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THE MECHANISMS REGULATING CO_2 NARCOSIS IN *BOMBUS IMPATIENS*

A Thesis in

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by

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ABSTRACT

Carbon dioxide (CO_2) is commonly used to immobilize insects and to induce reproduction in bees. However, despite its wide use and potential off-target impacts, its underlying mechanisms are not fully understood. Here I used *Bombus impatiens* to examine whether CO_2 impacts are mediated by anoxia and whether these mechanisms differ between female castes or following mating.

I examined the behavior, physiology, and gene expression of workers, mated and virgin queens following exposure to anoxia (lack of oxygen), hypoxia (reduced oxygen levels), full and partial hypercapnia (elevated carbon dioxide levels), and control. Hypercapnia and anoxia caused immobilization, but only hypercapnia resulted in behavioral, physiological, and molecular impacts in bees. Recovery from hypercapnia resulted in increased abdominal contractions and took longer in queens. Additionally, hypercapnia activated queens'- but inhibited workers' ovaries in a dose-dependent manner and caused a depletion of fat-body lipids in both. All responses of hypercapnia were weaker following mating in queens. Analysis of gene expression related to hypoxia and hypercapnia supported the physiological findings in queens, demonstrating that the overall impacts of CO_2, excluding virgin queen ovaries, were unique and were not induced by anoxia. This study contributes to our understanding of the impacts and the mechanistic basis of CO_2 narcosis in insects and its impacts on bees' physiology.

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Introduction
Chapter 1

Being able to handle live insects with ease is necessary for conducting certain experimental procedures and multiple methods for anesthetizing insects have been developed over the years. It is critical for these methods to not have side effects that may interfere with the results of the experiment and influence its outcomes. This is particularly important in studies addressing questions related to insect physiology, which could be influenced by the choice of the anesthetic method.

Cold chilling and CO_2 narcosis are the most common methods of anesthesia followed by the use of isoflurane [1-5]. While the ideal form of anesthesia would limit the unwanted effects it has on the study organism, numerous studies have shown unwanted side effects [6]. Cold chilling is easy to apply due to the access to freezers or ice in a laboratory [7, 8]. In this method, live insects are typically exposed to a low temperature (<5°C) until movement ceases [9]; however the duration and temperature of exposure are species-specific. Exposure to low temperatures induces chill coma [10], a stupor state during which insects lose neuromuscular function [11] and that is reversible upon rewarming [10]. The chill coma temperatures depend on the insect's biology as was shown by [12] who used electrical activity from flight muscles to determine the chill coma temperatures in various orders of insects. Although easily accessible, cold chilling may cause a wide range of side effects in invertebrates [9]. For example, it causes a decrease in learning and memory in *Apis mellifera* [9, 13], a decrease in foraging time in *Bombus terrestris* [4], and affects offspring development time in *Drosophila melanogaster* [14].

Another anesthetic method makes use of isoflurane which is a halogenated ether that has been used in various groups of animals, including reptiles, birds, and mammals [15], but rarely used in insects [1, 16, 17]. A small amount of isoflurane put on a cotton ball in a sealed container can create an isoflurane filled atmosphere which knocks out the insect rapidly [1]. Studies in *Apis mellifera* show that isoflurane can alter their circadian rhythm [16, 18], but no other side effects have been reported. Despite being a commonly used anesthetic in the veterinary sciences, isoflurane and its potential side effects have yet to be tested across a broad range of insect species [17].

Lastly, carbon dioxide (CO_2) has also been used for decades for anesthesia, and has been documented as early as the 1920s [19]. CO_2 is an easy alternative for anesthetizing organisms, but it is known to have numerous effects on insect metabolism, reproduction, and behavior [1, 6, 20-25]. Collectively, there is evidence that CO_2 impacts metabolism across wide range of insect species [26-30] and that this impact may translate into changes in reproduction in a species-specific manner. In *Drosophila melanogaster,* CO_2 narcosis impacts the concentrations of several metabolites up to 14 hours after exposure [31]. In *Bombus* impatiens queens, overall fat body lipids are also reduced after CO_2 exposure [32]. Change in macronutrient allocation (e.g., lipids, proteins and carbohydrates) from the fat body to the ovaries and other tissues in the body was also found in *B. impatiens* queens [29, 37]. While the impacts on metabolism across insects seem conserved, the impacts on reproduction are more diverse. Studies show that CO_2 impacts insect reproduction in several orders, including Coleoptera [27], Hymenoptera [24, 26, 33], Diptera [34, 35] and Blattodea [20, 36]. This includes effects on ovary size, number of offspring and the number of viable offspring in numerous species [17]. In honey bee workers, CO_2 inhibits

ovarian activation [24, 25], whereas in honey bee queens and several species of bumble bee queens, CO_2 accelerates ovarian development and egg laying [23, 32, 33]. A recent study teased apart the metabolic and reproductive shifts after CO_2 narcosis in *B. impatiens* [29] and showed that the metabolic effect of CO_2 is primary, while the reproductive shift is secondary [29]. In this study, both ovariectomized and control queens showed a reduction in their fat body lipids, demonstrating that the metabolic shift in queens' macronutrients caused by CO_2 is not mediated by a reproductive shift, but overall, the mechanistic knowledge about the CO2 mode of action is limited. Examining these mechanisms is immensely important for mapping other unwanted side effects of CO_2 and for understanding the molecular mechanisms underlying the metabolic and reproductive changes caused by CO_2 in *Bombus* species.

There have been several proposed hypotheses on how CO_2 may be causing its pleiotropic impacts in insects. Earlier studies [2, 6] suggested that CO_2 produces acidification of the hemolymph using its hydrate form of carbonic acid, thereby causing downstream effects on the central nervous system, such as changes to endocrine and neurological processes [6, 32]. Another cause of acidification could be anoxia, or the lack of oxygen [38]. Oxygen delivery may be impaired following the administration of CO_2 leading to an anoxic environment. When insects are exposed to such environment, they may switch to anerobic ATP production and a suppression of metabolism [39, 40], which could explain the downstream effects of CO_2 in some insects. An alternative hypothesis suggests that CO_2 could directly affect the central nervous system by decreasing sensitivity to glutamate receptors, causing reduction in locomotor activity [41]. CO_2 may also be affecting biogenic amine levels in the central nervous system [42]. Biogenic amines act as neurotransmitters and neuromodulators regulating insect behavior and

reproduction [43-47]. This has been shown in previous studies which showed that CO_2 causes a reduction in brain dopamine of worker and queen honey bees [42], and an increase in the expression of dopamine and octopamine receptors in queen honey bee ovaries [48]. The overall data supporting these hypotheses is scattered and more research is needed to address how CO_2 operates.

In this thesis, I investigated the mode of action of CO_2 in *Bombus impatiens* by testing the hypotheses that CO_2 operates via anoxia. *Bombus impatiens* is an excellent model organism to explore the mechanisms underlying CO_2 since queen reproduction is regularly induced by CO_2 narcosis and vast information is available about the impacts of CO_2 in queens. This information can be used to tease apart the effect of CO2 and anoxia. Additionally, *B.* impatiens is an economically important species that contributes to pollination services in both agriculture and the wild. By examining how *B. impatiens* is impacted by CO_2 narcosis, we can gain a better understanding of how to use this method (if at all) as an alternative to cold storage to initiate new colonies in the lab and for pollination services. Currently, cold storage is being used in place of diapause but incurs high rates of mortality [49].

In wild populations, *B, impatiens* queens undergo a winter diapause, an arrested state of development allowing insects to overcome the harsh conditions of the winter [50]. Previous studies have shown that CO_2-treated queens are phenotypically similar to queens exiting diapause [51]. While the factors determining the termination of natural diapause are still under investigation, the hypercapnic levels queens may experience in their hibernaculum may also play a role in the termination phase, and understanding the mode of action of CO_2 could also contribute to understanding these processes Here, I used behavioral assays along with

physiological and molecular techniques to examine the suite of changes caused by CO_2 and anoxia to determine whether the physiological and behavioral shifts caused by CO_2 are mediated by anoxia.

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CHAPTER 2

Impacts and mechanisms of CO_2 narcosis in bumble bees: Narcosis depends on dose, caste and mating status and is not induced by anoxia

Introduction

Carbon dioxide (CO_2) is a prevalent gas present in the atmosphere that can vary in concentration in different microclimates. Low concentrations of CO_2 are often used as an attractant or repellent and are identified by designated receptors in insects (1). They can guide host plant finding and increase foraging efficiency when flowers are open in herbivores (2), induce nest digging in ants (3, 4) and nest maintenance behaviors in social bees and termites (5, 6). In contrast, hypercapnic concentrations (above 0.04%) of CO_2 can result in pervasive physiological, molecular, and behavioral changes (2, 7). Full hypercapnia (100% CO_2) is a commonly used anesthetic during experimental procedures in insects but may result in unwanted side effects. Despite its wide use and importance in insects, the mechanisms underlying the mode of action of CO_2 are not fully understood.

