Thermal hazards for queen honey bees

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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 globally distributed micro-livestock; therefore, they could serve as environmental biomonitors for heat-induced reductions in fertility. Here, we found that queens have two potential routes of temperature-stress exposure: within colonies and during routine shipping. Our data suggest that temperatures of 15 to 38°C are safe for queens at a tolerance threshold of 11.5% loss of sperm viability, which is the viability difference between failed and healthy queens collected from beekeepers. Heat shock activates expression of specific ATP-independent heat-shock proteins in the spermatheca, which could serve as biomarkers for heat stress. This protein fingerprint may eventually enable surveys

for the prevalence of heat-induced loss of sperm viability in diverse landscapes as part of a biomonitoring program.

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].

Insects and other ectothermic animals are especially vulnerable to changes in local temperatures because, unlike mammals, they are less able to thermoregulate. Insects and are critical components of ecosystems and agriculture, with economic estimates placing the global value of insect pollination at approximately €153 billion annually[28]. Worryingly, declines in insect populations[29] and pollinating species[30-34] have already been reported, with habitat destruction, range compression, pesticide residues, diseases, and their interactions commonly reported as potential drivers[28,35,36]. However, there has been relatively little research on the effects of heat on pollinating insect reproduction[18,19].

Young honey bee queens have one brief mating period early in life, and store the sperm they acquire for the rest of their lives (up to 5 years)[37]. As the sole egg-layer, colony productivity directly depends on the queen's reproductive output, which in turn depends on the viability and abundance of her stored sperm[18,38,39].

Like other insects, individual honey bees are ectothermic, but colonies can thermoregulate and maintain stable core temperatures of around 35°C in the brood nest[40]. Although colonies can persist in regions with extreme heat, evidence suggests that extreme ambient temperatures (38 - 46°C) are associated

with colony losses[41]. Furthermore, internal hive temperatures positively correlate with ambient temperatures above 18°C, and brood nest temperatures can rise upwards of 37°C, both under natural conditions[42] and during simulated heatwaves[43]. Hives occasionally experience spikes of > 40°C[44], suggesting that queens could be vulnerable to temperature extremes even inside the hive.

Honey bees have excellent potential for being temperature stress biomonitors. They are a globally distributed, managed species, so they are readily available in almost any geographic region, and they are already accepted as effective biomonitors for pollution[45]. If we begin to observe signs of heat stress in honey bees colonies, that would signal a worrisome risk of reduced reproductive output for ectothermic species.

Here, we begin to explore honey bees' utility as temperature stress biomonitors. We monitored temperature fluctuations in colonies under extreme weather conditions, establishing that damaging intra-hive temperatures can occur. Next, 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 sperm viability reduction in queens and drones using quantitative proteomics, which showed how heat stress alters protein expression of reproductive tissues. The specific set of upregulated proteins we identified may eventually serve as diagnostic tools to elucidate causes of queen failure and eventually enable regional surveys of heat stress as part of an environmental temperature biomonitoring program.

Results and Discussion

Stored sperm viability of failed and healthy queens

To establish how much of a reduction in sperm viability is associated with field-observable reduced reproductive output (and associated economic losses for beekeepers), we collected queens rated as 'failing' (N = 58) and 'healthy' (N = 55) by beekeepers and measured the queens' stored sperm viability

(**Fig 1a**). We found that the failed and healthy viability data were normally distributed (Shapiro test, P = 0.18 and 0.11, respectively), and that failed queens had significantly lower sperm viability (Student's t test, P = 5.8E-06, F = 28.16), with an average drop of 11.5%. We then set an 11.5% viability drop as the tolerance threshold in subsequent experiments aimed at identifying critical temperatures beyond which queens are at risk of substantial loss of stored sperm viability.

Temperature exposure thresholds

Previous research has shown that both cold (4°C) and hot (42°C) temperatures reduce stored sperm viability in queens[18]. To determine critical temperature and duration thresholds, we compared stored sperm viability across a temperature and duration gradient (5, 10, 15, 25, 38, 40 and 42°C, exposed for 1, 2 or 4 h followed by a 2 d recovery period) (**Fig 2a**). Not all experimental groups' data were normally distributed (Shapiro test, $P < 0.05$); therefore, we analyzed it with a Kruskal-Wallis test for non-parametric data. There was a significant effect of temperature for the 2 h ($\chi^2 = 15.6$, P = 0.016) and 4 h ($\chi^2 = 17.9$, P = 0.0065) treatments, while not at 1 h ($\chi^2 = 9.12$, P = 0.17). A Dunnett's post hoc test revealed that the only temperatures that were significantly different from the control (25°C) were the 2 h, 10°C treatment (P = 0.045) and the 4 h, 42°C treatment (P = 0.00057), at a family-wise error rate of $\alpha = 0.05$. The 2 h and 4 h data were then pooled and optimally fit to a cubic polynomial regression ($R^2 = 0.092$, P = 0.012; **Fig 2b**) to find the temperature tolerance thresholds for queens, given a pre-defined maximum acceptable drop in sperm viability (11.5%). This model suggests that 15.2 – 38.2°C is the suggested "safe zone" with minimal loss of viability for 2 - 4 h exposures.

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 2c)**. These data were normally distributed (Shapiro test, P = 0.18); therefore, we used a two-way ANOVA for analysis. We found that responses differed depending on the colony source, but heat dramatically decreased viability by 35% after both 2 and 4 h

(factors: time and colony, $P_{(time)}$ = 1.2E-07, $F_{(time)}$ = 24 $df_{(time)}$ = 2; $P_{(colony)}$ = 0.00015, $F_{(colony)}$ = 11, $df_{(colony)}$ = 2). Heat-shock therefore affects stored and ejaculated sperm viability at similar magnitudes. Drones could also be appropriate biomonitors of heat-stress, as their sperm is also sensitive to changes in temperature. However, drones are not as long-lived as queens and are only seasonally available.

Shipment and hive temperature fluctuations

To document if routine shipping poses a threat of adverse temperature exposure to queens, we tracked the temperatures of eight domestic queen shipments (seven via ground transportation, one via air; **Fig 1b**). We found that even in these shipments, which were not deliberately timed to occur during extreme weather events, one package experienced a temperature spike to 38°C and one dropped to 4°C. Since honey bees cannot adequately thermoregulate in queen cages, extreme ambient temperatures are a hazard for shipping. However, little is known about a whole colony's ability to thermoregulate in the face of extreme heat.

To gain a more complete picture of temperature fluctuations within colonies, we recorded temperatures throughout the brood nest (loggers placed between each frame of three 10-frame hives) during extreme heat in August in El Centro, California. The ambient temperatures, measured in the shade beneath each hive, reached up to 45°C (**Fig 1c**). In all three hives, the temperature at the two outer-most frames spiked to upwards of 40°C for 2 - 5 h, and in two of the hives, temperatures exceeded 38°C even one or two frames closer to the core. Therefore, the colony's ability to thermoregulate begins to break down in extreme heat, and queens can be vulnerable to temperature stress inside the hive.

Queen survival through heat stress

Sturup *et al.* previously reported that drones are mortally sensitive to heat[19]; however, queens are typically tolerant of stressful conditions. As a biomonitor, a favourable feature would be to survive through heat stress while accumulating a physiological and molecular record of the stress event(s). We

115 compared drone, queen, and worker survival over time at 25°C, 38°C, and 42°C, confirming that drones,
116 but not queens, are indeed mortally sensitive to heat (Log-rank test, $P < 0.00001$; **Fig 3a, FigS1)**. Fifty-
117 four percent of drones died over the course of 6 h at 42°C, whereas every queen survived. We also
118 found that drones are more sensitive to heat than workers, which have a similar lifespan to drones but
119 are non-reproductive females.

120 In this experiment, drones and workers were from local colonies, while queens were either from a local
121 origin or imported from Hawaii and Australia. If bees are to be used as a global biomonitor for
122 temperature stress, an important experiment will be to determine if there are differences in survival,
123 physiological response, or biochemical response to heat between genetic stocks that may be adapted to
124 hotter or cooler climates.

125 Honey bees' sex-biased heat sensitivity is puzzling because drones and queens spend most of their lives
126 inside the hive and can avoid participating in mating flights during hot weather. Therefore, they are
127 exposed to similar environmental conditions, and based on this, one might expect them to have similar
128 physiological tolerance thresholds. However, the ability of a colony to survive and produce reproductive
129 individuals directly depends on the survival of individual queens (there is only one per colony), and not
130 individual drones (hundreds per colony). Natural selection may have favoured high survivorship of
131 queens, as evidenced by not only their long lifespan (up to 5 y) but also their high tolerance to heat
132 stress, despite the death of their stored sperm. To our knowledge, sex biases in thermal tolerance for
133 other social insects has not yet been investigated, as critical thermal tolerance studies have focused on
134 workers or on male fertility alone.[46-55]

135 Next, we investigated sex biases in heat tolerance for two solitary insect species—fruit flies (*Drosophila*
136 *melanogaster*) and brown marmorated stink bugs (*Halyomorpha halys*). The stink bugs have a similar
137 body size and thermal tolerance range as honey bees[56] and their global distribution has expanded to

cover four continents, making them a potential candidate biomonitor. Fruit flies have a much smaller body size but are also distributed in laboratories around the world, so they could be candidate biomonitors too. We found that both male and female stink bugs and fruit flies readily die with heat (**Fig 3b and c)**, meaning that they cannot accumulate a physiological or molecular record of a heat stress event and have limited utility for observing impacts of heat on fertility through time. However, these data do indicate another worrying trend: that exceptionally extreme temperatures may reduce insect populations through direct kills, in addition to reducing reproductive output if they survive. We found no difference between males' and females' heat sensitivities for fruit flies and a slight opposite (female) biased heat sensitivity in stink bugs. In contrast, the high survivorship of honey bee queens makes them a good candidate biomonitor, should they possess molecular signatures indicative of heat stress when it occurs.

