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RESEARCH ARTICLE

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

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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, we used *Bombus impatiens* to examine whether CO_2 impacts are mediated by anoxia and whether these mechanisms differ between female castes or following mating in queens. We examined the behavior, physiology and gene expression of workers, mated queens and virgin queens following exposure to anoxia, hypoxia, full and partial hypercapnia, and controls. 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 the ovaries of queens, but inhibited those of workers in a dose-dependent manner and caused a depletion of fat-body lipids in both castes. All responses to 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 bee physiology.

This article has an associated ECR Spotlight interview with Anna Cressman.

KEY WORDS: Carbon dioxide, Insects, Reproduction, Metabolism, Anesthesia

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 (Kwon et al., 2007). They can guide host plant finding and increase foraging efficiency when flowers are open in herbivores (Nicolas and Sillans, 1989), and induce nest digging in ants (Hangartner, 1969; Römer et al., 2018) and nest maintenance behaviors in social bees and termites (Seeley, 1974; Korb, 1999). In contrast, hypercapnic concentrations (above 0.04%) of CO_2 can result in pervasive physiological, molecular and behavioral changes (Nicolas and Sillans, 1989; Guerenstein and Hildebrand, 2008). 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 (Fuzeau-Braesch et al., 1982), Diptera (Barron, 2000; Perron et al., 1972), Hemiptera (Kumar and Saxena, 1978) and Hymenoptera (Stec and Kuszewska, 2020; Czekońska, 2009; Amsalem and Grozinger, 2017; Berger et al., 2015). These impacts include, for example, changes in fecundity and longevity, and reduced climbing behavior following CO_2 treatment in *Drosophila melanogaster* (Perron et al., 1972; Bartholomew et al., 2015); reduced feeding and drinking in *Empoasca devastans* (Kumar and Saxena, 1978); and reduced social aggregation in *Locusta migratoria* (Fuzeau-Braesch et al., 1982; Bartholomew et al., 2015). The specific impacts of CO_2 on reproduction, such as initiating egg laying in queen bees (Roseler, 1985; Mackensen, 1947), 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 (Moloo and Kutuza, 1975), suppressed ovarian development in adult *Tribolium castaneum* (Press et al., 1973), reduced egg laying in *Pyrrhocoris apterus* (Hodkowa and Fuzeau-Baresch, 1988) and lowered ovarian activation in honey bee workers (Amsalem and Grozinger, 2017; Berger et al., 2015; Karsli and Gurel, 2013). 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 (Amsalem et al., 2015a; Engels et al., 1976; Thompson et al., 2007), insulin and juvenile hormone (JH) signaling pathways (Amsalem et al., 2015a) and oxidative stress (Amsalem and Grozinger, 2017; Amsalem et al., 2015a).

Earlier studies examining the mechanisms underlying CO_2 suggested that it affects the nervous system through rapid changes in intracellular pH (Nicolas and Sillans, 1989). Although the hemolymph acidity was shown to increase following CO_2 in numerous species (Sillans and Biston, 1979; Clark and Eaton, 1983; Badre et al., 2005), 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, which could lead to acidic intracellular pH (Nicolas and Sillans, 1989; Woodring et al., 1978; Fuzeau-braesch and Nicolas, 1981; Chuda-Mickiewicz et al., 2012). However, the data supporting this hypothesis are controversial. Although several