CO_2 narcosis has pleiotropic impacts on insect physiology and behavior. These effects have been documented in multiple orders including Orthoptera (8), Diptera (9, 10), Hemiptera (11), and Hymenoptera (12-15). These impacts include, for example, changes in fecundity and longevity (10) and reduced climbing behavior (16) following CO_2 treatment in *Drosophila melanogaster*; reduced feeding and drinking in *Empoasca devastans* (11), and reduced social aggregation in *Locusta migratoria* (8, 16). The specific impacts of CO_2 on reproduction, such as initiating egg laying in queen bees (17, 18), are often used for practical purposes. However, these impacts are not limited to stimulation and may also include suppression of reproduction in some species. For example, exposure to CO_2 led to a reduction in insemination frequency in *Glossina* females (19), suppressed ovarian development in adult *Tribolium castaneum* (20), reduced egg laying in *Pyrrhocoris apterus* (21), and lower ovarian activation in honey bee workers (14, 15, 22). Similar differences following CO_2 were found in the gene expression profile of multiple species

within hours of exposure, demonstrating changes in genes related to vitellogenesis (23-25), insulin and juvenile hormone (JH) signaling pathways (23) and oxidative stress (14, 23).

Earlier studies examining the mechanisms underlying CO_2 suggested that it affects the nervous system through rapid changes in intracellular pH (2). While the hemolymph acidity was shown to increase following CO_2 in numerous species (26-28), it has never been directly linked to the unique effect of CO_2 and can be caused by several potential mechanisms. One of these is anoxia, whereby CO_2 operates via the lack of oxygen (2, 29-31). This too could lead to acidic intracellular pH; however, the data supporting this hypothesis are controversial. While several studies have claimed that adverse effects of CO_2 narcosis are caused by low oxygen concentrations (29, 32, 33), others provided indirect evidence that hypercapnia and anoxia induced such a different suite of changes that it makes little sense to conclude that CO_2 operates via anoxia. For example, in honey bees, both anoxia and hypercapnia caused age-related changes and early foraging (32), and in termites, both CO_2 and hypoxic conditions increased the reproductive output in *Reticulitermes speratus* (34) and also induced similar changed in reproduction-related genes (35). On the other hand, a nitrogen treatment (anoxia) failed to induce a shift from a gregarious to a solitary state in *Locusta migratoria*, as did CO_2 (8). The same was found in crayfish where a nitrogen treatment did not produce the same behavioral (avoidance response) and physiological (increase in heart and ventilatory rates) responses caused by CO_2 (36). Likewise, anoxia resulted in a decreased metabolism and increase in the production of reactive oxygen species (37, 38), both contradicting the effects CO_2 induce in bumble bees, i.e., down regulation of antioxidant genes that typically respond to the influx of ROS production, reduced fat body lipids and increased aggression, which may reflect increased metabolic rate (39). Finally, in cricket larvae, some of the impacts of CO_2 could be mimicked by nitrogen (feeding and drinking inhibition), but not all of them (e.g., metabolic rate) (29), and overall more research is needed to settle the question whether CO_2 is mediated via anoxia.

Another hypothesis proposed that CO_2 operates directly on the central nervous system, but how exactly and whether the mechanism is conserved across species remain open. Recent studies in *Drosophila melanogaster* and crayfish demonstrated that CO_2 blocks glutamate receptors at the neuromuscular junction (NMJ) and inhibits the recruiting motor neurons within the CNS (28,

40). In crayfish, NMJ responds to exogenous glutamate and to saline that was adjusted to pH 5.0 by depolarization. However, the synaptic transmission is shut down in response to CO_2-saturated water and does not respond to either exogenous glutamate or to acidic pH (5.0). Similar evidence for CO_2 blocking the glutamate receptors were obtained also in Drosophila (28). There, it was also shown that CO_2 has actions not only in the CNS, but also in the periphery.

Additional studies provided some more information about the cascade process between CO_2 administration and its diverse effects. First, changes in brain biogenic amines follow CO_2 narcosis. The brain dopamine, tryptophan and tyrosine levels of pollen-fed honey bee workers were significantly reduced compared to untreated controls, whereas in caged queens, dopamine expression levels decreased by 45% compared to controls (41). Levels of octopamine, content of cAMP, sensitivity of their receptors, and the neuroendocrine system were modified also in *Locusta migratoria* following CO_2 narcosis (30). Second, JH, a gonadotropin in most insects, was shown to spike following CO_2 narcosis in bumble bees (23), and when queens were fed with JH inhibitor, CO_2 had no effect on their ovaries and fat body macronutrient amounts as compared to controls (42). Combined, these changes likely follow the neural changes and the decrease in intracellular pH leading to the diverse behavioral and physiological effects exhibited by CO_2, but the cascade of reactions and the overall mechanism are not fully understood. Furthermore, this question has never been examined before in bees, where CO_2 induces unique and caste-dependent effects on reproduction.

In this study we examined whether CO_2 causes immobilization and transition to reproduction by inducing anoxia and whether these mechanisms differ between female castes or change throughout the life cycle of the bee using the social bumble bee *Bombus impatiens*. This bee goes through an annual life cycle where a single mated queen produces workers until the nest reaches several hundreds of bees, and then switches to produce virgin queens and males that will mate and go into a winter diapause. The effects of CO_2 were extensively studied in honey bees and bumble bees where it induced a transition to reproduction in queens (17, 18) and inhibition of reproduction in honey bee workers (43). It was further shown to induce a metabolic shift in bumble bee queens (both *B. terrestris* and *B. impatiens*), reducing lipid levels in the fat body and increasing glycogen and protein in the ovaries (14, 23, 42), as well as increasing the hemolymph

levels of JH (23) and acting as an accelerator of metabolism (44). The effects of CO_2 on queen metabolism in *B. impatiens* persist even when queen ovaries were removed but did not persist when queens were fed with JH inhibitor (42), suggesting CO_2 impacts are mediated via JH and its effect on reproduction is secondary. It was also shown that when combined with cold storage, the positive effect of CO_2 on reproduction in queens diminished with the length of cold storage (45), showing once again that the effect on reproduction is a byproduct of other processes. CO_2 also increased aggression and flight behavior in virgin queens (14) and changed the gene expression profile of the queen's following administration (14, 23). However, data on the impacts of CO_2 throughout the different queen life stages and in workers are lacking. Altogether, due to the many tools and knowledge available on the impacts of CO_2 in bumble bees, they serve as an excellent system model to examine the mechanisms underlying the mode of action of CO_2.

To examine whether CO_2 is induced by anoxia, we assigned workers, virgin, and mated queens to anoxia (100% nitrogen), hypoxia (14% oxygen and 86% nitrogen), and full and partial hypercapnia (100% and 50% CO_2), as compared to controls and examined the behavior, physiology, and gene expression of bees. We hypothesize CO_2 narcosis is induced through the physiological effects of a lack of oxygen. If this hypothesis is correct, we expect both hypercapnia and the lack of oxygen (anoxia) to have similar effects on behavior, physiology and gene expression in bees. Alternatively, if CO_2 is not mediated via the lack of oxygen, we expect both hypercapnia and anoxia to have unique and non-overlapping effects.

Methods

Bees and treatments. Colonies were obtained from Koppert Biological Systems (Howell, MI) and maintained in environmental chambers under darkness, 28-30° C, 60% relative humidity, and supplied with *ad libitum* 60% sugar solution and fresh pollen purchased from Koppert. Newly emerged workers and gynes (< 24 hours old and distinguished by their silvery appearance) were collected from multiple source colonies (n=12), tagged with a colored number, and randomly assigned a cage (11 cm diameter and 7 cm high). Workers were kept in groups of three while gynes were kept alone. All cages were supplied with unlimited sugar and fresh pollen as the full-size colonies. Cages were randomly assigned to one of five gas treatments including

full hypercapnia (FH, 100% CO_2), partial hypercapnia (PH, 50% CO_2, 50% N_2), hypoxia (HYP, 14% O_2, 86% N_2), anoxia (AN, 100% N_2), and control (C, no gas exposure). Gas treatments were conducted for one full minute of air flow into a nearly sealed plastic cage. Queens and workers typically lose mobilization within seconds when exposed to full hypercapnia, partial hypercapnia and anoxia, but do not lose mobility in hypoxic or control conditions. The seal was left on the container for an additional 30 minutes after exposure, allowing the cage to reach an equilibrium with the ambient air and was removed afterwards. Following treatments, cages were placed back in the environmental chamber.

Workers: newly emerged workers (n=534, the split of sample size to treatments and variables is provided in Table S1) were collected upon emergence and kept in group of three. In the first trial ("single treatment"), workers (n=309) were treated with gas on day 1 of age and were kept for six- and ten-days post treatment. Following recovery from the gas treatment, workers were immediately observed for abdominal contractions and for aggressive behavior between nestmates for 20 min per day on days 1-6. They were then stored in -80 ºC until further analyses. Six-day-old workers were examined for ovary activation, fat body lipid amount, and head gene expression, and 10-day-old workers were examined for total number of eggs laid per cage. The second trial of workers ("multiple treatments", n=225) was identical to the first trial with two differences: first, workers were treated every day on days 1-6 of age (multiple gas treatments) to account for age of exposure being a possible factor in the effectiveness of the gas treatment and second, workers were flash-frozen at the age of 7 instead of 6 days to allow them more time to activate their ovaries. The workers of the second trial were examined for ovarian activation, fat body lipid amount and head gene expression.

