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Successful Indoor Mass Storage of Honeybee Queens (*Apis mellifera*) during Winter

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Abstract: The production of young, mated honeybee queens (*Apis mellifera*) is essential to replace dead queens or to start new colonies after wintering. Mass storage of mated honeybee queens during winter and their use the following spring is an interesting strategy that could help fulfill this need. In this study, we investigated the survival, fertility, and fecundity of young, mated queens stored massively in queenless colonies from September to April (eight months). The queens were kept in environmentally controlled rooms at temperatures above and below cluster formation. The results show that indoor mass storage of mated queens can be achieved with success when queen banks are stored above cluster temperature. Significantly higher survival of queens was measured when wintering queen banks at 16 °C. Surviving queens wintered at different temperatures above or below cluster formation had similar fertility (sperm viability) and fecundity (egg laying and viable worker population). This study shows the potential of indoor overwintering of honeybee queen banks. The technique we describe could be applied on a commercial scale by beekeepers and queen breeders.

Keywords: *Apis mellifera*; queen storage; queen wintering; queen banks; queen fertility; queen fecundity

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1. Introduction

Young, mated honeybee queens (*Apis mellifera*) are essential to replace dead queens or to start new colonies when multiplying livestock. In many countries, such as Canada, the demand for young queens is at its highest after the wintering period because of colony mortality levels, which have averaged 28% since 2007 [1]. Unfortunately, early spring is accompanied by cold climate and poor foraging environment that are unfavorable for queen and drone rearing [2,3]. Thus, the locally produced queens become available later in spring when environmental conditions improve [4,5] and queens must be imported early spring from warmer climate countries to fulfill industry demand [6]. This importation of honeybee stock is associated with several abiotic and biotic risks and issues [7], such as unwanted genotypes (e.g., the Africanized bee), exotic pathogens and parasites (e.g., the small hive beetle, *Aethina tumida*) or pathogens and parasites resistant to existing treatments (e.g., the foulbrood causing bacteria resistant to oxytetracycline). Furthermore, shipped queens may be exposed to thermohydrometric conditions during transport that negatively affect queen fertility [8,9]. Along with the rising price of imported queens, all these factors justify efforts to increase the domestic supply of queens early spring.

Mass storage of mated honeybee queens during winter that can be used in the following spring is a very interesting strategy. There is scarce scientific information on this practice, but different methods have been tested for long-term mass winter storage of queens: laboratory systems [10–12], four- or five-frame nucleus queen banks or mating nuclei [13,14] and complete Langstroth hive queen banks [13,15,16]. On the other hand, summer queen storage is common practice. Beekeepers often "bank" their mated queens

until needed. Young, mated queens can be caged individually and placed into holding frames within specially prepared colonies called queen banks [13,17]. Queen bank colonies are usually queenless to avoid aggressivity towards queens [15,16] and have many nurse bees as well as an abundant supply of stored honey and pollen. A summer queen bank can keep up to 96 queens successfully for three months [18,19]. Winter mass storage of queens for extended periods presents greater challenges: cluster formation by bees during colder months and an increasing scarcity of nurse bees during winter will result in poor queen survival rates [20]. In 1993, Wyborn et al. [15] tested several systems to massively store queens in colonies from November to April in Canada. Highest survival levels (60%) were obtained when queens were stored individually in small screened wooden cages within a strong queenless colony. They identified three critical colony management strategies: (1) the importance of having a considerable number of adult workers within the banking colony, (2) the necessity of abundant sucrose reserves in colonies, and (3) the importance of keeping the banked queens in the center of the cluster or preventing the formation of a winter cluster of bees. Gençer [16] also found that strong colonies with large numbers of workers are essential to prevent that the honeybee cluster diameter constricts during prolonged winter confinement and that it withdraws from the position of the stored queen bank. If this happens solo queens will suffer chill-coma and die [21].

In this study, we investigated the survival of young, mated queens stored massively in queenless colonies from early September 2018 to late April 2019 (eight months). These queen banks were kept in environmentally controlled rooms at temperatures above and below cluster formation. We also measured the impact of mass storage on queen fertility and fecundity.

