The Pennsylvania State University

The Graduate School

ENVIRONMENTAL AND SOCIAL FACTORS AFFECTING GYNE AND BROOD PHYSIOLOGY IN THE BUMBLE BEE *BOMBUS IMPATIENS*

A Thesis in

Entomology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

May 2022

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ABSTRACT

Bumble bees have become widely used for pollination services and as a model species in research; as a result, over a million colonies are reared each year. However, the knowledge about factors determining brood body size and caste and how common methods of rearing affect queen physiology is still partial. This study examines the physiological effects of CO_2 narcosis, and factors influencing bumble bee brood growth and development. First, I evaluated the metabolic influence of CO_2 narcosis on *Bombus impatiens*. CO_2 narcosis is a common rearing practice used to initiate egg-laying in gynes but the mechanisms by which CO_2 causes these physiological changes are poorly understood. My findings demonstrate that CO_2 induces reallocation of macronutrients in gyne tissues and that these effects are independent from the impact of CO_2 on reproduction. I further confirmed the role of juvenile hormone in mediating macronutrient allocation. In addition, I investigated the mechanisms regulating body size and caste in *Bombus impatiens* colonies. Particularly, I examined development time and weight gain in larvae females (queen/worker). My results show that the number of nurses, but not their caste, influenced body size. However, neither the number of nurses, nor their caste, affected caste determination. Overall, these data broaden our understanding of the metabolic changes induced by CO_2 and the mechanisms determining size and caste in bumble bees. These findings can be further used to inform and optimize bumble bee rearing practices.

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ACKNOWLEDGEMENTS

I would like to thank my committee and members of the Amsalem lab for all their feedback and advice over the course of this project. Funding for this work was provided by United States-Israel Binational Agricultural Research and Development Fund and The Penn State Graduate Training Program in Integrative Pollinator Ecology, funded by the USDA-NIFA-NNF Program (2017-38420-26766). Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author and do not necessarily reflect the views of the United States Department of Agriculture.

CHAPTER 1
Introduction

An estimated 70% of the world's agricultural crops rely on bee pollination (Klein, Vaissière et al. 2007). As a result, some bee species are commercially managed to provide pollination services. Commercial rearing of bumble bees began in the late 1980s and has since increased significantly (Velthuis and Van Doorn 2006). Commercial bumble bees are utilized to pollinate a variety of crops such as peppers, eggplant, strawberries, and blueberries (James, James et al. 2008, Yankit, Rana et al. 2018, Nayak, Rana et al. 2020). As bumble bees started being reared for pollination purposes, they also became a popular model system to study questions related to social behavior, insect learning and memory, sexual selection, and threats to bee populations (Baer 2003, Riveros and Gronenberg 2009, Woodard, Lozier et al. 2015). A recent study demonstrated a dramatic growth in peer-reviewed papers about bumble bees in the last century (Treanore, Barie et al. 2021). The increased use of bumble bees for pollination and research has resulted in over a million bumble bee colonies being commercially reared each year (Velthuis and Van Doorn 2006). Despite the extensive use of bumble bees, many questions related to their sociobiology, development and rearing remain open.

Bumble bees have an annual colony cycle. In temperate climates, bumble bee gynes initiate colonies in the spring after emerging from a months-long winter diapause (Alford 1969). After finding a place to nest, gynes lay their first batch of eggs. At the beginning of the colony development, queens are responsible for all nest tasks such as foraging and caring for developing brood. Following the emergence of workers, queens stop foraging and focus on laying eggs, while workers become the foragers and the primary caretakers of the brood (Alford 1975). Towards the end of the colony life cycle, reproductive individuals (males and gynes) are produced (Alford 1975). The queen is the only individual in the colony able to lay female eggs which may be developed into new gynes. However, male eggs can be laid by both the queen and workers (Cnaani, Schmid-Hempel et al. 2002). Sexuals will disperse and mate whereas gynes will also accumulate energy stores before entering the winter diapause. Shortly after, the colony declines; the workers and the old queen will eventually die.

During their life cycle, bumble bees go through a winter diapause allowing them to sustain the low temperatures and lack of floral resources. Ovarian activation and colony initiation generally occur after diapause (Alford 1975, Denlinger 2008). The winter dormancy period is achieved in the laboratory and commercial rearing facilities by placing gynes in cold storage for 2-3 months (Beekman and Van Stratum 2000, Lindsay 2020). However, this cold storage period lengthens the process of producing colonies and results in high mortality (Lindsay 2020). A more efficient method of stimulating ovarian activation and colony initiation in gynes is to anesthetize them with 100% carbon dioxide gas (CO_2 narcosis) (Roseler 1985, Amsalem and Grozinger 2017) for one minute. CO_2 narcosis is often used in bumble bee rearing because it is a fast and effective way to induce colony initiation without the added costs associated with cold storage, but its impacts on gyne physiology are not entirely clear.

CO_2 narcosis results in a variety of physiological changes in bumble bee gynes. Gynes treated with CO_2 display an increased production of juvenile hormone (JH), ovarian activation, and suppression of lipid accumulation (Amsalem and Grozinger 2017). These physiological changes are similar to the changes gynes experience naturally following the termination of diapause and before they initiate a colony. These changes are not specific to bumble bee queens; the induction of reproduction by CO_2 has been documented in other social insect species such as honey bees and termites (Seeley 1974, Roseler 1985, Tasaki, Komagata et al. 2020). In addition to being used to trigger ovarian activation, CO_2 narcosis is commonly used in insect research as a means of anaesthetization (Fuzeau-Braesch and Nicolas 1981, Nicolas and Sillans 1989). Acute exposure to CO_2 has been shown to influence insect behavior, reproduction, and survival (Nicolas and Sillans 1989, Guerenstein and Hildebrand 2008, Amsalem and Grozinger 2017). Additionally, CO_2 narcosis has also been shown to have unintended effects on immunity (Helenius, Krupinski et al. 2009, Amsalem and Grozinger 2017), and honey bees and drosophila exposed to CO_2 have incurred memory loss and changes to climbing and flying behaviors (Bartholomew, Burdett et al. 2015, Amsalem and Grozinger 2017). Many of CO_2 pleiotropic impacts in insects are related to changes in metabolism and reproduction (Perron, Huot et al. 1972, Faucher, Forstreuter et al. 2006, Helenius, Krupinski et al. 2009, Colinet and Renault 2012, Bartholomew, Burdett et al. 2015, Amsalem and Grozinger 2017). CO_2 narcosis alters metabolites in fruit flies (Colinet and Renault 2012), lipid levels in bees (Amsalem and

Grozinger 2017), and metabolic rate in moth pupae and crickets (Woodring, Clifford et al. 1978, Zhou, Criddle et al. 2000). CO_2 exposure has differential effects on reproduction across insects, increasing ovarian activation in some insects while suppressing ovarian activation in others (Roseler 1985, Thompson, Yockey et al. 2007, Tasaki, Komagata et al. 2020). Juvenile hormone has been shown to increase in insects exposed to CO_2 narcosis (Amsalem, Galbraith et al. 2015), and mediates a variety of physiological processes including reproduction and metabolism (Williams 1956, Wyatt and Davey 1996). JH serves as a gonadotropin in most insects (Santos, Humann et al. 2019), and treatment with JH analogs causes a decrease in fat body stores of honey bee queens, *Bombus terrestris,* and crickets (Bühler, Lanzrein et al. 1983, Zera and Zhao 2004, Wang, Brent et al. 2012, Shpigler, Magory Cohen et al. 2021). Given the similar effect of JH and CO_2, JH may be mediating the changes associated with CO_2 narcosis, however, this has yet to be tested. Despite the widespread use of CO_2 narcosis in bumble bee rearing and the ubiquitous effects of CO_2 across insects, the physiological mechanisms underlying the effects of CO_2 in insects are not well understood.

Chapter 2 examines the metabolic changes induced by CO_2 narcosis in *Bombus impatiens* gynes and the physiological mechanisms regulating these changes. This study examined how macro-nutrient amounts change in various tissues of gynes exposed to CO_2 narcosis. To get a better understanding of the mechanism regulating CO_2 impact, I further teased apart the impact of CO_2 on metabolism and reproduction and tested the role of juvenile hormone in mediating the impact of CO_2 on gyne physiology. I evaluated the metabolic changes following CO_2 treatment in non-reproductive gynes by removing gynes ovaries. To determine if JH mediates gynes response to CO_2, JH titer was blocked using a JH antagonist before and after receiving CO_2 treatment.

