially vulnerable during routine shipments[18]. The queen breeding industry depends on shipping live, newly mated queens across long distances to deliver them to beekeepers, and temperature stress while in transit could undermine the industry. Queens are routinely shipped from New Zealand, Australia, and Chile to countries in North America and Europe; therefore, risk of queen temperature stress during shipment is a global industrial issue. While in transit, even relatively minor heatwaves can easily push temperatures into damaging ranges (upwards of 40 °C). Such temperatures also occur in many regions where honey bee colonies are kept; therefore, extreme exposures could also occur outside of routine shipping.

Young honey bee queens have one brief mating period (when they are ~1 week old) and store the sperm they acquire to use for fertilizing eggs for the rest of their lives (up to 5 years)[47]. As the sole egg layer, colony productivity directly depends on the queen's reproductive output, which in turn depends on not only the queen's physiology but the quality of her stored sperm[18,48,49]. Previous research has shown that temperature stresses on mated queens significantly reduce stored-sperm viability in laboratory experiments and in commercial shipments[18]. However, critical thresholds (temperatures and durations of exposure) have not yet been determined, and the biochemical basis of temperature-induced reductions in sperm viability is unknown. Understanding the physiological processes underlying this loss of sperm viability is not only relevant to the apiculture industry, it will also help identify molecular biomarkers and establish honey bees as biomonitors of environmental temperature stress.

Here, we tested a range of temperatures and exposure durations to determine thresholds above which queen quality is likely to be compromised. We then investigated the biochemical basis of heat-induced

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sperm viability reduction in queens and drones using quantitative proteomics. Next, we investigated sex biases in heat tolerance and found that drones (males), but not queens, are highly sensitive to heat. This sex-biased heat tolerance was absent in two solitary insect species—stink bugs (*Halyomorpha halys*) and fruit flies (*Drosophila melanogaster*)—suggesting that this may be a sperm quality control strategy for insects with long-lived queens and nuptial flight mating systems. Finally, we analyzed spermathecae and ovaries from healthy and failing queens in the field and, using the five spermathecal heat-shock proteins (HSPs) and single ovarian HSP as markers, we found that the protein expression patterns in field-failed queens cluster together with laboratory heat-shocked queens, demonstrating that they possess the heat-shock signature. The remaining queens show divergent stress signatures in the ovaries, suggesting an alternate cause of failure. This work provides some of the first insights into how heat stress alters the biochemistry of reproductive tissue to influence honey bee fecundity and begins to develop diagnostic tools to elucidate causes of queen failure, enabling regional surveys of heat stress.

## Results and Discussion

### *Queen temperature exposure thresholds*

Previous research has shown that both cold (4 ˚C) and heat (42 ˚C) stress reduces stored sperm viability in honey bee queens[18]. To determine critical temperature and duration thresholds, we compared stored sperm viability across a temperature range between 5 ˚C and 42 ˚C with exposures ranging from 1 - 4 hours followed by a 2-day recovery period at a well-tolerated (25 ˚C) control temperature (**Fig 1A**). We found that queen sperm viability responses were highly variable, but there was a significant effect of temperature and time, as well as an interactive effect (two-way ANOVA; $P_{(temperature)} = 0.024$, $F_{(temperature)} = 2.5$, $P_{(time)} = 0.014$, $F_{(time)} = 4.4$, $P_{(interaction)} = 0.037$, $F_{(interaction)} = 2.0$). The interactive effect was driven by the consistent decrease in viability observed for the 4 h time points at hot temperatures (38 ˚C, 40 ˚C, and 42 ˚C), which was not observed for the cold temperatures (5 ˚C, 10 ˚C, and 15 ˚C). A Tukey honest significant difference (HSD) post hoc test revealed that the only temperature that was significantly different from the control was the 42 ˚C treatment—a difference that was clearly driven by the 4 h exposure. The 2 h and 4 h exposures were not significantly different from each other, so the data were pooled and optimally fit a cubic polynomial regression ($R^2 = 0.092$, $P = 0.012$; **Fig 1B**). Considering a ≤ 20% drop in viability to be tolerable, this model reveals that 13 - 39 ˚C is the "safe zone" with minimal viability reductions, if any, for 2 - 4 h exposures. This is consistent with previous observations.[18]

### *Male-biased mortal heat sensitivity*

Baer *et al.* previously reported that drones are mortally sensitive to heat[19]; however, queens have remarkably high survival rates at high temperatures, despite the death of their stored sperm. The success of a colony depends on virgin queens obtaining high-quality sperm on their nuptial flights; therefore, we hypothesized that mortal heat sensitivity of drones could be a quality control mechanism to prevent queens from being inseminated with damaged sperm. If this is true, a) the sperm in drone ejaculates should also be heat-sensitive, b) queens should tolerate the temperatures at which drones perish, c) there should be no selection for worker heat-sensitivity (workers have similar lifespans to drones but are non-reproductive), and d) solitary insects, which do not rely on long-term sperm storage, should not have sex-biased heat tolerance.

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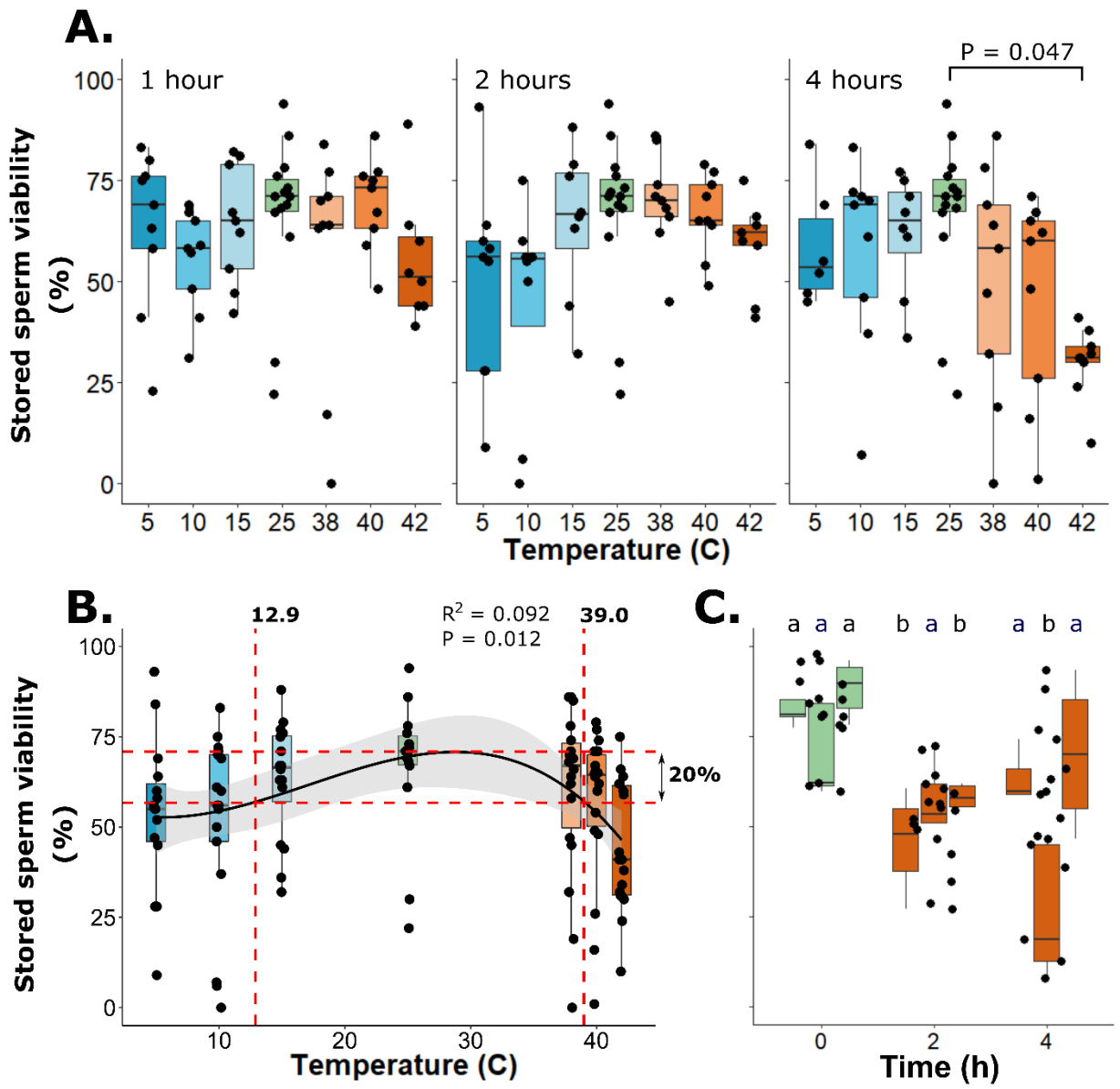


