

The Potential of Bee-Generated Carbon Dioxide for Control of Varroa Mite (Mesostigmata: Varroidae) in Indoor Overwintering Honey bee (Hymenoptera: Apidae) Colonies

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ABSTRACT The objective of this study was to manipulate ventilation rate to characterize interactions between stocks of honey bees (*Apis mellifera* L.) and ventilation setting on varroa mite (*Varroa destructor* Anderson and Trueman) mortality in honey bee colonies kept indoors over winter. The first experiment used colonies established from stock selected locally for wintering performance under exposure to varroa ($n = 6$) and unselected bees ($n = 6$) to assess mite and bee mortality and levels of carbon dioxide (CO₂) and oxygen (O₂) in the bee cluster when kept under a simulated winter condition at 5°C. The second experiment, used colonies from selected bees ($n = 10$) and unselected bees ($n = 12$) that were exposed to either standard ventilation (14.4 liter/min per hive) or restricted ventilation (0.24 liter/min per hive, in a Plexiglas ventilation chamber) during a 16-d treatment period to assess the influence of restricted air flow on winter mortality rates of varroa mites and honey bees. Experiment 2 was repeated in early, mid-, and late winter. The first experiment showed that under unrestricted ventilation with CO₂ concentrations averaging <2% there was no correlation between CO₂ and varroa mite mortality when colonies were placed under low temperature. CO₂ was negatively correlated with O₂ in the bee cluster in both experiments. When ventilation was restricted, mean CO₂ level ($3.82 \pm 0.31\%$, range 0.43–8.44%) increased by 200% relative to standard ventilation ($1.29 \pm 0.31\%$; range 0.09–5.26%) within the 16-d treatment period. The overall mite mortality rates and the reduction in mean abundance of varroa mite over time was greater under restricted ventilation ($37 \pm 4.2\%$) than under standard ventilation ($23 \pm 4.2\%$) but not affected by stock of bees during the treatment period. Selected bees showed overall greater mite mortality relative to unselected bees in both experiments. Restricting ventilation increased mite mortality, but did not affect worker bee mortality relative to that for colonies under standard ventilation. Restricted ventilation did not affect the overall level of *Nosema* compared with the control. However, there was an interaction between stock, season, and time of the trial. Unselected stock showed an increase in *Nosema* over time in the late winter trial that did not occur in the selected stock. In conclusion, these findings suggested that restricted ventilation has potential to suppress varroa mite in overwintering honey bee colonies via a low-cost and environmentally friendly measure.

KEY WORDS *Apis mellifera*, *Varroa destructor*, carbon dioxide, restricted ventilation, mite mortality

Carbon dioxide (CO₂) has a vital role in arthropod physiology. In addition to its role in respiration, it influences longevity and behaviors such as orientation, reproduction, and cold resistance (Engels and Ramamurty 1976, Barrozo and Lazzari 2004). The concentration of CO₂ in social insect colonies varies considerably (Dietlein 1985, reviewed in Nicolas and Sillans 1989). CO₂ concentration ranges from 1 to 2% in ant colonies (Portier and Duval 1929, Nielsen et al. 2003), 0.8–5.2% in termite colonies (Luscher 1961, Matsumoto 1977), and 0.2–9.9% in honey bee (*Apis mellifera* L.) colonies (Hallund 1956, Taranov and Mikailov 1960, Free and Simpson 1963, Seeley 1974, Sugahara and Sakamoto 2009).

The honey bee is well adapted to thermoregulate and manage the hive microclimate to survive under severe winter conditions in temperate climates (Fahrenholz et al. 1989; Stabentheiner et al. 2003, 2010). Changes in colony-level ventilation can affect levels of respiratory gases in the cluster. The level of CO₂ in the bee cluster correlates with changes in temperature (Free and Simpson 1963, Nagy and Stallone 1976). However, bees are able to adjust atmospheric CO₂ concentration to some degree and maintain concentrations of 0.1–4.25% in small colonies and 5–6% in full-size wintered colonies (Seeley 1974, Van NERUM and Buelens 1997). The CO₂ concentration in the cluster in winter is higher than in summer (0.5–2%; Van NERUM and Buelens 1997). There also may be daily fluctuations in CO₂ within the hive. Ohashi et al. (2009) found two peaks in CO₂ concentration, one in early morning (~5%) and another in afternoon (4%) in late summer due to daily changes in bee ventilation behavior. Oxygen level is typically inversely correlated with CO₂

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(Nagy and Stallone 1976, Southwick and Moritz 1987). However, O₂ concentrations of ~15% can be found in the winter cluster even when ambient O₂ is >15% (Van Nerum and Buelens 1997).

Little is known about the effects of colony-level respiratory changes on varroa mites, *Varroa destructor* Anderson & Trueman. Varroa mites are important parasites influencing *A. mellifera* winter colony losses worldwide (Currie et al. 2010, van der Zee et al. 2014). Varroa parasitism decreases the weight of newly emerged bees, diminishes worker life span, reduces hypopharyngeal glands, and reduces protein and carbohydrate levels in hemolymph (De Jong et al. 1984, Schneider and Drescher 1987) in addition to vectoring several pathogenic microorganisms of honey bees (Ball 1985, Chen et al. 2004, de Miranda and Fries 2008). The impact of varroa mites on colonies varies with location, climate, and the strain of bees (De Jong et al. 1984, Anderson 1994). Varroa mites also evolved in conjunction with a tropically adapted honey bee, *Apis cerana* F., prior to transferring to *A. mellifera* (Anderson and Trueman 2000). Little is known about what ranges or durations of CO₂ concentrations might be typically found in *A. cerana*, but owing to the warmer tropical climates these bees inhabit, the concentrations are likely to be lower than those found in bee colonies in the more temperate climates where *A. mellifera* typically occurs. If the phoretic stage of varroa mites is susceptible to the higher levels of CO₂ found in wintering clusters of *A. mellifera*, it may provide opportunities for controlling the mite through manipulation of CO₂ levels or enhancing the effectiveness of other defensive responses such as grooming.

CO₂ fumigation has been suggested as a nonpolluting and an environmentally friendly tactic for control of grain pests because it leaves no residue in food products (Aliniaze 1971, White and Jayas 1991). The potential to control varroa mites with CO₂ through manipulation of ventilation has been investigated in caged-bee studies. Kozak and Currie (2011) showed increased CO₂ levels (1.1–2.5% in the air surrounding the cluster) enhance varroa mite mortality in small clusters of caged bees at 10°C and 25°C. However, nothing is known about the influence natural bee-generated CO₂ would have on mite mortality at the levels of gas that would be found in wintering bee clusters under the colder temperatures experienced during indoor wintering.

Although CO₂ fumigation has been suggested for control of some honey bee pests, for example, the greater wax moth, *Galleria mellonella* L., in beekeeping equipment (Shimanuki and Knox 1997) and varroa mite in colonies (Kozak and Currie 2011), it can have negative effects on bees (Ebadi et al. 1980, Mardan and Rinderer 1980) and could cause unintentional increases of populations of other pathogens. Czekonska (2007) found that *Nosema apis* Zander spores rapidly proliferate when caged bees are inoculated with CO₂-treated *Nosema* spores which, in turn, dramatically decreases survival rates of *Nosema*-inoculated bees. This could pose potential problems for winter fumigation with CO₂ for varroa mite if it also facilitates *Nosema*

development. There is currently no information about how high CO₂ might affect microsporidian diseases in overwintering honey bee colonies. Other honey bee behavior or physiology affected by changes in CO₂ concentration include the development of hypopharyngeal glands, fat body, wax glands, ovaries, corpora allata, brain chemistry, juvenile hormone titer, pollen collection and consumption, hoarding behavior, polyethism, lifespan, and egg production in worker bees (Ribbands 1950, Mardan and Rinderer 1980, Buhler et al. 1983, Koywiwattrakul et al. 2005, Czekonska 2009). Thus, it could be disadvantageous to implement CO₂-based fumigation if the CO₂ negatively influences colony performance.