Worker body size and gyne production are also important in rearing colonies for pollination and research purposes (Jauker, Speckmann et al. 2016, Holland, Nakayama et al. 2021, Treanore, Barie et al. 2021). Bumble bees have two female castes (queens and workers) in addition to males (Michener and Michener 1974, Goulson 2010). Queens are the reproductive caste that initiates a colony and produces most of the offspring (Michener and Michener 1974). However, bumble research relies on purchasing commercial colonies in advanced age or on the natural and highly variable production of gynes in already established colonies. Gyne production varies not

only between colonies, but also across species (Duchateau 2004), and stimulating gyne production is difficult due to a limited understanding of caste determination mechanisms and how environmental factors influence gyne production. The body size of workers is also variable, and its regulation is not well understood. The worker caste is primarily responsible for nest tasks and foraging. Though workers are always smaller than the queen, their body size is highly variable, with body mass varying up to 10-fold between workers. For most crops, larger bodied workers are more effective at pollinating (Kapustjanskij, Streinzer et al. 2007). Additionally, large-bodied workers are better at foraging, increasing the amount of pollen and nectar brought back to the colony (Spaethe and Weidenmüller 2002, Willmer and Finlayson 2014, Jauker, Speckmann et al. 2016), which in turn influences colony growth and gyne production (Williams, Regetz et al. 2012, Malfi, Crone et al. 2019). Improving rearing practices to produce gynes and larger workers would improve research and pollination services provided by bumble bees.

Optimizing bumble bee rearing is challenging since our understanding of the environmental factors impacting gyne production and worker body size is incomplete. Cross fostering experiments (i.e., swapping genetically identical brood between different environmental conditions) in *Bombus terrestris* suggest neither body size nor caste are genetically determined (Cnaani, Robinson et al. 2000), and are likely mediated by environmental variables. The mechanisms and the environmental factors influencing caste determination differ across bumble bee species. Research on *Bombus hypnorum*, *Bombus rufocinctus*, and *Bombus ternarius* suggests queen determination occurs late in development and depends on food quantity and feeding regimes during development (Röseler 1970, Röseler and Röseler 1974, Plowright and Jay 1977, Plowright and Pendrel 1977). In *B. terrestris* however, caste is determined early in larval development (Pereboom, Duchateau et al. 2003). In social insects, brood fate is often modulated by the adult members of the colony who care for the brood. Brood care is largely influenced by a colony's social environment, which is a combination of variables such as colony age, size, and interactions between the queen, workers, and brood (Chole, Woodard et al. 2019). The impact of the social environment on caste and worker body size was mainly studied in *Bombus terrestris*, and anecdotally also in several other species, but due to the variation in these mechanisms across species, data obtained so far cannot be applied to *B. impatiens*.

Research on bumble bee brood growth and development could aid in manipulating colonies to produce gynes and larger workers. Chapter 3 investigates how the social environment influences gyne production and brood development and body size in *Bombus impatiens*. Particularly, this chapter examines how the number and identity of caretakers affect the brood's developmental duration, weight, and resulting caste.

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CHAPTER 2

CO_2 narcosis induces a metabolic shift mediated via juvenile hormone in *Bombus impatiens* gynes

Introduction

Carbon dioxide is an environmental stimulus commonly encountered by insects that induces a diverse range of behavioral and physiological effects (Coviella and Trumble, 1999; Guerenstein and Hildebrand, 2008; Jones, 2013; Nicolas and Sillans, 1989). Elevated concentrations of CO_2 in the atmosphere trigger various behavioral responses such as fanning in honey bees (Seeley, 1974), nest digging in ants (Hangartner, 1969; Römer et al., 2018), feeding behavior in herbivorous insects (Couture et al., 2015), and attraction in blood-feeding insects (Jones, 2013; Vale, 1980). Insects are able to sense minor atmospheric differences using CO_2-sensitive external-designated receptors in the peripheral sensory system (Kwon et al., 2007) that are conserved across species (Robertson and Kent, 2009) and tightly regulate CO_2 levels in their habitats, as was demonstrated in several social insect species (Römer et al., 2018; Seeley, 1974; Weidenmüller et al., 2002).

The response to acute exposure to CO_2 is significantly less understood compared to atmospheric CO_2 despite its wide use as a method to immobilize insects in the lab (Nicolas and Sillans, 1989). High levels of CO_2 induce a fairly stressful response in insects due to increased hemolymph acidity and reduced oxygen delivery to tissues (Badre et al., 2005; Harrison, 2001; Nicolas and Sillans, 1989). Insects exposed to high concentrations of CO_2 exhibit diverse changes in physiology, such as changes in ovary activation and reproduction (Press et al., 1973; Roseler, 1985; Tasaki et al., 2020; Thompson et al., 2007), but also display changes in immunity, hormone levels, lipid storage, gene expression and insemination frequency (Amsalem et al., 2015a; Amsalem and Grozinger, 2017; Helenius et al., 2009; Moloo and Kutuza, 1975). Behavior changes following CO_2 include an increase in aggression and flight behavior in bumble bees (Amsalem and Grozinger, 2017) and, conversely, impaired climbing and flight behavior in *Drosophila* (Bartholomew et al., 2015). Often, desired impacts of CO_2 cause additional unintended effects. For example, honey bee queens treated to induce reproduction and

cockroaches immobilized using CO_2 incurred memory loss (Freckleton and Wahlsten, 1968; Stec and Kuszewska, 2020). Similarly, bumble bee workers treated with CO_2 eject healthy larvae out of the colony (Skyrm et al., 2009). While widely used, the mechanisms underlying the acute response to CO_2 narcosis, and whether they are conserved across insects are mostly unknown.

Many of the known impacts of CO_2 narcosis are associated with metabolism and reproduction, but so far, no attempt was made to tease apart their effects following CO_2 narcosis. In fruit flies, CO_2 narcosis alters whole body metabolites up to 14 hours following exposure (Colinet and Renault, 2012). In bumble bee queens (*Bombus terrestris*), lipids were depleted 24 hours following CO_2 treatment (Amsalem and Grozinger, 2017), and moth pupae (*Platynota stultana*) showed a rapid decrease in metabolic heat rate when exposed to 20-79% CO_2 (Zhou et al., 2000). Ovarian activation is altered by CO_2 in a wide range of species, with accelerated ovary development in honey bee and bumble bee queens (Amsalem and Grozinger, 2017; Seeley, 1974), and termites (Tasaki et al., 2020), and suppression of oocyte development in beetles (*Tribolium castaneum*) (Press et al., 1973), *Drosophila* (Helenius et al., 2009), and honey bee workers (Thompson et al., 2007).

Insect reproduction is often associated with reallocation of stored macronutrients (Wu et al., 2021) such as lipids, proteins and carbohydrates in various tissues. Lipid and glycogen are the main fat body reserves and the main source of metabolic fuel insects use during diapause (Arrese and Soulages, 2010). Lipid composition and amount change with life stage and activity (Amsalem et al., 2015a; Arrese and Soulages, 2010; Votavova et al., 2015). Several proteins are involved in the shift to reproduction: vitellogenins are synthesized in the fat body, transported to the hemolymph, and incorporated into the ovaries (Wu et al., 2021). Storage proteins are used as a reservoir for amino acids, and lipid carriers are responsible for the transport of lipids from one tissue to another (Arrese and Soulages, 2010). Others, such as heat shock proteins may be produced in preparation for, during, and sometimes after diapause (Denlinger, 2002). Proteins also surround lipid droplets, and are involved in their storage and metabolism (Walther and Farese, 2012). Carbohydrates are a common fuel for flight (Suarez et al., 2005) and glycogen, in particular, is often used to initiate flight in insects, a behavior that is often associated with reproduction. Bumble bee gynes, for example, increase their mobility to forage and look for a nest before initiating a colony (Amsalem and Grozinger, 2017). Finally, crop sugars can be used

as a carbohydrate source during diapause, as was demonstrated in several bumble bee species (Alford, 1969a). Gynes transitioning to reproduction in response to CO_2 may no longer accumulate sugar stores in their crop. Studying shifts in these macronutrients in response to CO_2 can be used to characterize the metabolic changes following CO_2 and identify whether CO_2 primer effect is on reproduction or metabolism.

A potential mediator of metabolic changes that can also explain the reproductive changes caused by acute exposure to CO_2 in social insects is juvenile hormone (JH), which serves as a gonadotropin in most insects and regulates molting in insect larvae (Santos et al., 2019). JH titers have been shown to increase in response to CO_2 narcosis in bumble bees (Amsalem et al., 2015a), honey bees (Robinson et al., 1991), and locusts (Fuzeau-Braesch et al., 1982). Treatment with JH analogs also caused a decrease in fat body stores and an increase of lipid and carbohydrates in the hemolymph of honey bee and crickets (Bühler et al., 1983; Wang et al., 2012; Zera and Zhao, 2004). Similar results were obtained in *Bombus terrestris* (Shpigler et al., 2021). The presence of JH during larval molting prevents metamorphosis and ensures that the molt will produce another immature instar, directly affecting the metabolic state of the larva and its ability to acquire and store nutrition (Jindra et al., 2013). For example, JH presence during larval development in *Bombus terrestris*, delays molting and leads to the production of larger individuals (queens) (Cnaani et al., 1997). Given the positive link between CO_2 and JH and their similar effects on insect physiology, it has been suggested that CO_2 impact is mediated through elevated JH levels, but this has not been tested yet.