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studies have claimed that adverse effects of CO_2 narcosis are caused by low oxygen concentrations (Woodring et al., 1978; Ribbands, 1950; Hooper, 1970), others have provided indirect evidence that hypercapnia and anoxia induced such a different suite of changes that it is unlikely that CO_2 operates via anoxia. For example, in honey bee workers, both anoxia and hypercapnia caused age-related changes and early foraging (Ribbands, 1950), and in termites (*Reticulitermes speratus*), both CO_2 and hypoxic conditions increased reproductive output (Tasaki et al., 2018) and induced similar changes in reproduction-related genes (Tasaki et al., 2020). In contrast, a nitrogen treatment (anoxia) failed to induce a shift from a gregarious to a solitary state in *L. migratoria*, as did CO_2 (Fuzeau-Braesch et al., 1982). 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 (Bierbower and Cooper (2010). Likewise, anoxia resulted in decreased metabolism and an increase in the production of reactive oxygen species (ROS) (Callier et al., 2015; Wegener and Moratzky, 1995), both contradicting the effects CO_2 induced in bumble bees, i.e. downregulation 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 (Amsalem and Grozinger, 2017). 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) (Woodring et al., 1978), 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 questions. Recent studies in *D. melanogaster* and crayfish demonstrated that CO_2 blocks glutamate receptors at the neuromuscular junction and inhibits the recruiting motor neurons within the central nervous system (Badre et al., 2005; Bierbower and Cooper, 2013). In crayfish, the neuromuscular junction 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 acidic pH (5.0). Similar evidence for CO_2 blocking the glutamate receptors was obtained also in *Drosophila* (Badre et al., 2005). There, it was also shown that CO_2 acts not only in the central nervous system, 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 with untreated controls, whereas in caged queens, dopamine expression levels decreased by 45% compared with controls (Harris et al., 1996). Levels of octopamine, content of cAMP, sensitivity of their receptors and the neuroendocrine system were also modified in *L. migratoria* following CO_2 narcosis (Fuzeau-braesch and Nicolas, 1981). Second, JH, a gonadotropin in most insects, was shown to spike following CO_2 narcosis in bumble bees (Amsalem et al., 2015a), and when queens were fed with JH inhibitor, CO_2 had no effect on their ovaries and fat body macronutrient amounts as compared with controls (Barie et al., 2022). Combined, hormonal changes likely follow the neural changes induced by CO_2 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 mechanisms are not fully understood.

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 sexuals 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 (Roseler, 1985; Mackensen, 1947) and inhibition of reproduction in honey bee workers (Koywiwattrakul et al., 2005). It was further shown to induce a metabolic shift in bumble bee queens (in both *B. terrestris* and *B. impatiens*), reduce lipid levels in the fat body, and increase glycogen and protein in the ovaries (Amsalem and Grozinger, 2017; Amsalem et al., 2015a; Barie et al., 2022), as well as to increase the hemolymph levels of JH (Amsalem et al., 2015a). The effects of CO_2 on queen metabolism in *B. impatiens* persisted even when queen ovaries were removed but did not persist when queens were fed with JH inhibitor (Barie et al., 2022), suggesting that CO_2 impacts are mediated via JH and that 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 (Treanore and Amsalem, 2022), 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 of *B. impatiens* (Amsalem and Grozinger, 2017) and changed the gene expression profile of queens following administration in both *B. terrestris* and *B. impatiens* (Amsalem and Grozinger, 2017; Amsalem et al., 2015a). However, data on the impacts of CO_2 throughout the different queen life stages and in workers are lacking. The only other species where the impact of CO_2 was examined across castes is *Apis mellifera*, where JH has lost its gonadotropin role (Amsalem et al., 2014), making it less ideal to test questions related to CO_2 as compared with bumble bees, where JH maintained its gonadotropin function as in other insect species. Altogether, the knowledge available on the impacts of CO_2 in bumble bees and their hormonal similarities to other insects make them 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 queens and mated queens to anoxia (100% nitrogen), hypoxia (14% oxygen and 86% nitrogen), and full and partial hypercapnia (100% and 50% CO_2) treatments, and examined the behavior, physiology and gene expression of the bees as compared with controls. If CO_2 narcosis is induced through the physiological effects of a lack of oxygen (anoxia), we would expect both hypercapnia and 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 would expect both hypercapnia and anoxia to exhibit unique and non-overlapping effects.

MATERIALS AND METHODS

Bees and treatments

Colonies of *Bombus impatiens* Cresson 1863 were obtained from Koppert Biological Systems (Howell, MI, USA) and maintained in environmental chambers under darkness at 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 h 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

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7 cm high). Workers were kept in groups of three, whereas gynes were kept alone. All cages were supplied with unlimited sugar and fresh pollen, the same as the full-size colonies. Cages were randomly assigned to one of five gas treatments: 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 min 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.

Experimental design in 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 groups of three. In the first trial ('single treatment'), workers (n=309) were treated with gas on day 1 of age and were kept for 6 and 10 days post treatment. Following recovery from the gas treatment, workers were immediately observed for abdominal contractions (20 min total) and for aggressive behavior between nestmates (20 min per day) on days 1–6. They were then stored in −80°C 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: (1) workers were treated daily 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 (2) workers were flash-frozen at the age of 7 days 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 attempts to mate (virgin). The mating procedure is described below. Queens were randomly assigned to a gas treatment 24 h post-mating or the attempt to mate and were observed for abdominal contractions immediately after recovery (20 min total). 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 homogeneous in terms of their reproductive status. Queens were flash-frozen and stored at −80°C 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 (35×12×12 cm) with 3–4 males for every queen. Queens were given a 3-h 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 Treanore et al. (2021). The arena was checked every 15 min to observe for mating pairs and pairs were removed while mating and placed back into an individual cage. The pair remained connected by their genitalia for approximately 30 min 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 (ACs) are performed by insects to facilitate gas exchange throughout the body (Tartes et al., 2002; Weis-Fogh, 1967), and typically start upon recovery from anesthesia. The number of ACs was video-recorded and quantified for 20 min 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 time points after the gas treatments.