**Figure 1. Viability of stored and ejaculated sperm after temperature stress.** A) Honey bee queens were heat- and cold-shocked for 1 - 4 hours, then held at 25 ˚C prior to assessing stored sperm viability. The 25 ˚C treatment is the negative control (the same control data is replotted in each panel). Only the 42 ˚C treatment resulted in a significant drop in sperm viability (two-way ANOVA, factors: temperature and time). Differences among specific groups were evaluated with a Tukey HSD post hoc test. B) Combined 2 h and 4 h treatments were fit with a cubic model ($R^2$ = 0.092, P = 0.012). Thresholded at 20% loss of viability, 13 - 39 ˚C can be considered safe for queens. C) Ejaculated honey bee semen from three different colonies (5-6 drones per colony, illustrated as different boxes) was subject to heat-shock at 42 ˚C for 2 h and 4 h, then kept at 25 ˚C for 2 d. Letters indicate significant differences (two-way ANOVA, factors: colony and time, followed by a Tukey HSD post hoc test).

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To test effects of heat on ejaculated sperm viability, we exposed single-drone ejaculates to 42 ˚C for 0, 2, or 4 h, followed by a 2 d recovery period at 25 ˚C **(Fig 1C; Fig S2)**. We found that responses differed depending on the colony source, but heat dramatically decreased viability by 35% after both 2 and 4 h (two-way ANOVA, factors: time and colony, $P_{(time)} = 0.00000012$, $F_{(time)} = 24$; $P_{(colony)} = 0.00015$, $F_{(colony)} = 11$). Heat-shock therefore affects stored and ejaculated sperm viability at similar magnitudes.

Next, we compared drone, queen, and worker survival over time at 25 ˚C, 38 ˚C, and 42 ˚C. We confirmed that drones, but not queens, are indeed mortally sensitive to heat **(Fig 2A)**. 54% of drones died over the course of six hours at 42 ˚C, whereas every queen survived. We also found that drones are more sensitive to heat than workers, which have a similar lifespan to drones but are non-reproductive. Finally, we looked for sex-biased heat tolerance in two solitary insect species—fruit flies (*Drosophila melanogaster*) and brown marmorated stink bugs (*Halyomorpha halys*) —but we found no difference between males' and females' sensitivities for fruit flies and an opposite (female) biased heat sensitivity in stink bugs **(Fig 2B-C)**. Therefore, the extreme male sensitivity to heat in honey bees may be a unique quality control feature of eusocial insects. Most highly eusocial insects rely on a nuptial flight as a single opportunity to acquire high-quality sperm for fertilizing eggs for the rest of their lives (up to five years for honey bees), and males that perish by heat-shock would never have the opportunity to inseminate a queen with already-damaged sperm.

*Queens combat heat stress with ATP-independent sHSPs*

Sperm longevity is enabled by molecular processes that reduce oxidative damage and maintain sperm in a quiescent metabolic state. For example, the spermatheca is a highly anaerobic environment, which helps prevent reactive oxygen species (ROS) formation[50]. Enzymes that further limit damage from ROS are also upregulated in mated queens compared to virgins[51], and ROS damage leads to infertility in mammals[52,53]. Heat is well known to lead to oxidative stress[54]; therefore, we hypothesized that queens may combat heat-stress by upregulating enzymes that mitigate oxidative damage. Additionally, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been previously implicated in stored sperm longevity, since supplementing artificial spermathecae with its substrate (GAP) improves viability and produces the highest molar ratio of ATP via anaerobic catabolism[50]. Heat-induced changes in GAPDH expression could therefore also impact sperm viability.

To test these hypotheses, we compared expression of ROS mitigating enzymes (superoxide dismutases (SOD1, 2, and 3) and catalase) as well as GAPDH in heat-shocked and non-heat-shocked virgin spermathecae, mated spermathecae, and ejaculated semen. Stored sperm are reportedly transcriptionally active[50], and analyzing all three tissues enabled us to disentangle the male versus female origins of expression in sperm-filled, mated spermathecae. All enzymes were significantly upregulated in mated spermathecae compared to the other tissues (**Fig 3A**), which is consistent with previous observations[51]. Most markedly, GAPDH expression was massively up-regulated in mated queens approximately 2,000 times relative to virgin spermathecae and approximately five times relative to semen. However, heat-shock did not upregulate expression of these proteins (**Fig 3B-D**); rather, all enzymes were consistently (but not significantly) downregulated with heat (by 10-30%). Heat, therefore, neither induces expression of ROS-mitigating enzymes nor does it interfere with GAPDH expression. Queens must therefore employ other strategies to combat heat stress.

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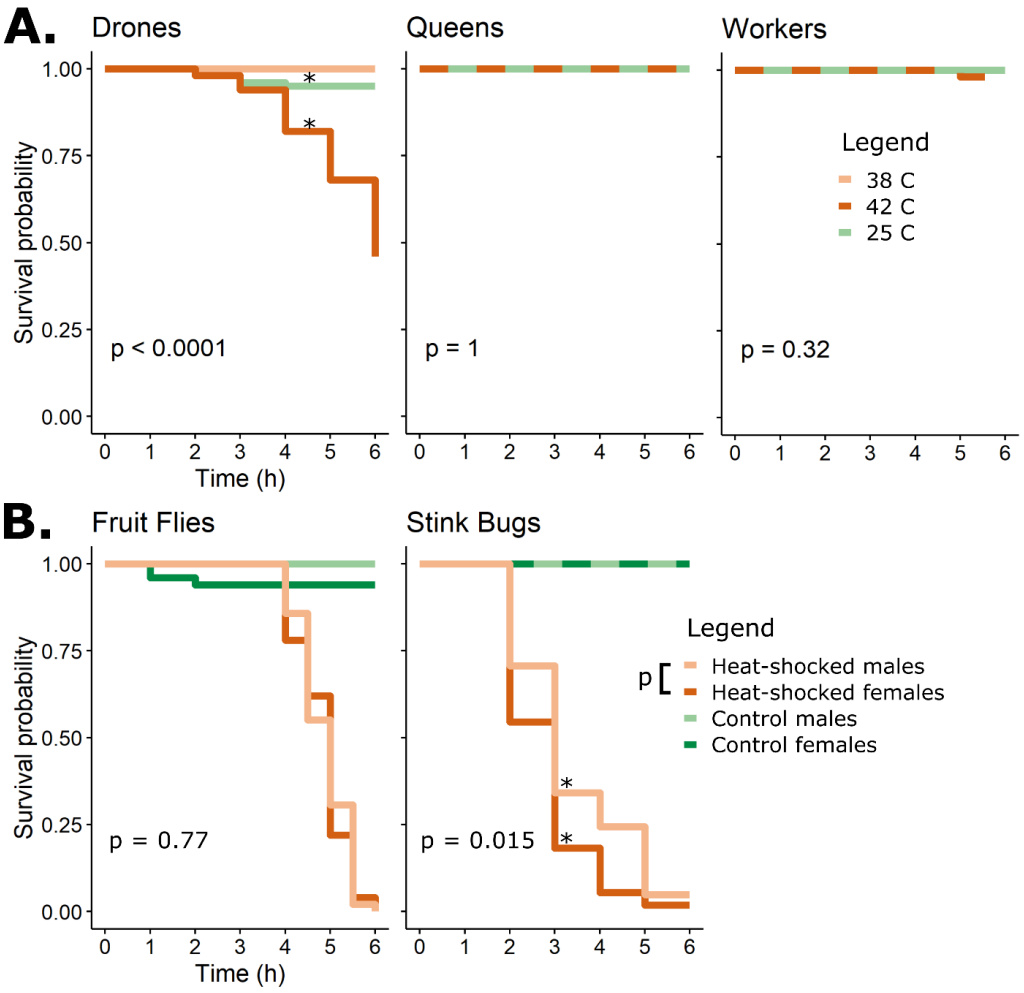