Although much is known about the critical roles of ventilation and thermoregulation in wintering honey bees, no information is available on how variation in respiratory gases could affect ectoparasitic mites in honey bee stocks that vary with regard to wintering performance. The goals of this study were to manipulate ventilation rate to increase bee-generated CO₂ levels in the bee cluster, and to assess interactions between restricted ventilation (which elevates CO₂) and stock of bees on varroa mite mortality rates, worker bees mortality rates, and changes in mean abundance of varroa mites and *Nosema* during indoor wintering of honey bee colonies.

Materials and Methods

Two experiments were carried out at the University of Manitoba, Winnipeg, Manitoba, Canada (49° 54' N, 97° 14' W). Experiment 1 was performed in summer 2008 and experiment 2 in winter 2011. In each of the two experiments, colonies were headed by either queens from the Manitoba Queen Breeders Association that had been characterized as "selected" stock or from colonies headed by either New Zealand queens (Arataki Honey, Havelock North, New Zealand) or Hawaiian queens (Kona Queens, Captain Cook, HI) classified as "unselected" stock. Selection was hypothesized to select honey bee colonies for mechanisms associated with mite tolerance or mite resistance through a cooperative breeding program initiated in 2002 (Bahreini 2014). The objectives of experiment 1 were to characterize mite and bee mortality, and to measure CO₂ and O₂ levels in the bee cluster in selected and unselected stocks maintained under a simulated winter condition. The objectives of experiment 2 were to assess the influence of restricting ventilation (from the standard rate of 14.4 liter/min per hive to 0.24 liter/min per hive) on the levels of CO₂ and O₂, varroa mite and *Nosema* spore mean abundance and mortality of mites and bees in stock strongly selected for wintering performance and unselected stocks during indoor wintering.

Experiment 1. In summer 2008, small hives (5-frame Langstroth boxes containing Hoffman frames) (Shimanuki et al. 2007) were established from colonies at the University of Manitoba that contained mixed-age worker bees from either selected stock ($n = 6$) or unselected stock ($n = 6$). Each hive was queenless but contained a plastic release device containing synthetic

queen mandibular pheromone (Pseudo Queen, Con-tech Enterprises Inc., Victoria, BC, Canada) as a queen substitute. All colonies were inoculated with 200 live varroa mites. Mites for this experiment were collected from a separate set of highly infested colonies using a modification of the CO₂ method (Ariana et al. 2002). In this method, bees infested with varroa mites were placed in a hive with a screen bottom and put in a plastic (3.5 L TakeAlongs, Rubbermaid, Mississauga, ON, Canada) container. The container was then agitated at 400 rpm for 10 min on a Labline (Fisher, Ottawa, ON, Canada) orbital shaker table while being exposed to CO₂ (5 liter/min) (for more details, see Currie and Tahmasbi 2008). Mites falling onto the bottom of the container were collected with the tip of a soft paint brush, placed in Petri dishes lined with a moist paper towel. The mites were then introduced into the hives by a fine-tipped paint brush through a wire mesh screen (eight squares per inch) that covered the top of the hive and prevented bees from flying out. The mites were placed directly on the bees. The hives from each of the two stocks (selected and unselected) were randomly placed in an indoor, temperature-controlled chamber (208 by 208 by 273 cm³ = 11.81 m³) and held in constant darkness at 5°C for 6 d. CO₂ and O₂ concentrations (%) were measured twice per day, morning and afternoon, in room air and inside the cluster of bees using a headspace gas analyzer (Model 900141, Bridge Analyzer Inc., Alameda, CA). The temperature (°C) and absolute humidity (g/m³) inside the room were monitored every 2 h using HOBO C-8 (Onset Computer Corporation, Bourne, MA) data loggers. Each hive had a completely open bottom entrance (19 by 2.5 cm). To measure CO₂ and O₂ concentrations, polyethylene air sample tubes (outer diameter: 6.35 mm; inner diameter: 4.32 mm, Watts company, North Andover, MA) were fitted with one end positioned at either the center of the bee cluster or in the room space and with the other end of the tubes on the outside the room so gases could be sampled without entering the room, as described by Underwood and Currie (2004) for sampling of formic acid. Tubes were sealed with a rubber sleeve stopper (Size No. 11-1/2, 10.70 mm length, plug diameter 5.16–6.73 mm, Red, Fisher Limited, Ottawa, ON, Canada). Air samples were collected after removing and discarding the entire volume of “dead air” (the volume of space in the tube was determined by first filling each tube with water) from the tubes using a disposable 50-ml syringe (Underwood and Currie 2004) before using the headspace gas analyzer to quantify CO₂ and O₂.

Daily varroa mite and worker bee mortality rates were assessed (Martin 1998) based on dead bees and dead mites collected from bottom board of hives and dead bee traps. A piece of white poster board (19 by 61 cm) covered with wax paper was placed on the bottom board of each hive so varroa mites and worker bees that fell from the bee cluster could be collected daily. Additional dead bees and dead mites that exited the hive were collected in a dead bee trap affixed to the hive entrance; the trap was an aluminum, three-sided tray that was 29 by 19 by 8 cm³. Bees and mites

that fell onto the bottom board and dead bee trap of hives were also subjected to alcohol wash (Gatien and Currie 2003) and those bees and mites were included when calculating mortality rates. At the end of the experiment, the bee population was assessed visually from both top and bottom and the numbers of frame seams completely covered with bees was counted (each frame seam = ~2,430 worker bees; Burgett and Burikam 1985, Underwood and Currie 2005). Mites remaining in the colony were determined by multiplying the estimated total number of bees in the colony by the percentage mite infestation as determined by alcohol wash.

Experiment 2. To tightly control ventilation for this experiment and simultaneously monitor respiratory gases and bee activity a special Plexiglas chamber was constructed. The Plexiglas chamber (65 by 130 by 130 cm³ = 1.0985 m³, Acrylic sheet with 6.0 mm thickness, Evonik Industrial, Parsippany, NJ) was designed and constructed to hold a volume of air approximately equal to the space required for overwintering hives (Gruszka 1998). The air flow to the chamber was provided by a heavy duty air compressor (Speedaire, Model 4B233E, 135 PSI, 30 gallons, Grainer Inc., IL) supplied with an air compressor filter (HDA70400AV, Husky, Cuajimalpa, Mexico, Mexico). Inlet air flow to the chamber was regulated by a flow meter (CF-4000, 50 PSI, National Torch Tip Co., Pittsburgh, PA). The flow meter was connected to the chamber via a clear vinyl tube (outer diameter: 9.52 mm, inner diameter: 6.35 mm, Watts company, North Andover, MA). The Plexiglas chamber was located in a small treatment room (170 by 270 by 300 cm³) of an overwintering building, as described in Underwood and Currie (2004; Fig. 1). Air flowed throughout the small room via a ventilation tube at a rate of 14.4 liter/min per hive (Underwood and Currie 2004). Temperature inside the treatment room was controlled by a baseboard heater and a thermostat maintained temperature at 5–7°C.

Three or four hives were placed in the ventilation chamber during experiments. The effect of hive position in the chamber (two hives in top row vs. two hives in bottom row) on the amount of CO₂ in the bee cluster was tested in a preexperiment assay. In the preexperiment, small hives ($n=4$) from unselected stock were randomly placed at each of four positions in the chamber (hives were placed in two rows and two columns) and maintained for 7 d under a low ventilation rate (0.24 liter/min per hive; as determined by preliminary experiments) in a temperature-controlled chamber at 5°C, as described in experiment 1. Then, the positions of hives were switched for the next 7 d. These queenless hives (supplied with pseudo queen as a queen substitute) were fitted with polyethylene air sample tubes in the center of the cluster as described in experiment 1. In the preexperiment, only the percentage of the concentration of CO₂ and O₂ in the bee cluster were measured; mite and bee mortality were not assessed. No differences by position were found (for details, see Bahreini 2014).