Aggression in worker groups

Aggressive behavior in workers was observed between days 1 and 6 following exposure in the workers of trial 1 (single treatment). Each cage was observed under red light for 20 min in the morning between 08:00 h and 12:00 h. We recorded and summed four aggressive behaviors typically seen in queen-less groups of *B. impatiens* (Amsalem and Grozinger, 2017): (1) humming (a series of wing vibrations lasting less than 3 s), (2) climbing (one bee crawls on top of another bee), (3) darting (a sudden movement in the direction of another bee) and (4) 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 because they were kept alone.

Egg laying

Cages of workers at the age 10 days and queen cages were checked daily for newly constructed egg cells throughout the experiment. Ten days after the gas treatment (for both queens and workers), 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 (e.g. Amsalem and Grozinger, 2017; Amsalem et al., 2015a).

Fat body lipid analysis

Worker and queen fat body was dissected out and homogenized in 5 ml of 2% sodium sulfate, as in Amsalem and Grozinger (2017). A volume of 200 µl was transferred to a new vial, with 2.8 ml of a 1:1 chloroform:methanol solution. Vials were centrifuged for 5 min at 1000 ***g*** to separate the lower and the upper phase. The supernatant was transferred to a new vial, where 2 ml of distilled water was added, and samples were centrifuged for 5 min at 1000 ***g***. 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 (Amsalem and Grozinger, 2017). 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 to 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 found to be regulated by CO_2 in previous studies, but their response to anoxia is unknown (*vitellogenin*, *FOXO* and *PHGP*) (Amsalem and Grozinger, 2017; Amsalem et al., 2015b), and three hypoxia inducible factors (HIFs) that were regulated by the lack of oxygen in previous studies (Hardy et al., 2012; Cervoni et al., 2017), but their impact on CO_2 is unknown. Genes with known responses 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 with the untreated controls (Amsalem et al., 2015a). *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 (Amsalem et al., 2015a). 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 (Amsalem and Grozinger, 2017). 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 (Cervoni et al., 2017). The forward and reverse primers for each gene were designed 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 manufacturer's instructions (Qiagen, Valencia, CA, USA) and its quality was assessed using NanoDrop One. The RNA of queens was extracted from individual samples whereas heads of workers from the same cage were pooled together. A total of 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 the QuantStudio 5 real-time PCR system (Applied Biosystems). A volume of 2 µl of the diluted cDNA was 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 *B. impatiens* (Amsalem and Grozinger, 2017; Treanore and Amsalem, 2020; Treanore et al., 2020). 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 ACs were compared using a two-way ANOVA mixed model with caste (virgin, mated and workers) and treatment (FH, PH and AN) as fixed effects and colony and cage as random factors. The sum of aggressive behavior in workers was compared using a Kruskal–Wallis test followed by a multiple comparison test using the Wilcoxon method. Ovarian activation, fat-body lipid amount, the total egg production and gene expression levels were compared using an 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* comparisons were performed using a Tukey HSD test. Statistical significance was accepted at α=0.05. Data are presented as means±s.e.m. Data not already included in this paper are available upon request.

RESULTS

The time it took bees to become immobile, the recovery time from the gas treatments and the number of ACs bees exhibited upon recovery were only observed following recovery from anoxia (AN), and 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 with the other treatments, and workers lost mobility quicker than queens following FH but slower than queens following AN (Fig. 1; see Table S3 for means±s.e.m. and results of 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 with queens, but recovery from FH took longer than the other treatments. Finally, we quantified the number of ACs in 20 min 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 number of ACs following PH, then FH, and the lowest number of ACs 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, χ^2_4=8.98, P=0.06, followed by *post hoc* comparisons for FH versus PH (P=0.037), FH versus HYP (P=0.004), FH versus control (P=0.029) and FH versus 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; Fig. 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 versus HYP and control, PH versus HYP and control, and P<0.04 for AN versus 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 with