**Figure 2. Sex-biased heat mortality in honey bees, stink bugs, and fruit flies**. Statistical differences were evaluated using a log-rank test. Asterisks indicate significant differences. A) Honey bees (drones, queens, and workers) were held at different temperatures and mortality was recorded hourly. Drones are sensitive to heat, whereas queens and workers are not. B) Fruit flies (*Drosophila melanogaster*; heat-shock = 38 ˚C) and stink bugs (*Halyomorpha halys*; heat-shock = 42 ˚C) do not have male-biased heat sensitivity. For fruit flies, loss of motility was used as the endpoint.

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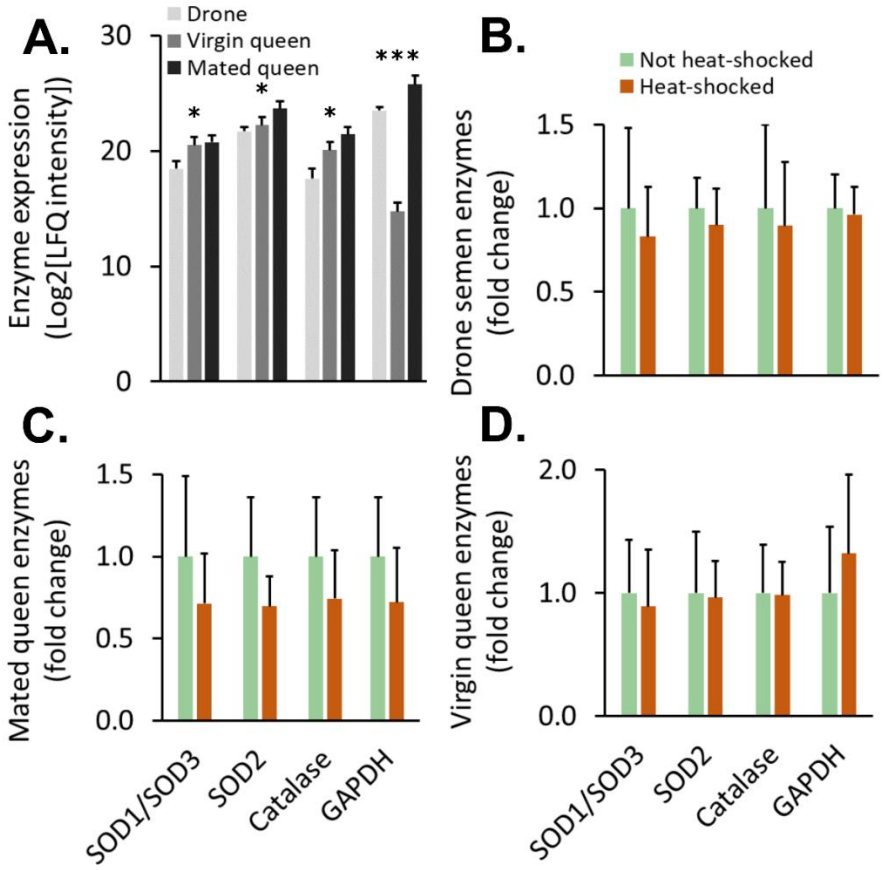


**Figure 3. Heat-shock does not affect expression of superoxide dismutase, catalase, and GAPDH.** A) Isoforms of superoxide dismutase (SOD1, SOD2, and SOD3; accessions XP_026298263.1, NP_001171519.1, and ANS15098.1, respectively), catalase (XP_026296889.1), glutathione-S-transferase (GST1-1; accession XP_026296300.1), and glyceraldehyde-3-phoshate dehydrogenase (GAPDH; accession XP_397363.4) were evaluated in drone, virgin queen, and mated queen reproductive tissues by intensity-based, label-free quantitative tandem mass spectrometry (permutation-based FDR = 10%). * P < 1.0E-5, ***** P < 1.0E-10. SOD1 and SOD3 were indistinguishable based on the observed peptides. B-D) Protein fold-change was evaluated between heat-shocked and non-heat-shocked treatments. Heat-shock tended to decrease enzyme abundance, but not significantly (one-way MANOVA).

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A multitude of other proteins could also be responsible for mitigating damage from heat—most obviously, members of the heat-shock protein (HSP) family[24,55-57]. Some HSPs contain an ATPase domain and require ATP to function (*e.g.,* HSP70s, HSP90s, HSP110s), while others operate independently of ATP (*e.g.,* HSP10s, HSP20s, HSP60s). In addition, since GAP yields the most ATP per unit via anaerobic catabolism of any tested substrate and significantly increased sperm longevity[50], this implies that ATP usage economy is a critical factor for maintaining viable sperm. Therefore, we expect that ATP-independent HSPs should be upregulated in spermathecae, and ATP-dependent HSPs should be upregulated in other tissues. In addition, some ATP-independent short HSPs (sHSPs, typically 20-28 kDa) suppress ROS generation while preventing protein aggregates and apoptosis[54,58]. Therefore, we hypothesized that heat should induce expression of sHSPs in the spermatheca.

To determine which HSPs were upregulated with heat-shock, we compared global protein expression profiles in heat-shocked and non-heat-shocked spermathecae, ejaculates, and ovaries **(Fig 4A, Fig S2)**. We hypothesized that the heat-shock response should conserve ATP in the spermatheca, but this should not be as critical in the ovaries. Therefore, we expected heat to induce ATP-independent sHSPs in the spermathecae; conversely, we expected ATP-dependent HSPs to become upregulated in the ovaries. Of the 2,778 protein groups identified in the spermathecae and ejaculates, only five were significantly up-regulated with heat (5% FDR), all of which were in the spermathecae. All five of the proteins were unique, ATP-independent sHSPs (accessions: XP_001120194.1, XP_001119884.1, XP_395659.1, XP_001120006.2, and XP_026294937.1) (**Fig 4B**). Two of the same sHSPs (XP_395659.1 and XP_001120006.2) were also upregulated in heat-shocked virgin spermathecae, indicating that this is a queen-derived, and not a sperm-derived, response **(Fig 4C and D**). By contrast, the most strongly upregulated protein in heat-shocked ovaries was HSP70—an ATP-dependent HSP— and no sHSPs were upregulated in this tissue, supporting our initial hypotheses **(Fig 4E)**.

