Supplemental Table S1. Timeline of queen development

| Day | 1 | 2 | 3 | 4 | 5 | 6 | 7* | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16-17 |
|-----|---|---|---|---|---|---|----|---|---|----|----|----|----|----|----|-------|
| Cell state | open | capped (sealed) | emergence |
| Developmental stage | egg | larva | pre-pupa | pupa | imago |

*7 – day of queen larva exposure to THI

Supplemental Table S2. Glossary of beekeeping terminology

| Term | Definition |
|------|------------|
| Capped brood | at the end of larval stage the cells are sealed with wax caps to allow pupation |
| Frame | structural component of a hive that supports wax comb |
| Foundation | plastic plate with imprinted hexagonal pattern which is used by bees to build wax comb for food storage (honey and pollen) and rearing of brood (eggs, larvae, pupae) |
| Grafting | the process of transferring a larva (<24 hours post hatching) into a plastic queen cup during artificial queen rearing process |
| Grafting frame | frame containing queen cups in which larvae (<24 hours post hatching) are transferred during artificial queen rearing process |
| Late larva | larval stage starts with egg hatching at day 3 post oviposition and it is completed by day 8-9 with capping. Accordingly, in this study, we refer to larvae at day 7 post oviposition as late larvae |
| Mating nucleus | small colony used to house virgin queen during the first three weeks of life while mating flights takes palace; these are used for commercial queen production |
| Queen-right colony | colony with a present laying queen |
| Sealed brood | capped brood |
| Standard Langstroth frame | the most commonly used frame size in Langstroth bee hive with following standardized dimensions 24.3x48.0x23.0x3.5 cm |
| Strong colony | highly populated colony |
| Unsealed (uncapped) brood | eggs and developing larvae that have not been sealed off with a wax cap |
Supplemental Materials and Methods

Rearing of queens

A detailed day-by-day description of the experimental timeline is provided in Supplemental Table S3. The queens were reared in highly populated (strong) colonies in the presence of a laying queen (queen right). Each queen-rearing colony consisted of two Langstroth brood chambers with 19 frames total (used to store food and raise brood). The queen in each colony was restricted in a cage in the middle of the top chamber made of queen excluder mesh. All frames of unsealed worker brood (uncapped larvae under 8-9 days post oviposition) were removed from the colony to prevent unwanted spontaneous queen cell formation, and returned to the colony prior to worker bee emergence to sustain a vibrant nurse bee population. The caged queen was given empty foundation drawn with wax every 24 hours to generate frames with eggs of synchronized age, which were used as a source of larvae to produce queens. Newly hatched larvae were grafted (transferred) into artificial plastic queen rearing cell cups (Mann Lake Ltd. Hackensack, MN, USA) suspended from the bars of a grafting frame. After larval transfer was complete, the grafting frames were incubated in the top chamber of the queen-rearing colony (starter colony), on either side of the queen cage; accordingly, the queen cells were accepted and raised in the queen right queen-rearing colony until day 7 post-oviposition, at which time they were exposed to treatment. Grafting frames with treated queen larvae were moved to an incubator colony (finisher colony) for capping and maturation until day 14. The incubator colonies were strong and occupied a standard hive composed of two 10-frame Langstroth brood-chambers divided by a queen excluder to separate the queen in the bottom brood chamber from the experimental grafting frame in the top brood chamber. On day 14, the queen cells were removed from the incubator colony and individually placed into mating nucleus colonies (small, queen-less colonies) prepared for queen emergence and mating.
Supplemental Table S3. Experimental design and timeline

| Day of the experiment | Description |
|-----------------------|-------------|
| 0                     | Insert wax-drawn worker frame into a one-frame queen cage with a queen; |
| 1                     | Remove the worker frame with eggs from the queen cage; |
| 4                     | Graft (transfer) newly hatched bee larvae from the worker frame into plastic queen cups on a grafting frame; Place grafting frames with grafted larvae into a starter colony for initial rearing of honey bee queen larvae; |
| 7                     | Remove grafting frame from the starter colony; Pipette 4μl of test solution with incremental doses of THI (0, 5, 50 ng/larva) into each queen cell; Place the grafting frame with treated larvae into an incubator (finisher) colony for further development; |
| 8-14                  | Record queen survival daily by examining the integrity of the queen cells on the grafting frame; Record capping rate on day 9 post oviposition; |
| 13                    | Assemble mating nucleus colonies by placing 3 frames into nucleus boxes, including 1) a foundation frame, 2) a full frame of honey and bee bread with bees, and 3) a frame of fresh and capped brood with bees; |
| 14                    | Place one experimental queen cell into each mating nucleus; |
| 37                    | Examine each mating nucleus for the presence of eggs and larvae to identify the number of mated queens; |
| 38-42                 | Collect mated queens from mating nucleus colonies for further evaluation (weight, sperm analysis, histology). |