Journal of Experimental Biology (2023) 226, jeb244746. doi:10.1242/jeb.244746

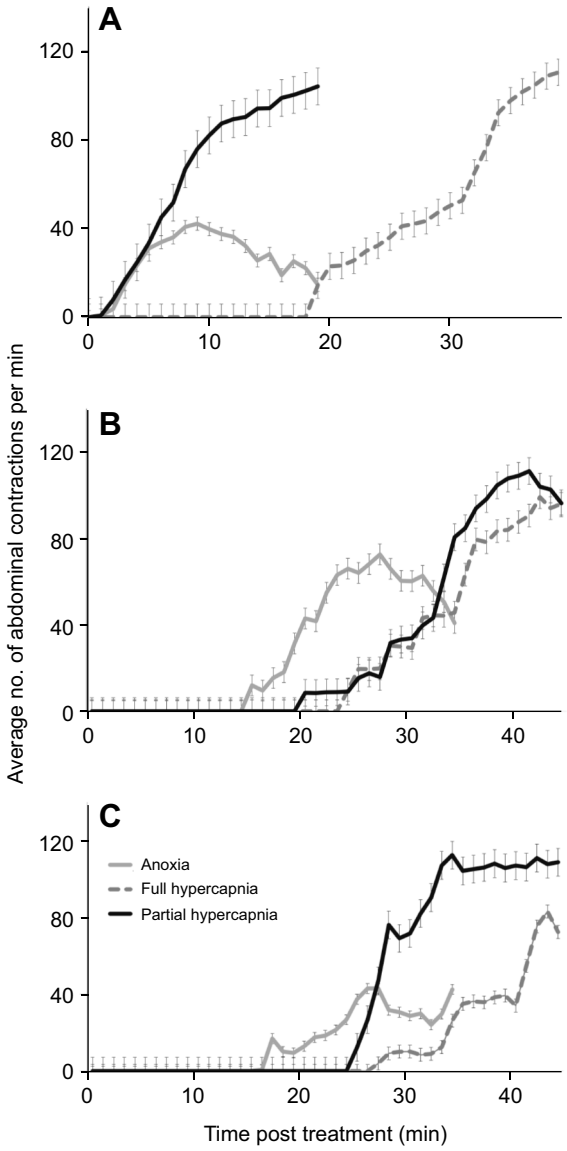

Fig. 1. The recovery time from anesthesia and the effect of gas treatments on the number of abdominal contractions in bumble bees *Bombus impatiens*. (A) Workers, (B) virgin queens and (C) mated queens. Bees were observed for 20 min from their recovery (first movement) in the three treatments that induced anesthesia. Data are presented as means ±s.e.m. Additional data and statistical analyses are provided in Table S3.

hypercapnia and control. In mated queens, the data show a similar pattern to the one of virgin queens with a significant effect of the treatment (ANOVA mixed model, $F_{4,87}$=4.7, P=0.0013, followed by a Tukey *post hoc* test, P=0.03 for AN versus FH, P=0.009 for FH versus control, and P<0.001 for FH versus HYP) (Fig. 3D). Egg laying was observed in all 10-day-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 because queen-less workers start laying eggs within approximately 7 days of emergence, whereas queens require 2–3 weeks to initiate a colony following CO_2 treatment. Thus, 10-day-old workers had more time to lay eggs compared with the queens, which 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, 10 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 with the mated queens.

Lipid percentage in the fat body was overall lower in workers (on average of 4%) compared with queens (average 20%). The treatment did not affect the lipid percentage of workers that were treated once (ANOVA mixed model, $F_{4,45}$=1.3, P=0.297; Fig. 3A), but did affect workers that 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 for AN and PH versus C, and P=0.02 for FH versus 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 with 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 with control (P=0.023), AN (P=0.042) and HYP (P=0.035); Fig. 4C], whereas 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 (*vitellogenin*, *FOXO* and *PHGP*) or anoxia (*sima*, *tango* and *fatiga*) were tested across treatments in workers, virgin queens 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 *tango*: $F_{2,29}$=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 *B. impatiens* workers and queens. Our goal was to determine whether 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 AC 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 owing to the smaller sample size in workers), but overall, these differences 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