As expected, the significantly enriched Gene Ontology (GO) terms were all related to responses to heat and stress in the spermathecae (**Fig 5A and B**). However, the only significantly enriched GO term in the semen analysis was related to the electron transfer activity (**Fig 5C**), driven by a heat-induced down-regulation of the proteins linked to this GO term (**Fig 5D**), suggesting that the sperm may be less able to produce the large amounts of ATP necessary for flagellar beating. This is consistent with the findings of Gong *et al.*, who found that heat stress at 42˚C impaired mitochondrial function, reduced electron transport chain complexes' activities, and lowered total cellular ATP[23].

Examining all HSPs identified in this study, we found that 20 HSPs were identified in spermathecae overall and only 13 were identified in the semen **(Fig 6**); however, the precise functions of specific honey bee HSPs are largely unknown. By contrast, mammalian HSPs have been studied extensively; research on human sHSPs has focused on their roles in many types of cancer (osteosarcoma, leukemia, breast, colorectal, hepatic, gastric, and prostate cancer)[59,60], where their upregulation is associated with both pro- and anti-apoptotic properties and mitigating ROS production[54,57,61,62].

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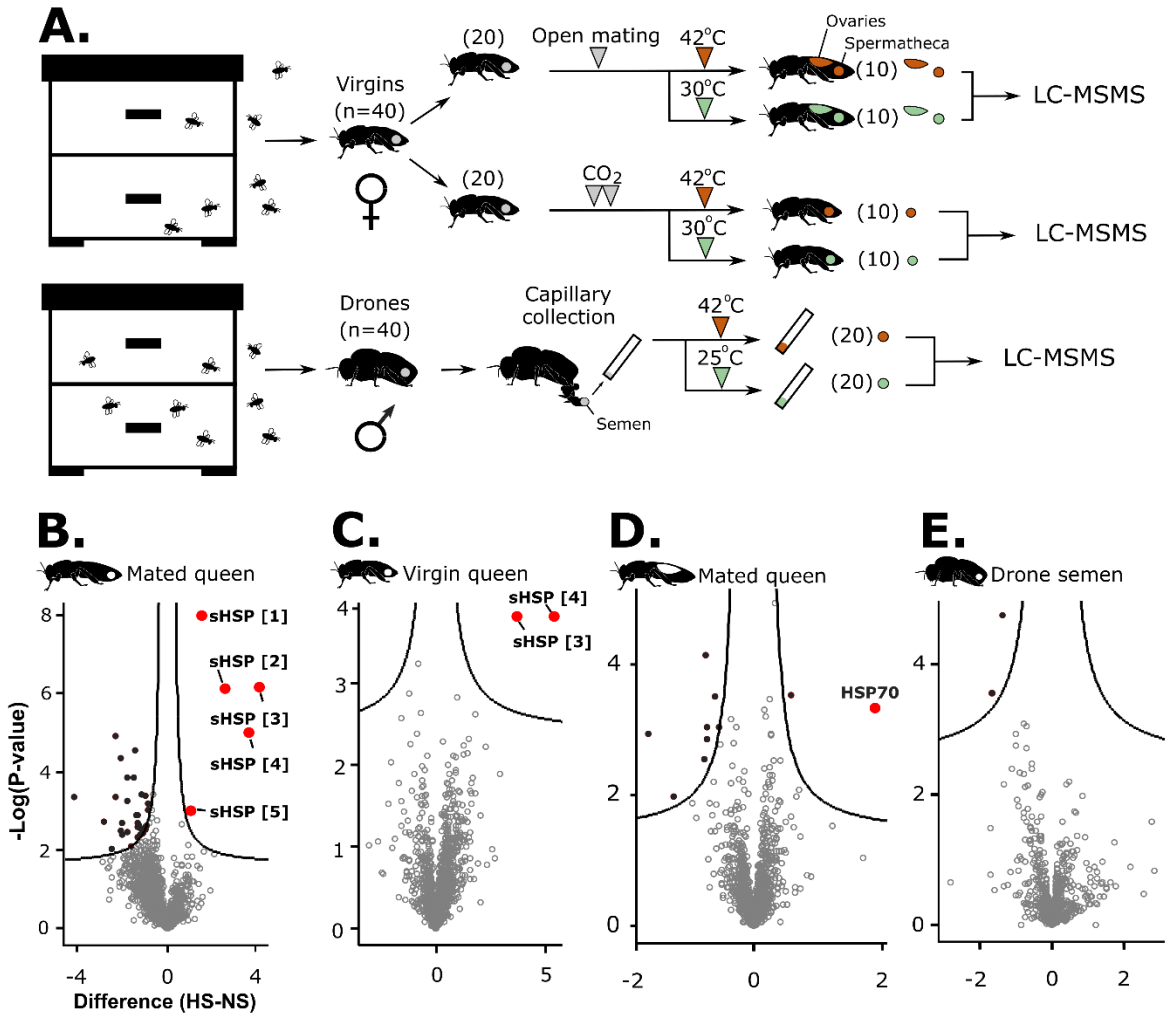


**Figure 4. Differential protein expression comparing heat-shocked (HS) and not heat-shocked (NS) reproductive tissues.** A) Samples included mated queen spermathecae, virgin queen spermathecae, ejaculated semen, and mated queen ovaries, which were all analyzed by intensity-based, label-free quantitative tandem mass spectrometry. The significance cut-off for volcano plots (B-E) was 10% FDR (false discovery rate, permutation-based). Significant proteins are labelled red. Accessions for sHSP [1-5] are XP_001120194.1, XP_001119884.1, XP_395659.1, XP_001120006.2, and XP_026294937.1, respectively. The accession for HSP70 is NP_001153536.1.

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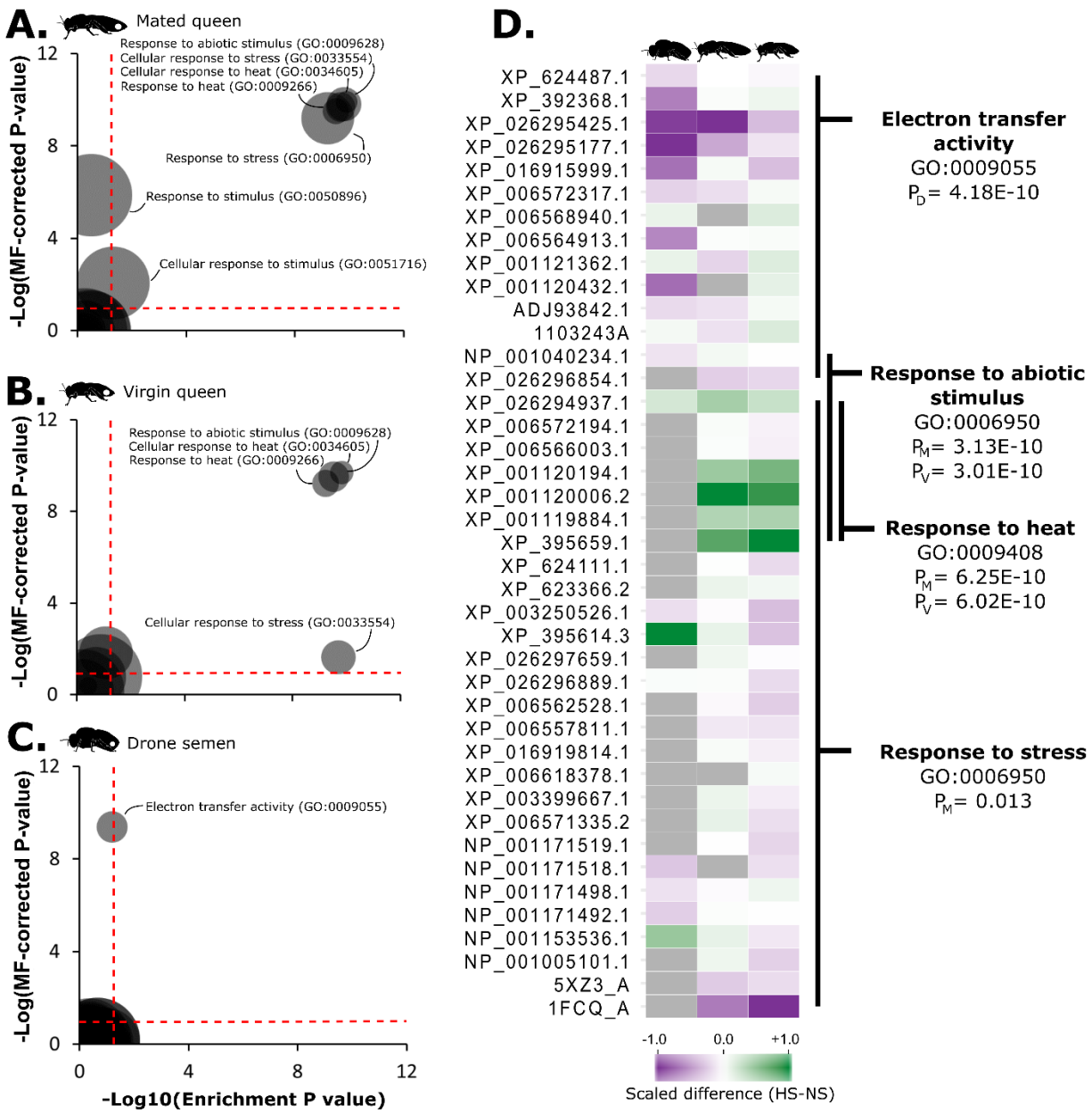