2. Materials and Methods

2.1. Queen Rearing and Shipment

In August 2018, a total of 630 queens were produced by three local queen breeders: Miels d'Anicet (Available online: https://mielsdanicet.com (accessed on 23 April 2021); Ferme-Neuve, Québec), Rayons de miel (Available online: https://rayons-de-miel.business.site/ (accessed on 23 April 2021); Saint-Adrien, Québec, QC, Canada) and our research center (Centre de Recherche en Sciences Animales de Deschambault-CRSAD, Available online: http://crsad.qc.ca/ (accessed on 23 April 2021); Deschambault, Québec, QC, Canada). Queens from each local breeder were sister queens from their selected hybrid Italian stock. were raised using the grafting technique [22], open mated in mating nucs and harvested a few days after the beginning of egg laying. These young, mated were received at our research center 27–29 August 2018 and kept in a dark room at 30 °C until creation of the queen banks (30 August 2018).

2.2. Queen Banking Systems and Control Queens

Experiments were conducted at our research center. On 28 August 2018, the queens of 30 of our colonies (standard Langstroth hives) were removed. These colonies were kept queenless for 48 h and used to create 15 strong colonies "banking colonies", each comprising of two brood chambers with 9, 10 frames of brood (approximately 20,000 brood cells) and 8 kg of a mixture of young adherent bees and older bees (approximately 40,000 bees). On 30 August, a modified frame with 40 individually caged queens (California Mini Queen Cages, Mann Lake Ltd., Hackensack Minnesota USA #HD-398) was placed in the center position of the upper brood chamber of each banking colony. These modified queen holding frames were made from Langstroth frames cut in the center to accommodate 20 queen cages held together back-to-back (Figure 1). Data loggers (Hobo data logger U12-O13, Hoskin Scientific, Saint-Laurent, Quebec Canada) were placed next to the banked queens to record temperature and relative humidity during storage of queen banks. A total of 600 queens were stored in a total of 15 banking colonies, each holding 40 queens on a single frame.

Figure 1. Modified Langstroth wooden frame with 40 banked queens, each in a California Mini Queen Cage (20 on each side) and a Hobo data logger (left side). This frame was placed in the center position of the upper brood chamber of each banking colony.

2.3. Mass Storage of Queens

From September to November 2018: The 15 queen banks (queens and banking colonies) were kept outdoors in the same apiary from the time they were created (30 August) until 9 November. Queen banks were treated against varroa with Apivar® as prescribed per label starting on September 14 (2 strips/brood chamber, 4 strips per queen bank) and fed 40 L of 2:1 sucrose syrup using Miller top feeders (#FE-1100, Propolis etc.) at the same time. No additional syrup was given throughout the entire protocol (September to April). In addition to the 15 queen banks, 20 queens (control queens) were introduced individually in colonies composed of 10 standard Langstroth frames with brood and honey. These control queens were able to lay eggs and move freely on frames. They were also treated against varroa with Apivar®, fed sucrose syrup (20 L of 2:1 sucrose syrup using Miller top feeders) and kept outdoors as per queen banks.

From November 2018 to April 2019: On November 9, the 15 queen banks were randomly distributed into three groups (N = 5 queen banks/group) and assigned to three different environmentally controlled rooms: 6 °C ± 1 °C; 11 °C ± 1 °C; 16 °C ± 1 °C. Control queens in standard colonies were placed in an environmentally controlled room at 6 °C ± 1 °C. In each room, the relative humidity was set at 55% ± 10%.