**Mating nucleus set up**

Mating nucleus is a small hive that houses a colony with low population of bees without a queen; the mating nuclei are used as starter colonies for a newly emerged queen to mate. The mating nuclei in our study were made up of a single standard Langstroth box divided into three completely separate compartments. Thus, each Langstroth box housed three mating nuclei with three standard Langstroth frames (24.3 x 48.0 x 23.0 x 3.5 cm) in each: one frame of capped honey, one frame of sealed brood with bees, and a new empty frame with plastic foundation. These mating nuclei were made on day 13 with frames donated by healthy, non-experimental colonies. On day 14 post-oviposition, each box containing three mating nuclei was randomly assigned three queen cells representing each treatment (control, 5 ng, and 50 ng THI), with one cell inserted in each mating nucleus. After 2 weeks and 3 weeks post queen emergence, the experimental nucleus colonies were checked to determine mating success of
queens based on the presence of eggs and worker brood. All mated queens were harvested 22-26 days post emergence for further evaluation.
Supplemental Table S4. Summary of the original data collected from all measured parameters described

|                | June (A)                                      | August (B)                                 |
|----------------|----------------------------------------------|--------------------------------------------|
|                | Control | 5ng THI | 50ng THI | Control | 5ng THI | 50ng THI |
| 1. Total treated |         |         |          |         |         |          |
| 29             | 26      | 36      | 18       | 25      | 24      |
| 2. Total capped |         |         |          |         |         |          |
| 29             | 26      | 32      | 18       | 22      | 21      |
| 3. Total emerged |        |         |          |         |         |          |
| 29             | 26      | 25      | 18       | 17      | 13      |
| 4. Total mated |         |         |          |         |         |          |
| 9              | 13      | 9       | 12       | 12      | 13      |
| 5. Queen weight (mg) |     |         |          |         |         |          |
|                |         |         |          |         |         |          |
|                | 215.3   | 196.1   | 231.4    |         |         |
|                | 252.6   | 228.5   | 246.4    |         |         |
|                | 245.6   | 217.8   | 237.3    |         |         |
|                | 211.8   | 202.6   | 241.3    |         |         |
|                | 256.4   | 216.9   | 190.9    |         |         |
|                | 257.4   | 246.2   | 193      |         |         |
|                | 220.9   | 261.3   | 211.5    |         |         |
|                | 244.7   | 258     | 203.4    |         |         |
|                | 248.6   | 231     | 234.4    |         |         |
|                | 199.9   | 238.2   | 201.3    |         |         |
|                | 221.3   | 230.8   | 259.2    |         |         |
|                | 228.1   | 209.3   | 190.9    |         |         |
|                |         |         |          |         |         | 215.4    |
Supplemental Table S4. (continued)

|                | June (A)       | August (B)     |
|----------------|----------------|----------------|
|                | Control 5ng THI 50ng THI | Control 5ng THI 50ng THI |
| 6. Total sperm count | 2,024,000 2,915,000 1,276,000 3,936,000 | 5,072,000 4,752,000 |
|                | 2,453,000 1,232,000 1,529,000 5,232,000 | 4,140,000 5,448,000 |
| .              | 2,255,000 2,255,000 5,316,000 6,480,000 | 4,788,000 |
| 3,619,000      | 2,189,000 2,222,000 4,920,000 4,812,000 | 3,852,000 |
| 2,761,000      | 1,386,000 2,156,000 6,288,000 4,800,000 | . |
| 3,949,000      | 2,222,000 3,179,000 4,272,000 5,748,000 | 3,168,000 |
| 2,277,000      | 4,367,000 5,467,000 4,832,000 5,936,000 | 4,020,000 |
| 1,925,000      | 4,818,000 1,595,000 4,448,000 5,824,000 | 5,312,000 |
| 1,969,000      | 3,553,000 1,958,000 2,384,000 5,120,000 | 5,408,000 |
| .              | 3,916,000 3,200,000 6,224,000 4,080,000 | . |
| 1,727,000      | 800,000 4,416,000 3,280,000 4,416,000 | . |
| 2,101,000      | 4,864,000 4,416,000 4,416,000 | . |
| 2,926,000      | 4,256,000 | . |
### Supplemental Table S4. (continued)