Queens: Newly emerged queens (n=192, Table S1) were kept in individual cages for 6 days and were introduced into a mating arena at the age of 6-11 days. Queens were put back in their individual cages following mating (mated) or unsuccessful attempt to mate (virgin). Mating procedure is described below. Queens were randomly assigned to a gas treatment 24 hours post-mating or the attempt to mate and were observed for abdominal contractions immediately after recovery. Queens were kept for an additional 10 days post treatment, resulting in all queens being 17-22 days old at the completion of the experiment. Queens of that age range are quite

homogenous in terms of their reproductive status. Queens were flash-frozen and stored in -80 ºC and were examined for ovary activation, egg laying, fat body lipids and head gene expression.

Mating. At the age of 6 days, queens were placed in a mating arena (35 x 12 x 12 cm) with 3-4 males for every queen. Queens were given a three-hour window to mate, during which they were constantly observed. If queens did not mate, a similar attempt was conducted daily until a successful mating was observed or the queen reached the age of 11 days. This range was chosen based on the optimal age for mating found in (46). The arena was checked every 15 minutes to observe for mating pairs and pairs were removed while mating and placed back into an individual cage. The pair remains connected by their genitalia for approximately 30 minutes and males were removed from the cage upon the completion of mating. Whenever a mated queen was sampled, a same-age unmated queen (virgin) was removed from the arena and sampled as well.

Abdominal contractions. Abdominal contractions (AC) are performed by insects to facilitate gas exchange throughout the body (47, 48), and typically start upon recovery from anesthesia. The number of abdominal contractions was video-recorded and quantified for 20 minutes in queens and workers from the first movement and only in treatments that caused immobility (full hypercapnia, partial hypercapnia, and anoxia). We also quantified the time it took bees to enter anesthesia (in seconds) and the recovery time from anesthesia (in minutes). Recovery time differed between treatments and between queens and workers (see results), and therefore recording began at different timepoints after the gas treatments.

Aggression in worker groups. Aggressive behavior in workers was observed between days 1 to 6 following exposure in workers of trial 1 (single treatment). Each cage was observed for 20 minutes per day under red light between the hours of 8:00-12:00 AM. We recorded and summed four aggressive behaviors typically seen in queen-less groups of *Bombus impatiens* (14): humming (a series of wing vibrations lasting less than 3 s), climbing (one bee crawls on top of another bee), darting (a sudden movement in the direction of another bee) and attacking (biting, pushing, dragging by legs or wings, and an attempt to sting). The identity of the bee performing and receiving each behavior was recorded. The total aggressive behaviors performed by

individual workers during days 1-6 was used in further analyses. Queens were not observed for aggression since they were kept alone.

Egg laying. Cages of workers at the age of 10 days and queen cages were checked daily for newly constructed egg cells throughout the experiment. Ten days after the gas treatment, egg cells were carefully opened using a micro spatula. Eggs were counted and summed for each cage.

Ovarian activation. All workers and queens were dissected under stereomicroscope. The three largest ovarioles were measured using an ocular scale (mm) and at least one ovariole per ovary was measured. Mean terminal oocyte length was used in further analyses. The average of the terminal oocytes was measured in numerous previous studies as an index for female reproduction; examples: (14, 23).

Fat body lipid analysis. Worker and queen fat body was dissected out and homogenized in 5 ml of 2% sodium sulfate, as in (14). 200µl was transferred to a new vial, with 2.8 ml of a 1:1 chloroform-methanol solution. Vials were centrifuged for 5 minutes at 3000 rpm to separate the lower and the upper phase. The supernatant was transferred to a new vial, where 2 ml of distilled water were added, and samples were centrifuged for 5 minutes at 3000 rpm. The bottom layer (lipids) was separated from the remaining fraction and was placed on a heating plate at 100° C to evaporate the solvent. Lipid amount was determined by using a vanillin-phosphoric reaction (14). A standard curve was created using five different concentrations of vegetable oil in chloroform. Absorbance values (OD 525) were determined using Biotek Synergy LX plate reader and converted into micrograms per bee based on the standard curve equation. The amounts of lipids were normalized for the tissue mass, which was measured on electronic scale prior to analysis.

Gene expression. The effect of gas treatments on gene expression levels was quantified for six candidate genes and three treatments (full hypercapnia, anoxia and control) in the two trials of workers and in the virgin and mated queens (see Table S1 for sample size). Analysis included three genes that were regulated by CO_2 (but not for anoxia) in previous studies (*vitellogenin*,

FOXO, and *PHGP*) (14, 49), and three hypoxia inducible factors (HIFs) that were regulated by the lack of oxygen in previous studies (50, 51), but their impact on CO_2 was not tested. Genes with known response to anoxia or hypercapnia were chosen to examine whether the effect of CO_2 on gene expression is unique to the gas or not. Vitellogenin (*vit*) codes for the major egg yolk protein in the ovaries and has been shown to be upregulated in CO_2 treated queens compared to the non-treated controls (23). *FOXO* is a conserved transcription factor that regulates insulin and insulin-like growth factor signaling. It has been shown to be downregulated after CO_2 treatment. Finally, *PHGP* is a gene coding to an antioxidant enzyme and was previously shown (together with other antioxidant enzymes) to be downregulated in queens in response to hypercapnia (14). HIF-1α (*sima*) and β (*tango*) are two units of a transcription factor involved in the response to low oxygen concentration and *fatiga* is part of a family of prolyl hydroxylases, which plays a vital role in the degradation of HIF-1α. All three genes are expected to be regulated in response to anoxia (51). Design of the forward and reverse primers for each gene was done using primerBLAST. The list of primers and accession numbers is provided in Table S2. Genes with multiple variants had primers designed to target the most conserved region between the sequences. RNA was extracted from queen and worker heads using the RNeasy mini kit according to the manufactural instructions (Qiagen, Valencia, CA) and its quality was assessed using NanoDrop One. Queens' RNA was extracted from individual samples whereas heads of workers from the same cage were pooled together. 600 ng of RNA was used for cDNA synthesis with Reverse Transcriptase (Applied Biosystems). After PCR, cDNA was diluted using 85 µl of nuclease free water and stored at -20° C until real time PCR was performed. Gene expression was measured using QuantStudio 5 real-time PCR system (Applied Biosystems). Two microliters of the diluted cDNA were used in a reaction with 5 µl SYBR Green, 0.2 µl of the forward and reverse primers, and 4.6 µl of nuclease free water. *Arginine kinase* and *Phospholipase A2* were used as housekeeping genes to control for PCR efficiency and differences across samples. These control genes have been used in previous studies with *Bombus impatiens* (52). Each reaction was performed in triplicate and averaged for data analysis. Each plate had a water control and a negative control (cDNA reaction without Reverse Transcriptase enzyme). Expression levels of each gene were normalized using the geometric means of the two housekeeping genes using the $2^{-\Delta\Delta Ct}$ technique.

Statistical Analysis. All statistical analyses were performed in JMP Pro 16. The time to enter anesthesia, the recovery time following anesthesia and the number of abdominal contractions were compared using Two-way ANOVA mixed model with caste (virgin, mated, workers) and treatment (FH, PH, and AN) as fixed effect and colony and cage as random factors. The sum of aggressive behavior in workers was compared using Kruskal Wallis test followed by multiple comparison test for each pair using Wilcoxon method. Ovarian activation, fat-body lipid amount, the total egg production and gene expression levels were compared using ANOVA mixed model with colony as a random effect (in queens) or with colony and cage as random effects (in workers). Gene expression data were log transformed prior to analysis. Post-hoc comparison was performed using Tukey HSD test. Statistical significance was accepted at $\alpha=0.05$. Data are presented as means ± S.E.M

Results

The time it took bees to become immobile, the recovery time from the gas treatments and the number of abdominal contractions bees exhibited upon recovery were only observed following recovery from anoxia (AN), full and partial hypercapnia (FH, PH), because hypoxia (HYP) or control did not result in anesthesia. The time to enter anesthesia differed between all treatments (Two-way ANOVA Mixed model, $F_{2,158}=134.3$, $p<0.0001$ followed by Tukey HSD $p<0.0001$) and between workers and virgin queens ($F_{2,158}=5.18$, $p=0.006$ followed by Tukey HSD $p=0.004$). Losing mobility was the fastest following FH compared to the other treatments, and workers lost mobility quicker than queens following FH but slower than queens following AN (Fig. 1, Table S3 for means, S.E.M and statistical tests). The recovery time following anesthesia also differed between all treatments ($F_{2,158}=72.6$, $p<0.0001$ followed by Tukey HSD $p<0.0001$) and between workers and queens ($F_{2,158}=50.1$, $p<0.0001$ followed by Tukey HSD $p<0.001$). Workers were faster to recover compared to queens, but recovery from FH took longer than the other treatments. Finally, we quantified the number of abdominal contractions (AC) in 20 minutes beginning with the first movement across treatments and castes. Here, differences were found between treatments ($F_{2,158}=14.8$, $p<0.0001$ followed by Tukey HSD $p<0.03$ for all groups) but not between castes ($F_{2,158}=1.16$, $p=0.31$), with the highest AC following PH, then FH and the lowest AC following AN.