ATP-independent sHSPs are upregulated in heat-shocked queen spermathecae

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[57]. Enzymes that further limit damage from ROS are also upregulated in mated queens compared to virgins[58,59], and ROS damage leads to infertility in mammals[60,61]. Heat is well known to lead to oxidative stress[62]; 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 metabolic activity measurements of stored sperm supplemented with its substrate (glyceraldehyde-3-phosphate) improved viability and produced the highest molar ratio of ATP via anaerobic catabolism[57]. 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 in queens to some degree[57], although whether this is true in general has been a matter of debate across species[63]. It is not known with certainty whether ejaculated sperm are transcriptionally active; nevertheless, analyzing ejaculates, virgin spermathecae and mated spermathecae helped us to disentangle the male versus female origins of expression in sperm-filled, mated spermathecae. We found that heat-shock did not upregulate expression of ROS-mitigating enzymes, nor GAPDH (**Fig S2**); rather, all enzymes were consistently (but not significantly) downregulated with heat (by 10-30%). Queens must therefore employ other strategies, if any, to combat heat stress.

A multitude of other proteins could also be responsible for mitigating damage from heat—most obviously, members of the heat-shock protein (HSP) family[24,64-66]. HSPs generally function as molecular chaperones that stabilize proteins, refold damaged proteins, and prevent protein aggregation, but they can have diverse functions in specific contexts and their precise role in the honey bee spermatheca is unknown. 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 glyceraldehyde-3-phosphate yields the most ATP per unit via anaerobic catabolism of any tested substrate and significantly increased sperm longevity[57], this implies that ATP usage economy is a critical factor for maintaining viable sperm. Therefore, we expect that ATP-independent HSPs should be upregulated with heat stress in spermathecae, and ATP-dependent HSPs (or a mix of ATP-dependent and ATP-independent HSPs) should be upregulated as a result of heat stress in other tissues. In addition, some ATP-independent short HSPs (sHSPs, typically 20-28 kDa) suppress ROS generation while preventing protein aggregates and apoptosis[62,67]. Since molecular processes that reduce oxidative

185 damage favour sperm longevity, we hypothesized that heat should induce expression of sHSPs in the
186 spermatheca.

187 To determine which HSPs were upregulated with heat-shock, we compared global protein expression
188 profiles in heat-shocked and non-heat-shocked mated and virgin spermathecae, ejaculates, and ovaries
189 **(Fig 4a, Fig S3)**. We hypothesized that the heat-shock response should both mitigate ROS production
190 and conserve ATP in the spermatheca, but this ATP-conservation may not be as critical in the ovaries
191 where the ATP economy is not expected to be as tightly controlled. Of the 2,778 protein groups
192 identified in the spermathecae and ejaculates, only five were significantly up-regulated with heat (5%
193 FDR), all of which were identified in the mated and virgin spermathecae. All five of the proteins were
194 unique, ATP-independent sHSPs (accessions: XP_001120194.1, XP_001119884.1, XP_395659.1,
195 XP_001120006.2, and XP_026294937.1) (**Fig 4b**). In heat-shocked virgin spermathecae, two of the same
196 sHSPs (XP_395659.1 and XP_001120006.2) were also significantly upregulated, and all mirrored the
197 expression patterns in the spermathecae of heat-shocked mated queen (even if not significant),
198 indicating that this is a queen-derived, and not a sperm-derived, response **(Fig 4c and d**). By contrast,
199 the most strongly upregulated protein in heat-shocked ovaries was HSP70—an ATP-dependent HSP—
200 and no sHSPs were upregulated in this tissue, supporting our initial hypotheses **(Fig 4e)**.

201 As expected, the significantly enriched Gene Ontology (GO) terms were all related to responses to heat
202 and stress in the spermathecae (**Fig 5a and b**). However, the only significantly enriched GO term in the
203 semen analysis was related to the electron transfer activity (**Fig 5c**), driven by a heat-induced down-
204 regulation of the proteins linked to this GO term (**Fig 5d**), suggesting that the sperm may be less able to
205 produce the large amounts of ATP necessary for flagellar beating. This is consistent with the findings of
206 Gong *et al.*, who found that heat stress at 42°C impaired mitochondrial function, reduced electron
207 transport chain complexes' activities, and lowered total cellular ATP[23]. Numerous proteins (none of
208 which were HSPs) were downregulated in heat-shocked spermathecae, but based on the GO term

209 enrichment analysis and manual inspection of their functions, it is unclear what their biological
210 significance is. It is possible that they are degradation products of heat-killed sperm, but since these
211 proteins were largely absent in the semen samples, we cannot confirm this hypothesis.

212 Twenty HSPs were identified in spermathecae overall and 13 were identified in the semen **(Fig 6**);
213 however, the precise functions of specific honey bee HSPs are largely unknown. To gain insight into
214 potential roles of the five differentially expressed HSPs we identified, we compared their sequences to
215 annotated sequences in other species and identified putative protein domains using NCBI's basic local
216 alignment search tool (BLAST)[68]. All five of the HSPs contain one or more alpha crystallin domains, which
217 is characteristic of small HSPs (sHSPs). Four of these HSPs are within the expected molecular weight
218 range, and one of the proteins (XP_026294937.1) is predicted to be 56.7 kDa (and contains two alpha
219 crystallin domains instead of one), highlighting that HSPs should not necessarily be categorized based on
220 molecular weight alone. All five of the honey bee sHSPs are orthologous to the *D. melanogaster* gene
221 *l(2)efl* (also known as CRYAB). In *Drosophila*, upregulation of this gene causes increased lifespan of
222 individual flies[69]. CRYAB and other sHSPs are highly conserved in both vertebrates and invertebrates,[70]
223 but is by far the best studied in human. In humans, sHSP upregulation is associated with anti-apoptotic
224 properties, as well as mitigating ROS production[62,66,71,72]. Their up-regulation in heat-shocked testes is
225 thought to help compensate for the damaging effects of heat[71], and we speculate that they are playing a
226 similar role in the spermathecae. Queens with strongly up-regulated sHSPs may therefore be better able
227 to counter-act ROS production, sperm death, and ultimately maintain longevity.

228 Other researchers have suggested that in mammals, some ATP-dependent HSPs (*e.g.,* HSP70s and
229 HSP90s) may be important for maintaining male fertility via quality control of sperm[66,73]. Unlike sHSPs,
230 these HSPs appear to be pro-apoptotic factors and could theoretically help prevent damaged sperm
231 from being able to fertilize an egg. Neither queen spermathecae nor drone ejaculates provide evidence
232 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**)[66]. Rather, HSP70 was only upregulated in the ovaries, which does not directly participate in sperm maintenance.

In mammals, sHSPs are upregulated in testes after heating, but so are ATP-consuming proteins like HSP105, HSP70-1, HSP70-2, and HSP90[74-76]. We speculate that the energetic cost associated with ensuring high sperm quality is advantageous in mammals 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 in the spermatheca is consistent with a tightly controlled ATP usage economy, as well as 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 and conserving ATP. 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 uniquely upregulated in this tissue (**Fig 5 and 6**). This observation is consistent with selection for ATP-consuming quality control mechanisms when a failed fertilization event fails to produce offspring (in this case, when a non-viable egg meets a viable sperm). 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.

Finally, we compared protein expression in spermathecae, fat bodies, and ovaries between 11 queens that failed due to unknown causes and 11 age-matched, apiary-matched healthy queens. We identified 1,219, 1,640, and 1,782 proteins, respectively, but did not find any significant expression differences at 10% FDR, indicating that there is not one universal signature of failure (**Fig S4**). Rather, we suspect that different stressors alter protein expression in these tissues in different ways, and that no significant differences were found because it is highly unlikely that the queens all failed from the same cause.

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 include a blind heat-shock 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 that overlap with the heat-shock signature.

Conclusion

Our experiments show that temperature stress deeply damages both stored and ejaculated honey bee sperm viability, that queens are vulnerable to temperature changes both in colonies and during transport, and that temperatures ranging between 15°C and 38°C for 2 - 4 h are generally safe for queens. Honey bees have a strong sex bias in heat tolerance, with females being highly tolerant—a bias which does not exist in the two solitary insect species we tested (*H. halys* and *D. melanogaster*). Future research should investigate if this sex-biased heat tolerance is also present in other Hymenopterans in order to better understand the evolutionary origin. Upon heat-shock, queens upregulate ATP-independent HSPs in their spermathecae, which both minimizes ATP consumption and could provide beneficial anti-apoptotic properties. In contrast, HSP70 (an ATP-consuming HSP) is upregulated in

279 ovaries. Once validated in field trials, these protein signatures could serve as biomarkers for heat stress
280 enabling longitudinal surveys for the prevalence of heat-induced loss of sperm viability in diverse
281 landscapes as part of a biomonitoring program.