Experiment 2 compared selected and unselected stocks under either standard or restricted ventilation

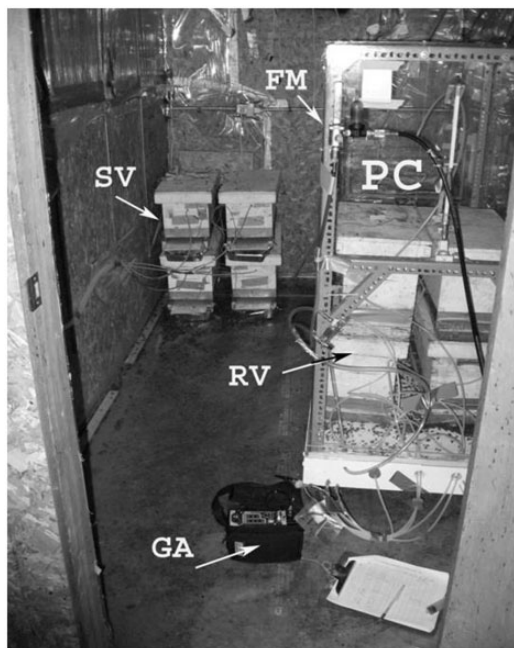


Fig. 1. Setup of experiment 2. Hives from selected stock and unselected stock were arranged either in a Plexiglas ventilation chamber (PC) for application of restricted ventilation (RV) or in a wintering room for standard ventilation (SV). CO₂ and O₂ concentrations (%) in the bee cluster and spaces of chamber and room were measured through vinyl tubes via a gas analyzer (GA). During the restricted ventilation (treatment period), inlet air flow into the chamber was regulated using a flow meter (FM) set on 0.24 liter/min per hive. The standard ventilation rate was set to 14.4 liter/min per hive in the wintering room and in the Plexiglas chamber.

using hives with a five-frame capacity. The experiment was repeated using four colonies from selected stock and four colonies from unselected stock in each of the first two trials (trial 1: 6 February–3 March 2011; trial 2: 10 March–4 April 2011). In the third trial (9 April–4 May, 2011) there were only two selected and four unselected colonies ($n=10$ selected colonies and 12 unselected colonies). Different colonies were used for each trial. Two selected and two unselected colonies were randomly positioned in either a small room within the overwintering building or were arranged in the Plexiglas chamber at 5°C. Once the hives were placed in the chamber or in the room, polyethylene air sampling tubes (6.35–4.32 mm) were fitted to the center of the bee cluster in each hive and inside the spaces of the Plexiglas ventilation chamber and wintering building room to allow for gas sampling as described earlier. The colonies were maintained in the room (standard ventilation) or in the chamber (restricted ventilation) for 26 d (6 d as stabilizing period, 2 d as pretreatment period, 16 d as treatment period, and 2 d as posttreatment period). Inlet air flow to the chamber was controlled with a flow meter set on 0.24 liter/min per hive during 16 d of treatment. However, hives in the

chamber were exposed to standard ventilation (14.4 liter/min per hive) during stabilizing, pretreatment, and posttreatment periods. Colonies under standard ventilation were sampled in the same way as for restricted ventilation, but the rate of air flow was consistently 14.4 liter/min per hive over all periods. CO₂ and O₂ concentrations were measured two times per day, morning and afternoon, as described in experiment 1. Daily mite and bee mortality rates were quantified, as described in experiment 1. The main door of the chamber during the experiment was sealed to maintain gas balance inside the chamber except for a short period when dead bees and dead mites were collected from either the bottom board of hives or dead bee traps. In the Plexiglas chamber, this was done through a small gate (18 by 48 cm) on the main door of the chamber. In this experiment, the mean abundance of varroa mites and *Nosema* spores (arithmetic mean of the number of varroa mite per bee or the number of *Nosema* spores per bee, respectively; Rozsa et al. 2000) were also assessed. Mean abundance of varroa mite at the end of the stabilization period (days 7 and 8) and at the end of the ventilation manipulation (days 25 and 26) was determined by dividing the total number of mites present at the time by the total number of bees. Mean abundance of *Nosema* spores was assessed in samples of 100 bees collected from each colony at the beginning and end of each trial. Samples were processed according to the method of Cantwell (1970) using maceration and homogenization of the bees in water (1 ml of distilled water per bee). The number of *Nosema* spores was counted on a hemocytometer (Bright-Line, American Optical, Buffalo, NY). Then, the number of spores per bee was calculated. To estimate the total number of mites remaining in the colony, hives were treated with the acaricide fluralanate (Apistan, Wellmark International, Guelph, ON, Canada) for 4 wk to collect remaining mites on the bees after each trial was over. Bees were sampled after the end of the Apistan treatment by alcohol wash to assess efficacy and confirm that acaricide resistance was not influencing the result. Mite infestation levels at the end of the acaricide treatment averaged 0.033 ± 0.013 mites per 100 bees. Then, the initial number of mites in each colony at the beginning of the experiment was calculated by the summing all mites that fell during the trial and during and after acaricide treatment. The initial number of worker bees in the hives was also estimated by summing the number of bees that fell during each trial and adding this number to the estimated number of live bees remaining after the trial. Bee population size at the end of each trial was visually scored as described in experiment 1.

In the first experiment, the daily mortality rates for worker bee and varroa mites and concentrations of CO₂ and O₂ were analyzed by a repeated measure analysis of variance (ANOVA) using an autoregressive covariance structure with stock of bees as main effect (PROC MIXED, SAS Institute Inc. 2011, Cary, NC). In the preexperiment, the effects of hive position in the chamber on CO₂ and O₂ levels were analyzed by a repeated measures ANOVA using an autoregressive

covariance structure with position of hive as main effect (PROC MIXED, SAS Institute Inc. 2011). For experiment 2, the percentage change in mean abundance of varroa mite from the beginning of treatment (on days 7 and 8 of the pretreatment period) to the end of treatment (on days 25 and 26 of the posttreatment period) was calculated and analyzed by a two-way ANOVA with ventilation and stock of bees as main factors (PROC MIXED, SAS Institute Inc. 2011). The percentage change in log-transformed mean abundance of *Nosema* spores over the entire experiment was analyzed by the same method. For changes in mean abundance of varroa mites and *Nosema* spores, "trial" (the time of winter when experiment 2 was replicated) and interactions between trial and main effect factors were removed from model because they were not significant. A before and after control impact (BACI) design was used to assess the effects of the ventilation treatment and stock of bees on bee and mite mortality rates, CO₂ and O₂ concentrations and *Nosema* spores, where colonies within each chamber were treated as replicates and the interaction between main effects and period was used as criteria to declare significant treatment effects (Stewart-Oaten et al. 1986, Smith 2002, Underwood and Currie 2005). Ventilation setting, stock of bees, and trial were treated as main effects, and period and day within periods were treated as repeated measures with colony as the subject. Nonsignificant higher order interactions were deleted from the model. An autoregressive covariance structure was used for the analysis. Where significant interactions were found an analysis of simple effects was used to test for differences within trials and periods (Slice procedure, PROC MIXED, SAS Institute Inc. 2011) followed by Bonferroni adjusted contrasts (to examine difference within trials when required). Prior to analyses, concentrations of CO₂ and O₂ were square root transformed, and mean abundance of varroa mites and the rates of mite and bee mortality for each day were arcsine-transformed. All data are presented as untransformed means. The correlations among CO₂ and O₂ concentrations, and daily mortality of bees and mites were analyzed using partial correlation controlling for all other variables (PROC CORR, SAS Institute Inc. 2011).

Results

Experiment 1. In the experiment simulating winter conditions, the concentration of gases measured in the core of the bee cluster was consistent for both stocks of bees throughout the experiment. The average CO₂ concentration in the bee cluster was similar in selected bees (average: $1.42 \pm 0.14\%$; maximum: 3.90%) and unselected bees (average: $1.39 \pm 0.14\%$; maximum: 2.62%; $F = 0.04$; $df = 1, 10$; $P = 0.840$) and did not vary over time (stock of bees $\times$ time: $F = 1.24$; $df = 5, 50$; $P = 0.303$; Fig. 2A). The average O₂ concentration was also similar in the cluster of selected bees ($19.50 \pm 0.12\%$) and unselected bees ($19.54 \pm 0.12\%$; $F = 0.07$; $df = 1, 10$; $P = 0.303$) and did not vary with time (stock of bees $\times$ time: $F = 1.35$; $df = 5, 50$; $P = 0.258$; Fig. 2B). CO₂ was negatively correlated with

O₂ ($r = -0.96$; $P < 0.0001$). Under these ventilation conditions, neither daily mite mortality nor daily bee mortality were correlated with CO₂ concentration ($P > 0.05$).