The effect of the gas treatments on aggression was examined in groups of workers that were treated a single time on the day of emergence. Comparison of all groups showed nearly significant increase in aggression following CO_2 (Kruskal Wallis test, x^2_4=8.98, p=0.06 followed by post-hoc comparisons between FH vs.PH (p=0.037), FH vs. HYP (p=0.004), FH vs. control (p=0.029), and FH vs. AN (p=0.07) (Fig. 2).

Worker ovarian activation was not affected by a single treatment of CO_2 (ANOVA Mixed model, $F_{4,140}$=0.25, p=0.96, Figure 3A), but was affected by the treatment in the multiply-treated workers (ANOVA Mixed model, $F_{4, 70}$=20.2, p < 0.0001; followed by a Tukey post-hoc test p<0.001 for FH vs. HYP and control, PH vs. HYP and control, and p<0.04 for An vs. PH, HYP and control, Fig 3B). Ovarian activation was also affected by the treatment in the virgin queens (ANOVA Mixed model, $F_{4,95}$=19.3, p<0.001, Fig 3C), where both full and partial hypercapnia treatments differed significantly from the remaining treatments (p<0.001), but not from the anoxia treatment (p>0.05) which displayed intermediate levels of ovarian activation as compared to hypercapnia and control. In mated queens, the data show a similar pattern to the ones of virgin queens with significant effect of the treatment (ANOVA Mixed model, $F_{4,87}$=4.7, p=0.0013 followed by Tukey post- hoc test between=0.03 for AN vs. FH; p=0.009 for FH vs. control, and p<0.001 for FH vs. HYP (Fig. 3D). Egg laying was observed in all 10-days old worker cages that were treated once regardless of treatment (n=50), but only in a small fraction of the queen cages (7.7% of all queens, n=154, data not shown). This has no practical meaning since queen-less workers start laying eggs within approximately 7 days from emergence, while queens require 2-3 weeks to initiate a colony following CO_2 treatment. Thus, 10-days old workers had more time to lay eggs compared to the queens who were sampled 10 days after the gas treatments. Egg laying in workers did not significantly differ across treatments (ANOVA Mixed model, $F_{4,45}$=1.8, p=0.15). In mated queens, two queens laid eggs (in full hypercapnia and anoxia treatments) and in virgin queens, ten queens laid eggs, eight of them in the full and partial hypercapnia treatments. These results are in line with the stronger impact of CO_2 on the virgin queen ovaries compared to the mated queens.

Lipid percentage in the fat body was overall lower in workers (on average of 4%) compared to queens (on average of 20%). The treatment did not affect the lipid percentage of workers who

were treated once (ANOVA Mixed model, $F_{4,45}$=1.3, p=0.297, Fig. 3a), but did affect workers who were treated multiple times (ANOVA Mixed model, $F_{4,30}$ =3.79, p=0.013 followed by a Tukey post-hoc test p=0.05 between AN and PH vs. C; and p=0.02 between FH vs. C, Fig. 3b). Virgin and mated queens showed a similar pattern of response to the treatments with lower lipid percentages in FH and PH compared to the other treatments, but the differences were significant only in the virgin queens (ANOVA Mixed model, $F_{4,59}$=3.8, p=0.009 followed by lower lipids in FH compared to control (p=0.023), AN (p=0.042) and HYP (p=0.035), fig. 4C) ,while the differences in the mated queens were insignificant (ANOVA Mixed model, $F_{4,45}$=1.77, p=0.15, Fig 4D).

Gene expression of six candidate genes, previously shown to be regulated by CO_2 (HIFs) (*vitellogenin, FOXO* and *PHGP*), or by anoxia (*sima, tango, and fatiga*) (51) were tested across treatments in workers, virgin and mated queens. Analysis focused on comparing the control, full hypercapnia, and anoxic treatments. In workers, there was no significant effect of treatment on either the HIF or the CO_2 genes. This was the case in workers that were treated once (ANOVA Mixed model for *fatiga*: $F_{2,15}$=1.45, p=0.27; for *tango*: $F_{2,15}$=0.55, p=59; for *sima*: $F_{2,15}$=1.16, p=0.34; for *PHPG*: $F_{2,15}$=0.8, p=0.46; for *FOXO*: $F_{2,15}$=0.27, p=77; for *vitellogenin*: $F_{2,15}$=1.1, p=0.35, data not shown), and also in workers that were treated multiple times (ANOVA Mixed model for *fatiga*: $F_{2,14.4}$=1.98, p=0.17; for *tango*: $F_{2,14.2}$=2.6, p=0.11; for *sima*: $F_{2,14.5}$=2.1, p=0.16; for *PHGP*: $F_{2,12.6}$=3.5, p=0.06; for *FOXO*: $F_{2,14.5}$=2.2, p=0.15; for *vitellogenin*: $F_{2,13.4}$=0.41, p=0.67, Fig. 5A). Queen groups were combined, as there was no effect of mating on gene expression levels. In queens, there was a significant effect of treatment on gene expression level in each of the HIF genes, *sima, tango,* and *fatiga* (ANOVA Mixed model for *fatiga*: $F_{2,30}$=3.5, p=0.04; for *tang*o: $F_{2,29}$=3.5, p=0.04; for *sima*: $F_{2,27}$=9.08, p=0.0009). Additionally, there were significant effects of treatment on the expression levels of *FOXO* and *PHGP*, but not *vitellogenin* (ANOVA Mixed model for *PHGP*: $F_{2,20}$=4.8, p=0.02; for *FOXO*: $F_{2,29}$=5, p=0.01, Fig. 5B).

Discussion

In this study, we examined the behavioral, physiological and gene expression effects caused by different gas treatments in *Bombus impatiens* workers and queens. Our goal was to determine if the effects caused by CO_2 narcosis are mediated through anoxia. Our data demonstrate that

overall, this is not the case. Hypercapnia, but not hypoxia or anoxia, caused immobilization in a dose-dependent manner and the recovery from hypercapnia, but not from anoxia, was associated with increased abdominal contraction behavior. Hypercapnia also had marginal impacts on aggression in workers. Physiologically, hypercapnia, but not anoxia or hypoxia, affected ovarian activation and fat-body lipids. Finally, gene expression differences were significant only in queens, although trends were apparent also in workers (likely due to the smaller sample size in workers), but these differences overall indicated different impacts of anoxia and hypercapnia on gene expression, suggesting again that hypercapnia is unlikely to be mediated via anoxia. More specifically, contrary to our expectations, the three genes associated with anoxia (*fatiga, tango* and *sima*) were downregulated in queens following hypercapnia, but not following anoxia. And, as expected, two out of the three genes associated with CO_2 in previous studies (*FOXO* and *PHGP*) were significantly downregulated after hypercapnia in queens. Thus, each gene was uniquely impacted by the gas treatments, and we did not observe any similar changes across both hypercapnia and anoxia. Overall, the behavioral, physiological and gene expression differences following hypercapnia were unique to CO_2 and were not induced by anoxia. The only exception were the ovaries of virgin queens that were significantly increased by both CO_2 and anoxia which we discuss below.

A second goal of this study was to understand the impacts of CO_2 across female castes and following mating. Here we discovered that the recovery from hypercapnia was slower in queens compared to workers, and that also workers exhibit increased aggression following CO_2 narcosis (14), likely due to their increased ovary activation and competition. We found that full hypercapnia had a stronger effect compared to partial hypercapnia, and that CO_2 impacts on ovary activation were opposite in queens and workers, and also weaker following mating in queens. We also noted that while queens respond to CO_2 narcosis, regardless of their age, duration, and number of exposures (14), workers did not respond to CO_2 when treated upon emergence and required multiple exposures. These caste differences were only shown before in *Apis mellifera*, an advanced eusocial species where JH, the factor likely mediating the response to CO_2 (42), evolved to no longer act as a gonadotropin as it does in most insect species (including bumble bees) (53). CO_2 is likely increasing JH levels (23, 42). It was therefore supposed to have no effect on reproduction in honey bee workers, and activate the ovaries in

bumble bee workers, yet in both species it does the opposite, calling for alternative explanation for the way CO_2 operates in queens vs. workers. These caste differences are also valuable to understanding the mechanisms by which CO2 operates, but future research should likely focus on generic models (like bumble bees) where the function and roles of JH are similar to other insects. Overall, the effects of CO_2 were dose-, caste-, and mating status dependent. These results demonstrate the diverse effects of CO_2 in bumble bees, their regulation by caste and mating, and preclude anoxia as a potential mechanism explaining how CO_2 operates.

Our findings demonstrated that the effects of CO_2 narcosis are not mediated through anoxia, meaning that the CO_2 has a unique impact on cells, such as through a reduction in extra- and intra- cellular pH either by blocking glutamate receptors in the neuromuscular junction (28, 40) or by altering the levels of other neurotransmitters and/or neuromodulators in the nervous system. Studying these questions requires experimentation at the cell level rather than at the level of the entire organism. For example, the caste-differences induced by CO_2 could be due to different buffering abilities of queens and workers. Examining this idea requires the ability to measure the fluxes of sodium and potassium over range of pH (54). Other neurotransmitters and/or neuromodulators that could be directly affected by CO_2 are endogenous biogenic amines (BAs) such as dopamine, octopamine, serotonin and tyramine. These low molecular substances are not only involved in reproduction and ovipositing in numerous insects (55-63), but were also shown to be directly affected by CO_2. In honey bee queens and workers, CO_2 narcosis was found to upregulate dopamine levels in the brain and also dopamine receptors in both the ovaries and the brain (64). Selected BAs such as dopamine also correlate with reproductive status in bees in a caste-dependent manner (62). Finally, BA levels can also modify the level of JH and explain its elevated levels following CO_2 narcosis (23).