282 **Methods**

283 *Sperm viability assays*

284 Honey bee queens (Kona Queens supplied in a single shipment) were treated at one of five different
285 temperatures (5, 10, 15, 25, 38, 40, and 42°C) for 1, 2, or 4 hours, then held at 25°C for 2 d. The
286 temperature range was chosen because previous research showed that 4°C and 8°C were sufficiently
287 cold to reduce sperm viability, and that 40 - 42°C was sufficiently hot.[18] Therefore, more temperatures
288 were chosen at the cold (5, 10, and 15°C) and hot (38, 40, and 42°C) extremes to try to capture the
289 critical point at which the viability begins to drop. We chose 25°C as the control temperature, rather
290 than 35°C, because these queens were obtained from a commercial supplier and queens are not held at
291 hive temperature during transport. While a 35°C treatment group would be an appropriate control, it
292 would not be meaningful in these circumstances.

293 Fourteen queens (replicates) were included in the 25°C treatment (negative control), whereas 8 or 9
294 queens were included in all other temperatures and exposure durations (see **Table 1** for specific
295 replication information). Following this, queens were beheaded, and their spermathecae were dissected
296 with fine forceps. The spermathecae were gently agitated with a pipet tip in 100 µl Buffer D (17 mM D-
297 glucose, 54 mM KCl, 25 mM $NaHCO_3$, 83 mM $Na_3C_6H_5O_7$) to break them open and release the sperm.
298 Sperm viability was determined using a live/dead sperm viability kit (Thermo) following the protocols of
299 Collins and Donoghue[77]. Briefly, the SYBR14 dye was diluted 1:9 in DMSO. Two microlitres of the diluted
300 SYBR14 dye and 4 µl of propidium iodide were gently mixed with the sperm and incubated for 15
301 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 tested for normality using a Shapiro test. The data was not normally distributed, we used a non-parametric test (Kruskal Wallis) to investigate the effects of temperature on viability for each duration separately. When a significant effect of temperature was identified, we performed a post-hoc Dunnett's test to identify significant contrasts to the 25°C control. To identify the best-fitting linear model, we pooled the 2 h and 4 h data and performed linear regressions 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 headed by Kona queens with unknown relatedness. The colonies were kept in Beaverlodge, Alberta. Semen was collected with glass capillaries according to the methods of Collins and Donoghue[77]. 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 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[78]. Viability assays for these semen samples was performed following the same methods as for spermathecae. Data were analyzed by a Shapiro test and Levene's median test to confirm normal distribution and evaluate the validity of the assumption of equal variance, respectively. The data passed both tests, so a two-way ANOVA (factors: time, colony) was performed, followed by a Tukey HSD test.

Recording temperatures during shipments

We arranged seven shipments of locally produced (BC) queens via ground transportation from Kettle Valley Queens, Nicola Valley Honey, Wild Antho, Campells Gold Honey, Heather Meadows Honey Farm, Six Legs Good Apiaries, and Wildwood Queens (located in Grand Forks, Merrit, Armstrong, Abbotsford, Telka, Surrey, and Powell River, respectively) to the University of British Columbia, Vancouver, in the summer of 2019. These queens (7-8 queens per shipment) were all considered to be "good" queens by the donating bee breeders based on their laying pattern. These queens cumulatively make up the "healthy" queen cohort (N = 55 queens total). An additional shipment travelled from Edmonton to UBC Vancouver via air freight. All shipments were accompanied by two temperature data loggers (B-series WatchDog loggers, Spectrum Technologies) set to record the temperature every 10 minutes for the duration of the shipment.

Failed queen viability survey

We solicited samples of failed queens, as rated by beekeepers, from throughout BC in the summer of 2019. Reported reasons for designating the queens as 'failed' included poor brood pattern, drone laying, poor population build-up, disease symptoms, injury, small size, and worker rejection. These queens (N = 58) make up the "failed" queen cohort. We measured the sperm viability of all failed and healthy queens using the same live/dead sperm staining kit as described under "Sperm Viability Assays."

Hive temperature recording

To record the internal and external temperatures of colonies, we placed temperature loggers inside three standard, 10-frame colonies in El Centro, California, from August 23rd to September 6th, 2017. The hottest day (the data shown) was August 26th. Loggers were set to record the temperature every 10 minutes and were placed between each frame of the hive. Hives consisted of 10 deep frames in a single

wooden Langstroth brood box with a medium depth box on top of the brood chamber and a migratory wooden cover. The ambient temperatures were recorded with a temperature logger placed in the shade beneath each hive.

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%, but this did not have an effect of survival (100% of queens survived in both cases). Eight queens were held at 42°C for 6 h at a constant 60% relative humidity specifically for survival analysis. See **Fig S1** for sample sizes and risk tables for each treatment group. No queens died in these tests; therefore it was not necessary to account for geographic origin in statistical analyses.

Male and female 3- to 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

377 used this as the endpoint because fruit flies will become paralyzed with heat, but recover motility after
378 several hours (in some cases, overnight) at cooler temperatures. Control female flies were maintained
379 for a further week after the experiment to confirm that the majority of them had successfully mated
380 prior to the heat stress tests.

381 Male and female stink bugs were reared in the laboratory according to standard protocols[79]. Stink bugs
382 which transitioned from nymphs to adults 3 - 7 d prior were transferred to ventilated 500 ml volume
383 plastic cages with a piece of moist paper towel (3 - 6 stink bugs per cage). They were heat stressed at
384 42°C (55 females and 41 males) or held at 25°C (36 females and 24 males) for 6 h. Only mated females
385 carrying eggs were included in the experiment, as determined by post-stress dissection.

386 In all cases, Kaplan-Meier survival curves were generated in R and compared using log-rank tests. See **Fig**
387 **S1** for risk tables and sample size information for survival tests for all species.

388 *Heat-shock for proteomics*

389 Our experimental design for the proteomics experiments was to compare heat-shocked and non heat-
390 shocked treatments of three tissues: virgin spermathecae, mated spermathecae, and ejaculated semen
391 (N = 10 each for all but the semen, which was N = 5 where each N was a pooled sample of five drones
392 from one colony). For the mated spermathecae, expression differences induced by heat-shock would be
393 the sum of any expression differences that may have occurred in the queen's cells and in the sperm
394 cells. The virgin spermathecae, however, do not contain sperm so that response to heat-shock is purely
395 from the queen.

396 Honey bee colonies were maintained in an apiary at the University of British Columbia. During the
397 summer of 2018, 40 queens were reared from a single colony of local origin and half were allowed to
398 open mate, while the other half were kept as virgins in plastic queen cages. Two weeks after emergence,
399 the virgin queens were given two, eight-minute carbon dioxide treatments on sequential days, then re-

400 introduced to their nucleus colonies. This process stimulates virgin queens to begin laying[80], and we
401 conducted these treatments in order to minimize the physiological differences between virgin and
402 mated queens.

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

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

415 *Proteomics sample preparation*

416 Semen and spermatheca samples were homogenized in 2 ml screw-cap tubes containing 100 µl of lysis
417 buffer (6 M guanidinium chloride, 100 mM Tris, pH 8.5) and four ceramic beads. The homogenizer
418 (Precellys 24, Bertin Instruments) was set to 6,500 s^{-1} for 30 s, then samples were centrifuged (16,000
419 rcf, 10 min, 4°C) to remove debris. Supernatants were transferred to a new tube and diluted 1:1 with
420 dH_2O. Protein was precipitated by adding four volumes of ice-cold acetone and incubating overnight at -
421 20°C. The precipitated protein was pelleted and washed twice with 500 µl of 80% acetone, then the

422 pellet was allowed to air dry (ca. 5 min) prior to solubilization in 50 µl of digestion buffer (6 M urea, 2 M
423 thiourea).

424 Approximately 25 micrograms of protein were reduced (0.5 µg dithiothreitol, 20 min), alkylated (2.5 µg
425 iodoacetamide, 30 min, dark), and digested (0.5 µg Lys-C for 3 h, then 0.5 µg trypsin overnight). Digested
426 peptides were acidified with one volume of 1% trifluoroacetic acid and desalted with high-capacity
427 STAGE tips as previously described[81]. Eluted samples were dried (SpeedVac, Eppendorf, 45 min) and
428 resuspended in Buffer A (0.1% formic acid). Peptide concentrations were determined using a NanoDrop
429 (Thermo, 280 nm) and sample orders were randomized for liquid chromatography-tandem mass
430 spectrometry (LC-MS/MS) analysis.