Daily mite mortality was greater in selected bees (0.029 ± 0.004) than in unselected bees (0.015 ± 0.004 ; $F = 20.06$; $df = 1, 10$; $P = 0.0012$), but did not vary with time (stock of bees $\times$ time: $F = 2.28$; $df = 5, 50$; $P = 0.060$). Daily bee mortality was significantly lower in selected stock (0.004 ± 0.001) than unselected stock (0.009 ± 0.001 ; $F = 9.85$; $df = 1, 10$; $P = 0.011$). The interaction between bee stock and time was not significant, indicating that treatment differences in bee mortality were consistent during the experimental period ($F = 0.07$; $df = 5, 50$; $P = 0.620$).

Experiment 2. Overall, concentration of CO₂ in the bee cluster differed based on ventilation settings (restricted: $2.85 \pm 0.23\%$ vs. standard: $1.43 \pm 0.23\%$; $F = 24.01$; $df = 1, 10$; $P = 0.0006$). However, stock of bees (selected: $1.99 \pm 0.25\%$ vs. unselected: $2.29 \pm 0.22\%$) did not affect the concentration of CO₂ in the bee cluster ($F = 0.76$; $df = 1, 10$; $P = 0.403$) during the 26 d of the experiment. There was a significant interaction between ventilation and period for CO₂ concentration ($F = 4.79$; $df = 3, 42$; $P = 0.0058$). The three-way interaction ventilation $\times$ trial $\times$ period was not significant ($F = 1.77$; $df = 12, 42$; $P = 0.086$). The mean CO₂ concentration in the bee cluster during 16 d of treatment period was significantly higher under restricted ventilation ($3.82 \pm 0.31\%$) than under standard ventilation ($1.29 \pm 0.31\%$; $F = 41.44$; $df = 1, 10$; $P < 0.0001$), but this variable did not differ over days within ventilation period (ventilation setting $\times$ time: $F = 0.45$; $df = 15, 270$; $P = 0.963$). CO₂ concentration in both stocks of bees (selected: $2.43 \pm 0.33\%$, unselected: $2.67 \pm 0.28\%$) was similar through all treatment periods ($F = 0.27$; $df = 1, 10$; $P = 0.616$; Fig. 3). The maximum concentration of CO₂ in the core of the bee cluster was 8.44% and 7.76% in restricted and standard ventilation, respectively.

During the 26 d of experiment, the concentration of O₂ in the bee cluster also differed within ventilation settings (restricted: $17.76 \pm 0.18\%$ vs. standard: $19.03 \pm 0.18\%$; $F = 29.35$; $df = 1, 10$; $P = 0.0003$), and stock of bees did not affect the concentration of O₂ in the bee cluster (selected: $18.45 \pm 0.19\%$ vs. unselected: $18.34 \pm 0.16\%$) ($F = 0.21$; $df = 1, 10$; $P = 0.66$). However, the three-way interaction ventilation $\times$ trial $\times$ period was significant ($F = 6.28$; $df = 12, 42$; $P < 0.0001$). Partitioning by trial and period showed significant differences in O₂ concentration between ventilation treatments (Table 1; Fig. 5A). Average O₂ concentration measured in the bee cluster during the 16-d treatment period was significantly lower under restricted ventilation ($17.00 \pm 0.21\%$) than under standard ventilation ($19.16 \pm 0.21\%$; $F = 51.08$; $df = 1, 10$; $P < 0.0001$), but this variable was similar between selected bees ($18.10 \pm 0.22\%$) and unselected bees ($18.07 \pm 0.19\%$; $F = 0.01$; $df = 1, 10$; $P = 0.916$). The minimum concentrations of O₂ in the core of bee cluster were 13.40 and 15.48% in restricted and standard ventilation, respectively.

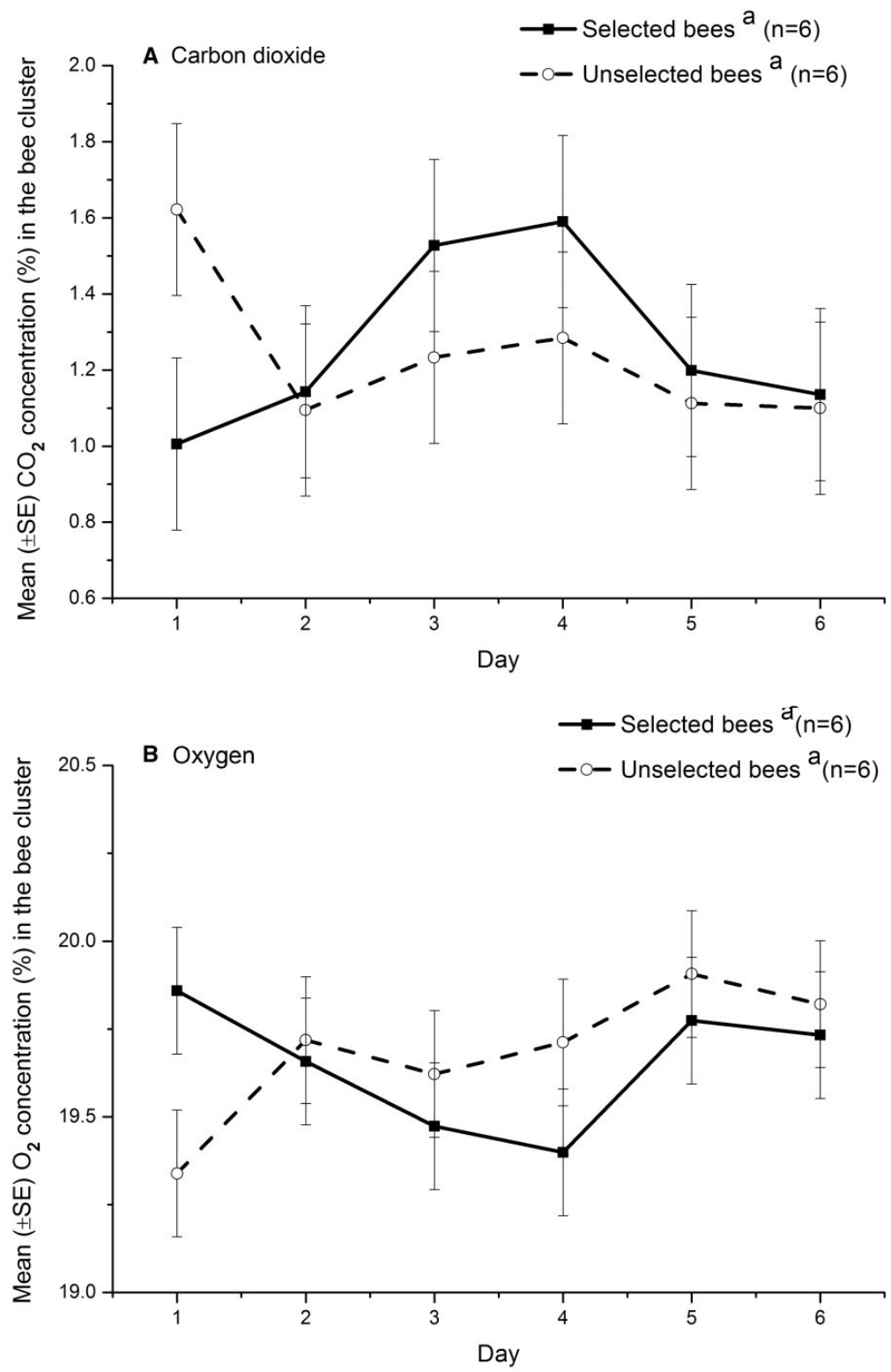


Fig. 2. Mean ($\pm$ SE) CO₂ (A) and O₂ (B) concentrations (%) in the bee cluster within selected stock and unselected stock in experiment 1. Hives were located in a temperature-controlled environmental chamber and held in constant darkness at 5°C. Each point represents an average of two measurements (morning and afternoon). Means followed by the same letter between stocks of bees are not significantly different.