Understanding these mechanisms can also clarify why the impacts of CO_2 are caste dependent in both bumble bees (current study) and honey bees (15, 25, 65). If CO_2 activates network of genes related to JH and vitellogenesis that were decoupled during the evolution of social behavior (53, 66, 67), then the downstream effects on worker and queen reproduction are easier to explain. For example, if CO_2 regulates JH and JH decoupled during the evolution of sociality to regulate reproduction in honey bee queens and task allocation in honey bee workers, its caste-dependent

effects on reproduction are intuitive. However, while this explanation makes sense for honey bees, it does not make sense in bumble bees, where JH maintained its gonadotropin role in both queens and workers.

In contrast to its caste-dependent impact on reproduction, CO_2 causes a reduction in lipids in the fat body of both queens and workers. Typically in insects that reproduce, lipid stores correlate negatively with reproductive status, as lipids are reallocated to build the oocytes (68). However, workers of social species who remain sterile may have low lipid reserves if they use them for maintenance tasks that require energy, like foraging (69-71). Foragers show lower reproductive capacity compared to house bees in both the honey bee (70) and bumble bees (53, 72). The relationship lipid reserves, and task may explain how the same impact (on lipid levels) can translate into different reproductive status in queens and workers. It also emphasizes that CO_2's primary effect on insect physiology is on metabolism with reproduction being a secondary byproduct of it (42). The metabolic shift caused by CO_2 was demonstrated in several species (14, 73, 74). Focusing on the molecular mechanisms that affect metabolism and macronutrient allocation may narrow down the possible mechanisms to explore. One candidate is the insulin signaling pathway that could be triggered by neural changes and affect metabolism.

The weaker effects of CO_2 on mated queens were observed across all the parameters examined in the study. Post-mating changes occur widely in insects (75-77) and include changes in immunity (76, 78), stimulation of egg laying (79), and a decrease in the response to sex pheromones (75). Since CO_2 also induces some of these changes in bumble bees (14), its impact following mating may be redundant. It should be noted that the only effect of anoxia in this study was observed in the ovary of virgin queens which could reflect a higher sensitivity to lack of oxygen during earlier life stages. Interestingly, response to anoxia in other insects is indeed graded, tissue and age dependent (80).

Overall, our data show that the behavioral, physiological and gene expression differences following hypercapnia were unique to CO_2 and were not induced by anoxia, with the exception of ovarian activation being affected by both hypercapnia and anoxia in virgin queens. We also demonstrated that CO_2 affects queens and workers differently alongside a conserved effect of

CO_2 on lipid level in both castes and likely also across species. Our results contribute to understanding of the mechanisms of CO_2 narcosis in bees. Further studies at the cell level could help identify the molecular mechanisms underpinnings CO_2 effect on the neural system and how these are translated into metabolic differences in insects.

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Author's contributions

AC and EA designed the experiments, AC collected and analyzed the data, AC and EA wrote the manuscript.

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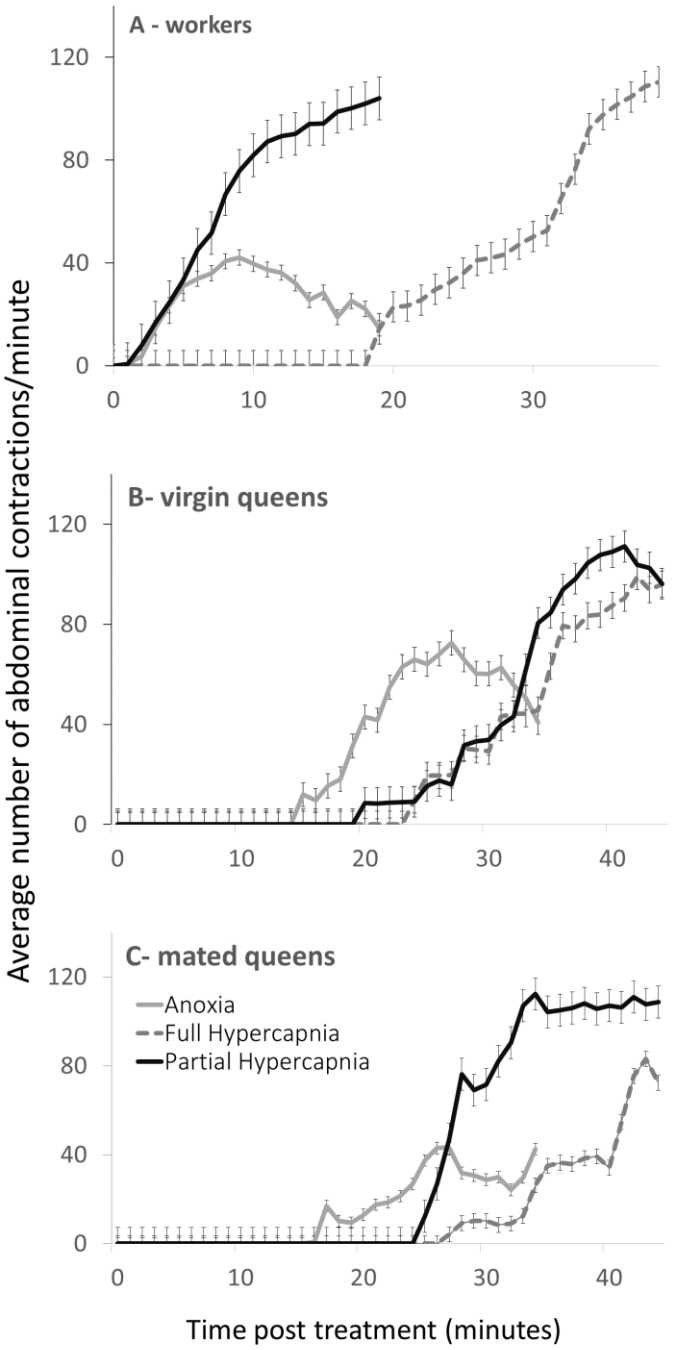

Figure 1. The recovery time from anesthesia and the effect of gas treatments on the number of abdominal contractions in workers (A), virgin queens (B), and mated queens (C). Bees were observed for 20 minutes from their recovery (first movement) in the three treatments that induced anesthesia. Data are presented as means ± S.E.M. Additional data and statistical analyses are provided in Table S3

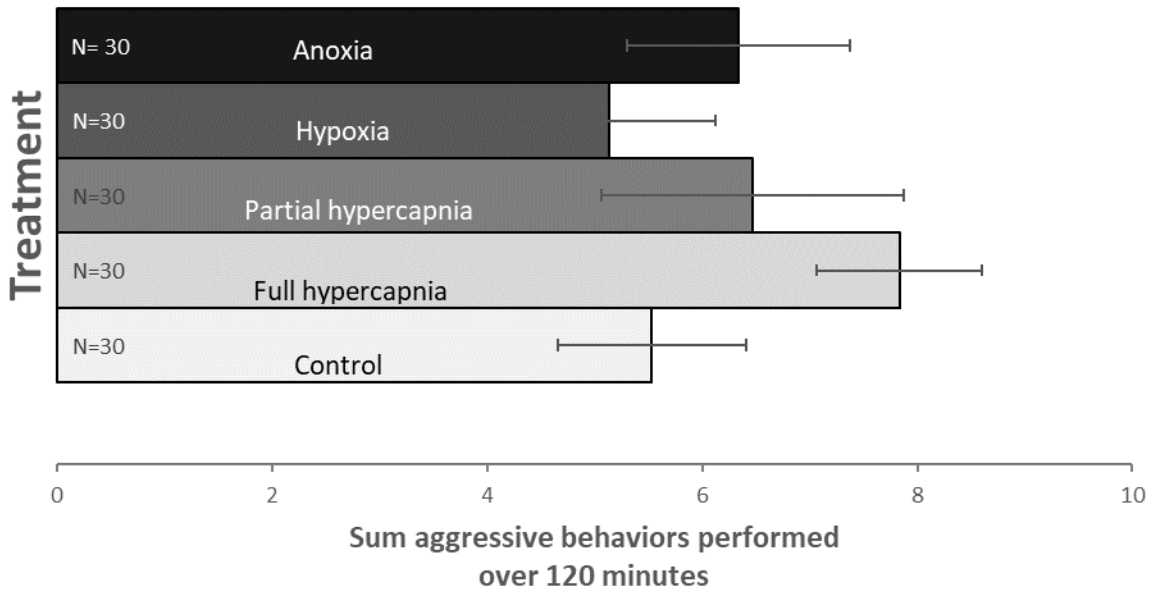

Figure 2. The effect of treatment on the sum aggressive behaviors performed by workers during days 1-6 following emergence. Workers were assigned to five gas treatments on the day they emerged. They were kept in groups of 3 (n=10/treatment) and were observed daily for 20 minutes. Four aggressive behaviors were documented and summed for each worker. Data are presented as means ± S.E.M.