431 *LC-MS/MS data acquisition and analysis*

432 Peptides (0.5 µg for each sample) were injected on an EASY-nLC 1000 liquid chromatography system
433 (Thermo) coupled to an Impact II Q-TOF mass spectrometer (Bruker), essentially as previously
434 described[82]. The LC system included a fused-silica (5 µm Aqua C18 particles (Phenomenex)) fritted 2 cm
435 trap column connected to a 50 cm analytical column packed with ReproSil C18 (3 µm C18 particles (Dr.
436 Maisch)). The separation gradient ran from 5% to 35% Buffer B (80% acetonitrile, 0.1% formic acid) over
437 90 min, followed by a 15 min wash at 95% Buffer B (flow rate: 250 µL/min). The instrument parameters
438 were: scan from 150 to 2200 m/z, 100 µs transient time, 10 µs prepulse storage, 7 eV collision energy,
439 1500 Vpp Collision RF, a +2 default charge state, 18 Hz spectral acquisition rate, 3.0 s cycle time, and the
440 intensity threshold was 250 counts.

441 Mass spectrometry data were searched using MaxQuant (v1.5.3.30) using default parameters, except
442 "match between runs" was enabled. Peptide spectral matches, peptide identifications and protein
443 identifications were controlled at 1% false discovery rates (FDRs). The protein search database was the
444 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). Differential expression analysis was performed in Perseus (v1.6.1.1) essentially as previously described[82]. Histograms of protein counts across LFQ intensities were first inspected for normality. Then 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 two-tailed t-tests and an ANOVA, respectively. Only P values surviving the 10% FDR threshold (permutation-based method) were considered significant.

GO term enrichment analysis

Gene Ontology (GO) terms were retrieved using BLAST2GO (v4.1.9)and subsequent enrichment analyses were conducted using ErmineJ[83]. We used the gene score resampling (GSR) method (with P values as scores). Unlike conventional over-representation analyses, this method does not depend on submitting a 'hit list,' the composition of which is sensitive to arbitrary significance cut-offs. Rather, the GSR method uses p-values as a continuous variable and looks for GO terms that are enriched in proteins with low p-values along the continuum. More documentation about the GSR method and ErmineJ can be found at https://erminej.msl.ubc.ca/help/tutorials/running-an-analysis-resampling/. Enrichment false discovery rates were controlled to 10% using the Benjamini-Hochberg correction method. 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. Differential expression analysis was performed in Perseus as described above.

References

1 Bellard, C., Bertelsmeier, C., Leadley, P., Thuiller, W. & Courchamp, F. Impacts of climate change on the future of biodiversity. *Ecol Lett* **15**, 365-377, doi:10.1111/j.1461-0248.2011.01736.x (2012).

2 Thomas, C. D. *et al.* Extinction risk from climate change. *Nature* **427**, 145-148, doi:10.1038/nature02121 (2004).

3 Bálint, M. *et al.* Cryptic biodiversity loss linked to global climate change. *Nature Climate Change* **1**, 313 (2011).

4 Sales, K. *et al.* Experimental heatwaves compromise sperm function and cause transgenerational damage in a model insect. *Nat Commun* **9**, 4771, doi:10.1038/s41467-018-07273-z (2018).

5 Walsh, B. S. *et al.* The Impact of Climate Change on Fertility. *Trends Ecol Evol*, doi:10.1016/j.tree.2018.12.002 (2019).

6 Zeh, J. A. *et al.* Degrees of disruption: projected temperature increase has catastrophic consequences for reproduction in a tropical ectotherm. *Global Change Biology* **18**, 1833-1842 (2012).

7 Jannes, P. *et al.* Male subfertility induced by acute scrotal heating affects embryo quality in normal female mice. *Hum Reprod* **13**, 372-375 (1998).

8 Pérez-Crespo, M., Pintado, B. & Gutiérrez-Adán, A. Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. *Mol Reprod Dev* **75**, 40-47, doi:10.1002/mrd.20759 (2008).

9 Setchell, B. The effects of heat on the testes of mammals. *Anim Reprod* **3**, 81-91 (2006).

10 Thonneau, P., Bujan, L., Multigner, L. & Mieusset, R. Occupational heat exposure and male fertility: a review. *Hum Reprod* **13**, 2122-2125 (1998).

11 Yaeram, J., Setchell, B. P. & Maddocks, S. Effect of heat stress on the fertility of male mice in vivo and in vitro. *Reprod Fertil Dev* **18**, 647-653 (2006).

12 Hurley, L. L., McDiarmid, C. S., Friesen, C. R., Griffith, S. C. & Rowe, M. Experimental heatwaves negatively impact sperm quality in the zebra finch. *Proc Biol Sci* **285**, doi:10.1098/rspb.2017.2547 (2018).

13 Breckels, R. D. & Neff, B. D. The effects of elevated temperature on the sexual traits, immunology and survivorship of a tropical ectotherm. *J Exp Biol* **216**, 2658-2664, doi:10.1242/jeb.084962 (2013).

14 Harvey, S. C. & Viney, M. E. Thermal variation reveals natural variation between isolates of Caenorhabditis elegans. *J Exp Zool B Mol Dev Evol* **308**, 409-416, doi:10.1002/jez.b.21161 (2007).

15 Porcelli, D., Gaston, K. J., Butlin, R. K. & Snook, R. R. Local adaptation of reproductive performance during thermal stress. *J Evol Biol* **30**, 422-429, doi:10.1111/jeb.13018 (2017).

16 Gasparini, C., Lu, C., Dingemanse, N. J. & Tuni, C. Paternal-effects in a terrestrial ectotherm are temperature dependent but no evidence for adaptive effects. *Functional Ecology* **32**, 1011-1021 (2018).

17 Saxena, B., Sharma, P., Thappa, R. & Tikku, K. Temperature induced sterilization for control of three stored grain beetles. *Journal of Stored Products Research* **28**, 67-70 (1992).

18 Pettis, J. S., Rice, N., Joselow, K., vanEngelsdorp, D. & Chaimanee, V. Colony Failure Linked to Low Sperm Viability in Honey Bee (Apis mellifera) Queens and an Exploration of Potential Causative Factors. *PLoS One* **11**, e0147220, doi:10.1371/journal.pone.0147220 (2016).

19 Stürup, M., Baer-Imhoof, B., Nash, D. R., Boomsma, J. J. & Baer, B. When every sperm counts: factors affecting male fertility in the honeybee Apis mellifera. *Behavioral Ecology* **24**, 1192-1198 (2013).

20 Zizzari, Z. V. & Ellers, J. Effects of exposure to short-term heat stress on male reproductive fitness in a soil arthropod. *Journal of insect physiology* **57**, 421-426 (2011).

21 David, J. R. *et al.* Male sterility at extreme temperatures: a significant but neglected phenomenon for understanding Drosophila climatic adaptations. *J Evol Biol* **18**, 838-846, doi:10.1111/j.1420-9101.2005.00914.x (2005).

22 Hansen, P. J. Effects of heat stress on mammalian reproduction. *Philos Trans R Soc Lond B Biol Sci* **364**, 3341-3350, doi:10.1098/rstb.2009.0131 (2009).

23 Gong, Y. *et al.* Heat Stress Reduces Sperm Motility via Activation of Glycogen Synthase Kinase-3α and Inhibition of Mitochondrial Protein Import. *Frontiers in physiology* **8**, 718 (2017).

24 Yang, R. C., Shen, M. R., Chiang, P. H., Yang, S. L. & Chen, S. S. A possible role of heat shock proteins in human sperm motility. *Gaoxiong Yi Xue Ke Xue Za Zhi* **8**, 299-305 (1992).

25 Luber, G. & McGeehin, M. Climate change and extreme heat events. *Am J Prev Med* **35**, 429-435, doi:10.1016/j.amepre.2008.08.021 (2008).

26 Hayhoe, K., Sheridan, S., Kalkstein, L. & Greene, S. Climate change, heat waves, and mortality projections for Chicago. *Journal of Great Lakes Research* **36**, 65-73 (2010).

27 Meehl, G. A. & Tebaldi, C. More intense, more frequent, and longer lasting heat waves in the 21st century. *Science* **305**, 994-997, doi:10.1126/science.1098704 (2004).

28 Potts, S. G. *et al.* Global pollinator declines: trends, impacts and drivers. *Trends Ecol Evol* **25**, 345-353, doi:10.1016/j.tree.2010.01.007 (2010).

29 Hallmann, C. A. *et al.* More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS One* **12**, e0185809, doi:10.1371/journal.pone.0185809 (2017).

30 Cameron, S. A. *et al.* Patterns of widespread decline in North American bumble bees. *Proc Natl Acad Sci U S A* **108**, 662-667, doi:10.1073/pnas.1014743108 (2011).

31 Rasmont, P. & Mersch, P. First estimation of faunistic drift by bumblebees of Belgium (Hymenoptera: Apidae). *Annalen van de Koninklijke Belgische Vereniging voor Dierkunde* (1988).

32 Winfree, R., Aguilar, R., Vázquez, D. P., LeBuhn, G. & Aizen, M. A. A meta-analysis of bees' responses to anthropogenic disturbance. *Ecology* **90**, 2068-2076 (2009).

33 Ricketts, T. H. *et al.* Landscape effects on crop pollination services: are there general patterns? *Ecol Lett* **11**, 499-515, doi:10.1111/j.1461-0248.2008.01157.x (2008).

34 Biesmeijer, J. C. *et al.* Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science* **313**, 351-354, doi:10.1126/science.1127863 (2006).