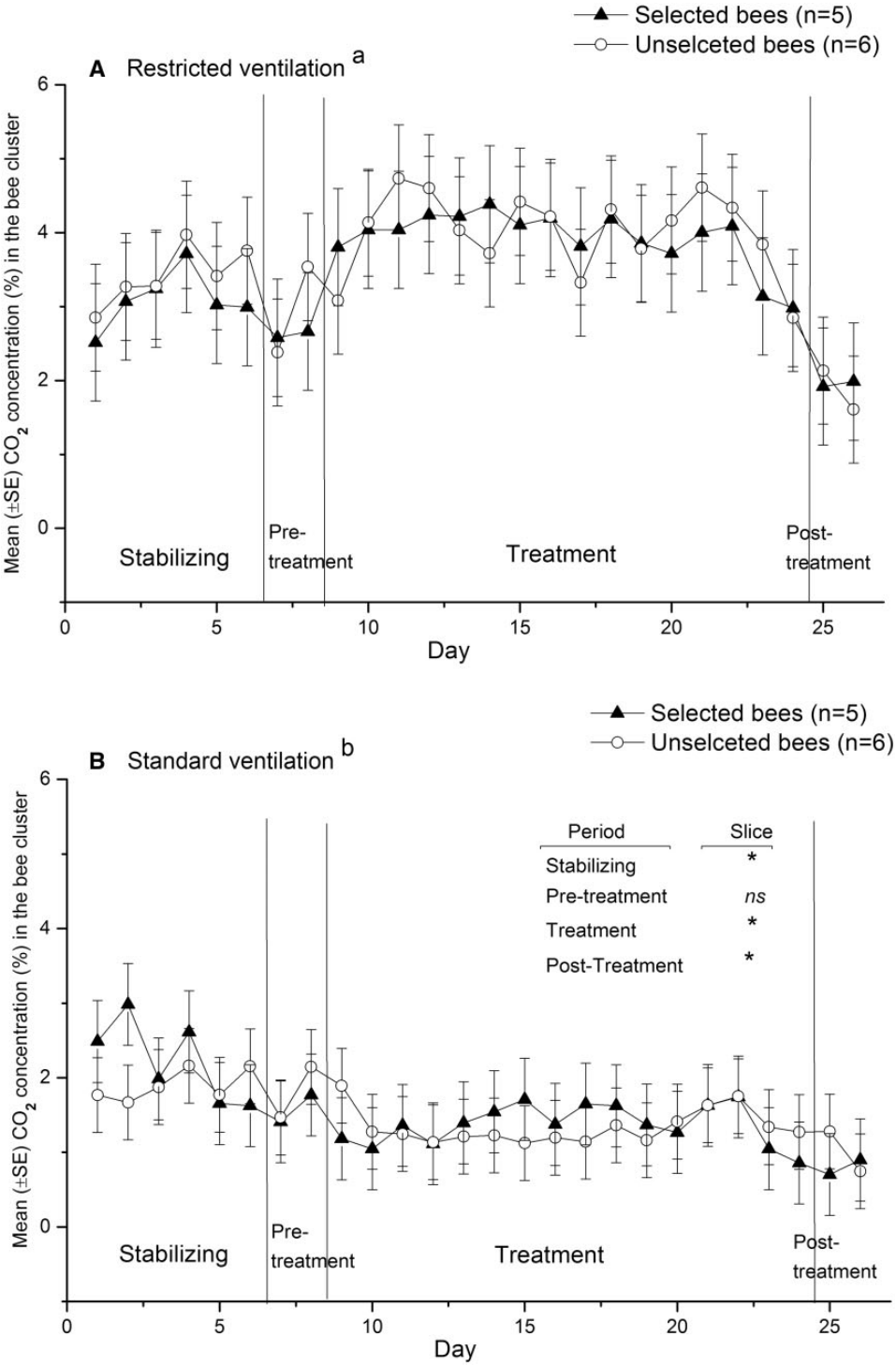


Fig. 3. Mean ($\pm$ SE) CO₂ concentration (%) in the core of the cluster of either selected bees or unselected bees in restricted ventilation ($n=11$) (A) and in standard ventilation ($n=11$) (B) treatment groups over the 26d of experiment 2. Restricted ventilation involved 6 d as a stabilizing period (14.4 liter/min per hive), 2 d as a pretreatment period (14.4 liter/min per hive), 16 d as a treatment period (0.24 liter/min per hive), and 2 d as a posttreatment period (14.4 liter/min per hive). The rate of air flow in standard ventilation was consistently 14.4 liter/min per hive over all periods. Each point represents pooled values for two measurements (morning and afternoon) and averages for all three trials. Asterisks (on legend) indicate significant differences between ventilations within each period ($P < 0.05$, slice) and *ns* represents a nonsignificant difference between ventilations within each period. Means followed by the same letter between ventilation settings are not significantly different.

Table 1. Partitioning results for the significant ventilation $\times$ trial $\times$ period interaction by trial and period for O₂ concentration in the bee cluster and daily varroa mite and bee mortality rates in experiment 2 (“–” under period indicates slice only by trial)

Variables	Trial	Period	F	df	P
Oxygen concentration	1	Treatment	47.46	1, 42	<0.0001
	1	Posttreatment	5.93	1, 42	0.019
	2	Stabilizing	19.72	1, 42	<0.0001
	2	Treatment	47.28	1, 42	<0.0001
Daily mite mortality	3	Treatment	8.02	1, 42	0.007
	2	Treatment	5.66	1, 42	0.022
	3	Stabilizing	4.53	1, 42	0.039
	3	Treatment	6.32	1, 42	0.016
Daily bee mortality	3	Posttreatment	17.64	1, 42	0.0001
	1	Stabilizing	8.10	1, 42	0.007
	1	–	3.99	7, 42	0.002
	2	–	2.25	7, 42	0.049

Ventilation setting and stock of bees both affected overall mite mortality rate during the experiment. Over all periods and trials, daily varroa mite mortality was greater in the restricted ventilation colonies (0.031 ± 0.003) than in the standard ventilation colonies (0.013 ± 0.003 ; $F = 14.63$; $df = 1, 10$; $P = 0.0033$), and was greater in selected bees (0.027 ± 0.003) than in unselected bees (0.016 ± 0.003 ; $F = 5.03$; $df = 1, 10$; $P = 0.049$; Fig. 4). However, the three-way interaction ventilation $\times$ trial $\times$ period was significant ($F = 2.35$; $df = 12, 42$; $P = 0.021$). Partitioning by trial showed significant treatment effects as indicated by changes in ventilation with period within the latter two trials (late winter) but not the first trial (Table 1; Fig. 5B). Overall, ventilation treatment had a significant effect on the change in mean mite abundance over the 16 d of restricted ventilation ($F = 5.27$; $df = 1, 18$; $P = 0.03$) relative to standard ventilation indicating an overall significant treatment effect. Mean abundance of mites decreased $37 \pm 4.2\%$ under restricted ventilation relative to only $23 \pm 4.2\%$ under standard ventilation. However, honey bee stock did not affect the change in mean abundance over the same time span ($F = 2.78$; $df = 1, 18$; $P = 0.113$).

Overall, ventilation setting and stock of bees did not affect bee mortality rate. Daily bee mortality did not differ between ventilation treatments (restricted: 0.0020 ± 0.0002 vs. standard: 0.0016 ± 0.0002 ; $F = 3.86$; $df = 1, 10$; $P = 0.078$), between stocks of bees (selected: 0.0019 ± 0.0002 vs. unselected: 0.0017 ± 0.0002 ; $F = 0.98$; $df = 1, 10$; $P = 0.35$) or between trials (Trial 1: 0.0022 ± 0.0002 ; Trial 2: 0.0017 ± 0.0002 ; Trial 3: 0.0014 ± 0.0002 ; ventilation treatment $\times$ trial: $F = 0.96$; $df = 2, 10$; $P = 0.42$). The interaction among ventilation, trial, and period was also significant ($F = 3.47$; $df = 12, 42$; $P = 0.0013$). Partitioning only by trial showed a significant interaction between ventilation and period within the first and second trials, but partitioning by trial and period showed that bee mortality differed between the two ventilations only for the stabilizing period in the first trial and differences between treatments within all other trials were not significant (Table 1; Fig. 5C).