2.4. Queen Survival, Morphometrics, and Fecundity

Survival of queens was recorded after outdoor mass storage from August 30 to November 9, 2018, and again after winter mass storage in environmentally controlled rooms, from November 9 to April 16, 2020. Queen fecundity and morphometric measurements were noted for a sample of queens prior to creation of queen banks (N = 10 queens), and again after winter mass storage in April of the following year in experimental groups and control (N = 15 queens per experimental group, N = 3 per queen bank and N = 7 control queens). Queen morphometric measurements (head, abdomen width/length and fresh weight) were taken with a digital precision scale (Model 938882, Mitutoyo SR44) and a precision balance (Model KHA 203, Kilotech Inc., Quebec, QC, Canada). Queen fecundity

was evaluated by measuring sperm count and sperm viability in spermatheca as described by Rousseau et al. [3], briefly, the abdomens of sampled queens were dissected to remove the spermatheca according to the methodology described by Collins and Donoghue (1999). Each spermatheca was ruptured and homogenized in 0.5 mL of modified Kiev Buffer (Moritz 1984; 0.3 g D+ Glucose, 0.41 potassium chloride, 0.21 g sodium bicarbonate, 2.43 g sodium citrate 2 hydrate in 100 mL of deionized water). Sperm dilution was stained with a Live/Dead Sperm Viability Kit (L-7011; Life Technology Inc., Carlsbad, CA, USA) using SYBR-14 and propidium iodide. Sperm count and viability were assessed using a Zeiss Observer Z1 microscope equipped with fluorescence filters by scoring live and dead sperm for 200 cells on four slides of 10 µL stained semen.

2.5. Queen Introduction Success and Fertility in Colonies

Mass storage of queen banks in the various experimental groups was maintained until May 3, 2019 and a sample of surviving queens (N = 19 from 6 °C group; N = 17 from 11 °C group: N = 16 from 16 °C group) were marked with a small spot of paint (Stannaby bee supplies #Q-910) on the back of the thorax and placed inside a Jz-Bz plastic cage with sugar candy. Each caged queen was randomly introduced in the middle of a nucleus colony composed of five standard Langstroth frames with two frames of brood, one frame of honey, some pollen bread, and two wax frames. Introduction success was verified after 7 to 10 days and was considered successful when the queen was seen laying eggs. On August 12, all of these colonies were visited and checked for the presence of the original marked queen. She was considered fertile when her progeny mas composed of >75% workers.

2.6. Statistical Analysis

Statistical analyses were performed using JMP Pro 15 software (version 15.1, from SAS). A survival analysis was performed using the log rank test to investigate the effect of temperature on mass storage queen banks. A linear mixed model was used to compare the different queen morphometric measurements and sperm viability between experimental groups (fixed effect = experimental group, random effect = bank). When the data was not normally distributed (abdomen length, sperm viability), we used a nonparametric test (Wilcoxon/Kruskal–Wallis) to identify contrasts between groups.

3. Results

3.1. Mass Storage of Queens from September to November 2018

3.1.1. Temperature and Relative Humidity

The average temperature recorded in queen banks was always above outdoor apiary temperature. It ranged from 13.4 to 34.7 °C in queen banks and followed the decreasing daily outdoor temperature, which ranged from −0.75 to 22.7 °C (Figure 2). The average relative humidity recorded inside queen banks was most often under outdoor apiary relative humidity and ranged from 48.7 to 71.5%, while the outdoor relative humidity ranged from 51.9 to 95.9% (Figure 3).