|                      | June (A)                      | August (B)                   |
|----------------------|------------------------------|------------------------------|
|                      | Control 5ng THI 50ng THI     | Control 5ng THI 50ng THI     |
| 7. Sperm viability (%)| 88.3 82.7 78.4 89.5 80.9 55.8 | 78.0 89.7 76.3 86.9 86.8 73.5 |
|                      | . 81.0 72.1 79.8 82.7 44.8   | 79.9 68.3 66.3 73.4 80.5 47.7 |
|                      | 74.0 67.8 60.5 75.3 77.0 44.4 | 74.0 66.7 60.5 75.3 77.0 44.4 |
|                      | 63.7 68.1 81.3 .               | 63.7 68.1 81.3 .               |
|                      | 78.2 60.7                     | 78.2 60.7                     |
| 8. Mandibular gland epithelial area (mm²) | 13.49 8.40 5.65 9.67 10.94 8.27 | 9.97 10.40 8.28 9.70 9.87 8.36 |
|                      | 8.20 9.11 8.58 12.46 8.97 8.38 | 10.95 8.23 7.52 11.30 8.20 9.49 |
| 9. Mandibular gland lumen total area (mm²) | 9.65 14.13 11.51 14.21 12.60 8.80 | 18.14 3.13 12.98 11.64 13.81 10.33 |
|                      | 11.10 14.58 11.39 14.27 8.67 10.01 | 9.49 8.52 3.89 13.24 5.04 14.61 |
| 10. Peri-mandibular gland fat body area (mm²) | 9.75 10.44 6.31 9.97 10.31 9.30 | 9.68 10.15 10.12 9.12 10.97 8.60 |
|                      | 9.53 11.51 8.95 12.97 11.02 7.56 | 10.82 9.70 9.70 9.47 9.01 10.90 |
| 11. Optic lobe total area (mm²) | 4.30 3.28 3.67 3.32 3.11 2.99 | 3.08 3.37 3.36 3.28 3.38 2.81 |
|                      | 4.01 4.08 3.86 3.20 3.42 2.79 | 4.17 3.79 3.32 2.89 3.19 3.35 |
Supplemental Table S4. (continued)

|                      | June (A)               | August (B)            |
|----------------------|------------------------|-----------------------|
|                      | Control    | 5ng THI | 50ng THI | Control | 5ng THI | 50ng THI |
| 12. Total ovariole count |           |         |          |         |         |          |
|                      | 296       | 292     | 280      | 294     | 343     | 294      |
|                      | 310       | 335     | 346      | 277     | 285     | 314      |
|                      | 340       | 274     | 303      | 272     | 305     | 317      |
|                      | 435       | 306     | 303      | 321     | 332     | 491      |
| 13. Number of apoptotic cells per mm² |           |         |          |         |         |          |
|                      | 96.2      | 114.3   | 100.1    | 140.9   | 118.6   | 237.4    |
|                      | 78.0      | 66.2    | 152.9    | 258.7   | 72.0    | 130.6    |
|                      | 30.7      | 232.9   | 56.8     | 138.7   | 87.6    | 55.0     |
|                      | 15.0      | 4.2     | 49.1     | 162.1   | 11.4    | 58.6     |
| 14. Total mandibular gland epithelial area in newly emerged honey bee queens; pilot study, 2017 |           |         |          |         |         |          |
|                      | 13.31     | 17.13   | 3.65     |         |         |          |
|                      | 19.57     | 15.38   | 13.14    |         |         |          |
|                      | 14.18     | 14.19   | 12.84    |         |         |          |
|                      | 18.67     | 16.07   | 12.78    |         |         |          |
|                      | 15.97     | 17.98   | 10.77    |         |         |          |
|                      | 18.11     |         | 9.30     |         |         |          |
|                      | 15.41     |         | 14.76    |         |         |          |
Supplemental Figure S1. Serial sectioning procedure of the ovary. Thirty consecutive 5 μm thick sections were cut, of which the first 10 sections were collected on a glass slide, stained with H&E and examined; the following 20 sections were discarded. In such way, we obtained for each queen 10 glass slides containing 10 serial consecutive cross sections of cranial ovary which were 100 μm apart.