35 Whitehorn, P. R., Tinsley, M. C., Brown, M. J., Darvill, B. & Goulson, D. Genetic diversity, parasite prevalence and immunity in wild bumblebees. *Proceedings of the Royal Society of London B: Biological Sciences*, rspb20101550 (2010).

36 McCallum, H. & Dobson, A. Disease, habitat fragmentation and conservation. *Proc Biol Sci* **269**, 2041-2049, doi:10.1098/rspb.2002.2079 (2002).

37 Baer, B., Collins, J., Maalaps, K. & den Boer, S. P. Sperm use economy of honeybee (Apis mellifera) queens. *Ecol Evol* **6**, 2877-2885, doi:10.1002/ece3.2075 (2016).

38 Delaney, D. A., Keller, J. J., Caren, J. R. & Tarpy, D. R. The physical, insemination, and reproductive quality of honey bee queens (Apis mellifera L.). *Apidologie* **42**, 1-13 (2011).

39 Tarpy, D. R. & Olivarez Jr, R. Measuring sperm viability over time in honey bee queens to determine patterns in stored-sperm and queen longevity. *Journal of Apicultural Research* **53**, 493-495 (2014).

40 Stabentheiner, A., Kovac, H. & Brodschneider, R. Honeybee colony thermoregulation–regulatory mechanisms and contribution of individuals in dependence on age, location and thermal stress. *PLoS One* **5**, e8967 (2010).

41 Alattal, Y. Impact of temperature extremes on survival of indigenous and exotic honey bee subspecies, Apis mellifera, under desert and semiarid climates. *Bulletin of Insectology* **68**, 219-222 (2015).

42 Fahrenholz, L., Lamprecht, I. & Schricker, B. Thermal investigations of a honey bee colony: thermoregulation of the hive during summer and winter and heat production of members of different bee castes. *Journal of Comparative Physiology B* **159**, 551-560 (1989).

43 Bordier, C. *et al.* Colony adaptive response to simulated heat waves and consequences at the individual level in honeybees (Apis mellifera). *Sci Rep* **7**, 3760, doi:10.1038/s41598-017-03944-x (2017).

44 Villa, J. D., Gentry, C. & Taylor, O. R. Preliminary observations on thermoregulation, clustering, and energy utilization in African and European honey bees. *Journal of the Kansas Entomological Society* **60**, 4-14 (1987).

45 Smith, K. E. *et al.* Honey as a biomonitor for a changing world. *Nature Sustainability* **2**, 223 (2019).

46 Mitchell, J. D., Hewitt, P. & Van Der Linde, T. D. K. Critical thermal limits and temperature tolerance in the harvester termite Hodotermes mossambicus (Hagen). *Journal of Insect Physiology* **39**, 523-528 (1993).

47 Clémencet, J., Cournault, L., Odent, A. & Doums, C. Worker thermal tolerance in the thermophilic ant Cataglyphis cursor (Hymenoptera, Formicidae). *Insectes sociaux* **57**, 11-15 (2010).

48 Baudier, K. M., Mudd, A. E., Erickson, S. C. & O'Donnell, S. Microhabitat and body size effects on heat tolerance: implications for responses to climate change (army ants: Formicidae, Ecitoninae). *J Anim Ecol* **84**, 1322-1330, doi:10.1111/1365-2656.12388 (2015).

49 Chirault, M. *et al.* A combined approach to heat stress effect on male fertility in Nasonia vitripennis: from the physiological consequences on spermatogenesis to the reproductive adjustment of females mated with stressed males. *PLoS One* **10**, e0120656, doi:10.1371/journal.pone.0120656 (2015).

50 Hidalgo, K., Beaugeard, E., Renault, D., Dedeine, F. & Lécureuil, C. Physiological and biochemical responses to thermal stress vary among genotypes in the parasitic wasp Nasonia vitripennis. *J Insect Physiol* **117**, 103909, doi:10.1016/j.jinsphys.2019.103909 (2019).

51 Macías-Macías, J. O. *et al.* Comparative temperature tolerance in stingless bee species from tropical highlands and lowlands of Mexico and implications for their conservation (Hymenoptera: Apidae: Meliponini). *Apidologie* **42**, 679-689 (2011).

52 Oberg, E., Del Toro, I. & Pelini, S. Characterization of the thermal tolerances of forest ants of New England. *Insectes sociaux* **59**, 167-174 (2012).

53 Verble-Pearson, R. M., Gifford, M. E. & Yanoviak, S. P. Variation in thermal tolerance of North American ants. *Journal of thermal biology* **48**, 65-68 (2015).

54 Andrew, N. R., Hart, R. A., Jung, M.-P., Hemmings, Z. & Terblanche, J. S. Can temperate insects take the heat? A case study of the physiological and behavioural responses in a common ant, Iridomyrmex purpureus (Formicidae), with potential climate change. *Journal of Insect Physiology* **59**, 870-880 (2013).

55 Abou-Shaara, H. F., Al-Ghamdi, A. A. & Mohamed, A. A. Tolerance of two honey bee races to various temperature and relative humidity gradients.
56 Scaccini, D., Duso, C. & Pozzebon, A. Lethal Effects of High Temperatures on Brown Marmorated Stink Bug Adults before and after Overwintering. *Insects* **10**, doi:10.3390/insects10100355 (2019).
57 Paynter, E. *et al.* Insights into the molecular basis of long-term storage and survival of sperm in the honeybee (Apis mellifera). *Sci Rep* **7**, 40236, doi:10.1038/srep40236 (2017).
58 Collins, A., Williams, V. & Evans, J. Sperm storage and antioxidative enzyme expression in the honey bee, Apis mellifera. *Insect molecular biology* **13**, 141-146 (2004).
59 Weirich, G. F., Collins, A. M. & Williams, V. P. Antioxidant enzymes in the honey bee, Apis mellifera. *Apidologie* **33**, 3-14 (2002).
60 Wagner, H., Cheng, J. W. & Ko, E. Y. Role of reactive oxygen species in male infertility: An updated review of literature. *Arab J Urol* **16**, 35-43, doi:10.1016/j.aju.2017.11.001 (2018).
61 Agarwal, A., Virk, G., Ong, C. & du Plessis, S. S. Effect of oxidative stress on male reproduction. *The world journal of men's health* **32**, 1-17 (2014).
62 Ikwegbue, P. C., Masamba, P., Oyinloye, B. E. & Kappo, A. P. Roles of Heat Shock Proteins in Apoptosis, Oxidative Stress, Human Inflammatory Diseases, and Cancer. *Pharmaceuticals (Basel)* **11**, doi:10.3390/ph11010002 (2017).
63 Ren, X., Chen, X., Wang, Z. & Wang, D. Is transcription in sperm stationary or dynamic? *Journal of Reproduction and Development*, 2016-2093 (2017).
64 Mayer, M. P. & Bukau, B. Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci* **62**, 670-684, doi:10.1007/s00018-004-4464-6 (2005).
65 Yue, L. *et al.* Genetic analysis of viable Hsp90 alleles reveals a critical role in Drosophila spermatogenesis. *Genetics* **151**, 1065-1079 (1999).
66 Ji, Z.-L. *et al.* Association of heat shock proteins, heat shock factors and male infertility. *Asian Pacific Journal of Reproduction* **1**, 76-84 (2012).
67 Bakthisaran, R., Tangirala, R. & Rao, C. M. Small heat shock proteins: Role in cellular functions and pathology. *Biochim Biophys Acta* **1854**, 291-319, doi:10.1016/j.bbapap.2014.12.019 (2015).
68 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J Mol Biol* **215**, 403-410, doi:10.1016/S0022-2836(05)80360-2 (1990).
69 Morrow, G. & Tanguay, R. M. Drosophila melanogaster Hsp22: a mitochondrial small heat shock protein influencing the aging process. *Front Genet* **6**, 1026, doi:10.3389/fgene.2015.00103 (2015).
70 Wójtowicz, I. *et al.* Drosophila small heat shock protein CryAB ensures structural integrity of developing muscles, and proper muscle and heart performance. *Development* **142**, 994-1005, doi:10.1242/dev.115352 (2015).
71 Kamradt, M. C., Chen, F. & Cryns, V. L. The small heat shock protein alpha B-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation. *J Biol Chem* **276**, 16059-16063, doi:10.1074/jbc.C100107200 (2001).
72 Paul, C. *et al.* Hsp27 as a negative regulator of cytochrome C release. *Mol Cell Biol* **22**, 816-834 (2002).
73 Izu, H. *et al.* Heat shock transcription factor 1 is involved in quality-control mechanisms in male germ cells. *Biol Reprod* **70**, 18-24, doi:10.1095/biolreprod.103.020065 (2004).
74 Rockett, J. C. *et al.* Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. *Biol Reprod* **65**, 229-239 (2001).
75 Biggiogera, M. *et al.* Localization of heat shock proteins in mouse male germ cells: an immunoelectron microscopical study. *Exp Cell Res* **229**, 77-85, doi:10.1006/excr.1996.0345 (1996).