Here we examine the effects of CO_2 treatment on nutrient storage and allocation and the mechanisms underlying CO_2 mode of action in the common eastern bumble bee *Bombus impatiens*. We further tease apart the effects of CO_2 on reproduction and metabolism and explore the role of JH in mediating these effects. Bumble bees are an excellent system to examine these questions since CO_2 narcosis is a common practice to induce reproduction and bypass diapause in newly emerged queens (gynes) (Roseler, 1985). Bumble bee gynes emerge towards the end of the season, mate, and typically enter a winter-diapause that may last 6-9 months (Alford, 1969b). Gynes then activate their ovaries and initiate a nest the following spring. In the laboratory, the winter diapause period can be replaced with cold storage, which prolongs colony establishment and results in high gyne mortality (Lindsay, 2020). As an alternative, gynes can be treated with

carbon dioxide gas (CO_2 narcosis). Exposure to 100% CO_2 results in profound changes to queen physiology and behavior, some of which are wanted (accelerated ovary development and egg-laying) and some of which may be unwanted, e.g., reduced lipid storage (Amsalem et al., 2015a; Amsalem and Grozinger, 2017). A previous study examined the effects of CO_2 narcosis in *Bombus impatiens* gynes showed a reduction in fat body lipids but no change in glycogen levels 24 hours after the CO_2 treatment (Amsalem and Grozinger, 2017). However, the involvement of other tissues and macronutrients over an extended period of time were not examined and the mechanisms underlying these changes remain elusive.

We examined macronutrient levels of protein, lipid, glycogen, and sugar in four tissues/body parts (fat body, ovaries, thorax, and crop) at three timepoints following the exposure of gynes to acute CO_2 as compared to untreated controls. We also compared gyne ovary activation following exposure to CO_2. We hypothesize that CO_2 treatment will result in metabolic changes associated with the transition to reproduction. To examine whether the observed metabolic changes are the result of CO_2 treatment or a byproduct of the transition to reproduction, we monitored the metabolic changes in gynes following ovary removal and CO_2 narcosis. We hypothesize that CO_2 causes metabolic changes independent of ovarian activation and its impact on lipids will not be affected by the removal of ovaries. Additionally, we examine if JH mediates the metabolic and reproductive changes associated with CO_2 treatment by manipulating the gyne diet using a JH antagonist (precocene) and examining fat body and ovary macronutrients. We predicted that the impact of CO_2 on metabolic changes will be blocked in gynes treated with precocene.

Methods

Bumble bee rearing

Colonies of *Bombus impatiens* were obtained from Koppert USA, Inc. (Romulus, MI). The colonies were kept in a dark chamber at 28-30° C and 60% relative humidity with an unlimited supply of pollen and 60% sugar solution. In the first experiment examining metabolic changes in gynes following CO_2 narcosis (Fig 1A), we used 54 gynes that were randomly sampled from 3 colonies. Gynes were collected upon emergence, assigned number tags, and individually placed in plastic cages (11 cm diameter × 7 cm height) that were maintained under the same conditions as the colonies. Seven days later, gynes were given two treatments: untreated controls (UT) or

CO_2 narcotized and were transferred to larger flight cages (35 x 21 x 12 cm). During the entire experiment gynes were maintained in individual cages. Gynes were then sampled at three timepoints: right after the treatment, 3 or 10 days later (thereafter, UT/0, UT/3, UT/10, CO_2/0, CO_2/3, CO_2/10). Sampling timepoints were chosen based on a previous study showing that ovarian activation occurs roughly 5 days after CO_2 treatment (Amsalem and Grozinger, 2017). After sampling, all gynes were kept at -80° C and were examined for ovarian activation and macronutrient concentrations in four tissues/body parts: the fat body (lipids, glycogen, proteins), ovaries (lipids, glycogen, proteins), thorax (proteins, glycogen), and crop (proteins and sugars). Gynes were starved for 2 hours prior to sampling to ensure the crop is empty. In the second experiment (Figure 1B), we sampled 64 gynes upon emergence from 4 colonies and assigned them to one of four treatments: (1) ovary removal (see below); (2) sham treated gynes who were dissected in the same manner as the ovariectomized gynes but without removing their ovaries; (3) precocene fed gynes (see below); (4) control gynes that remained untreated. All the gyne groups were divided randomly at the age of 7 days and half of them were treated with CO_2 narcosis. As in the first experiment, gynes were kept for an additional 10 days before they were frozen and analyzed for ovarian activation and body composition.

Carbon dioxide treatment

CO_2 (100%) was administered for 1 minute by placing individual gynes in a closed cage (11 cm diameter × 7 cm height). Gynes were kept in this cage for 30 minutes following the treatment, allowing CO_2 to slowly seep out until they were revived. After revival, gynes were either sampled (first timepoint in experiment 1) or transferred to flight cages and sampled 3 or 10 days later.

Ovary removal

Ovary removal was performed in newly-emerged gynes after removal from the parental colony. Ovary removal was preformed while gynes were callow because after emergence the cuticle of adult bumble bees is relatively soft and is easy to manipulate (Shpigler et al., 2014). Gynes were first anesthetized by chilling at 4° c for 5–10 min. Bees were fixed to a small sterile eraser with the ventral side up and a small incision was made between their 4^{th} and 5^{th} sternites (Doorn, 1989). The ovaries were then entirely removed using fine forceps. The tergites were then sealed together using nontoxic glue to shield the incision from bacteria. Both the tools and the incision

area were sterilized before and after the surgery using 70% ethanol. Tools were also sterilized by holding them into a flame for few seconds before every use. Sham-operated bees were handled and dissected similarly however, the ovaries were only gently touched and not detached. About a third (31%) of the gynes died following the surgery and mortality usually happened within a day. The sample size described in the text is of surviving queens.

Precocene treatment

Gynes were fed with precocene-I (Sigma-Aldrich, purity 99%) mixed into their sugar water similar to the methodology described in (Amsalem et al., 2014). A daily dose of 1.5 µg of precocene-I mixed with 90 µl of 60% sugar solution was provided to the gynes until they fully consumed it (typically within 8-10 h, each gyne was kept separately). Gynes were then provided with unlimited, untreated 60% sugar solution for the remainder of the day. Gynes were fed with precocene each day of the experiment from day 1 to 17. Untreated queens were also provided with 90 µl 60% sugar solution until they consumed it, but the sugar solution did not contain precocene. As in all experiments, queens were frozen and analyzed on day 17.

Dissection and tissue processing

Individual gynes were weighed using an electronic scale and dissected under a stereomicroscope. In the first experiment, four tissues/body parts were separated from each gyne and placed in 2% sodium sulfate solution with sterile zirconia beads: Fat body (1 ml), Thorax (1 ml), crop, and ovaries (500 ul each). The fat body tissue included the visceral cuticule excluding the abdomen content and the sting complex (Amsalem et al., 2015a; Treanore and Amsalem, 2020). The ovaries were measured prior to homogenization (see below). All samples were homogenized using fast prep machine and were stored at -20° C until further analyses. In the second experiment, we analyzed only the fat body and the ovary tissues based on the results of experiment 1.

Ovary activation

Oocyte size for each gyne was categorized as “activated” (fully mature oocytes ready to be laid, > 2 mm), “inactivated” (undeveloped oocytes, <0.5 mm) or “intermediate” (oocyte size between 0.5 to 2 mm) (Amsalem et al., 2015b).

Macronutrient analysis

All dissected tissues were weighed prior to analyses. Lipid, glycogen, and sugar were analyzed as in (Treanore and Amsalem, 2020). Briefly, 200 µl fraction of diluted homogenized tissue was added to 2.8 ml chloroform/methanol mix (v:v 1:1). Samples were centrifuged to separate the glycogen (precipitate) from the rest of the fraction. The upper layer in the remaining fraction was transferred to another glass vial and mixed with 2 ml of distilled H_20. Following centrifuging, the upper and lower fractions were transferred to two separate glass vials. Lipids (reside in the lower fraction) were quantified using a vanillin-phosphoric acid reaction whereas glycogen and sugars (reside in the precipitate and upper fraction, respectively) were quantified using a hot anthrone reaction (Van Handel and Day, 1988). Protein amount was measured using a Bradford assay reagent. Absorbance values (OD 525 for lipids, OD 625 for glycogen and sugars, and OD 595 for proteins) were determined using a BioTek Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT) in triplicates. Absorbance values were converted to micrograms using a formula calculated from standard curve regression lines for lipids, sugars and proteins. The standard curve for glycogen and sugars was created using 6 concentrations of 0.1% anhydrous glucose diluted in distilled H_2O. The lipids standard curve was created using 7 concentrations of vegetable oil diluted in chloroform. The protein standard curve was created using 9 concentrations of bovine serum albumin diluted in 2% sodium sulfate. Microgram values were multiplied by the dilution factor. The fat body tissue was diluted by 100x prior to lipid and glycogen analysis and 20x prior to protein analysis. Ovary tissue was diluted by 0.5x prior to protein analysis and diluted 2.5x for analysis of lipids and glycogen. The crop was diluted 250x for sugar analysis and remained undiluted for protein analysis. The thorax was diluted 2x for glycogen analysis and 10x for protein analysis. These dilutions were determined experimentally to make sure all absorbance values are within the standard curve range. To normalize for initial tissue mass, macronutrient amounts were divided by the tissue mass and reported as µg of macronutrient per mg of the wet tissue mass.