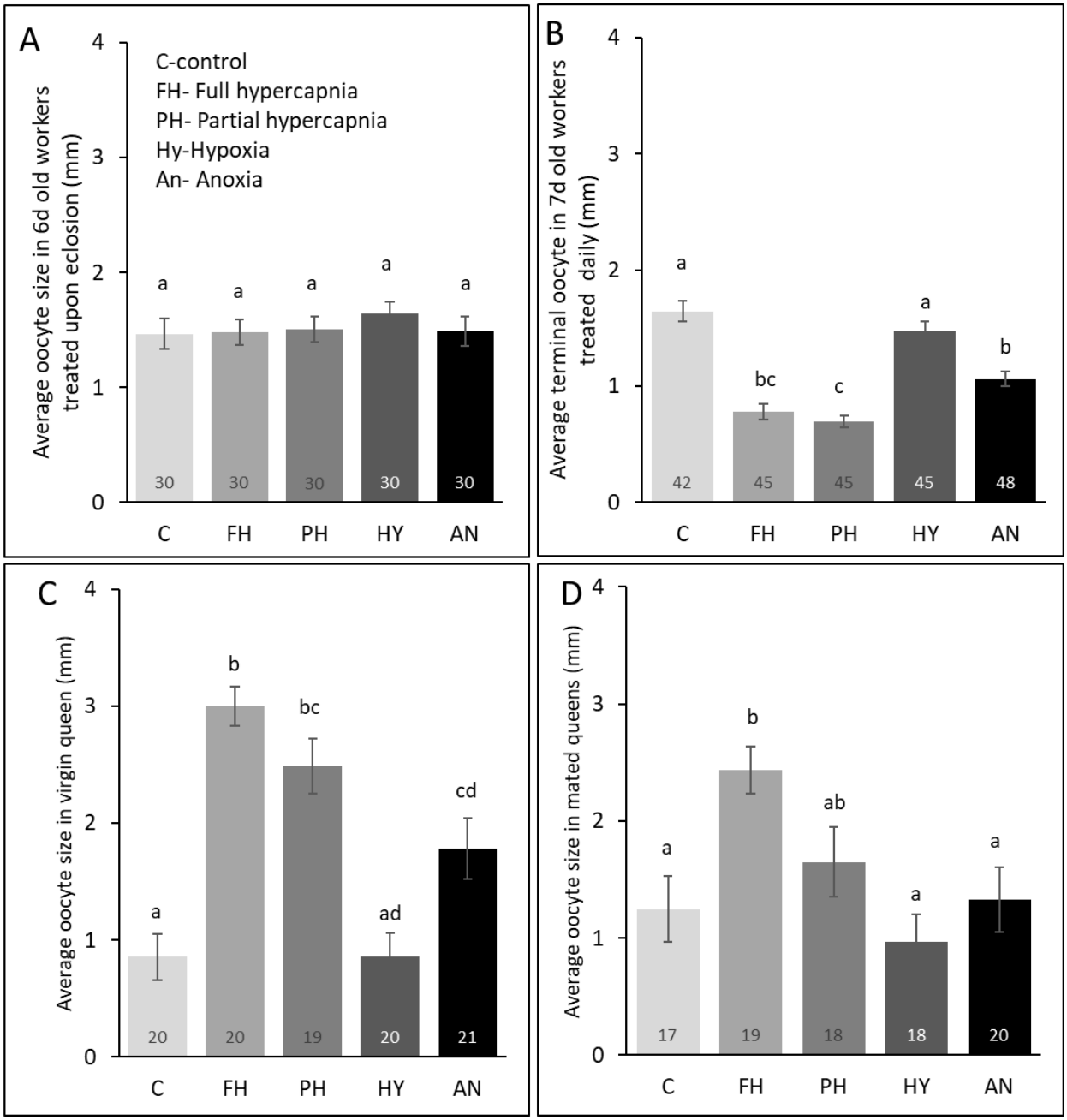

Figure 3. The effect of gas treatment on the average terminal oocyte size in All queens were frozen 10 days post treatment and were 17-20 days old. The sample size per treatment is denoted using the numbers within columns. Different letters denote significant differences at α=0.05. Data are presented as means ± S.E.M.

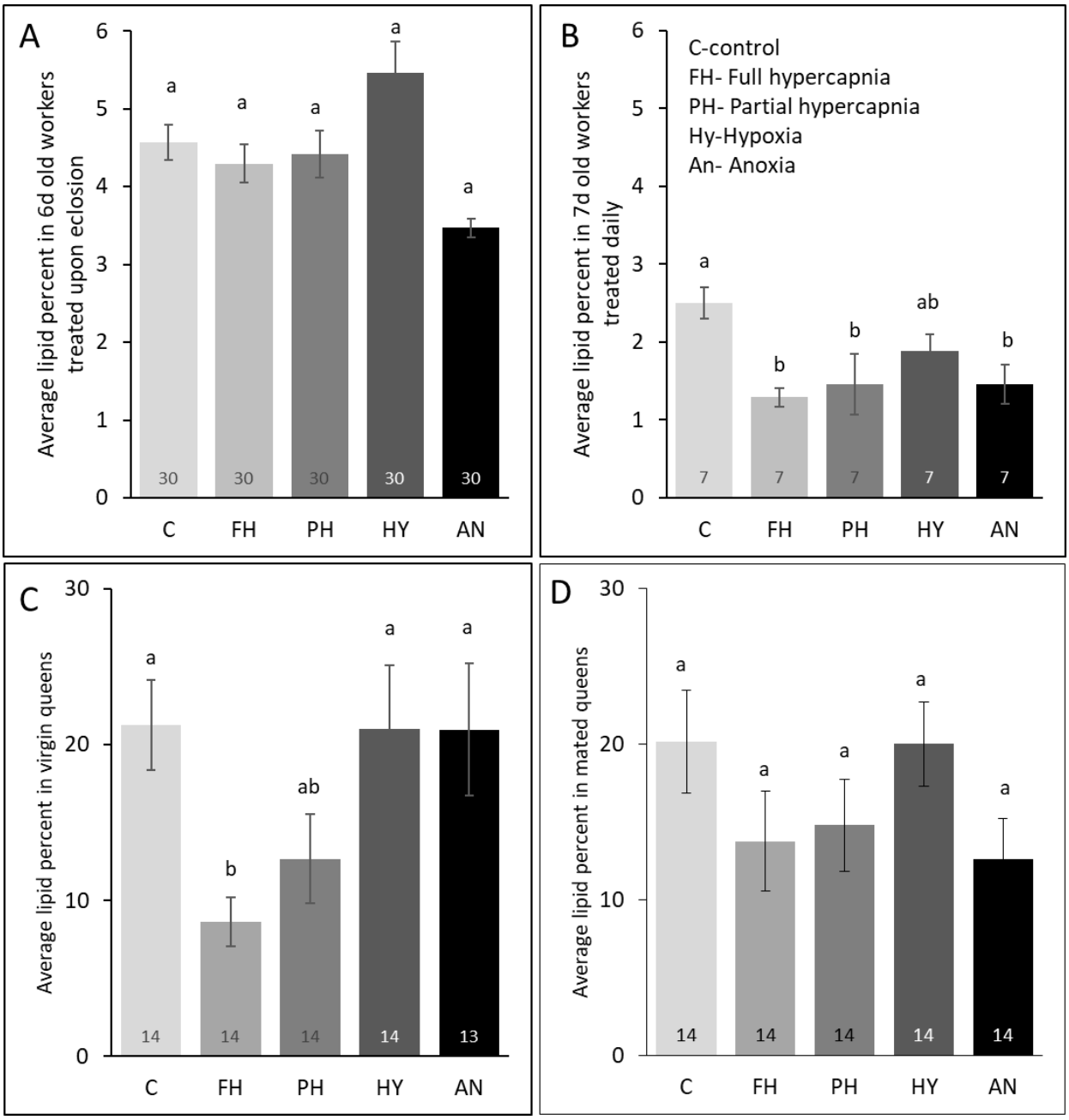

Figure 4. The effect of treatment on the average fat body lipid percent in (A) 6-day-old workers treated upon emergence, and (B) 7-day-old workers treated for six consecutive days after emergence, (C) virgin queens, and (D) mated queens. All queens were frozen 10 days post treatment and were 17-20 days old. The sample size per treatment is denoted using the numbers within columns. Different letters denote significant differences at $\alpha=0.05$. Data are presented as means ± S.E.M.