The percentage change in mean abundance of *Nosema* spores over the duration of the experiment did not differ with ventilation treatment ($F = 0.02$; $df = 1, 18$; $P = 0.89$) or stock of bees ($F = 2.12$; $df = 1, 18$; $P = 0.16$) and there was no interaction between period and ventilation through the experiment ($F = 0.01$; $df = 1, 18$; $P = 0.92$). However, the interaction among time, stock of bees and trial was significant ($F = 4.99$; $df = 2, 16$; $P = 0.027$). Partitioning by trial showed a significant interaction between time and stock of bees only within the third trial ($F = 9.38$; $df = 3, 16$; $P = 0.0008$; Fig. 6).

Measures of CO₂ concentration, O₂ concentration, temperature, and absolute humidity in the bee cluster and in the spaces of Plexiglas chamber and treatment room are summarized in Supp Table 1 [online only].

Discussion

Restricting ventilation to honey bee colonies during winter resulted in greater CO₂ levels and lower O₂ levels within the cluster and was associated with higher CO₂ levels, lower O₂ levels and higher humidity in the air space surrounding the colony relative to colonies held under standard ventilation. When honey bee colonies were exposed to restricted ventilation during winter, the overall rate of varroa mite mortality (but not mite abundance) increased relative to colonies held under standard ventilation but overall bee mortality rates were not affected. Although greater mite mortality rates were found in bees selected for resistance to varroa mites relative to unselected bees, there was no interaction between the ventilation treatment and bee stock.

A number of factors correlated with the restriction of ventilation could be responsible for the increased mite mortality rates that were observed. Kozak and Currie (2011) showed increases in humidity and CO₂ occur when caged bees are held under restricted ventilation and result in higher varroa mite mortality when ventilation is manipulated at high temperatures (25°C). However, in contrast to our results, they found that when caged bees are subjected to restricted ventilation at low temperature (10°C) greater mite mortality occurs at the highest ventilation rate with the lowest CO₂ and humidity. The greater mite mortality at high ventilation rates in that experiment was attributed to a higher probability of mite desiccation associated with low humidity in the bioassay chambers, which in turn, could affect mite survival and the ability of mites to locate their hosts in the bioassay chamber (Kozak and Currie 2011). Our experiment was conducted at slightly lower temperatures (~6–7°C), with much larger colonies and conducted within a wintering building rather than in a small-scale incubator trial. We found the highest mite mortality in the restricted ventilation treatment, which had the highest humidity in room air (absolute humidity was 12 g/m³ under restricted ventilation and only 7 g/m³ under standard ventilation). The high humidity in air surrounding the colonies likely resulted from moisture that accumulated in the chamber air space due to respiration of bees (Gruszka 1998).

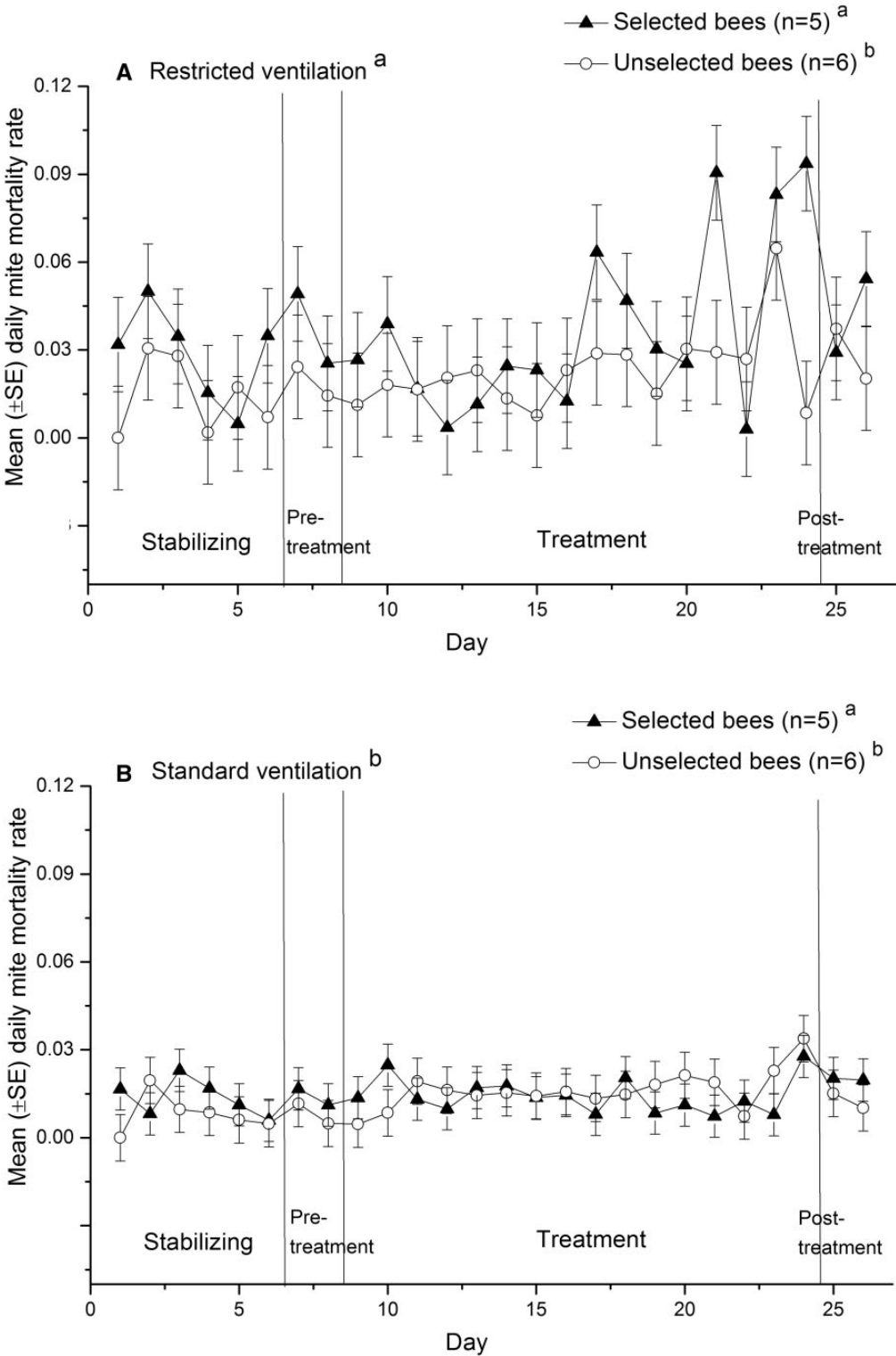


Fig. 4. Mean ($\pm$ SE) daily mortality rate of varroa mite in selected bees and unselected bees in the restricted ventilation (A) ($n=11$) and standard ventilation (B) ($n=11$) within the 26 d of experiment 2. Restricted ventilation involved 6 d as a stabilizing period (14.4 liter/min per hive), 2 d as a pretreatment period (14.4 liter/min per hive), 16 d as a treatment period (0.24 liter/min per hive), and 2 d as a posttreatment period (14.4 liter/min per hive). The rate of air flow in standard ventilation was consistently 14.4 liter/min per hive over all periods. Means followed by the same letter between ventilation settings or between stocks of bees are not significantly different.