**Figure 5. Gene ontology (GO) term enrichment analyses**. A-C) Enrichments were performed via gene score resampling, correcting for both multiple hypothesis testing and protein multifunctionality (MF). The x-axes depict enrichment P values (10% FDR, Benjamini-Hochberg correction) without multifunctionality correction. The y-axes depict enrichment P values after correction for both multiple hypotheses and multifunctionality. The red dotted lines indicate the P value significance cut-off that achieves 10% FDR. D) Summary of proteins associated with significantly enriched GO terms. Subscripts refer to comparisons within drones (D), mated queens (M), and virgin queens (V).

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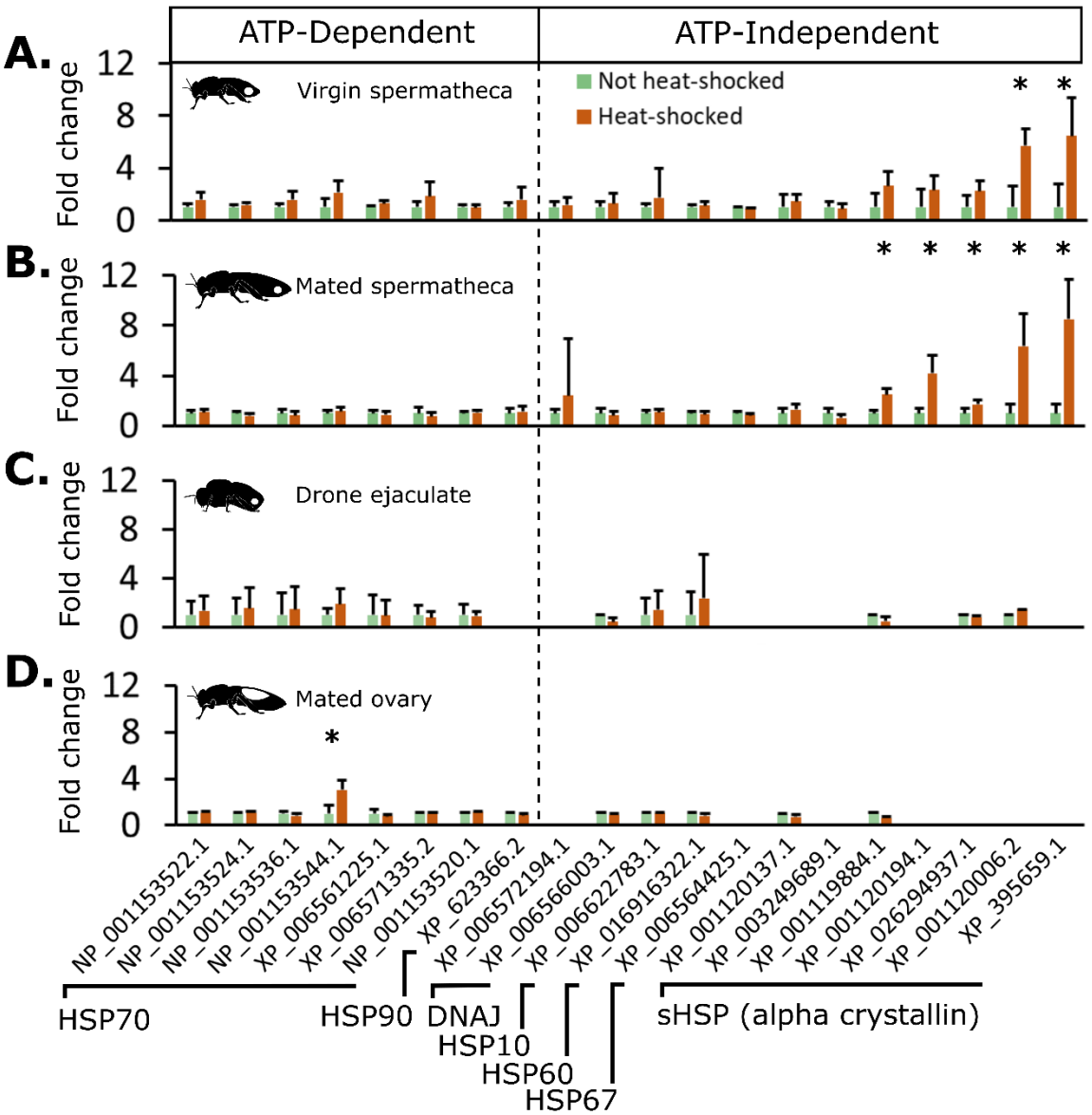


**Figure 6. Heat-shock protein expression profiles.** Proteins with GO terms for cellular response to heat were retrieved from the proteomics datasets. Fold-change is relative to the non-heat-shocked state. Proteins marked with an asterisk were significantly up-regulated in the global differential gene expression analysis (permutation-based FDR: 10%).

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To gain insight into potential roles of the five differentially expressed HSPs we identified, we compared their sequences to the human representative proteome and identified putative protein domains using NCBI's basic local alignment search tool (BLAST)[63]. All five of the HSPs contain one or more alpha crystallin domains, which is characteristic of small HSPs (sHSPs). Four of these HSPs are within the expected molecular weight range, and one of the proteins (XP_026294937.1) is predicted to be 56.7 kDa (and contains two alpha crystallin domains instead of one). One of the significantly differentially expressed honey bee sHSPs (XP_001120194.1) is most similar in sequence to a human sHSP beta-1 (HSPB1). In humans, this protein is considered to be testicle-specific and is only expressed in the Sertoli cells and spermatogonia, although not in the spermatozoa[64]. Surprisingly, here we find it is significantly up-regulated in heat-shocked mated queen spermathecae, and tends to be up-regulated in virgin spermathecae as well (although not significantly). We speculate that the queen bee may be creating a teste-like environment to house her sperm. In humans, HSPB1 has an anti-apoptotic effect, and its up-regulation in heat-shocked testes (like we observe in heat-shocked spermathecae) is thought to help compensate for the damaging effects of heat[61]. Queens with very strong up-regulation of XP_001120194.1 and other sHSPs may therefore be better able to counter-act ROS production, sperm death and ultimately maintain longevity.