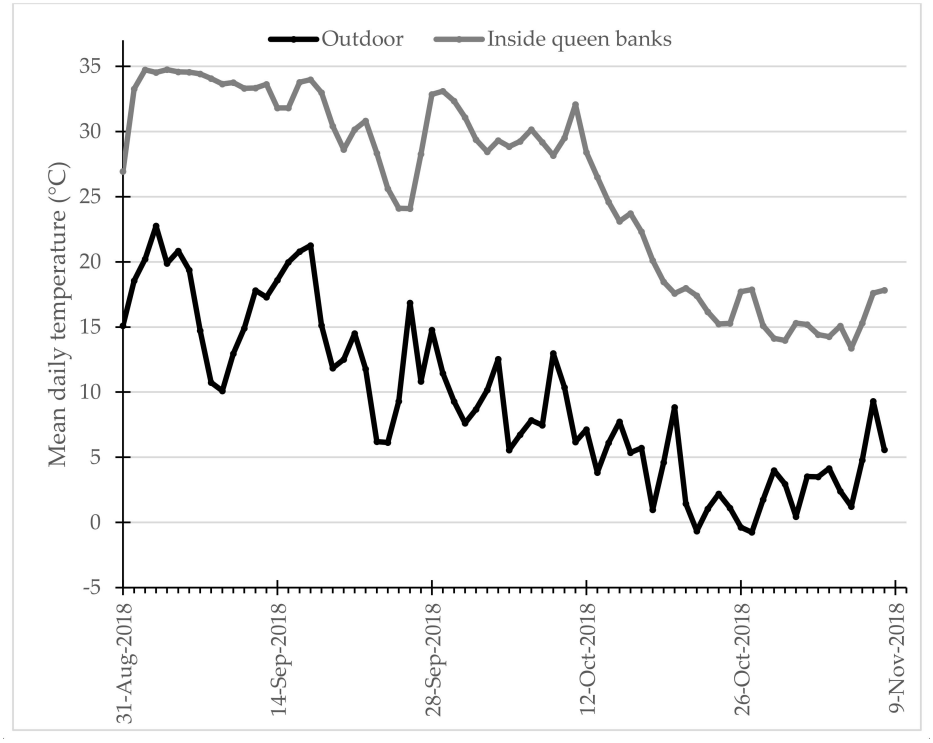

Figure 2. Average daily temperature (°C) outdoors and inside queen banks (15 queen banks) from 3 September to 9 November 2018.

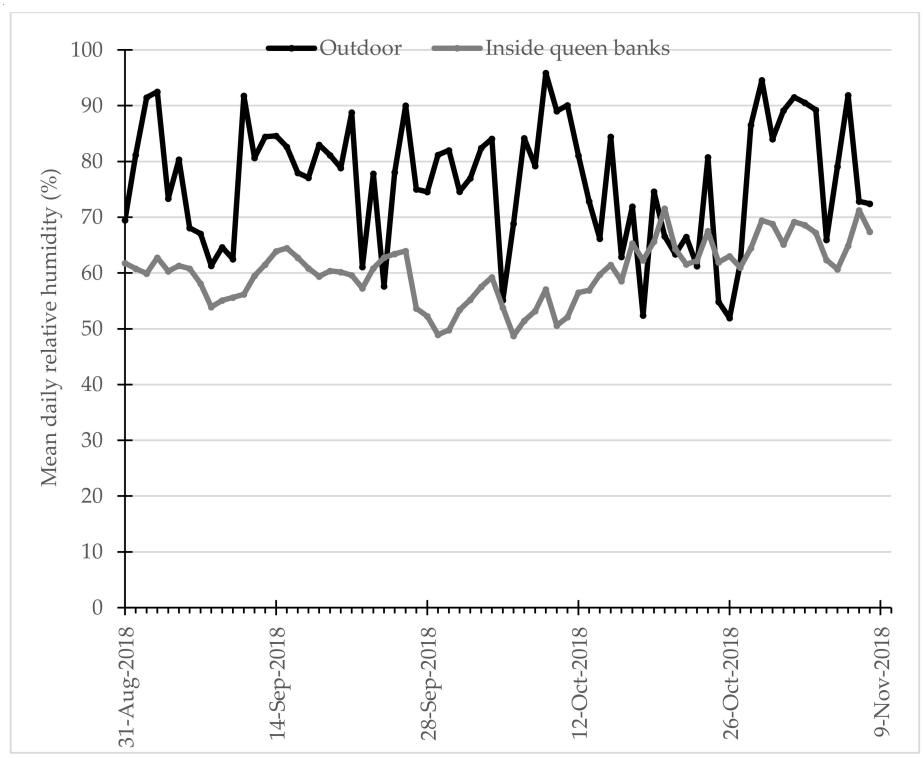

Figure 3. Average daily relative humidity (%) outdoors and inside queen banks (N = 15) from 3 September to 9 November 2018.