652 76 Zhang, X. S. *et al.* Dedifferentiation of adult monkey Sertoli cells through activation of
653 extracellularly regulated kinase 1/2 induced by heat treatment. *Endocrinology* **147**, 1237-1245,
654 doi:10.1210/en.2005-0981 (2006).
655 77 Collins, A. & Donoghue, A. Viability assessment of honey bee, Apis mellifera, sperm using dual
656 fluorescent staining. *Theriogenology* **51**, 1513-1523 (1999).
657 78 Cobey, S. W., Tarpy, D. R. & Woyke, J. Standard methods for instrumental insemination of Apis
658 mellifera queens. *Journal of Apicultural Research* **52**, 1-18 (2013).
659 79 Taylor, C. M., Coffey, P. L., Hamby, K. A. & Dively, G. P. Laboratory rearing of Halyomorpha halys:
660 methods to optimize survival and fitness of adults during and after diapause. *Journal of Pest*
661 *Science* **90**, 1069-1077 (2017).
662 80 Mackensen, O. Effect of carbon dioxide on initial oviposition of artificially inseminated and virgin
663 queen bees. *Journal of economic entomology* **40**, 344-349 (2014).
664 81 Rappsilber, J., Ishihama, Y. & Mann, M. Stop and go extraction tips for matrix-assisted laser
665 desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal*
666 *Chem* **75**, 663-670 (2003).
667 82 McAfee, A., Chan, Q., Evans, J. & Foster, L. J. A Varroa destructor protein atlas reveals molecular
668 underpinnings of developmental transitions and sexual differentiation. *Mol Cell Proteomics*,
669 doi:10.1074/mcp.RA117.000104 (2017).
670 83 Lee, H. K., Braynen, W., Keshav, K. & Pavlidis, P. ErmineJ: tool for functional analysis of gene
671 expression data sets. *BMC Bioinformatics* **6**, 269, doi:10.1186/1471-2105-6-269 (2005).

672

673 **Data availability statement**

674 All raw mass spectrometry data, databases, and search results are available on PRIDE ProteomeXchange
675 (accession: PXD013728). Figures with associated raw mass spectrometry data include Figs. 4, 5, 6, and
676 S4. Global protein abundances and p values for the laboratory heat-shock comparisons are available in
677 Table S1. Data used to generate other figures will be provided upon request.

678 **Code availability statement**

679 No specialized code central to our conclusions was used in this manuscript. R code for standard
680 statistical analyses and figure generation will be provided upon request.

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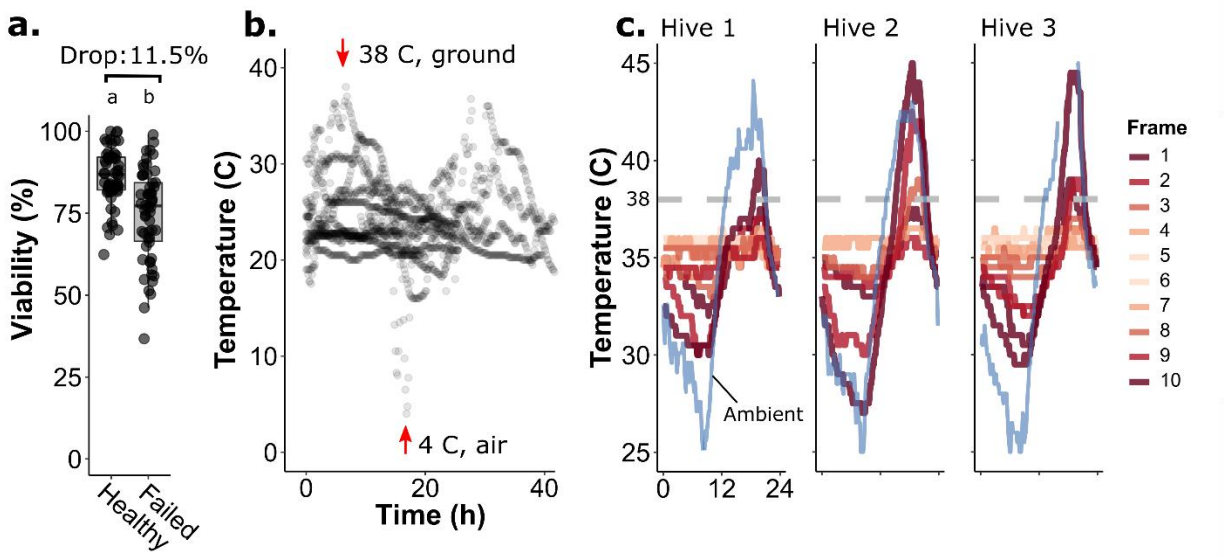
Author contributions

AM wrote the first draft of the manuscript and revisions, conducted all data analysis, made the figures, and performed the proteomics experiments. AC and AM conducted the failed queen survey, with assistance from HH and MMG. HH and MMG executed the queen shipment temperature tracking. JM performed the survival experiments. MMG and JSP performed the drone sperm viability analyses. JSP performed the queen sperm viability measurements across the range of temperatures and measured internal hive temperatures. Grants to LJF, JSP, MMG, and DRT funded the research. All authors contributed intellectually.

Competing interests

JSP owns a honey bee consulting business.

705 **Figures and Legends**



706

707 **Figure 1. Observational data on shipping temperatures, hive temperatures, and stored sperm viability.**
708 A) Healthy (N = 55) and failed queens (N = 58) were collected from BC beekeepers for sperm viability
709 analysis. Failed queens had significantly lower sperm viability (F = 28.16, P = 5.8E-06), with an average
710 drop of 11.5%. B) Temperatures of eight domestic Canadian queen shipments were recorded during the
711 summer of 2019 (7 via ground, 1 via air transportation). Temperature loggers were kept immediately
712 adjacent to the queen cages. C) Internal temperatures of three hives were recorded, with temperature
713 loggers placed between each frame. Ambient temperatures were recorded in the shade beneath each
714 hive.