Statistical analysis

Statistical analyses were performed with R (v. 1.3) using the nmle package. To examine the effect of CO_2 treatment and the time post treatment on ovarian activation, we created a contingency table and ran a Fisher's exact test to compare the number of individuals at each

ovary category across the different treatments. Fisher pairwise comparisons were performed between the treatment groups for each of the ovary categories. The effect of CO_2 treatment and the time post treatment on macronutrient amounts were examined by creating a General Linear Mixed Models with the parental colony as a random factor and running an ANOVA. A similar test was conducted to compare the effect of CO_2 treatment on macronutrient amounts in ovariectomized and precocene treated gynes. ANOVA analysis was then performed on the developed glmm to test for the overall significance of treatment in the model. Macronutrient amounts were square root transformed prior to running the analyses. An additional model was created to examine the effect of ovarian activation on macronutrient amounts. Post-hoc comparisons were conducted using Tukey's HDS.

Ethics

Adequate measures were taken to minimize pain or discomfort to bees. Sampling of bees was done by placing them at -80° C. The experiments were conducted in accordance with international standards on animal welfare.

Results

Experiment 1 - Ovarian activation

On the day of CO_2 administration, one untreated gyne (6%, n=18), had activated ovaries while 3 and 10 days later, 11% and 33% of the control and 78% and 100% of the CO_2 treated had activated ovaries, respectively (Figure 2). Gyne ovarian activation was compared across all 6 combinations of CO_2 treatment and timepoints and was found significantly different (Fisher's exact test, $P<0.001$). The CO_2/10 group had the highest number of individuals with activated ovaries compared to all the treatment groups ($p<0.001$) and fewer gynes with inactivated ovaries compared to all other treatment groups except CO_2/3 ($p<0.05$). The CO_2/3 group contained significantly more gynes with intermediate levels of ovarian activation than gynes in the UT/0, CO_2/0, and the CO_2/10 groups ($p<0.05$). The CO_2/3 group also contained fewer gynes with inactivated ovaries than the UT/0, CO_2/0, and CO_2/3 groups ($p<0.05$). Ovary category was not affected by the gyne colony of origin (One way ANOVA, $F_{2,51}= 0.108$, $p=0.9$).

Experiment 1 - Effect of CO_2 on tissue macronutrients

Macronutrients amounts were examined in the fat body (lipids, glycogen, proteins), ovaries (lipids, glycogen, proteins), thorax (proteins, glycogen), and crop (proteins and sugars). There was no significant impact of the gyne colony of origin on any of these macronutrient amount in any of the tissue/body parts (One way ANOVA, $p>0.05$). In the fat body (Figure 3), lipids were significantly affected by CO_2 treatment (two-way ANOVA, $F_{1,46} = 7.62$, $p=0.008$), but were not affected by time post-treatment ($F_{2,46} = 0.029$, $p=0.75$) and the interaction between treatment and time was not significant ($F_{2,46} = 1.08$, $p=0.35$). Post hoc analysis showed that lipid amount in CO_2/10 gynes was significantly lower compared to the UT/3 and UT/10 groups (Tukey's HDS, $P<0.05$). CO_2 treatment, days post CO_2 treatment, and the interaction between the two did not significantly impact the fat body protein (two-way ANOVA, $F_{1,46} = 0.45$, $p=0.5$) or glycogen levels (two-way ANOVA, $F_{1,46} = 0.16$, $p=0.69$). Ovarian activation level had a significant effect on fat body lipids ($F_{1,46} = 5.86$, $p=0.005$) and protein ($F_{1,46} = 3.52$, $p=0.04$). Gynes with activated ovaries had lower levels of fat body lipids than gynes with inactivated ovaries (Tukey's HDS, $P<0.05$). The protein levels in gynes with intermediate levels of ovarian activation were lower than in gynes with activated ovaries (Tukey's HDS, $P<0.05$). Fat body glycogen levels were not affected by ovarian activation levels ($F_{1,46} = 2.43$, $p=0.1$).

In the ovary tissue (Figure 4), glycogen amount was significantly affected by the treatment (two-way ANOVA, $F_{1,46} = 12.03$, $p=0.001$) and the time post treatment ($F_{1,46} = 3.44$, $p=0.04$), and there was a significant interaction between the two ($F_{1,46} = 3.26$, $p=0.04$). Post hoc analysis found that CO_2/10 gynes had significantly more glycogen than CO_2/3 and UT/0,3,10 groups (Tukey's HDS, $P>0.05$). The protein amount in the ovaries was significantly affected by CO_2 treatment (two-way ANOVA, $F_{1,46} = 9.19$, $p<0.001$), days post treatment ($F_{1,46} = 14.34$, $p<0.001$), and the interaction between them ($F_{1,46} = 10.99$, $p<0.001$). Post hoc analysis revealed that CO_2/10 gynes had significantly more proteins than all other groups (Tukey's HDS, $P<0.01$). Lipid amount in the ovaries was not affected by either the treatment (two-way ANOVA, $F_{1,46} = 0.46$, $p=0.49$) or the days post treatment ($F_{1,46} = 0.62$, $p=0.53$) and no significant interaction was found ($F_{1,46} = 2.09$, $p=0.13$). Lipid amount was also not affected by ovarian activation (two-way ANOVA, $F_{1,46} = 0.46$, $p=0.49$). Ovarian activation level did impact glycogen ($F_{1,46} = 16.89$, $p<.001$) and protein ($F_{1,46} = 54.7$, $p<0.001$) amount in the ovaries. Gynes with activated ovaries had significantly

more protein and glycogen in the ovary compared to gynes with inactivate ovaries (Tukey's HDS, $P<0.001$).

In the thorax (Figure 5), glycogen amount was significantly affected by the time post treatment (two-way ANOVA, $F_{1,46} = 5.15$, $p=0.009$) but not the CO_2 treatment ($F_{1,46} = 51.41$, $p=0.24$) and the interaction between the treatment and time was insignificant ($F_{1,46} = 1.84$, $p=0.17$). Post hoc analysis found that UT/3 gynes had significantly higher glycogen concentrations than CO_2/10 and UT/0 (Tukey's HDS, $P<0.05$). Protein amount was not affected by either the treatment ($F_{1,46} = 0.02$, $p=0.88$) or the days post treatment ($F_{1,46}=2.05$, $p=0.14$) and no significant interaction was found ($F_{1,46} = 1.84$, $p=0.17$). Ovarian activation did not affect thorax glycogen (two-way ANOVA, $F_{1,46} = 0.41$, $p=0.67$) or protein ($F_{1,46} = 0.13$, $p=0.87$) amounts.

In the crop (Figure 6), CO_2 treatment, time post treatment, and the interaction between the two did not significantly impact the sugar amount (two-way ANOVA, $F_{1,46} = 0.255$, $p=0.62$ for treatment, $F_{1,46} = 1.36$, $p=0.26$ for time post treatment and $F_{1,46} = 4.03$, $p=0.08$ for the interaction between treatment and time). However, crop sugar amount was affected by ovarian activation ($F_{1,46} = 3.87$, $p=0.03$), with lower amount of sugars in the crop of gynes with activated ovaries compared to gynes with inactivated ovaries (Tukey's HDS, $P<0.05$). CO_2 treatment (two-way ANOVA, $F_{1,46} = 0.39$, $p=0.54$). The interaction between treatment and time ($F_{1,46} = 0.11$, $p=0.89$) did not influence the crop protein amount, however, the time post treatment did positively affect the amount of protein in the crop ($F_{1,46} = 3.64$, $p=0.038$ followed by Tukey's HDS, $P<0.05$ for CO_2/10 vs. the other groups). The effect of ovarian activation on crop protein amount was also significantly higher in the CO_2/10 group vs. the other groups ($F_{1,46} = 4.89$, $p=0.01$ followed by Tukey's HDS, $P<0.05$).

Experiment 2 - Effect of ovary removal and CO_2 on tissue macronutrients

All the gynes with removed ovaries were confirmed by the end of the experiment to not have ovaries. As expected, gynes in the remaining groups (sham and control) activated their ovaries to an intermediate level in the groups that were not treated with CO_2 and fully activated their ovaries after CO_2 narcosis (Figure 7). Overall, the sham and control did not differ in their ovarian activation (Fisher's exact test, $p=0.73$). To examine the impact of CO_2 on macronutrients, we tested the effect of CO_2 treatment (CO_2 treated, control), the ovary treatment (ovary removal,

sham, control) and the interaction between them. CO_2 significantly reduced fat body lipids in all three ovary treatments (two-way ANOVA $F_{42,1}$= 61.42, p<0.001), and there were no significant differences between the ovary treatments (two-way ANOVA, $F_{2,42}$= 0.19, p= 0.83) or the interaction between the CO_2 treatment and ovary treatments (two-way ANOVA, $F_{2,42}$= 1.07, p= 0.35). Overall, the removal of ovaries did not change the impact of CO_2 on lipid amounts (Figure 7).