3.1.2. Queen Survival

At the end of the first week of the experiment (9 September, day 9), 48 of the 600 queens introduced initially in the 15 different banking colonies had died, and there was

no significant difference between the queen banks. No dead queens were measured in the control colonies and they were laying normally. After 70 days of mass storage of queens in banks kept outdoors the average queen survival in the 15 queen banks was 85.5 ± 6.8% and 100% for the 20 queens in control colonies (Table 1).

Table 1. Queen survival in queen banks and control colonies from 31 August to 9 November 2018, placed outdoors in a common apiary.

Variable	Dates		
	31 August 2018 (Day 0)	9 September 2018 (Day 9)	9 November 2018 (Day 70)
Queen banks (N = 15, 40 queens/bank)			
Live queens (total)	600	552	513
Live queens (mean/bank)	40 ± 0.0	36.8 ± 2.1	34.2 ± 2.7
Control colonies (N = 20, 1 queen/colony)			
Live queens (total)	20	20	20

3.2. Mass Storage of Queens from November 2018 to April 2019

3.2.1. Temperature and Relative Humidity

The average daily temperature in the queen banks were always above room temperature and showed little variation after an initial 3-day period of acclimatization (Figure 4). Queen banks in room at 6 °C started at 22.5 °C in November 2018 and gradually decreased to a low of 14.5 °C in mid-March 2019. Queen banks kept at 11 °C and 16 °C maintained similar average temperatures starting respectively at 26.0 °C and 27.5 °C in November 2018 and gradually decreasing to a low of 23.5 °C and 22.5 °C in April 2019. The average relative humidity in the queen banks showed some variation but were within set value of 55% ± 10% (Figure 5).

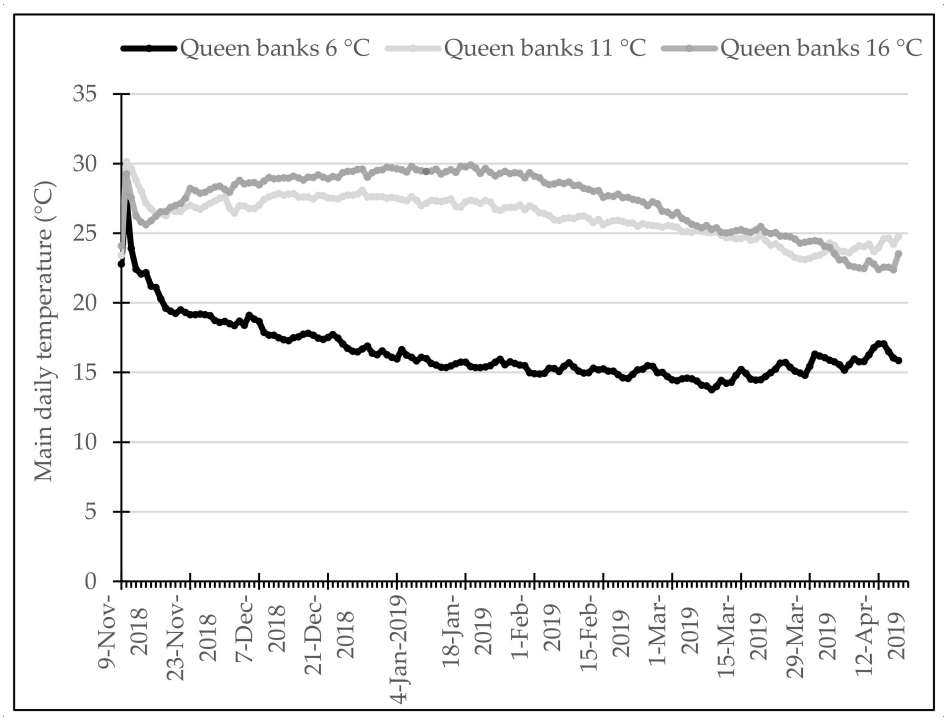

Figure 4. Average daily temperature in queen banks, in environmentally controlled rooms at 6 °C, 11 °C, and 16 °C, from 9 November 2018 to 16 April 2019.