Other researchers have suggested that in mammals, some ATP-dependent HSPs (*e.g.,* HSP70s and HSP90s) may be important for maintaining male fertility via quality control of sperm[57,65]. Unlike HSPB1, these HSPs appear to be pro-apoptotic factors and could theoretically help prevent damaged sperm from being able to fertilize an egg. Neither queen spermathecae nor drone ejaculates provide evidence supporting this strategy of quality control in honey bees; in this experiment, none of the HSPs that were up-regulated with heat in spermathecae or semen contained an ATP-binding nor ATPase domain, which are characteristics of HSP70s and HSP90s but not the sHSPs (**Figure 5 and 6**)[57]. Rather, HSP70 was only upregulated in the ovaries, which does not directly participate in sperm maintenance and suggests that pro-apoptotic strategies may be more important for egg, rather than sperm, quality control.

In mammals, sHSPs are upregulated in testes after heating, but so are ATP-consuming proteins like HSP105, HSP70-1, HSP70-2, and HSP90[66-68]. We speculate that in mammals, the energetic cost associated with ensuring high sperm quality is advantageous because it helps reduce the risk of an egg going unfertilized, which would produce no progeny. However, the honey bee's differing strategy of upregulating only the ATP-independent sHSPs is consistent with the their haplodiploid sex determination system: for honey bees, an unfertilized egg develops as a drone, rather than being non-viable. Therefore, honey bees may have experienced little selection for an ATP-consuming quality control measure, and instead combat heat damage to sperm in an ATP-conservative manner. The ATP-independent sHSPs have the dual purpose of also limiting oxidative damage. The significant enrichment for differentially expressed proteins involved in multiple nucleotide metabolic processes, including ATP, in the transition from virgin to mated spermathecae (**Fig S3**) supports the notion that regulating ATP production and consumption is critical for maintaining stored sperm viability.

Moreover, analyzing heat-shocked mated queen ovaries revealed that HSP70—an ATP-dependent HSP—was upregulated in this tissue, which never occurred in the spermathecae (**Fig 5 and 6**). This observation is consistent with selection for ATP-conservative quality control mechanisms when a failed fertilization event can still produce viable offspring (as is the case for honey bees when a non-viable sperm cell meets an egg, resulting in a drone) but not when a failed fertilization event fails to produce offspring (when a non-viable egg meets a viable sperm, and in non-haplodiploid organisms when dying sperm

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meets a viable egg). Overall, these data indicate that upregulation of specific sHSPs is not a general indicator of cellular stress, since it was not observed in the ovaries. Indeed, the ovaries appear not to express most sHSPs at all, whereas they were abundantly expressed even in non-heat-shocked spermathecae. We propose that the sHSP response of the spermathecae may serve as a post-queen-failure biomarker of heat stress which could help diagnose causes of colony failure in the field. Future experiments should investigate if other stressors (*e.g.,* pesticide exposure, cold-shock, or pathogen infection) yield the same or different stress signals.

*Proteome signatures of queens naturally failing in the field*

Queens can fail for a myriad of reasons ranging from heat stress, to pesticide poisoning, to disease. To examine if queens that failed in the field from unknown causes displayed a similar heat stress proteomic signature to queens that were heat-shocked in the laboratory, we sampled 11 failing and 11 age-matched healthy queens heading colonies in an apiary. Queens were rated as 'failing' if they had ceased to lay eggs, were 'drone layers' (*i.e.,* were not laying fertilized female eggs), or had otherwise inferior brood patterns. We used the six proteins up-regulated with laboratory heat-shock (the five spermathecal sHSPs and single ovarian HSP70) as a potential biomarker panel for heat stress. We found that five of the failed queens displayed spermathecal proteomic signatures with at least one of the five previously identified sHSPs upregulated (> 3-fold) relative to the queen's age-matched healthy partner (**Fig 7A**). Each of these queens also clustered with the laboratory heat-shocked queens in an unsupervised hierarchical clustering analysis considering all 6 candidate biomarkers simultaneously.

Of the queens that did not have an ostensible heat-shock signature, most presented a divergent stress signature in their ovaries, indicating that they may have suffered from an alternate cause of failure (**Fig 7B**). Indeed, these queens also had significantly reduced ovary mass compared to their age-matched healthy controls, a phenotype that we did not observe with laboratory heat-shock **(Fig S4)**. This indicates that ovary-based physiological failure may have contributed to the remaining queens' demise. With the present data, we cannot validate that queens in these samples (Field 2, 5, 8, 9, and 10) were actually heat-shocked in the past or not, but the data suggest this could have been the case. Future validation experiments should involve a blind heat-shock field trial to determine a) if previously heat-shocked queens can be reliably distinguished from non-heat-shocked queens based on these biomarkers, b) how long the heat-shock proteomic signature lasts, and c) if other stressors produce proteomic signatures mimicking the heat-shock signature. These data will help us solidify a panel of protein biomarkers for different stressors, so that in the future we can retroactively diagnose causes of queen failure. Such a molecular diagnostic tool would not only be useful for the beekeeping industry: measuring these heat-shock signatures would also enable us to survey heat-induced sperm damage in different regions as the climate changes with time.

**Conclusion**

Our experiments show that temperature stress deeply damages both stored and ejaculated honey bee sperm viability, and that temperatures ranging between 13 ˚C and 39 ˚C for 2 - 4 h is generally safe for queens. These temperatures should therefore not be exceeded during queen shipping and handling. Since drones[19], but not queens, are mortally sensitive to 42 ˚C temperatures, we suggest that this may be a mechanism for preventing queens from being inseminated with heat-damaged sperm. Future research should investigate if this sex-biased heat sensitivity is present in other eusocial insects, such as ants and wasps (Order: Hymenoptera) but not termites (Order: Blattodea), which have long-lived kings

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and queens. Queens combat heat-shock by upregulating ATP-independent HSPs, which both reduces ATP consumption and provides beneficial anti-apoptotic properties. In contrast, HSP70 (an ATP-consuming HSP) was upregulated in heat-shocked ovaries. We found similar patterns of HSP expression in queens that failed as a result of unknown causes in the field. Once validated, these signatures could serve as biomarkers for heat stress—enabling post-queen-failure diagnostics for the beekeeping industry—as well as for surveying the prevalence of heat-induced loss of sperm viability in diverse landscapes as part of a biomonitoring program. Honey bee colonies are thermoregulated superorganisms and environmental samplers; therefore, they have the potential to become sentinels for stress-induced losses in sperm viability for not only wild insect populations, but also for humans around the world.