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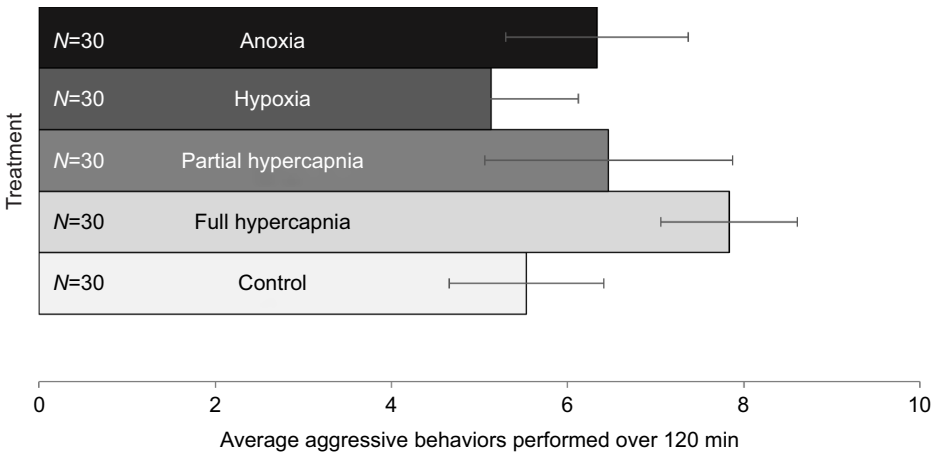

Fig. 2. The effect of treatment on the sum aggressive behaviors performed by *B. impatiens* workers during days 1–6 following emergence. Workers were assigned to five gas treatments on the day they emerged. They were kept in groups of three (n=10 per treatment) and were observed daily for 20 min. Four aggressive behaviors were documented and summed for each worker. Data are presented as means±s.e.m.

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 was the ovaries of virgin queens that were significantly increased by both CO_2 and anoxia, which we discuss below.