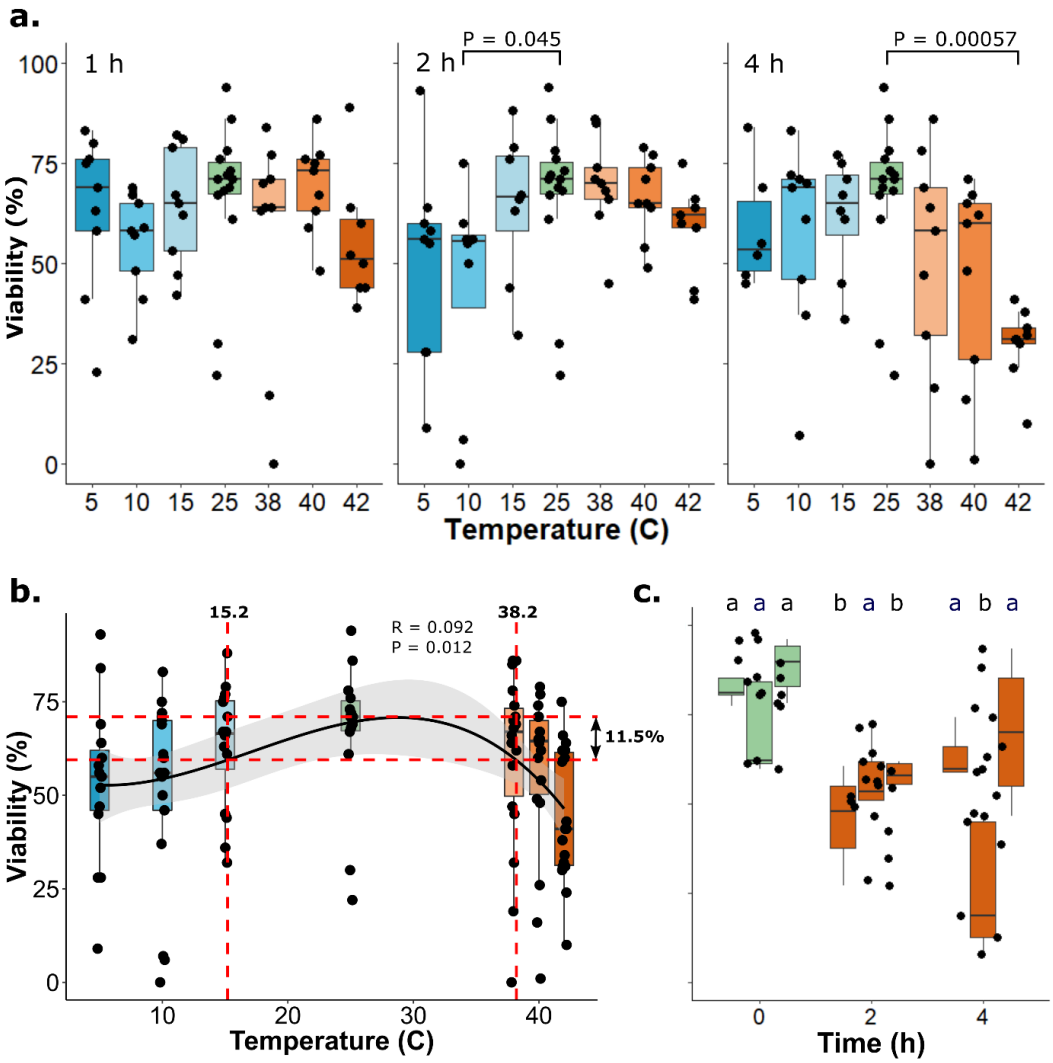
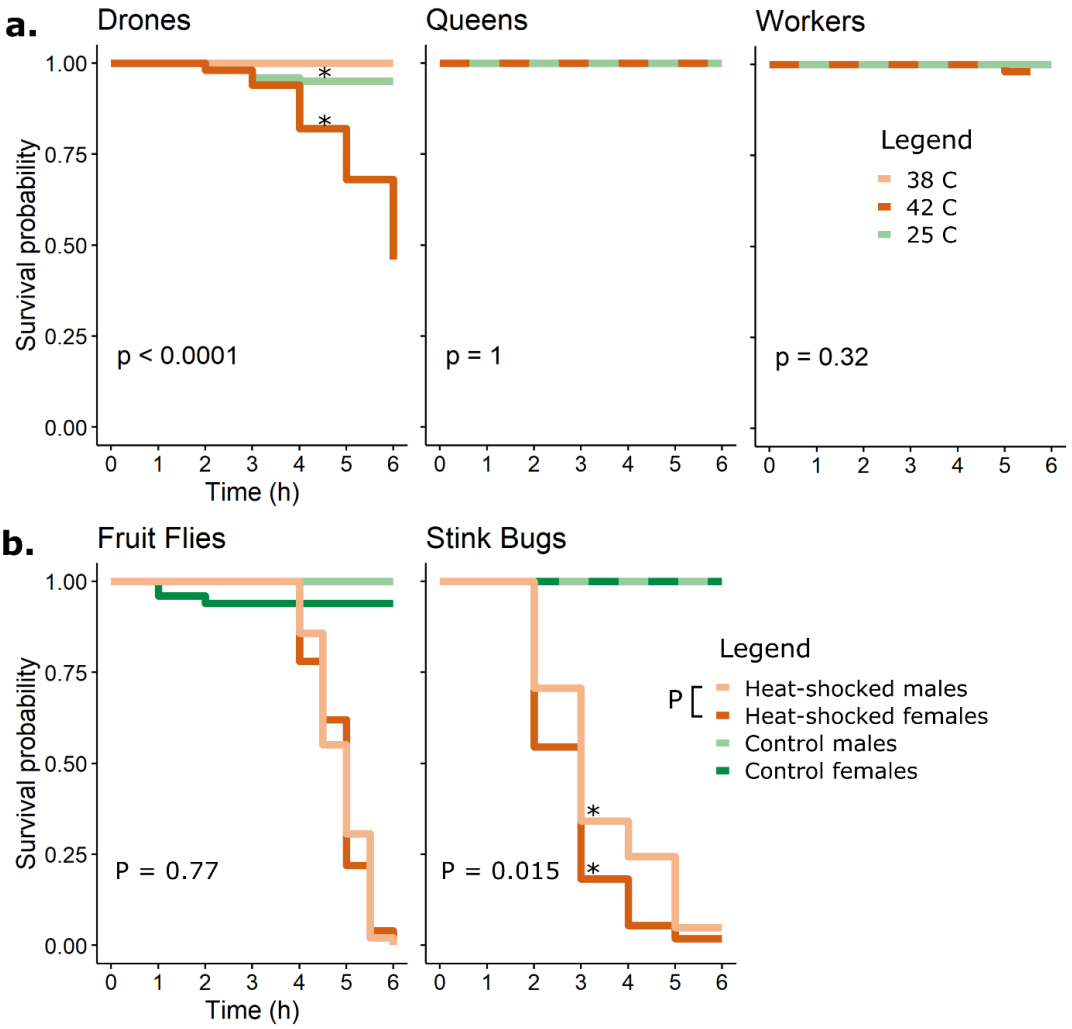


Figure 2. Viability of stored and ejaculated sperm after temperature stress. In all cases, boxes represent the bounds between the 2st and 3rd interquartile range (IQR), midlines represent the median, and whiskers are extended by 1.5*IQR. A) Honey bee queens were heat- and cold-shocked for 1 - 4 hours, 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). Each data point represents the viability measurement for a single queen. Data were analyzed with a Kruskal-Wallis test followed by a Dunnett's *post hoc* test. See **Table 1** for sample sizes. B) Combined 2 h and 4 h treatments were optimally fit with a cubic model (R^2 = 0.092, P = 0.012). Thresholded at 11.5% loss of viability, the line $y = y_{max} - 11.5$ intersects with temperatures 15.2 and 38.2°C. 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. The total number of drones evaluated for 0, 2, and 4 h treatments were 16, 16, and 18, respectively (see **Table 2** for more detail). Each data point represents the viability measurement for a single drone. Letters indicate significant differences at P < 0.05 (two-way ANOVA, factors: colony and time, followed by a Tukey HSD post hoc test).



730

731 **Figure 3. Sex-biased heat mortality in honey bees, stink bugs, and fruit flies**. Statistical differences
732 were evaluated using a log-rank test. Asterisks indicate significant differences. See **Fig S1** for risk tables,
733 including population sizes used to generate survival curves. A) Honey bees (drones, queens, and
734 workers) were held at different temperatures and mortality was recorded hourly. Drones are sensitive
735 to heat, whereas queens and workers are not. B) Fruit flies (*Drosophila melanogaster*; heat-shock =
736 38°C) and stink bugs (*Halyomorpha halys*; heat-shock = 42°C) do not have sex-biased heat sensitivity. For
737 fruit flies, loss of motility was used as the endpoint.

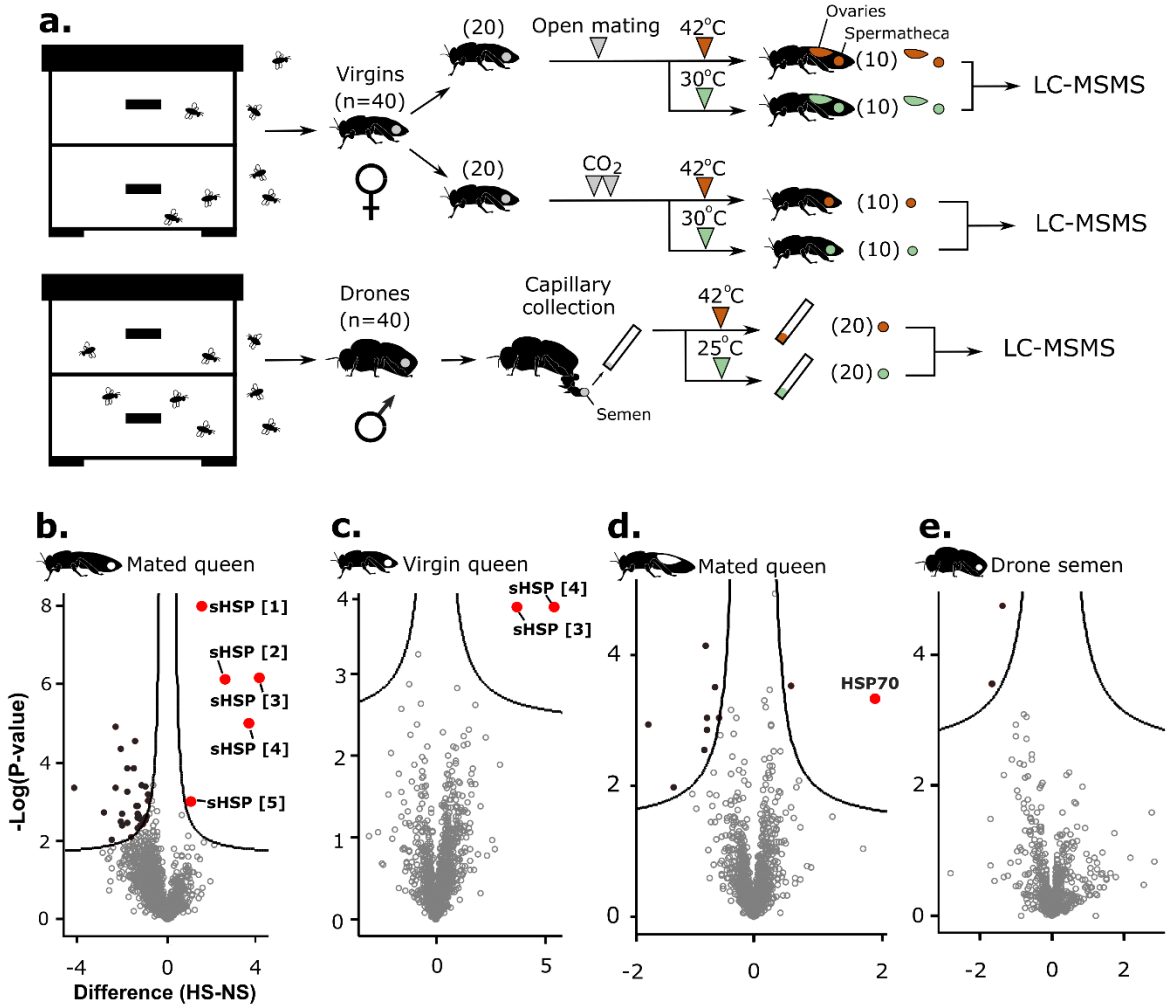


Figure 4. Differential protein expression comparing heat-shocked (HS) and not heat-shocked (NS) reproductive tissues. A) Samples included mated queen spermathecae, virgin queen spermathecae, ejaculated semen, and mated queen ovaries, which were all analyzed by intensity-based, label-free quantitative tandem mass spectrometry. For drones, 4 semen samples were pooled into one replicate, then fractionated into eight fractions by basic reverse phase chromatography prior to analyzing by mass spectrometry (final biological replicates: n = 5 heat-shocked and 5 non-heat-shocked). The significance cut-off for volcano plots (B-E) was 10% FDR (false discovery rate, permutation-based). Volcano plots represent mated queen spermathecae (B), virgin spermathecae (C), mated queen ovaries (D), and drone semen (E). Differentially expressed HSPs are red; other differentially expressed proteins are black. 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.