Effect of JH antagonist (precocene) and CO_2 on tissue macronutrients

The ovaries of gynes that were fed with precocene remained inactivated compared to control gynes (Fisher's exact test, p<0.001) and the impact of CO_2 on ovary activation in the control was greater than in all other treatment groups (p<0.05). Here too, in order to examine the effect of CO_2 on macronutrient amounts, we tested the effect of CO_2 (CO_2 treated, control) and JH treatment (precocene-fed, control) and the interaction between them. The macronutrients tested in this experiment were fat body lipids, and glycogen and protein in the ovaries (Figure 8). Analysis of fat body lipids showed no effect by the precocene treatment (two-way ANOVA, $F_{1,28}$= 1.94 p= 0.17). The effect of CO_2 treatment (two-way ANOVA, $F_{28,1}$=31.64. p<0.001) and the interaction between CO_2 and precocene treatment (two-way ANOVA, $F_{28,2}$=4.94, p=0.046) were significant. Gynes treated with CO_2 but not fed with precocene had lower fat body lipid concentrations than all other treatments (Tukey HDS, p<0.05). Analysis of glycogen levels in the ovaries showed a significant effect of the precocene treatment (two-way ANOVA, $F_{1,28}$= 12.03, p<0.001). The interaction between CO_2 and precocene treatment (two-way ANOVA, $F_{1,28}$= 8.71, p= 0.006), and CO_2 treatment (two-way ANOVA, $F_{1,28}$= 31.06, p<0.001) were both significant. Post hoc analysis showed that CO_2-treated gynes not fed with precocene had significantly more glycogen compared to all other treatment groups (Tukey's HDS, P<0.05). Finally, analysis of protein levels in the ovary found significant differences by the precocene treatment (two-way ANOVA, $F_{1,28}$= 7.74, p= 0.01), by the CO_2 treatment (two-way ANOVA, $F_{1,12}$= 20.53, p<0.001) and by the interaction between them (two-way ANOVA, $F_{1,28}$= 6.91, p= 0.013). Post hoc analysis revealed that gynes treated with CO_2 but not fed with precocene had significantly more protein compared to all other treatment groups (Tukey's HDS, P<0.05). Overall, feeding gynes with precocene blocked the impact of CO_2 on fat body lipids, ovary proteins and ovary glycogen.

Discussion

This study examined the metabolic shift induced by CO_2 treatment in bumble bee gynes and showed that CO_2 narcosis resulted in reproductive and metabolic changes. Gynes treated with CO_2 exhibit higher levels of ovarian activation than untreated gynes and a shift in macronutrient allocation, with lower fat body lipids and greater glycogen and protein in the ovaries. Changes in macronutrient amounts occurred also in glycogen levels in the thorax and protein levels in the crop but in both cases, these changes correlated with age and were not related to CO_2 treatment. Macronutrient levels in all tissues, except the thorax, were affected by the ovary activation level, suggesting a mutual impact between reproduction and metabolism, in line with a previous study showing that reproduction and aging are energetically linked through lipid metabolism (Hansen et al., 2013). Additionally, our study showed that the metabolic shift caused by CO_2 is not mediated through the reproductive changes gynes display, since even ovariectomized gynes exhibited a reduction in fat body lipids compared to ovariectomized gynes untreated with CO_2. Finally, gynes treated with JH inhibitor failed to show the metabolic shift associated with CO_2, confirming the role of JH in mediating the CO_2 effect. These findings suggest overall that fat body lipids are likely reallocated to support tissue-specific increases in protein and glycogen in the ovary as to enhance reproductive functions, following CO_2 narcosis. They further indicate that the metabolic changes are the primary changes caused by CO_2 and are not secondary changes caused by the impact on gyne reproduction. CO_2 likely induces an increase in JH that leads, independently, to both metabolic and reproductive shifts in gyne physiology. Since CO_2 impacts reproduction only in a limited number of insects, the reallocation of macronutrients it induces is likely to be a shared impact across insects. An effect of CO_2 on macronutrients may explain many of its pleiotropic effects across insects. Below we discuss the specific findings in bumble bees, as well as the broader relevance of the findings to the impact of CO_2 on insect physiology.

The changes observed in bumble bee gynes following CO_2 match the changes they exhibit in the transition to reproduction. When female bees activate their ovaries, fat body stores are reduced and levels of vitellogenin proteins in the hemolymph increase. These proteins, as well as lipids from the fat body, are then incorporated into the growing oocytes in the ovaries (Amsalem, 2020; Wu et al., 2021). Surprisingly, we didn't observe an increase in ovary lipids. Lipids are mostly

triacylglycerols that insects synthesize from free fatty acids and glycerol and since fatty acid production by the oocyte is low, insects must import these from the fat body (Arrese and Soulages, 2010; Ziegler and Van Antwerpen, 2006). Transport of lipids into the oocyte is done by lipoproteins in the hemolymph and at the oocyte surface (mostly lipophorin and vitellogenin), mechanisms that are still not fully understood. Instead of lipids, we found an increase in glycogen in the ovary. Glycogen is a glucose storage that is synthesized and stored in several tissues, the main ones are the fat body and the flight muscles (Elbein, 2010). However, some insects were shown to also store glycogen in the oocyte, possibly as a metabolic safeguard for sugar levels during early development of oocytes (Fraga et al., 2013; Santos et al., 2008). Our data suggest that this is also the case in bumble bee gynes.

The macronutrients in the crop were examined to address two questions. First, whether the CO_2 treated queens store less sugars in the crop, and second, to examine changes in macronutrients in the tissue itself. The first question follows a study showing that (wild) gynes of several bumble bee species stores sugars in the crop before they enter diapause (Alford, 1969b). We expected non-treated gynes, which are programed to go into diapause, to have more sugars in the crop than the CO_2-treated ones. However, we found no differences in crop sugar amounts, likely due to the nature of the experiment in the laboratory and the lack of environmental cues that signal gynes to enter diapause. The increase in proteins in the crop over time is unlikely to reflect the protein content in the crop since all gynes were treated the same, gynes were starved prior to sampling and the crops did not seem to contain visual pollen residues. Thus, the increased proteins are likely to reflect changes in the crop tissue and may indicate an increase in enzymatic activity as the gynes age. Perhaps in line with the transition of gynes to foraging as they age.

While the induction of reproduction by CO_2 is specific to a few social species (e.g., the honey bee, bumble bees, and termites) (Roseler, 1985; Seeley, 1974; Tasaki et al., 2020), the metabolic shift induced by CO_2 seems to be a more conserved phenomenon across CO_2-treated insects, and can even explain the reproductive inhibition CO_2 induces in other species (Helenius et al., 2009; Press and Flaherty, 1973; Press et al., 1973; Thompson et al., 2007). Correlative evidence for the link between CO_2 and metabolic changes exist in many species. For example, CO_2 treatment in the house cricket *Acheta domesticus* resulted in increased hemolymph lipids and carbohydrates and reduced metabolic rate (Woodring et al., 1978). CO_2 narcosis also decreased food conversion

efficiency in crickets and cockroaches (Brooks, 1957; Woodring et al., 1978), a depletion of glycogen (Friedlander and Navarro, 1979) and inhibition of the glutathione biosynthesis (Friedlander and Navarro, 1984) in pupae of the moth *Ephestia cautella,* and also an increase in neuronal glycogen as compared to controls in cockroaches (Wood et al., 1980). Similar phenomena were described in the corn earworm moth where CO_2 was found to modulate NADPH production (Edwards, 1968), in beetles where CO_2 increased the secretion of parabenzoquinones that are used for defense (Irwin et al., 1972), and in the honey bee where CO_2 doubled the metabolism of vitellogenin in queens (Engels et al., 1976). In the current study, CO_2 caused a metabolic shift even in gynes that were ovariectomized, allowing us to disentangle the metabolic and reproductive changes and to confirm that the primer effect of CO_2 in *B. impatiens* gynes is on macronutrient allocation. Furthermore, gynes fed with a JH inhibitor failed to demonstrate a metabolic shift following CO_2 treatment, demonstrating that the relationship between CO_2 and JH is not only correlative, but also causative. JH is not only a gonadotropin in bumble bees and other female insects (Hartfelder, 2000), but was also recently shown to act as a stimulator of metabolism in *B. terrestris* gynes (Shpigler et al., 2021). Altogether, these findings may indicate that CO_2 induces long term changes to metabolism in numerous species, and these changes translate in a species-specific manner to the pleiotropic effects CO_2 induces in insects which, according to the species life cycle, could be either an increase or decrease in reproduction, longevity, and activity.

How CO_2 and JH are linked together is an open question. The CO_2 effect on JH levels may be mediated through the nervous system. The metabolism (both synthesis and degradation) of JH is regulated by biogenic amines in a variety of insect species (Thompson, Yagi et al. 1990, Woodring and Hoffmann 1994, Rachinsky, 1994). Unfavorable environmental conditions can result in a neurohormonal stress response which leads to changes in the content of biogenic amines and JH (Hirashima et al., 2000; Chentsova et al., 2002). CO_2 treatment has been shown to increase levels of dopamine and octopamine and decreased serotonin levels, suggesting biogenic amines may play a role in insects' response to CO_2 (Fuzeau-braesch and Nicolas 1981; Harris et al. 1996; Puiroux et al. 1990; Vergoz et al. 2012).