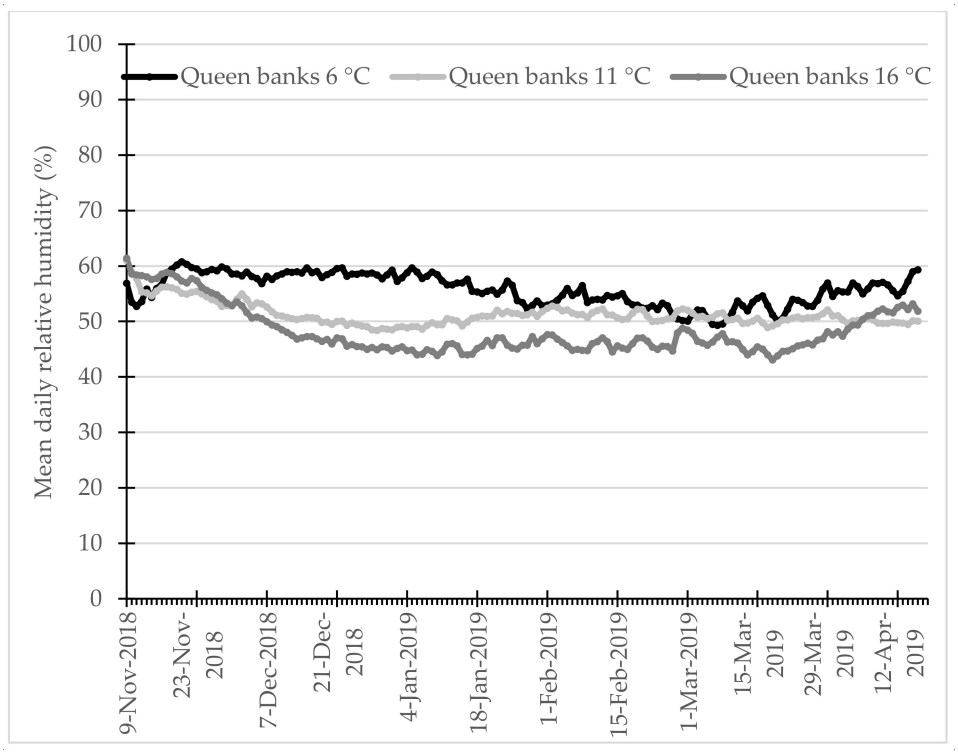

Figure 5. Average daily relative humidity (%) measured in queen banks in environmentally controlled rooms at 6 °C, 11 °C, and 16 °C, from 9 November 2018 to 16 April 2019.

3.2.2. Queen Survival

On April 16, all queen banking colonies had survived and had residual sucrose supplies from previous fall feeding. From 9 November 2018 to 16 April 2019 (day 70 to day 228 of mass storage), there was a significantly higher queen survival in banks kept at 16 °C than in banks kept at 6 °C and 11 °C ($\chi^2 = 23.7187$, $p < 0.0001$): 86.3 ± 4.1% of the queens survived at 16 °C compared to 56.9 ± 4.6% and 55.5 ± 15.0% at 6 °C and 11 °C degrees, respectively (Table 2).

Table 2. Queen survival in control colonies (N = 20) and queen banks (N = 5 banks per treatment and 40 queens/bank) while wintering in environmentally controlled rooms at three different temperatures (from 9 November 2018 to 16 April 2019). Different letters in the April 2019 line indicate significant differences of survival between treatment groups ($p < 0.0001$ log-rank test).

Variable	Control Colonies (N = 20, 1 Queen/Colony)	Treatment Groups (N = 5 Banks/Treatment, 40 Queens/Bank)		
	6 °C	6 °C	11 °C	16 °C
Live queens November 2018	20	34.6 ± 1.6	34.4 ± 1.4	33.6 ± 0.5
Live queens April 2019	14	19.8 ± 2.2 [a]	18.6 ± 4.8 [a]	29.0 ± 1.3 [b]

3.2.3. Queen Morphometrics and Sperm Viability

In April 2019, queens in control colonies had a greater body weight than queens banked at different temperatures (Figure 6) (χ^2 (4.55) = 19.668, $p = 0.0006$). They also had longer abdomens (F (4.55) = 21.3716, $p < 0.0001$), whereas abdomen width was similar between groups (F (4.55) = 0.6803, $p = 0.6085$). Sperm viability was similar between control queens and banked queens at different temperatures (χ^2 (4.55) = 3.4128, $p = 0.4913$), the overall average was 80.3% ± 1.2.