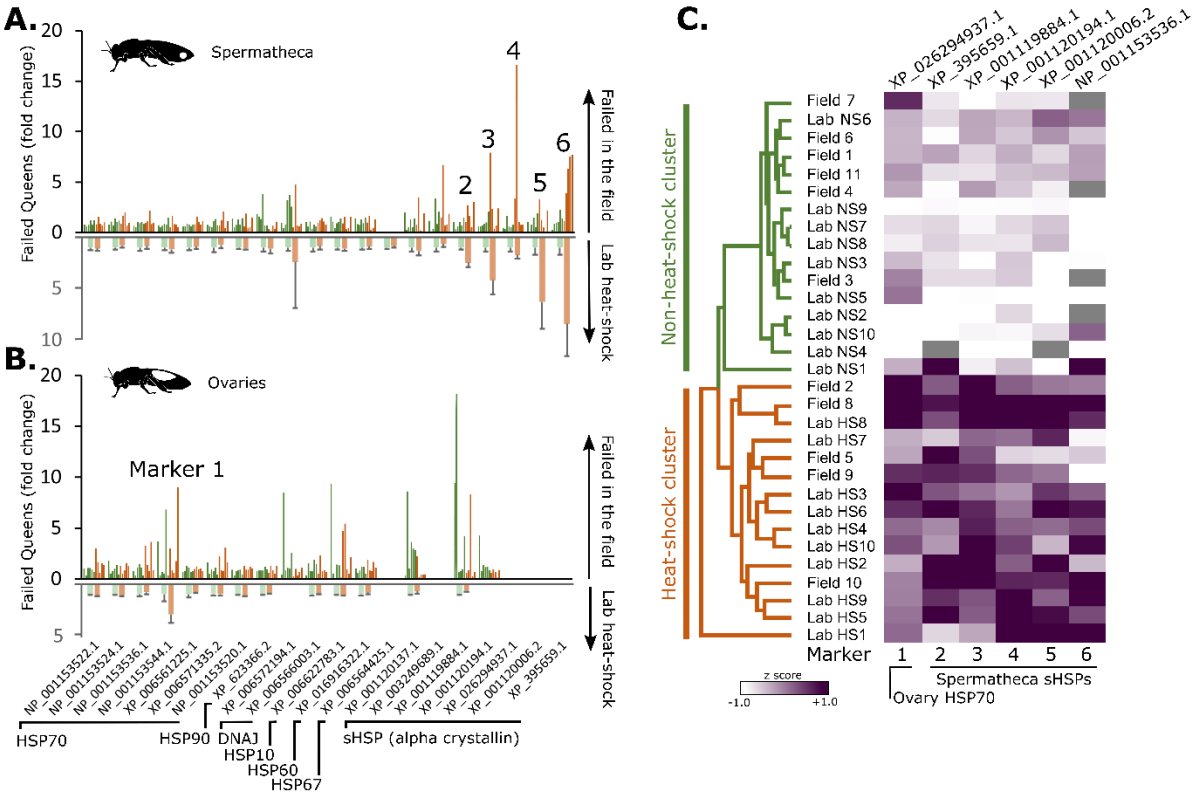


**Figure 7. Proteomic signatures of queens heading failing colonies.** We sampled 22 queens from the field (11 heading healthy colonies, and 11 heading failing colonies) and performed quantitative proteomics analysis on their spermathecae (A) and ovaries (B). Each bar on the upward-oriented graph represents one age- and apiary-matched queen pair. Queen pairs were colour coded based on if they expressed at least one up-regulated (> 3-fold) sHSP that was also significantly differentially expressed in the laboratory heat-shock experiment. The inverted graph represents data obtained from experimentally heat-shocking queens in the laboratory (from **Fig 6**). C) The field samples colour-coded orange in A and B (Field 2, 5, 8, 9, and 10) also cluster together with the laboratory heat-shock samples (unsupervised hierarchical clustering, Euclidian distance, rows are samples, columns are significantly upregulated proteins identified by laboratory heat-shock).

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## Methods

*Sperm viability assays*

Honey bee queens (Kona Queens supplied in a single shipment) were treated at one of five different temperatures (5, 10, 15, 25, 38, 40, and 42 ˚C) for 1, 2, or 4 hours, then held at 25 ˚C for 2 d. Fourteen queens (replicates) were included in the 25˚C treatment (negative control), whereas 8-9 queens were included in all other temperatures and exposure durations (see **Table 1** for replication information). Following this, queens were beheaded, and their spermathecae were dissected with fine forceps. The spermathecae were gently agitated with a pipet tip in 100 µl Buffer D (17 mM D-glucose, 54 mM KCl, 25 mM $NaHCO_3$, 83 mM $Na_3C_6H_5O_7$) to break them open and release the sperm. Sperm viability was determined using a live/dead sperm viability kit (Thermo) following the protocols of Collins and Donoghue[69]. Briefly, the SYBR14 dye was diluted 1:9 in DMSO. Two microlitres of the diluted SYBR14 dye and 4 µl of propidium iodide were gently mixed with the sperm and incubated for 15 minutes in the dark. Two microlitres of the mixture was then added to a glass microscope slide and viewed under a fluorescent microscope. Live (green) and red (dead) sperm were counted until 100 cells were observed, covering multiple fields of view. Unless otherwise reported, all statistical analyses were performed in R (v3.5.1). First, the data were analyzed by a two-way ANOVA (factors: temperature, time). Then a linear regression was performed, using temperature as a continuous variable, testing exponents 1-4 to identify the optimal fit (highest $R^2$).

Drones were harvested from three different colonies kept in Beaverlodge, Alberta. Semen was collected with glass capillaries according to the methods of Collins and Donoghue[69]. Briefly, we pinched drone abdomens with a rolling motion from the anterior to posterior end to expel the endophallus and semen. The semen was collected by first filling glass capillaries with 1 µl Buffer D, then drawing up the semen via capillary action (avoiding the white mucus secretions). Capillaries were then filled with a further 1 µl of Buffer D to prevent the sample from drying out, and both ends were wrapped with parafilm. This technique typically yielded 0.5-1.0 µl of semen per sample. Semen samples were then heat-shocked at 42 ˚C for 0, 2, or 4 h, then allowed to recover at 25 ˚C for 1-2 d (see **Table 2** for replication information). We chose a lower temperature for semen recovery because ejaculated drone semen can be maintained at room temperature for several weeks while retaining high viability[70]. Viability assays for these semen samples was performed following the same methods as for spermathecae. Data were analyzed by a two-way ANOVA (factors: time, colony) followed by a Tukey HSD test.

**Table 1. Biological replicates for queen temperature stress tests**

| Temperature | 0 h | 1 h | 2 h | 4 h |
|---|---|---|---|---|
| **5°C** | | 9 | 9 | 9 |
| **10°C** | | 9 | 8 | 9 |
| **15°C** | | 9 | 8 | 8 |
| **25°C** | 14 | | | |
| **38°C** | | 9 | 9 | 9 |
| **40°C** | | 9 | 9 | 9 |
| **42°C** | | 8 | 9 | 9 |
| **Total** | 14 | 53 | 52 | 53 |

**Table 2. Biological replicates for drone temperature stress tests***

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| Colony | 0 h | 2 h | 4 h |
|---|---|---|---|
| **1** | 5 | 5 | 6 |
| **2** | 6 | 6 | 6 |
| **3** | 5 | 5 | 6 |
| **4**** | 10 | 10 | |
| **Total** | 26 | 26 | 18 |

*Temperature stress for all replicates was 42 ˚C
**Data depicted in Fig S2

*Heat-shock survival*

For honey bee heat-shock tests, drones and workers were collected by retrieving a frame of capped brood from a hive and allowing the bees to emerge in an incubator. Newly emerged bees were marked with a paint pen and returned to the colony to age for one week, at which time they were recaptured and caged in California mini cages. For drone heat-shock tests, one drone was added per cage along with malleable candy (icing sugar-based) and five worker attendants. A total of 199 drones were caged in the following experimental groups: 42 ˚C heat (50), 38 ˚C heat (50), and 25 ˚C (99) for six hours (all at 60% relative humidity). Every hour, the number of drones that perished was recorded. Worker heat stress tests were conducted independently with six workers per cage (54 workers per group) held at 42 ˚C and 25 ˚C.