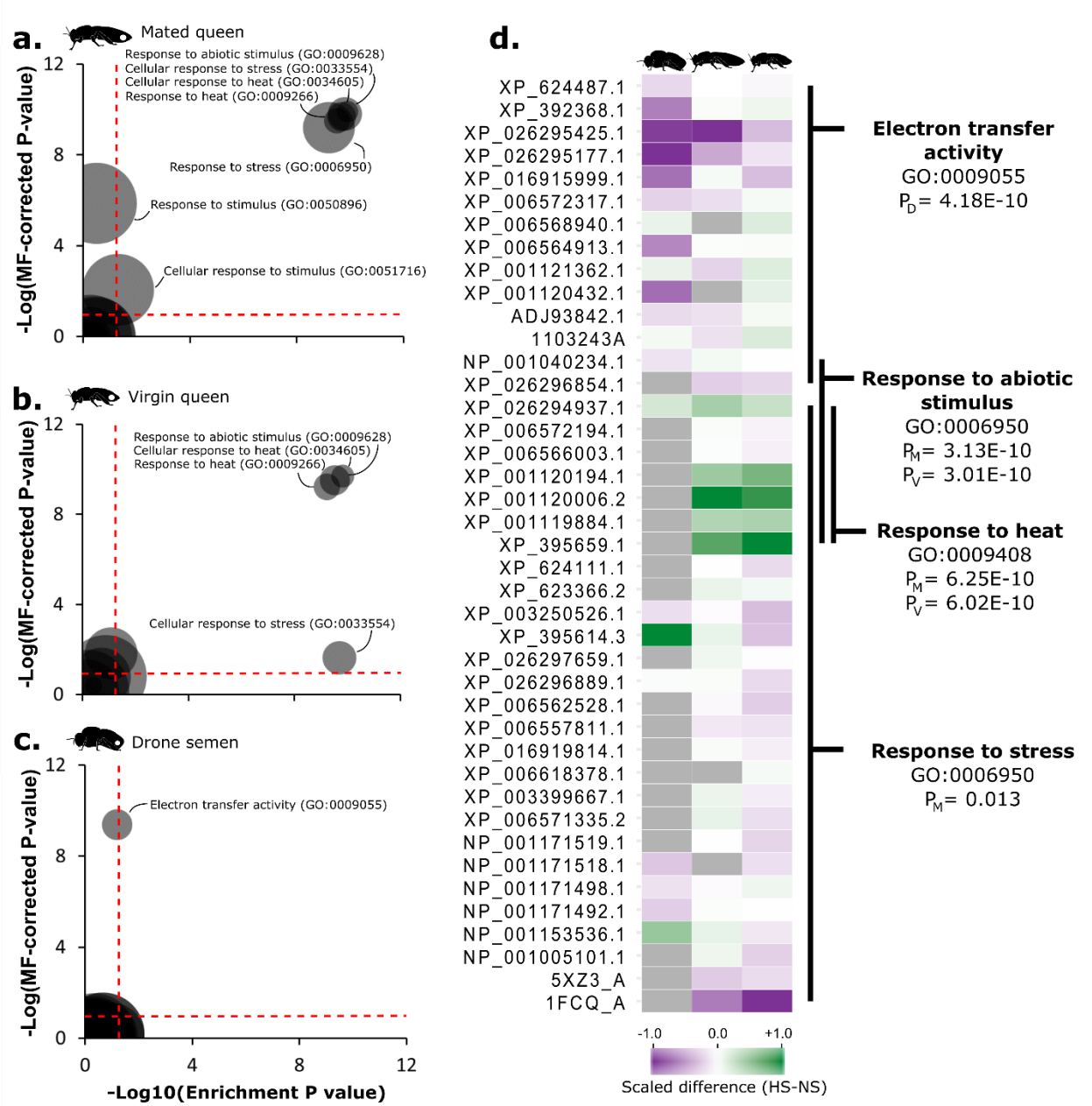


Figure 5. Gene ontology (GO) term enrichment analyses. A-C) Enrichments were performed via gene score resampling, correcting for both multiple hypothesis testing and protein multifunctionality (MF). The x-axes depict enrichment P values (10% FDR, Benjamini-Hochberg correction) without multifunctionality 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).

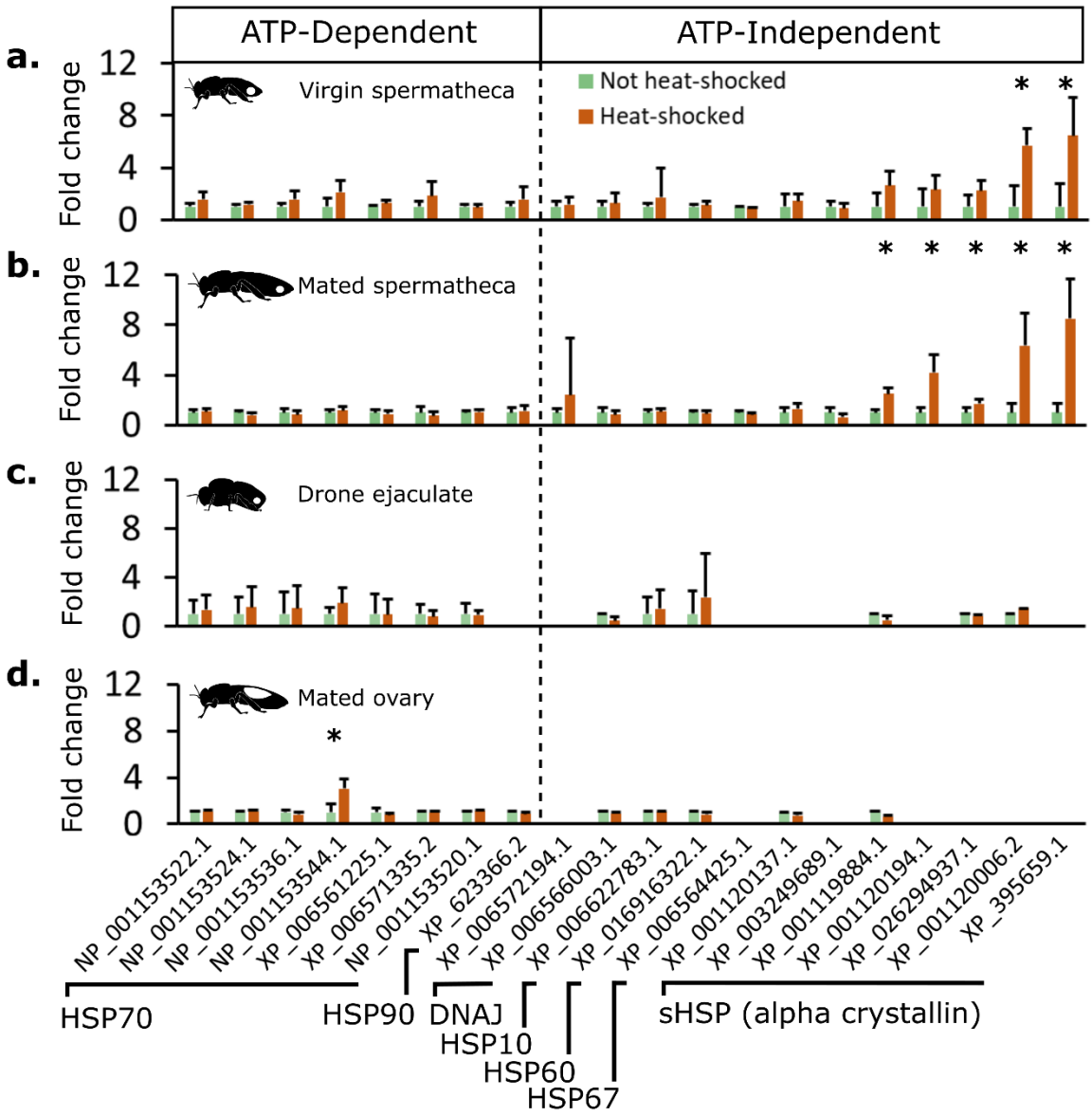


Figure 6. Heat-shock protein expression profiles. Proteins with GO terms for cellular response to heat were retrieved from the proteomics datasets. Fold-change is relative to the non-heat-shocked state. Proteins marked with an asterisk were significantly up-regulated in the global differential gene expression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

768 **Tables**

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*

Colony	0 h	2 h	4 h
1	5	5	6
2	6	6	6
3	5	5	6
Total	16	16	18

769 *Temperature stress for all replicates was 42°C