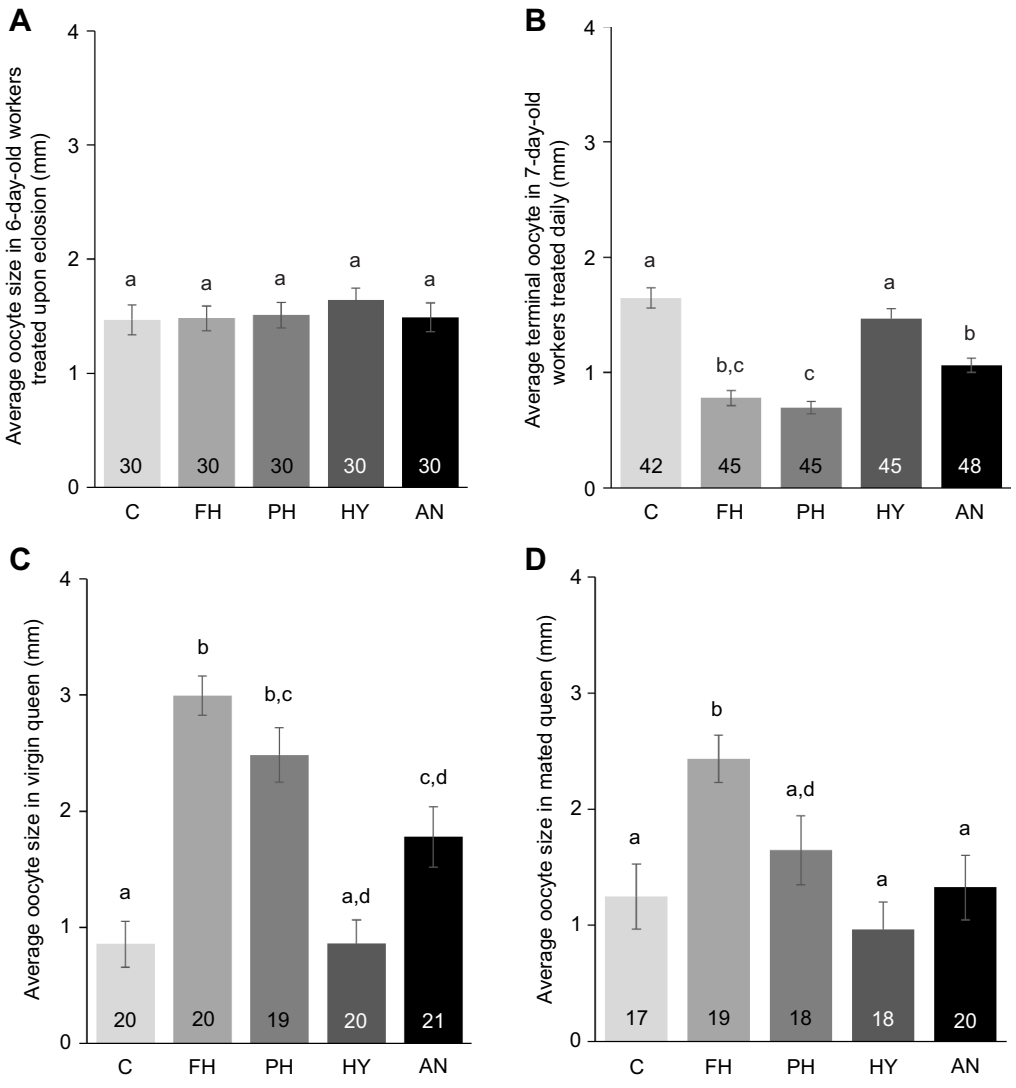

Fig. 3. The effect of gas treatment on the average terminal oocyte size in *B. impatiens*. All queens were frozen 10 days post treatment and were 17–20 days old. The sample size per treatment is denoted using the numbers within columns. Different letters denote significant differences at α=0.05. Data are presented as means±s.e.m. C, control; FH, full hypercapnia; PH, partial hypercapnia; HY, hypoxia; AN, anoxia.

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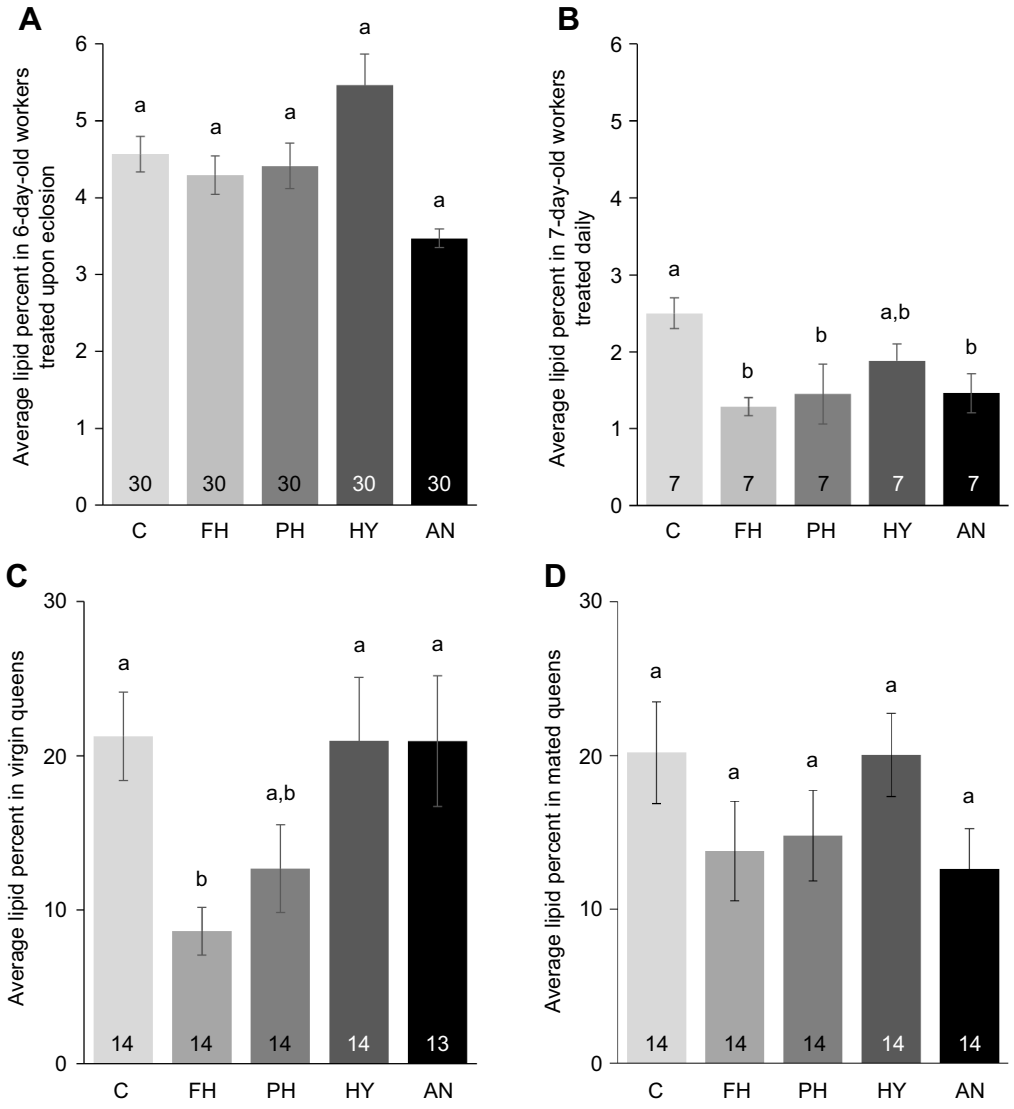