Figure 5a

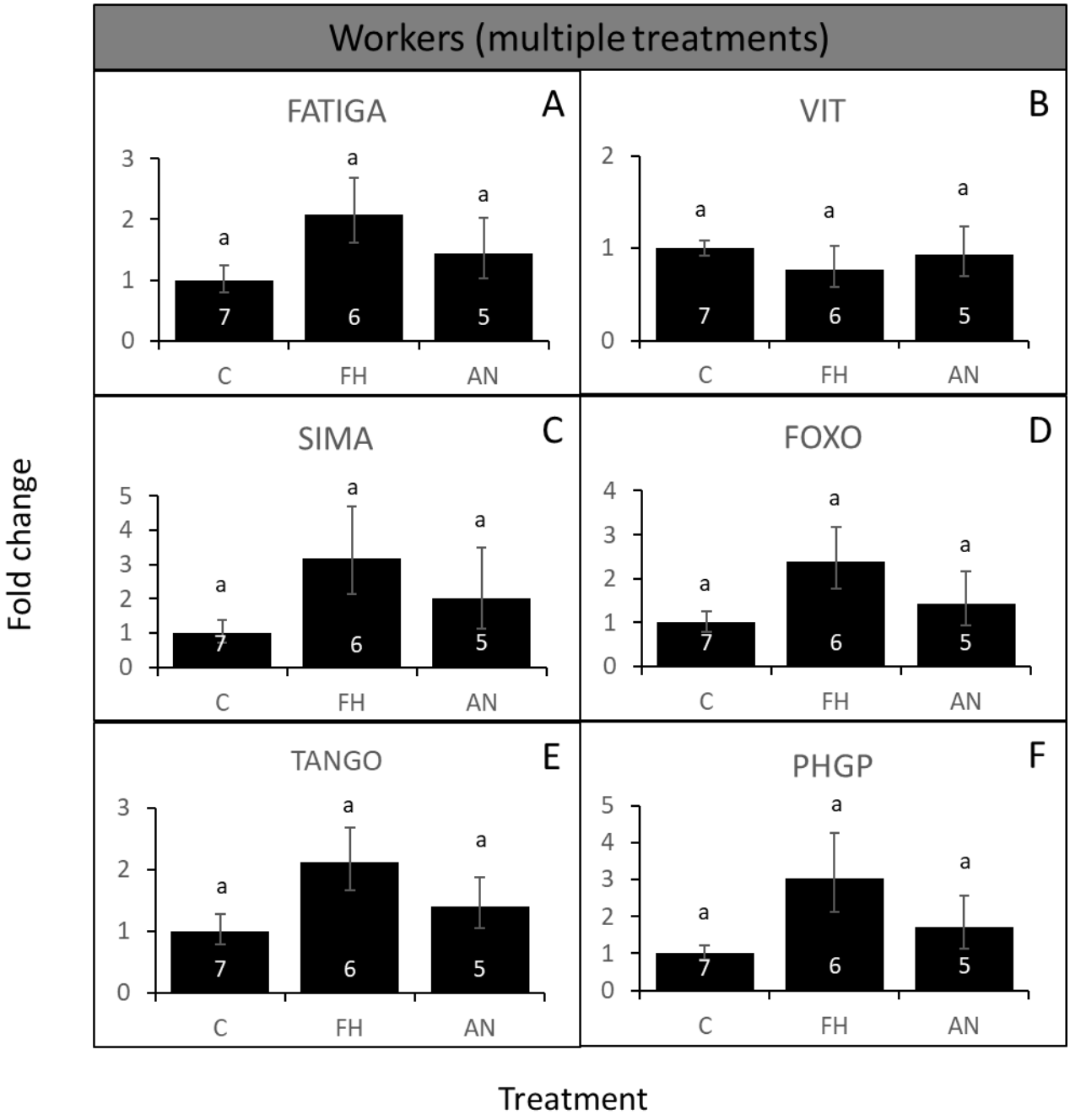
Workers (multiple treatments)
FATIGA
A
VIT
B
SIMA
C
FOXO
D
TANGO
E
PHGP
F
C
FH
AN
Fold change
Treatment

Figure 5b

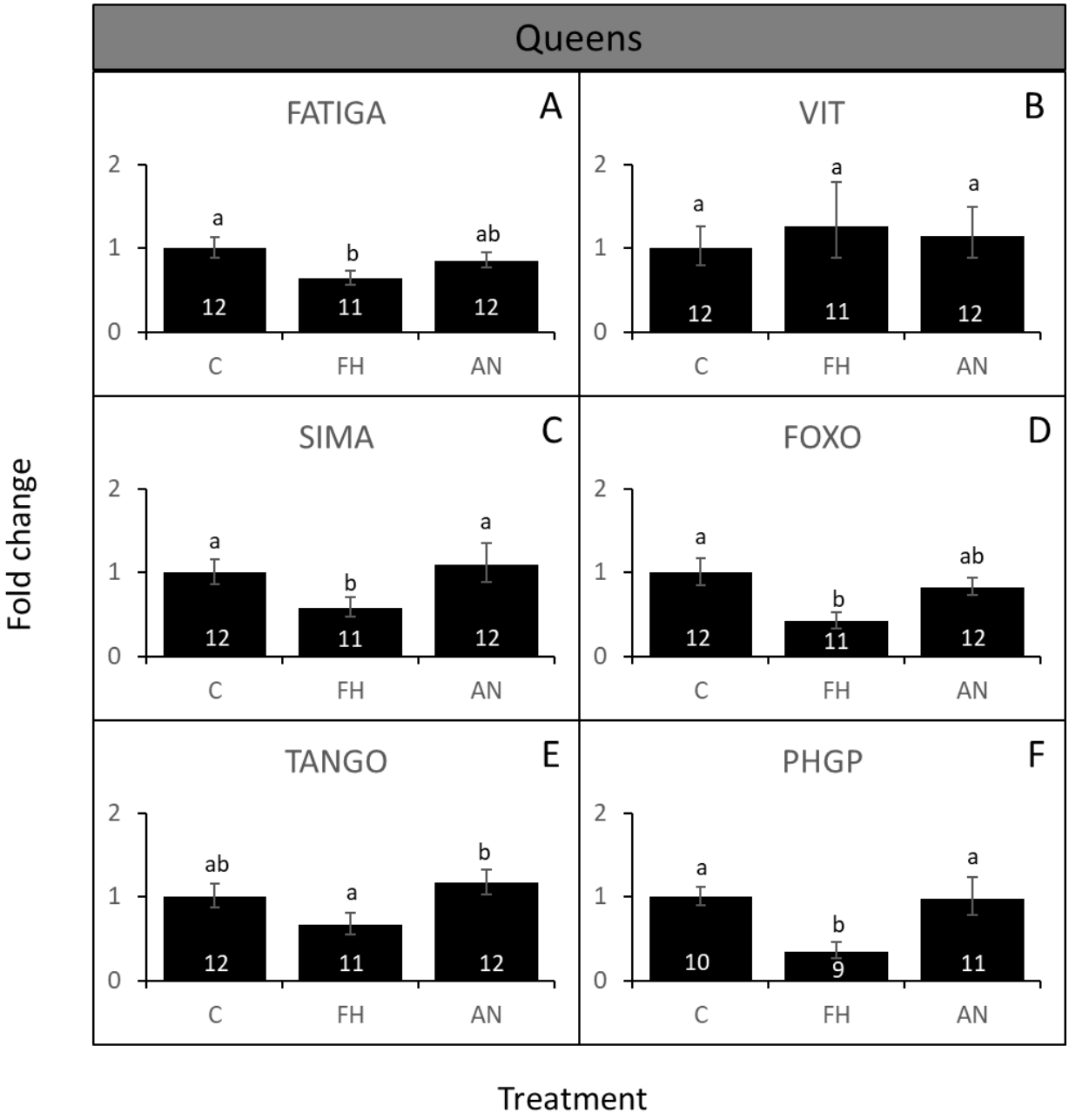

Figure 5. The effect of treatment on the expression level of six genes in *Bombus impatiens* workers (a) and queens (b). Workers were treated daily on days 1-6 after emergence and frozen on day seven. All queens were frozen 10 days post treatment and were 17-20 days old. No differences were found in gene expression of virgin and mated queens and therefore results were combined. The sample size per treatment is denoted using the numbers within columns. Different letters denote significant differences at $\alpha=0.05$. Data are presented as means ± S.E.M.

Supplementary materials

Table S1: The sample size of bees in the study split into the different treatments and variables

		Abdominal contractions (bees)	Aggression (bees)	Ovary activation (bees)	Egg laying (cages)	Fat-body lipids (bees)	Gene expression (bees)
Worker – single treatment (6d)	C	-	30	30	-	30	6
	FH	39	30	30	-	30	6
	PH	30	30	30	-	30	6
	HY	-	30	30	-	30	6
	AN	30	30	30	-	30	6
Worker – single treatment (10d)	C	-	-	-	10	-	-
	FH	-	-	-	10	-	-
	PH	-	-	-	10	-	-
	HY	-	-	-	10	-	-
	AN	-	-	-	10	-	-
Workers – multiple treatments (7d)	C	-	-	42	-	7	7
	FH	-	-	45	-	7	6
	PH	-	-	45	-	7	-
	HY	-	-	45	-	7	-
	AN	-	-	48	-	7	5
Virgin queens	C	-	-	20	20	14	6/4*
	FH	10	-	20	20	14	5/3*
	PH	12	-	19	20	14	-
	HY	-	-	20	20	14	-
	AN	11	-	21	21	13	6/5*
Mated queens	C	-	-	17	10	14	6/6*
	FH	10	-	19	11	14	6/6*
	PH	10	-	18	11	14	-
	HY	-	-	18	10	14	-
	AN	8	-	20	11	14	6/6*

*The first number applies to all genes whereas the second number applies to the gene PHGP, where four samples omitted due to a technical problem.

Table S2: primers sequences and accession numbers of *Bombus impatiens* used in the study.