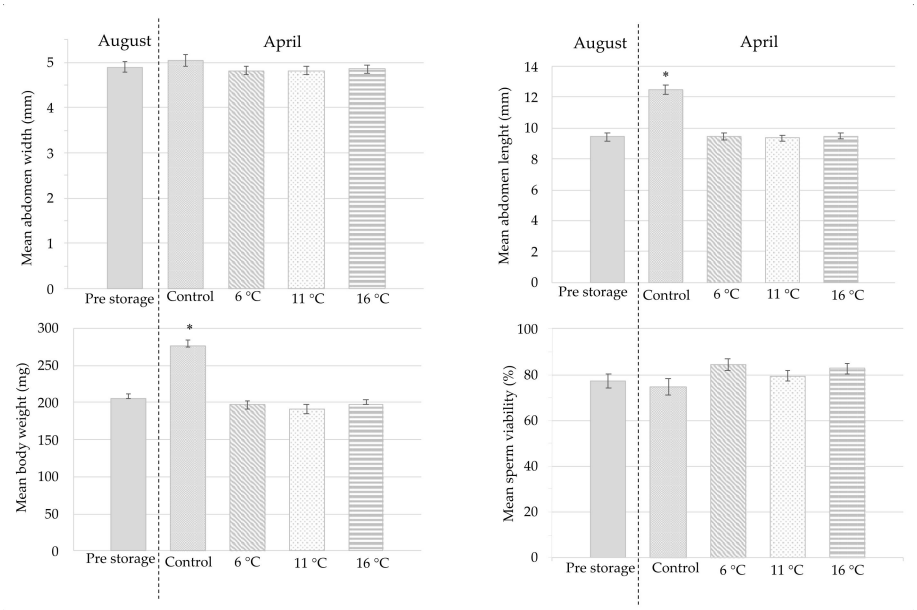

Figure 6. Queen morphometric and sperm viability (mean ± SD), measured before experiments (pre-storage 31 August 2018) and after wintering (April 2019) in control colonies and queen banks at 6 °C, 11 °C, and 16 °C. An asterisk (*) over bars indicates significant difference between groups ($p < 0.05$ Wilcoxon signed-rank test).

3.3. Queen Introduction Success and Fertility in Colonies

Queens from the 3 bank treatments, at 6 °C, 11 °C, and 16 °C, were introduced in nucleus colonies after wintering on 9 May 2019. After 7–10 days introduction success rate was 79%, 94%, and 88%, respectively (Table 3). Three months later, 12 August, most queens introduced from various queen banks were fertile: laying eggs and with worker progeny (53%, 71% and 68%, respectively). Of the 12 queens from all groups that failed during summer, ten colonies had fewer than 3 frames of bees and brood (4 from 6 °C group, 3 from 11 °C group, and 3 from 16 °C group), one colony was queenless (from 6 °C group), and one queen had superseded (from 11 °C group).

Table 3. Banked queen fertility after winter storage. Queens were introduced in colonies on 3 May 2019 and managed for honey production until 9 August.

Variable	Treatment Groups		
	6 °C	**11 °C**	**16 °C**
Queen from winter banks			
Queens introduced 3 May 2019	19	17	16
Queens accepted (after 7–10 days)	15 (79%)	16 (94%)	14 (88%)
Queens fertile August 12, 2019	10 (53%)	12 (71%)	11 (68%)

4. Discussion

In this study, we investigated three different temperatures for wintering queen banks in environmentally controlled rooms. The winter banking temperatures were chosen above and below cluster formation in order to verify the hypothesis that a temperature above cluster formation will increase survival of queens. The results showed that the long-term indoor mass storage of mated queens during winter could be achieved with success when queen banks are stored above cluster temperature at 16 °C.