Conclusions

Altogether, our data show a metabolic shift induced by CO_2 narcosis in bumble bee gynes is mediated through JH. This metabolic shift leads to a transition into reproduction in bumble bees and other social species, however, CO_2 narcosis may also result in reproductive inhibition, as demonstrated in other species. This link between CO_2 narcosis, hormone levels, reproduction, and metabolism may explain the pleiotropic impacts that CO_2 has in insects. However, the causal link between CO_2, JH, reproduction, and metabolism warrants further studies in additional species.

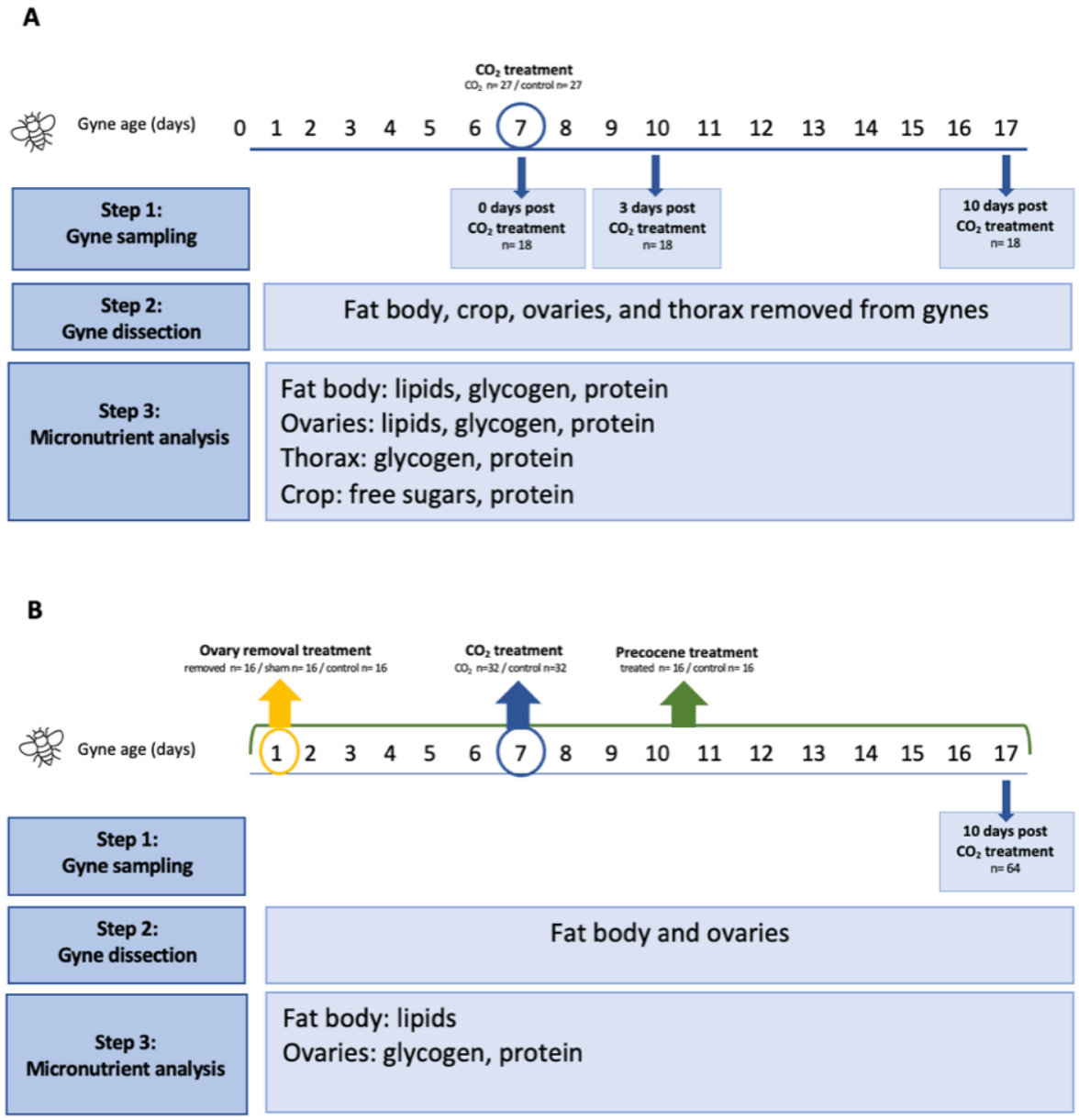

Figure 1: Overview of the experimental design. In experiment 1 (A), 54 gynes were sampled upon eclosion and kept in individual cages. On day 7, half of the gynes were randomly selected for CO_2 narcosis. Gynes were sampled immediately after the treatment (day 0), and on days 3 and 10. Four types of macronutrients (lipid, glycogen, protein, and sugar amounts) were examined in four tissues/body parts (fat body, ovaries, thorax and crop). In experiment 2 (B), 64 gynes were sampled upon eclosion and kept in individual cages. Gynes were assigned to one of 4 treatment (16 queens/treatment): fed with precocene from eclosion until 17 days of age, ovariectomy on day 1, sham ovariectomy on day 1 and control. Half of the gynes within each

treatment were randomly selected for CO_2 narcosis on day 7. All gynes were sampled 10 days following the CO_2 treatment.

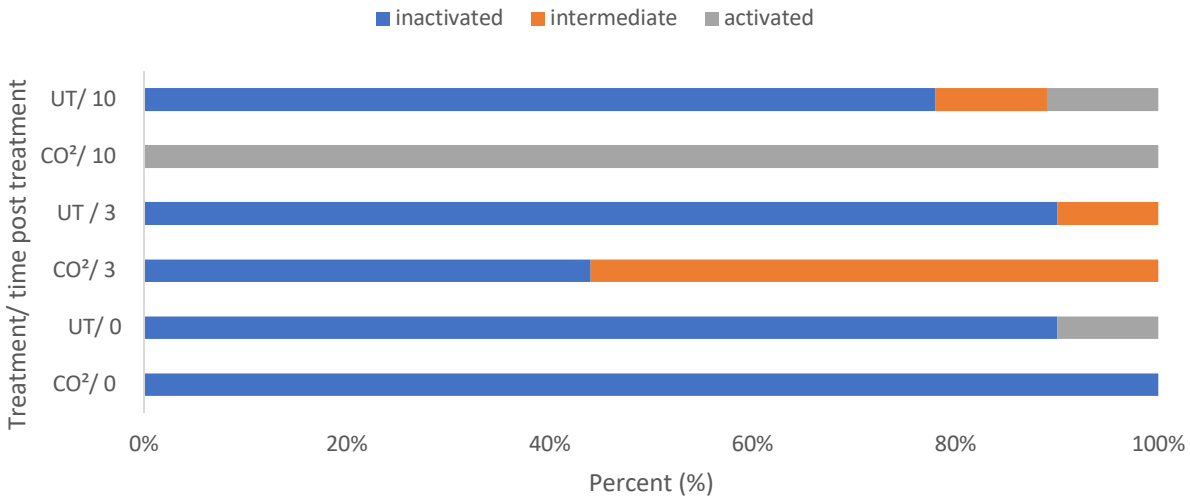

Figure 2: The effect of CO_2 narcosis on gyne ovarian activation. Gyne ovaries (n=54) were categorizes as activated (terminal oocyte larger than 2 mm, marked in blue), intermediate (0.5-2 mm, marked in grey) and inactivated (<0.5 mm, marked in orange) at three timepoints (0,3,10 days) following CO_2 exposure as compared to untreated controls (UT).