Queens were from a variety of sources (local, Hawaii, and Australia) and their ages ranged from approximately 3 - 5 weeks. They were kept in California mini-cages with five worker attendants each and held at 42 ˚C, 38 ˚C, or 25 ˚C for either 1, 2, or 4 h as part of other experiments (for viability measurements and proteomics). Relative humidity varied between 40 and 80%. Eight queens were held at 42 ˚C for 6 h at a constant 60% relative humidity specifically for survival analysis. See **Fig S2** for sample sizes for each treatment group.

Male and female 3-10 day old Canton S wild type fruit flies were held at 38 ˚C and 25 ˚C for six hours in *Drosophila* vials with media (50 males and 50 females). Loss of motility was recorded every hour. We used this as the endpoint because fruit flies will become paralyzed with heat, but recover motility after several hours (in some cases, overnight). Control female flies were maintained for a further week after the experiment to confirm that the majority of them had successfully mated prior to the heat stress tests.

Male and female stink bugs were reared in the laboratory according to standard protocols[71]. Stink bugs which transitioned from nymphs to adults 3-7 d prior were transferred to ventilated 500 ml volume plastic cages with a piece of moist paper towel (3-6 stink bugs per cage). They were heat stressed at 42 ˚C (55 females and 41 males) or held at 25 ˚C (36 females and 24 males) for 6 h. Only mated females carrying eggs were included in the experiment, as determined by post-stress dissection.

In all cases, Kaplan-Meier survival curves were generated in R and compared using log-rank tests. See **Fig S2** for sample size information for survival tests.

*Heat-shock for proteomics*

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For the proteomics experiments, honey bee colonies were maintained in an apiary at the University of British Columbia. During the summer of 2018, 40 queens were reared from a single colony and half were allowed to open mate, while the other half were kept as virgins in plastic queen cages. Two weeks after emergence, the virgin queens were given two, eight-minute carbon dioxide treatments on 2 sequential days, then re-introduced to their nucleus colonies. This process stimulates virgin queens to begin laying[72], and we conducted these treatments in order to minimize the physiological differences between virgin and mated queens.

Virgin and mated queens were retrieved from their nucleus colonies and half of each (10) were subjected to heat-shock (42 ˚C, 2 h), and then maintained at 30 ˚C for 2 d. The other half were held only at 30 ˚C for 2 d. Four to six weeks after mating, the queens were anesthetized with carbon dioxide, beheaded, then their spermathecae (including the tracheal net) were removed with fine forceps. Both ovaries were also removed and weighed.

During the same summer, 200 drones from a different colony in the same apiary were collected and maintained in the laboratory overnight at ambient temperature with excess syrup (50% sucrose). The next day, semen was harvested with glass capillaries according to the methods described above. Because many drones were not sexually mature, 60 semen samples (out of the 200 drones) were collected. Capillaries were placed in petri dishes and half (30) were heat-shocked as described above, then kept at 25 ˚C for 2 d. The other half were only kept at 25 ˚C for 2 d. Ten samples from each experimental group were used for sperm viability assays as described above.

*Proteomics sample preparation*

Semen and spermatheca samples were homogenized in 2 ml screw-cap tubes containing 100 µl of lysis buffer (6 M guanidinium chloride, 100 mM Tris, pH 8.5) and four ceramic beads. The homogenizer (Precellys 24, Bertin Instruments) was set to 6,500 $s^{-1}$ for 30 s, then samples were centrifuged (16,000 rcf, 10 min, 4 ˚C) to remove debris. Supernatants were transferred to a new tube and diluted 1:1 with $dH_2O$. Protein was precipitated by adding four volumes of ice-cold acetone and incubating overnight at -20 ˚C. The precipitated protein was pelleted and washed twice with 500 µl of 80% acetone, then the pellet was allowed to air dry (ca. 5 min) prior to solubilization in 50 µl of digestion buffer (6 M urea, 2 M thiourea).

Approximately 25 micrograms of protein were reduced (0.5 µg dithiothreitol, 20 min), alkylated (2.5 µg iodoacetamide, 30 min, dark), and digested (0.5 µg Lys-C for 3 h, then 0.5 µg trypsin overnight). Digested peptides were acidified with one volume of 1% trifluoroacetic acid and desalted with high-capacity STAGE tips as previously described[73]. Eluted samples were dried (SpeedVac, Eppendorf, 45 min) and resuspended in Buffer A (0.1% formic acid). Peptide concentrations were determined using a NanoDrop (Thermo, 280 nm) and sample orders were randomized for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

*LC-MS/MS data acquisition and analysis*

Peptides (0.5 µg for each sample) were injected on an EASY-nLC 1000 liquid chromatography system (Thermo) coupled to an Impact II Q-TOF mass spectrometer (Bruker), essentially as previously described[74]. The LC system included a fused-silica (5 µm Aqua C18 particles (Phenomenex)) fritted 2 cm trap column connected to a 50 cm analytical column packed with ReproSil C18 (3 µm C18 particles (Dr.

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Maisch)). The separation gradient ran from 5% to 35% Buffer B (80% acetonitrile, 0.1% formic acid) over 90 min, followed by a 15 min wash at 95% Buffer B (flow rate: 250 µL/min). The instrument parameters were: scan from 150 to 2200 m/z, 100 µs transient time, 10 µs prepulse storage, 7 eV collision energy, 1500 Vpp Collision RF, a +2 default charge state, 18 Hz spectral acquisition rate, 3.0 s cycle time, and the intensity threshold was 250 counts.

Mass spectrometry data were searched using MaxQuant (v1.5.3.30) using default parameters, except "match between runs" was enabled. Peptide spectral matches, peptide identifications and protein identifications were controlled at 1% false discovery rates (FDRs). The protein search database was the NCBI Identical Protein Groups database for *Apis mellifera* (downloaded Nov. 1st, 2018; 21,425 entries) plus all honey bee viral proteins contained within NCBI (a further 508 entries). Data were analyzed in Perseus (v1.6.1.1) as previously described[74]. Proteins differentially expressed between heat-shocked and non-heat-shocked samples, as well as among tissues (semen, virgin spermathecae, and mated spermathecae), were identified using t-tests and significant results were corrected to 10% FDR (permutation-based method). Raw data and search results are available on PRIDE proteomeXchange (www.proteomexchange.org; accession: PXD013728).

*GO term enrichment analysis*

Gene Ontology (GO) terms were retrieved using BLAST2GO and subsequent enrichment analyses were conducted using ErmineJ[75]. We used the gene score resampling (GSR) method (with P values as scores). False discovery rates were controlled to 10% using the Benjamini-Hochberg correction. Protein multifunctionality (MF) scores were also computed in ErmineJ based on the number of different GO terms with which the protein is associated. This process generates an "MF-corrected P value" (also limited to 10% FDR), which is the enrichment P value after correcting for multifunctionality.

*Field-failed queens*

Age-matched failing and healthy queens (11 each) were obtained from a research apiary in Pennsylvania. Queens were rated as 'failing' if they had ceased to lay eggs, were 'drone layers' (*i.e.,* were not laying fertilized female eggs), or had otherwise inferior brood patterns. Queens were frozen on dry ice in the field and stored at -80 ˚C until spermatheca and ovary dissection. Proteomics analysis was performed as described above. Sample handlers were blind to queen groups until all proteomics data was acquired. Heat-shock protein abundance is expressed as fold-change of abundance in the failed queen relative to her healthy queen pair. We then performed hierarchical clustering using the ratios of the six candidate heat-stress biomarkers (five spermathecal markers and one ovarian marker) identified in the laboratory heat-shock experiment. Hierarchical clustering with laboratory samples was performed in Perseus using average Euclidian distance (300 clusters, maximum 10 iterations).

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**Competing interests**

JSP owns a honey bee consulting business.

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