The internal temperature of the colonies harboring queen banks during the winter strongly influenced queen survival in this experiment. This factor was previously identified by other research along with the provision of a sufficient number of bees to nurse the stored queens [20,23,24]. During cold weather, worker bees vibrate their thoracic flight muscles

and gather together in their nest to form a cluster in the form of a ball. Bee behavior within the cluster aims to create heat and maintain a temperature gradient between the cluster's center and outside and it expands and contracts as the bees respond to the surrounding temperature [21,25,26]. The ambient temperature regulates cluster size [27] and the honeybees in the center generate most of the heat around their queen and brood, while older bees on the surface of the ball serve as insulators [28]. The cluster becomes well defined when the air temperature falls below 14 °C. The cluster also changes position during winter as the food reserves are consumed [29]. Thus, queens banked in a fixed position within a wintering colony risk to be abandoned by the bee cluster and die of chill coma. Wyborn et al. [15] mentioned the importance of abundant in-hive winter honey stores and the prevention of cluster formation to be critical to queen survival within banks. Previous experimental studies on long-term queen mass storage reduced contraction of cluster diameter by maintaining large worker population in colonies that harbor the queen banks. In our study, the storage at 16 °C, above that required for cluster formation, had a beneficial effect on queen survival (84% survival from November to April). Prabucki et al. [20] also found that increasing the storage temperature to above 15 °C had a favorable effect on queen survival.

Canadian and USA beekeepers living in northern cold climates winter honeybee colonies indoors in environmentally controlled buildings, or outdoors, wrapped in different materials for insulation [29,30]. For indoor-wintering, honeybee colonies are stored in a building under complete darkness with temperatures maintained at about 5 °C, the temperature at which honeybees are known to use their syrup/honey reserves most efficiently (Desai and Currie 2016). In our study, the temperature range chosen to maintain queen banks during winter was intended to test the effect of honeybee clustering on queen survival. Our results showed a queen survival rate of 57% at the standard temperature for indoor wintering of honeybee colonies.

While the temperature in each experimental room was controlled (at 6 °C, 11 °C, and 16 °C), the relative humidity of the rooms fluctuated but it was fairly stable within the banking colonies and ranged from 43 to 61%. The optimal humidity level within the bee nest is known to be high (>90%) for adequate brood development [31]. Colonies can tolerate a wide range of humidity and it is usually not tightly regulated in wintering rooms. Research has shown that when winter colonies are maintained at RH of 45%, 60%, and 80% they will have similar survival [29]. In our study, all three treatment rooms maintained the RH within this level.

Our study showed no significative impact of long-term indoor storage on sperm viability or abdomen width of queens, although stored queens incurred a length reduction of their abdomen following wintering compared to control queens. This is surely because the ovaries of the control queens were active and laying eggs within their colony at the end of wintering [24]. Wyborn et al. [15] found no correlation between queen weight measured in April after overwintering and colony performance measured in August: areas of brood, bees, honey, pollen and comb, and honey weight. There is still a need for long-term assessment of the impact of our method of long-term storage of queens on colony development and queen longevity, but initial data on introduction success of banked queens and survival throughout the ensuing season is encouraging.

5. Conclusions

This study shows the potential of indoor overwintering of honeybee queen banks to fulfill the high demand for queens in early spring. The technique described here allows a large quantity of queens to be stored in a single colony that could be applied on a commercial scale by beekeepers and queen breeders. Our results also support using this approach as an innovative and successful way to improve the self-sufficiency of the Canadian beekeeping industry, as well as conservation efforts of local honeybee diversity and honeybee breeding programs [32,33].

Author Contributions: A.R. and P.G. conceived and designed the described experiments; A.R. conducted the experiments and analyzed the resulting data; A.R. and P.G. wrote the paper and participated in its revision. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data set is available upon request to the corresponding author.

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