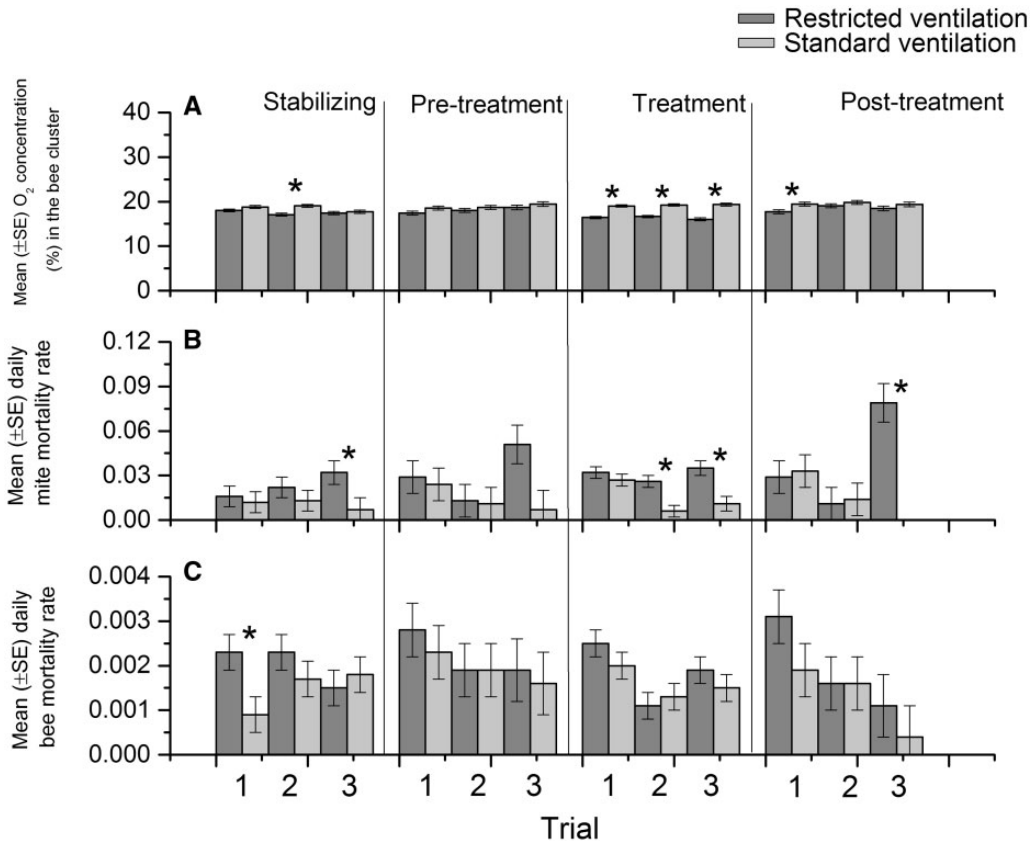


Fig. 5. Mean ($\pm$ SE) concentrations (%) of O₂ in the bee cluster (A), and daily varroa mite (B) and bee (C) mortality rates in wintering colonies in experiment 2, which were located in either a Plexiglas ventilation-controlled chamber (restricted ventilation, $n = 11$) or in a standard wintering room (standard ventilation, $n = 11$). Restricted ventilation involved 6 d as a stabilizing period (14.4 liter/min per hive), 2 d as a pretreatment period (14.4 liter/min per hive), 16 d as a treatment period (0.24 liter/min per hive), and 2 d as a posttreatment period (14.4 liter/min per hive). The rate of air flow in standard ventilation was consistently 14.4 liter/min per hive over all periods. Asterisks indicate significant difference between restricted ventilation and standard ventilation in each trial ($P < 0.05$, slice).

Although we did not measure humidity in the core of the bee cluster, it is likely that clusters also had high humidity as CO₂ is positively correlated with humidity in wintering bees (Van Nerum and Buelens 1997). Because high levels of humidity under constant ventilation do not increase mite mortality rates and because CO₂ at very high concentrations can be used to remove varroa mite from anesthetized bees (Currie and Tahmasbi 2008) it is likely that high levels of CO₂ (or low levels of O₂) rather than high humidity were primarily responsible for the increased rates of mite mortality. However, humidity cannot be excluded as a possible contributor to the increased mite mortality that was observed.

Hypercapnic (high CO₂) and hypoxic (low O₂) conditions affect insect longevity (Calderon and Navarro 1979; reviewed in Nicolas and Sillans 1989) and influence gas exchange and ventilation mechanisms in insects (Anderson and Ultsch 1987), and thus could be expected to have effects on either varroa mites or wintering honey bees. The mode of action for effects of CO₂ on varroa mites is not clearly understood.

However, CO₂ is known to influence feeding and locomotion activities in other arthropods by blocking signals at the neuromuscular junctions (Badre et al. 2005). This level of interference with the nervous system of varroa mites could be enough to result in greater numbers of mites dying, falling from the cluster or being more susceptible to grooming behavior of bees.

There are several factors that could affect colony CO₂ levels. Low ambient temperature increases endothermic activity in honey bee clusters, increasing metabolic rates in honey bees and O₂ consumption by colonies (Free and Spencer-Booth 1959, Nagy and Stallone 1976, Stabentheiner et al. 2003). This is followed by higher CO₂ concentrations in bee clusters especially when temperatures are below 10°C (Free and Spencer-Booth 1959, Nagy and Stallone 1976). However, CO₂ is regulated. Honey bees possess antennal sensory organs to distinguish different concentrations of CO₂ (Lacher 1967) and have the capacity to respond to differences in CO₂ concentration of less than 0.5% (Walla 1948). Fanning behavior is used to circulate fresh air into the colony in response to high CO₂

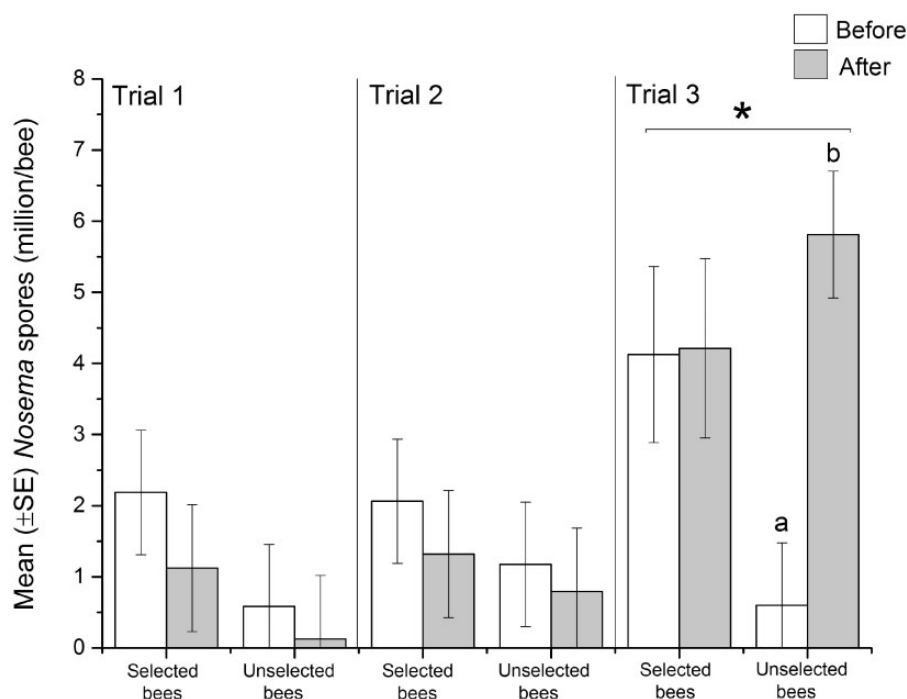


Fig. 6. Mean ($\pm$ SE) abundance of *Nosema* spores (million per bee) in stocks of selected bees and unselected bees at the start and end of each trial that each lasted for a period of 26 d. Trials 1 to 3 began on 6 February, 10 March, and 9 April, respectively. Each column represents pooled values for two ventilation treatments (restricted and standard). Horizontal bar with asterisk indicates significant interaction between stock and time within trials. Means followed by the same letter between within trials and bee stocks are not significantly different.