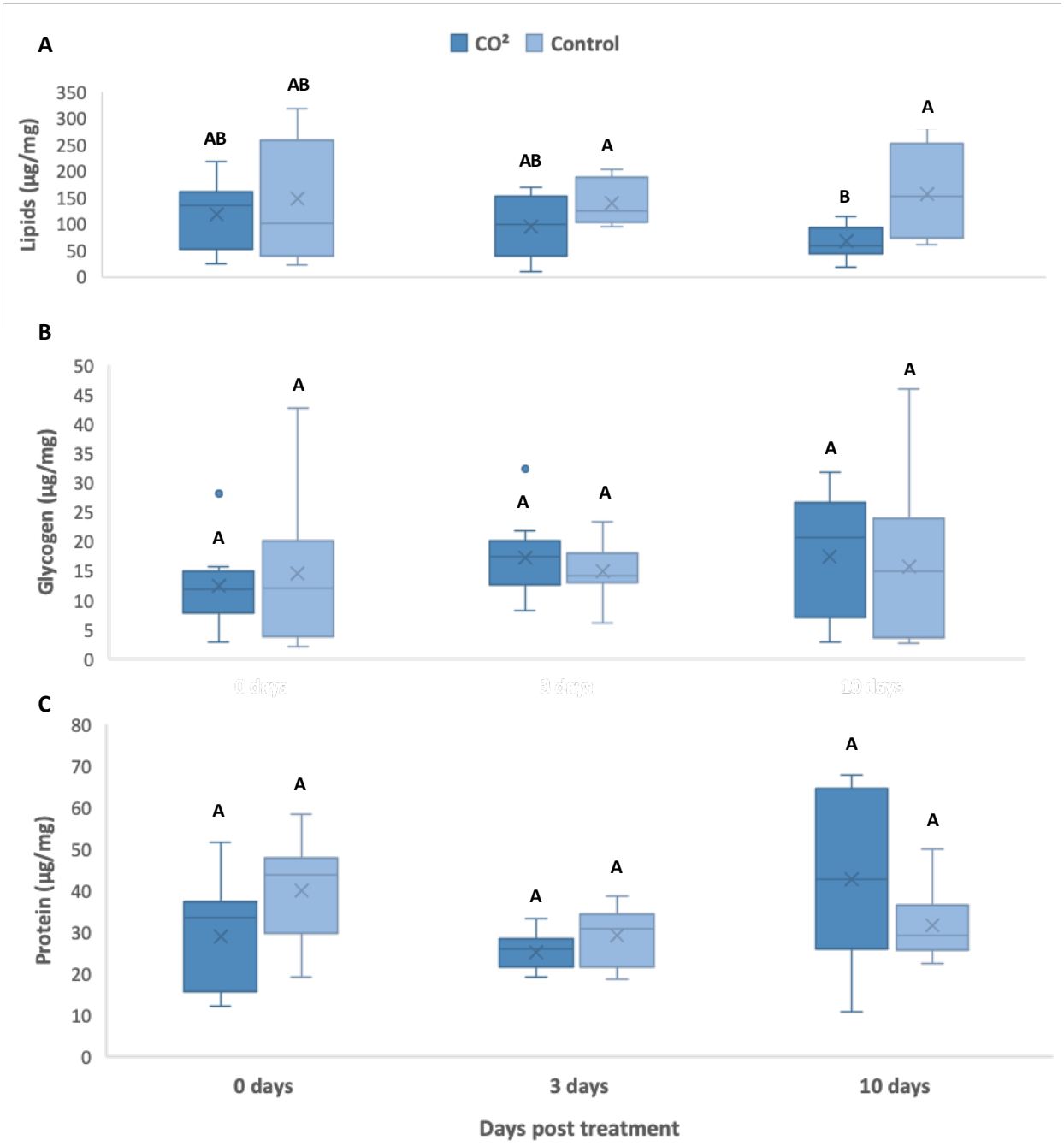

Figure 3: The effect of CO_2 narcosis on fat body macronutrients: lipids (a), glycogen (b) and protein amounts (c). Gynes (n=54) were necrotized with CO_2 7 days post eclosion and sampled immediately after the treatment, 3 or 10 days later. Different letters indicate statistical significances at α=0.05. Post hoc comparisons were preformed using Tukey's HDS.

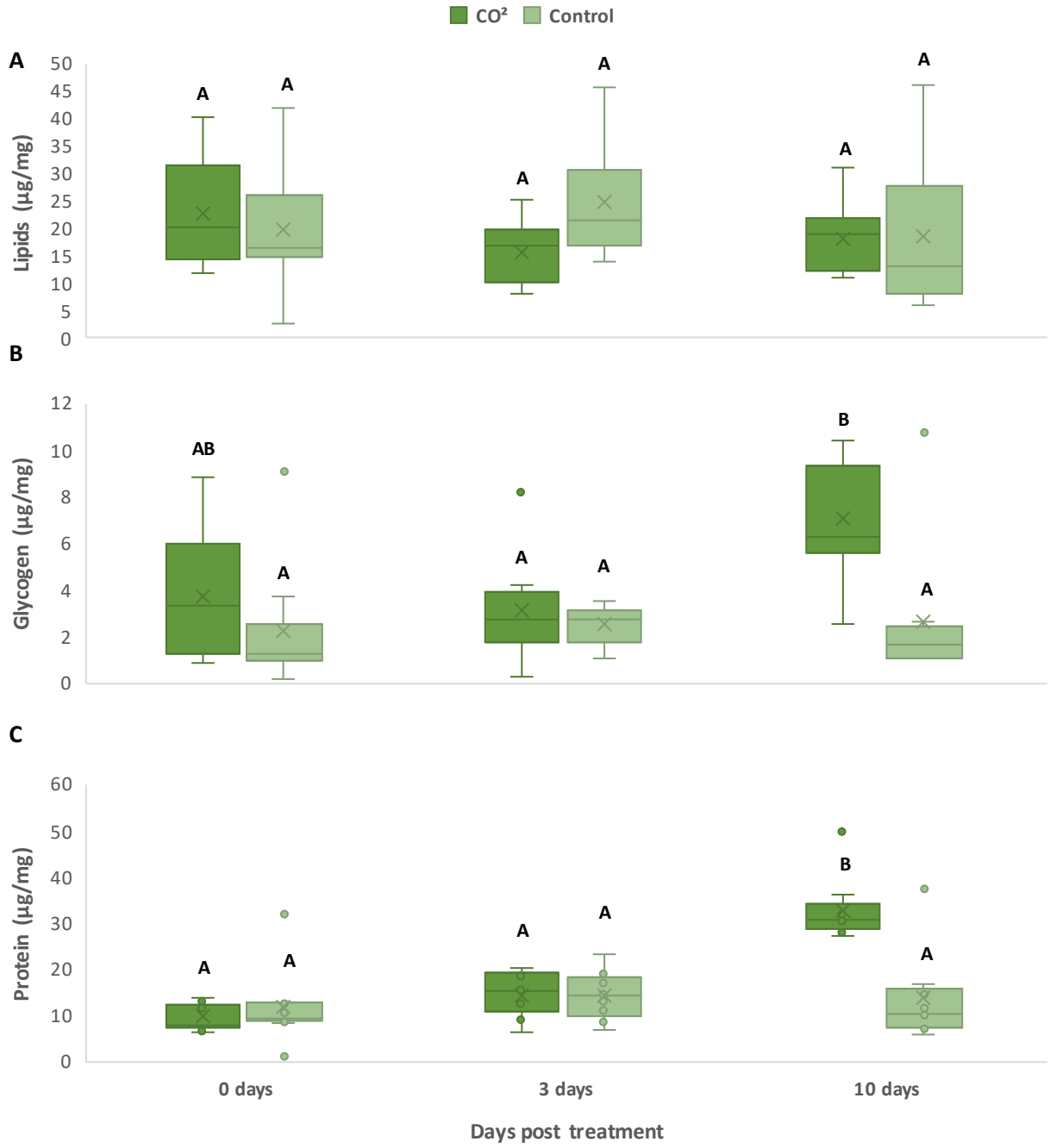

Figure 4: The effect of CO_2 narcosis on ovary macronutrients: lipid s (a), glycogen (b) and protein amounts (c). Gynes (n=54) were necrotized with CO_2 7 days post eclosion and sampled immediately after the treatment, 3 or 10 days later. Different letters indicate statistical significances at α=0.05. Post hoc comparisons were preformed using Tukey's HDS.