Fig. 4. The effect of treatment on the average fat body lipid percent in *B. impatiens* workers, virgin queens and mated queens. (A) Six-day-old workers treated upon emergence, (B) 7-day-old workers treated for six consecutive days after emergence, (C) virgin queens and (D) mated queens. All queens were 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 α=0.05. Data are presented as means±s.e.m. C, control; FH, full hypercapnia; PH, partial hypercapnia; HY, hypoxia; AN, anoxia.

A second goal of this study was to understand the impacts of CO_2 across female castes and following mating in queens. Here, we discovered that the recovery from hypercapnia was slower in queens compared with workers. We also found that full hypercapnia had a stronger effect compared with 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 although queens respond to CO_2 narcosis, regardless of their age, duration and number of exposures (Amsalem and Grozinger, 2017), workers did not respond to CO_2 when treated upon emergence and required multiple exposures. Similar caste differences were only shown before in *A. mellifera*, an advanced eusocial species where JH, the factor likely mediating the response to CO_2 (Barie et al., 2022), has evolved to no longer act as a gonadotropin as it does in most insect species (including bumble bees) (Amsalem et al., 2014). CO_2 is likely increasing JH levels (Amsalem et al., 2015a; Barie et al., 2022). 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 versus workers. These caste differences are valuable to understanding the mechanisms by which CO_2 operates, but future research should focus on generic models (such as bumble bees) where the function and roles of JH are similar to those in other insects. Overall, the effects of CO_2 were dependent on dose, caste and mating status. 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 demonstrate that the effects of CO_2 narcosis are not mediated through anoxia, meaning that CO_2 has a unique impact on cells, such as through a reduction in extracellular and intracellular pH either by blocking glutamate receptors in the neuromuscular junction (Badre et al., 2005; Bierbower and Cooper, 2013) or by altering the levels of other neurotransmitters and/or neuromodulators in the nervous system. Studying these questions

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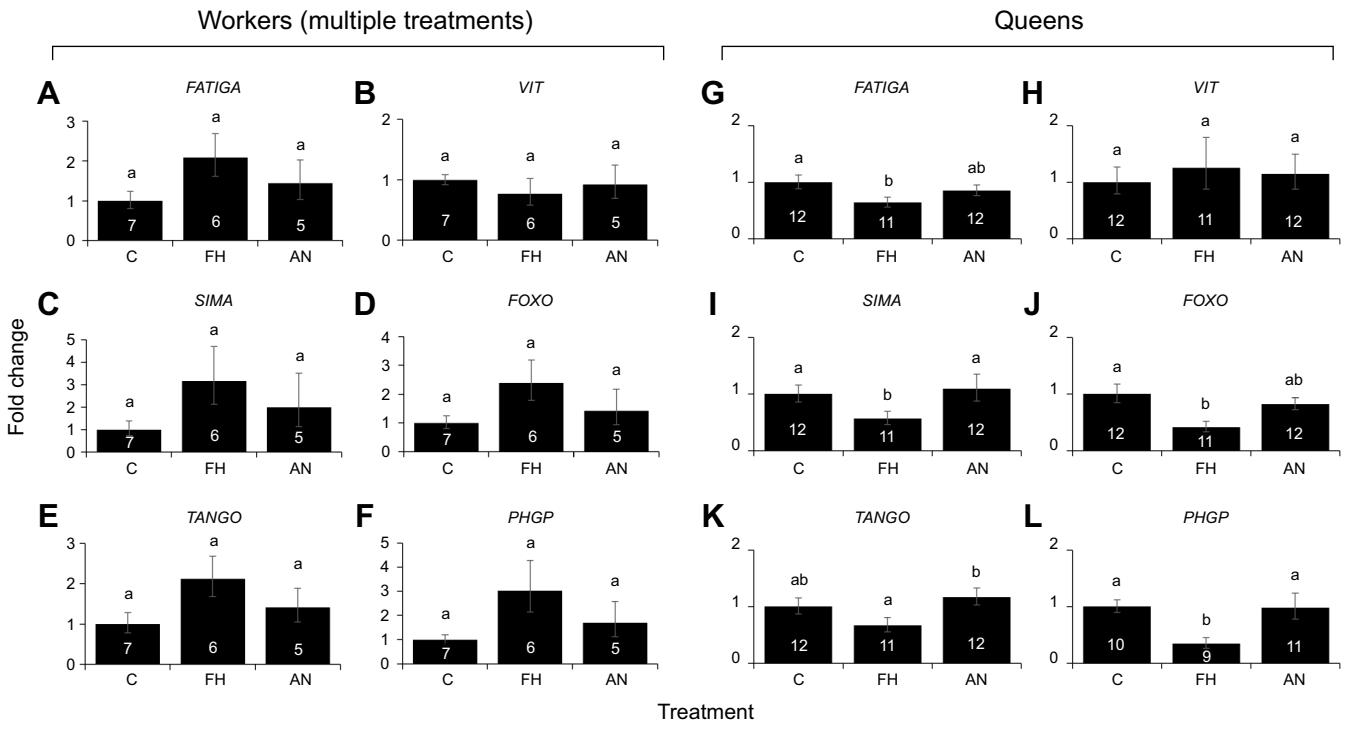