concentrations in the colony (Seeley 1974) and is typically initiated when CO_2 concentrations exceed 3.5% (Hazelhoff 1954). Colony size may influence CO_2 regulation with larger colonies being better able to regulate CO_2 than very small colonies (Seeley 1974, Van Nerum and Buelens 1997). Video analysis of colonies in preliminary experiments we conducted confirmed fanning behavior was induced as expected when high levels of CO_2 occurred in the experimental hives in the ventilation chamber used in this study (personal observation, data not shown). The concentration of CO_2 in the bee clusters in this study were within the range of published values but somewhat lower than those often reported for wintered colonies. CO_2 concentration averaged only 1.4% when honey bee colonies were maintained under standard ventilation, but was much higher ($\sim 4\%$) under restricted ventilation. This difference is in agreement with the study by Kozak and Currie (2011), which showed that restricting ventilation rate in caged bees in a confined system increases CO_2 concentration surrounding the bee cluster. Our results extend this finding and show that similar effects can be achieved in colonies during indoor wintering at low temperatures. The highest level of CO_2 in our experiments (9.3%) was similar to the highest levels reported in the literature (9–9.9%) (Hallund 1956, Free and Simpson 1963). In experiment 2, the standard ventilation treatment utilized a ventilation system typical for an overwintering building (Gruszka 1998, Underwood

and Currie 2004). This system likely prevented the CO_2 from accumulating in the room space where CO_2 levels ($\sim 0.01\%$) were similar to that in commercial buildings (Currie, personal observation) but slightly lower than CO_2 levels in the peripheral environment of hives (0.03%) (Southwick 1987; reviewed in Nicolas and Sillans 1989). However, CO_2 concentration in the chamber space under restricted ventilation (average 1.4%) was numerically higher than under standard ventilation during the treatment period. Absolute humidity in the room space was numerically higher under restricted ventilation than under standard ventilation but well within the range expected in commercial facilities (30–70%; Fingler 1980, Gruszka 1998). Oxygen concentration was slightly lower in the air space surrounding hives when restricted ventilation was applied than under standard ventilation.

Experiment 2 was set up using colonies in mid-winter with an older population of mixed bees and would be expected to have had a high proportion of endothermic winter bees with higher resting metabolism (Southwick 1982). Since the three trials were conducted at different periods during the winter, some differences in age distribution of the workers would be expected from mid to late winter (Harris 2010). There were overall differences between bee mortality rates in the different trials. However, effects of ventilation treatment on bee mortality were not significant and did not vary with trial date with the exception of the stabilizing period in one

trial, suggesting differences in bee mortality did strongly influence treatment effects on mite mortality rates.

Although the two stocks differed in rates of mite mortality, the use of restricted ventilation did not affect the relative differences in the rates. Stocks that were selected for resistance to varroa mites had similar CO₂ and O₂ levels during both experiments. This suggests that manipulation of ventilation would be beneficial for stocks with both high and low mite removal capacities.

Other factors, which could influence the ventilation of colonies, relate to changes that influence colony state. For example, the presence or absence of a queen and brood could also alter the dynamics of temperature regulation in the winter bee cluster and cause changes in bee activity and fanning behavior (Andino and Hunt 2011; Bahreini and Currie 2015). Our experiments were conducted using queenless and broodless colonies fitted with pseudo-queen pheromone lures. These colonies should be similar in population structure to typical wintering colonies, which also have little brood at that time of year (Kozak 2008). Interactions with parasites and pathogens are also possible but unlikely to have been a factor (Schafer et al. 2011). We did not measure viruses in this study, and significant differences in the numbers of other parasites or pathogens like *Nosema* in the colonies were not found to be influenced by ventilation.

While hypercapnic conditions stimulate antennal receptors in worker bees, hypoxia does not (Southwick and Moritz 1987). Thus, O₂ is indirectly controlled in the bee cluster (Van Nerum and Buelens 1997). Van Nerum and Buelens (1997) found honey bees tend to maintain the core O₂ concentration at ~15% in winter bee clusters, even when ambient O₂ concentrations are higher. This may be of benefit in helping bees maintain lower metabolic rates in the winter cluster. Our measurements showed higher average O₂ concentration in bee clusters than those found by Van Nerum and Buelens (1997), under both standard ventilation (19%) and restricted ventilation (17%). As others have noted, we found O₂ concentration was inversely correlated with CO₂ concentration (Seeley 1974, Van Nerum and Buelens 1997). Even though O₂ concentration decreased to a minimum of 13% in our study, it did not have a significant effect on worker bee mortality rates. Part of the greater varroa mite mortality we observed might be associated with the low O₂ availability, or synergistic effects of hypoxia and hypercapnia that are known to contribute to increased mortality in grain pests (Calderon and Navarro 1979). However, reactions of varroa mite to hypoxia and hypercapnia have not been studied.

Mid-winter acaricide treatments could be used to prevent winter colony loss when bees are stored during indoor wintering (Underwood and Currie 2003), but also are of potential value in ensuring that varroa mite infestation is below the spring mite treatment thresholds of 2 mites per 100 bees recommended for in the prairie region of Canada to prevent losses in honey yield (Currie and Gatién 2006). Treatment of indoor-wintered colonies with natural bee-generated CO₂ has

the potential to treat many colonies simultaneously with minimal labor and equipment costs and with no acaricide costs. One risk associated with indoor-wintering treatments using formic acid fumigation is increased queen loss (Underwood and Currie 2008). Since queenless colonies supplemented with queen lures were used in our study, any queen loss associated with CO₂ treatment could not be assessed. This would have to be examined in a full-scale trial in wintering buildings before the treatment should be considered for use by beekeepers. The level of efficacy using restricted ventilation was lower than that found with indoor fumigation of wintering buildings with formic acid (Underwood and Currie 2004, 2007) but the low cost and low hazard to beekeepers compared with the use of formic acid may make this an attractive management option. To improve the reliability and efficacy of varroa mite control with restricted ventilation, further work on the combinations of CO₂ or O₂ concentration (dose) and exposure time necessary to bring mean abundance of mites below spring economic threshold is required.

CO₂ narcosis has many effects on the behavior and physiology of bees (Ribbands 1950, Ebadi et al. 1980, Mardan and Rinderer 1980, Buhler et al. 1983, Koyiwattrakul et al. 2005). It is possible that the use of restricted-ventilation with high levels of CO₂ for treating varroa mite could influence factors associated with the longevity of bees during winter such as titers of vitellogenin (Koyiwattrakul et al. 2005), or juvenile hormone in the hemolymph of worker bees (Buhler et al. 1983). High levels of CO₂ affect cold tolerance of *Drosophila melanogaster* Meigen (Nilson et al. 2006, Milton and Partridge 2008) but are not likely to affect honey bees within the range found in winter clusters (Van Nerum and Buelens 1997). Free and Spencer-Booth (1960) showed that CO₂ treatment does not influence worker bee chill-coma temperature. Similarly, Kozak and Currie (2011) did not find higher bee mortality when elevated CO₂ concentrations occurred in the short duration of their experiment. In this study, CO₂ levels were within the range of those found in "normal" wintering colonies of honey bee. Although the duration of exposure to those levels may have been higher, we found only a small difference in bee mortality rates between ventilation treatments and only during the stabilizing period in the first trial. This was not attributed to a treatment effect as it occurred prior to manipulation of ventilation and there were no differences in CO₂ or O₂ concentrations between ventilation treatments through this period. It is likely that handling manipulations associated with placing the colonies in the ventilation chamber contributed to the pretreatment difference in bee mortality rate. The bee mortality rate during the restricted ventilation period was similar within both treatments (0.00174 bees per bee per day) and close to the those found in previous indoor-wintering studies when overwintering full size colonies placed under a standard ventilation (Bahreini and Currie, personal observation).

In conclusion, this study showed that the use of restricted ventilation during indoor-wintering of honey

bees has potential to decrease varroa mite levels in colonies without increasing bee mortality. Mite-infested colonies treated with CO₂ for 16d had mean abundance reduced by 37%. This method may be sufficient to reduce mean abundance of mites <2% recommended for spring mite treatment in the prairie region of Canada to prevent losses in honey yield (Currie and Gatién 2006) if used in conjunction with other integrated pest management approaches. The technique is attractive because it would be possible for beekeepers to manipulate the ventilation rates without costly modifications to their ventilation systems in their overwintering buildings. This approach is a low-cost, environmentally friendly approach to control varroa mite with no danger of residue accumulation in bee products.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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