Gene	Accession no.	F primer	R primers
Phospholipase A2 (HKG)	LOC100747217	CATTTCCGCAAGTGGTAGGT	GGTCACACCGAAACCAGATT
Arginine kinase (HKG)	LOC100741837	GTTGGTAGGGCAGAAGGTCA	AGGTCTACCGTCGTCTGGTG
sima	LOC100746032	GGAAAAGTCTCGTGATGCTGC	AGATGCCTGTTCCGATGGC
tango	LOC100740860	GAAGCACCTGATTCTGGAGGC	TGTAATTCAAAACAGGCGCCAC
fatiga	LOC100747684	CGGACCGATAGTCAGTCGTG	GCACCGTGAACACCTTAGGA
Vitellogenin	LOC10074717	CAGCCGCCAATATGATACCT	CCCTCCGTTCGAAGTGATAA
FOXO	LOC100745007	CAACAACAATCGCAAACAGG	CTCCCATCAATTGTCCCATC
PHGP	LOC100745584	ACTGTACGATGAGTATGCTGAC	TTCGGTACCTCCTGGTTCCT

Table S3: The time to enter anesthesia, the recovery time from anesthesia and the number of abdominal contractions exhibited by bees in 20 minutes from the initiation of movement. Data are presented as means ± S.E.M

	Workers	Virgin queens	Mated queens
Time to enter anesthesia (seconds)			
Full hypercapnia	14.3±0.3	17.8±0.7	18.7±0.4
Partial hypercapnia	34.7±1	29.8±2.2	31.2±1
Anoxia	29.9±0.9	21.5±1.2	22.3±1.3
Effect of treatment	ANOVA Mixed model, $F_{2,158}$=134.3, p<0.0001 followed by Tukey HSD p<0.0001 for all groups		
Effect of caste	ANOVA Mixed model, $F_{2,158}$=5.18, p=0.006 followed by Tukey HSD p=0.004 for workers vs virgin queens		
The recovery time from anesthesia (minutes)			
Full hypercapnia	29.7±1	33.9±1.9	38.4±0.7
Partial hypercapnia	7.07±0.6	31.5±1.4	28.8±0.8
Anoxia	4.33±0.4	21.6±1.1	24.6±2.2
Effect of treatment	ANOVA Mixed model, $F_{2,158}$=72.6, p<0.0001 followed by Tukey HSD p<0.0001 for all groups		
Effect of caste	ANOVA Mixed model, $F_{2,158}$=50.1, p<0.0001 followed by Tukey HSD p<0.001 for workers vs. queens		
Abdominal contractions number in 20 minutes			
Full hypercapnia	1215±116	1190±200	594±161
Partial hypercapnia	1264±162	1384±183	1764±73
Anoxia	526±42	955±198	475±170
Effect of treatment	ANOVA Mixed model, $F_{2,158}$=14.8, p<0.0001 followed by Tukey HSD p<0.03 for all groups		
Effect of caste	ANOVA Mixed model, $F_{2,158}$=1.16, p=0.31		

CHAPTER 3
Conclusions and future directions

Despite CO_2 being a commonly used anesthetic in entomological labs, it may cause many side effects that can impact insect physiology and behavior via the neurological and endocrine systems [1]. The impact on those systems can cause cascading effects throughout the organism [2]. To map these unwanted effects, it is critical to understand the mode of action of CO_2. Below, I will discuss the findings of my study regarding the hypothesis that CO_2 operates via anoxia. I will further discuss several remaining gaps in the field and suggest future experiments that will examine the role of CO_2 in regulating reproduction and metabolism in *Bombus impatiens* queens.

Examining whether the physiological and behavioral changes in *B. impatiens* after exposure to CO_2 narcosis are mediated through anoxia revealed that the effects of CO_2 are unique and are not mediated through anoxia. The effects of CO_2 are also dependent on dose, caste, and mating status of the queens. The impact of CO_2 on ovarian development and gene expression in queens and workers were opposite. CO_2 inhibited the ovarian activation of ovaries in workers, and accelerated ovarian development in queens, whereas genes related to CO_2 and hypoxia were differentially expressed between castes. Additionally, fat body lipid amount was overall higher in queens across treatments as compared to workers, likely because queens enter diapause and were selected to accumulate fat body reserves following emergence. Lastly, the effect of CO_2 on ovarian activation and fat body lipids was weaker in mated queens than in virgin queens. Overall, the impacts on physiology, behavior and gene expression were unique to CO_2 and different from anoxia, with few exceptions that were discussed in the main chapter.

Additionally, caste differences effects by CO_2 were apparent in specific variables, e.g., the impacts of CO_2 on ovary activation were opposite in queens and workers but were the same for lipid storage. In queens, it has been previously found that CO_2 increases juvenile hormone levels [3] which acts as a gonadotropin in bumble bees [4]. This explains the increased ovarian activation of queens, but not in workers. One explanation to settle this could be that while queens were selected to respond to CO_2 in an adaptive way, the same treatment induces stress in workers. This idea is supported by several pieces of evidence as follows: It has been previously hypothesized that CO_2 exposure could impact oxidative stress in tissues by altering reactive oxygen species (ROS) production [5]. Bursts of oxygen influxes upon recovery from CO_2 narcosis creates bursts of ROS production [6], a similar process to the natural changes occurring with aging. Indeed, studies in honey bee workers show that CO_2 narcosis leads to accelerated aging with workers shifting to field tasks (i.e., foraging) that are naturally performed by old bees [7]. In our study, gene expression of hypoxia-inducible factors differed between castes. While hypoxia-inducible-factor genes were downregulated after hypercapnia in queens (compared to control), those same genes were upregulated in response to high CO_2 in workers (though not significantly), suggesting that workers may be experiencing a stress response. Additionally, the antioxidant gene, *PHGP,* was also upregulated in workers, while downregulated in queens [2], supporting the idea that CO_2 may serve as a stressor in workers but not in queens. The differential gene expression we are seeing between castes may be due to different thresholds of low oxygen supply, where workers are more sensitive to the lack of oxygen than queens, or queens can sustain higher levels of oxidative metabolism [8]. These hypotheses, however, need to be tested directly.

While my research addresses a key mechanistic question about the mode of action of CO_2, showing that it acts uniquely, likely on the central nervous system [9], it doesn't address how exactly. Future questions in the field can be separated into two different categories **1)** how CO_2 is affecting the central nervous system? and **2)** from a bee management perspective, what are the long-term effects of CO_2 on colony development? CO_2 has been used as a method for colony foundation, however, the long-term impacts on colony development and the emerging individuals have not been examined.

To further explore the mechanisms underlying CO_2, it would be useful to focus on biogenic amines in the central nervous system that have been suggested to mediate the response to CO_2 and were also shown to regulate insect gonadotropins [8], such as juvenile hormone. Previous studies also indicate that CO_2 enlarges the corpora allata (the main endocrine gland to produce JH), and stimulates JH synthesis [2, 3]. A recent study in *B. impatiens* showed that CO_2-treated queens that were also treated with JH-inhibitor failed to induce reallocation of fat-body lipids, suggesting that JH mediates the effect of CO_2 [10]. The corpora allata is also regulated by biogenic amines, such as dopamine [7], which act as neurotransmitters and neurohormones in the central nervous system of insects [11-13]. Biogenic amine levels are also affected by CO_2 treatment [14-17]. This has been shown in *Apis mellifera* where CO_2 treatment increased dopamine and octopamine and caused a decrease in serotonin levels [14]. Overall, these combined findings may suggest that CO_2 directly alters brain biogenic amines in the central nervous system, which may be responsible for regulating JH and the further downstream effects of CO_2. We can test this hypothesis by manipulating different biogenic amines and their antagonists in conjunction with CO_2 and test whether they induced the typical suite of changes

normally induced by CO_2. Additionally, comparative studies of the mechanisms regulating CO_2 across species could be used to examine whether CO_2 has a conserved mode of action and whether it operates along a similar pathway across taxa [2].

From a management point of view, understanding the long-term effects of CO_2 on colony development could help in optimizing captive rearing of *Bombus* species. Previous studies (including the current) show that CO_2 causes a transition to reproduction in *Bombus impatiens* queens [2, 10, 18]. *Bombus impatiens* is the primary species reared for pollination and laboratory use in North America [19], however, replicating natural rearing conditions in the lab remain challenging. Bumble bees have an annual life cycle, where queens go through winter diapause and emerge in the spring to found colonies [17]. Cold storage has been used to replace natural diapause in the laboratory, but can result in increased mortality of queens [19, 20]. Thus, CO_2 narcosis could be used as an alternative to bypass diapause and stimulate reproduction [19, 21], but was only partially adopted. To fully adopt it, we need to know whether colonies headed by queens that went through CO_2 or cold storage are fundamentally different from each other. A previous study showed that colonies headed by CO_2-treated queens produce overall more males, workers and queens compared to the colonies headed by queens who were kept in cold storage [22]. Additionally, the onset of oviposition was accelerated in the CO_2 treated queens as compared to the cold-storage queens [2, 21, 22].These studies contributed to our understanding of the long-term impacts of CO_2 , however, many other long term effects were not looked at (i.e., differences in worker body size and in gyne quality). Following colony development after mated queens are treated with CO_2 could help cultivate a better management method for increasing the survival and productivity of managed bumble bee populations.

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