Fig. 5. The effect of treatment on the expression level of six genes in *B. impatiens* workers and queens. (A–F) Workers were treated daily on days 1–6 after emergence and frozen on day 7. (G–L) All queens were frozen 10 days post treatment and were 17–20 days old. No differences were found in gene expression 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 α=0.05. Data are presented as means±s.e.m.

requires experimentation at the cellular level rather than at the level of the entire organism, i.e. the caste differences induced by CO_2 could, for example, be due to different buffering abilities of queens and workers, but examining this idea requires the ability to measure the fluxes of sodium and potassium over range of pH (Breitwieser et al., 1987). 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 (Li et al., 2020; Cook et al., 2019; Sasaki et al., 2017; Brent et al., 2016; Salomon et al., 2012; Sasaki et al., 2009; Cuvillier-Hot and Lenoir, 2006; Bloch et al., 2000; Roeder, 1994), 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 (Vergoz et al., 2012). Selected BAs such as dopamine also correlate with reproductive status in bees in a caste-dependent manner (Bloch et al., 2000). Finally, BA levels can also modify the level of JH and explain its elevated levels following CO_2 narcosis (Amsalem et al., 2015a). All these suggestions, however, await further studies.

In contrast to its caste-dependent effect 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 (Arrese and Soulages, 2010). However, workers of social species that remain sterile may have low lipid reserves if they use them for maintenance tasks that require energy, such as foraging (Toth et al., 2009, 2005; Ament et al., 2011). Foragers show lower reproductive capacity compared with house bees in both the honey bee (Toth et al., 2005) and bumble bees (Amsalem et al., 2014, 2013). The relationship between lipid reserves and tasks may explain how the same impact (on lipid levels) can translate into different reproductive status in queens and workers. It is also in line with the finding that the primary effect of CO_2 on insect physiology is on metabolism, with reproduction being a secondary byproduct of it (Barie et al., 2022). The metabolic shift caused by CO_2 was demonstrated in several species (Amsalem and Grozinger, 2017; Colinet and Renault, 2012; Zhou et al., 2000). 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, which could be triggered by neural changes and affect metabolism.

The weaker effects of CO_2 on mated queens were observed across all parameters examined in the study. Post-mating changes occur widely in insects (Devescovi et al., 2021; Colgan et al., 2019; Gadenne et al., 2001), and include changes in immunity (Colgan et al., 2019; Kapelnikov et al., 2008), stimulation of egg laying (Herndon and Wolfner, 1995) and a decrease in the response to sex pheromones (Devescovi et al., 2021). Because CO_2 also induces some of these changes in bumble bees (Amsalem and Grozinger, 2017), 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 and is dependent on tissue and age (Harrison et al., 2018).

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 the

understanding of the mechanisms of CO_2 narcosis in bees. Further studies at the cellular level could help identify the molecular mechanisms underpinnings of the effect of CO_2 on the neural and endocrine systems and how these changes are translated into metabolic differences in insects.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.C., E.A.; Methodology: E.A.; Validation: E.A.; Formal analysis: A.C.; Investigation: A.C., E.A.; Resources: E.A.; Data curation: A.C.; Writing - original draft: A.C.; Writing - review & editing: E.A.; Visualization: A.C.; Supervision: E.A.; Project administration: E.A.; Funding acquisition: E.A.

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Data availability
All relevant data can be found within the article and its supplementary information.

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