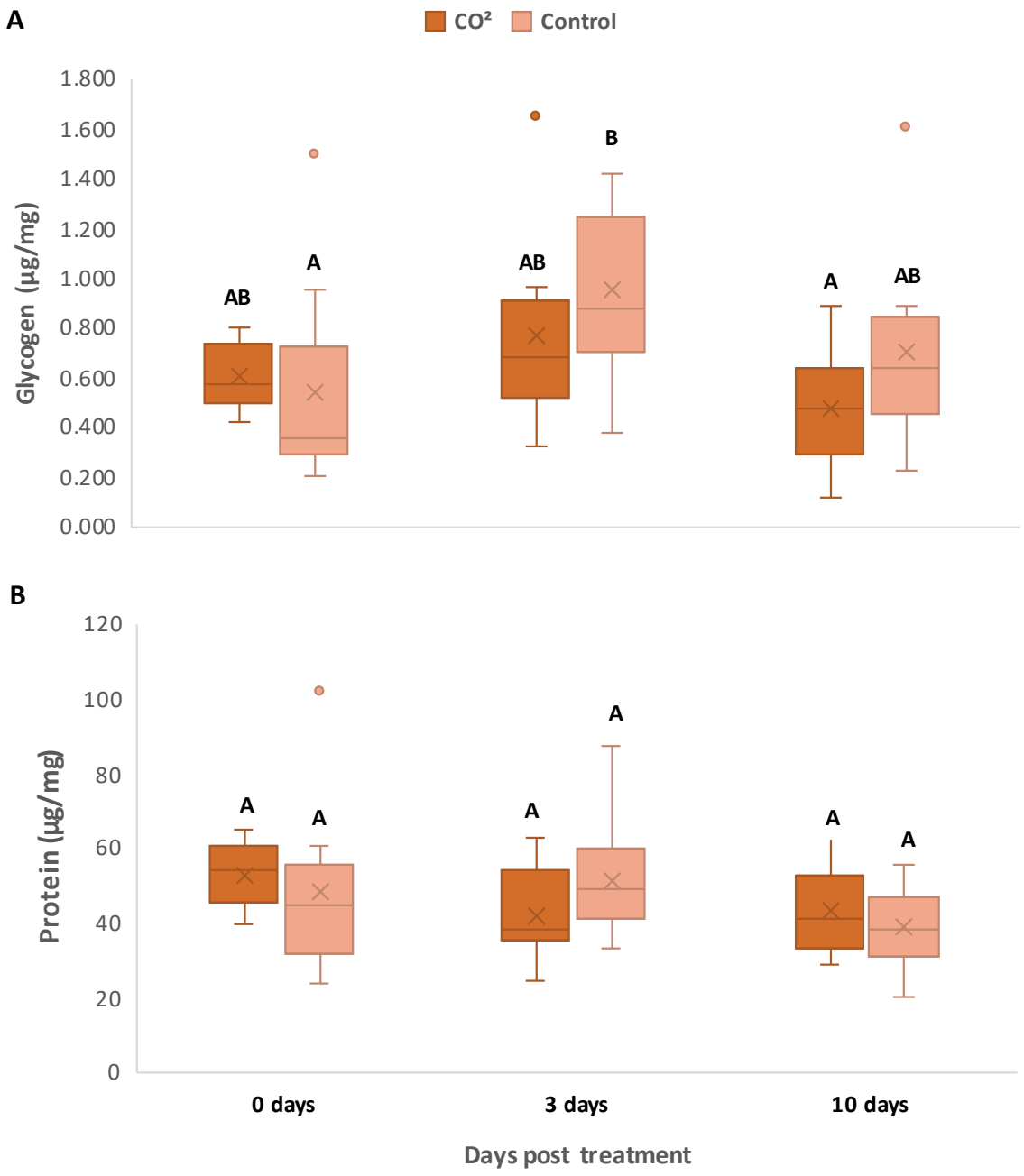

Figure 5: The effect of CO_2 narcosis on thorax macronutrients: glycogen (a) and protein amounts (b). Gynes (n=54) were necrotized with CO_2 7 days post eclosion and sampled immediately after the treatment, 3 or 10 days later. Different letters indicate statistical significances at α=0.05. Post hoc comparisons were preformed using Tukey's HDS.

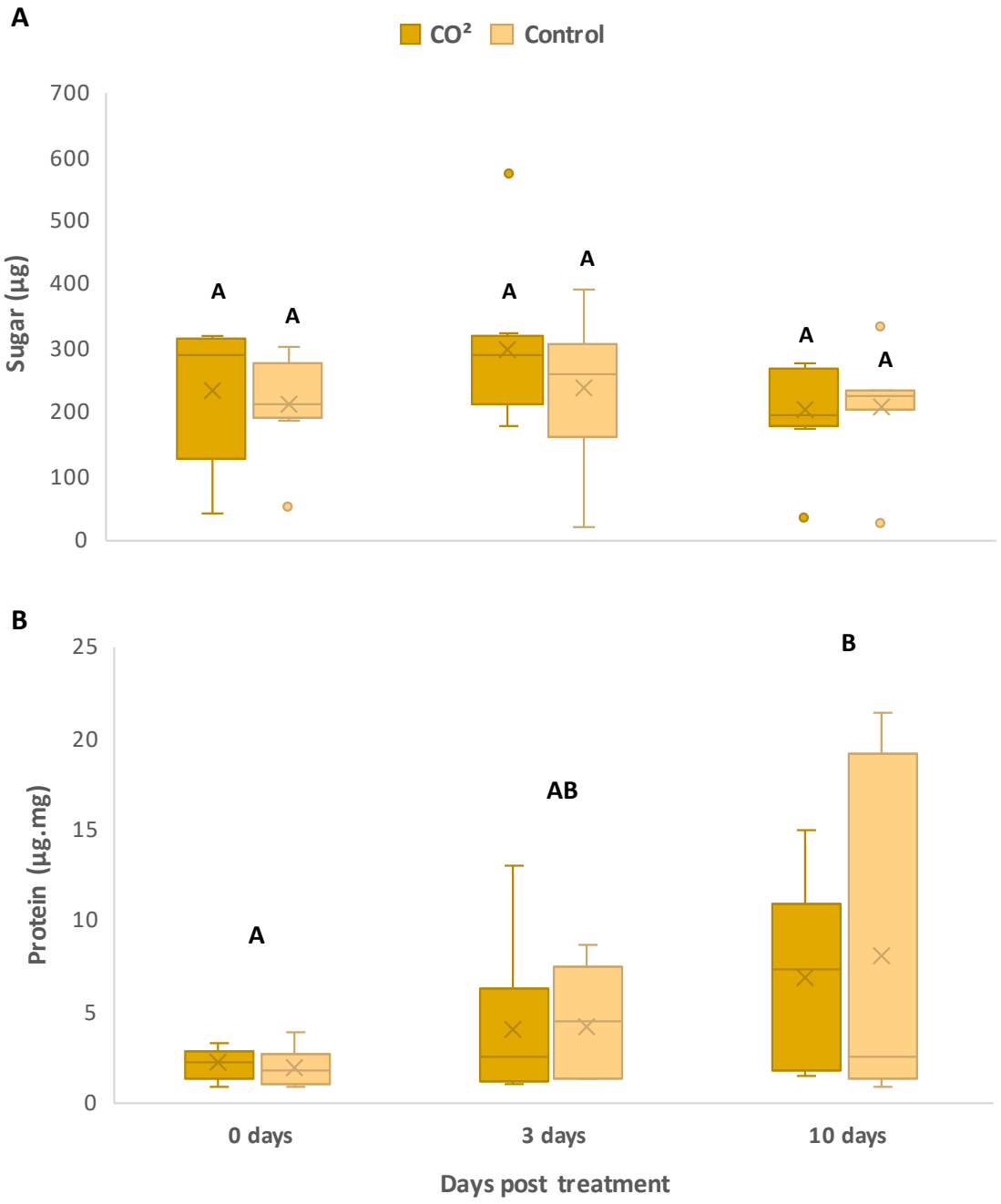

Figure 6: The effect of CO_2 narcosis on crop macronutrients: sugar (a), and protein amounts (b). Gynes (n=54) were necrotized with CO_2 7 days post eclosion and sampled immediately after the treatment, 3 or 10 days later. Different letters indicate statistical significances at α=0.05. Post hoc comparisons were preformed using Tukey's HDS.

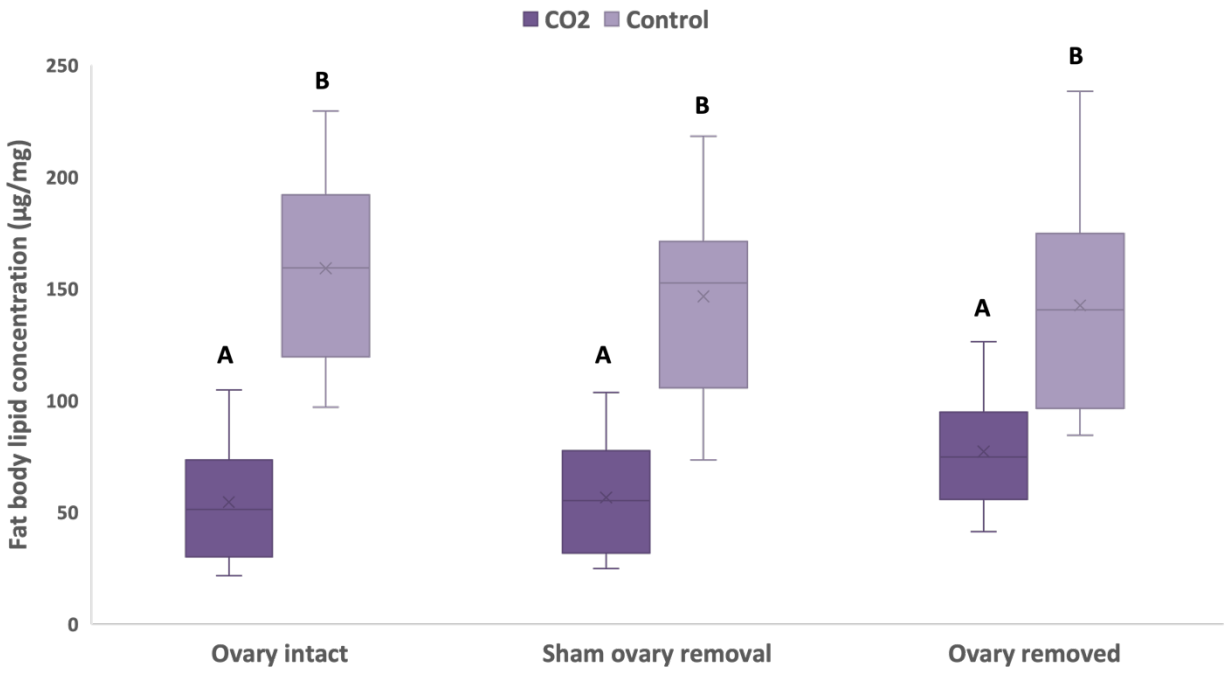

Figure 7: The effect of ovary removal and CO_2 treatment on fat body lipid amounts. Gynes (n=48) were narcotized with CO_2 7 days post eclosion and sampled 10 days later. 16 gynes per treatment were assigned to ovariectomy, sham ovariectomy or remain untreated directly after eclosion. Half of the gynes within each treatment were randomly selected for CO_2 narcosis on day 7. Different letters indicate statistical significances at α=0.05. Post hoc comparisons were preformed using Tukey's HDS.

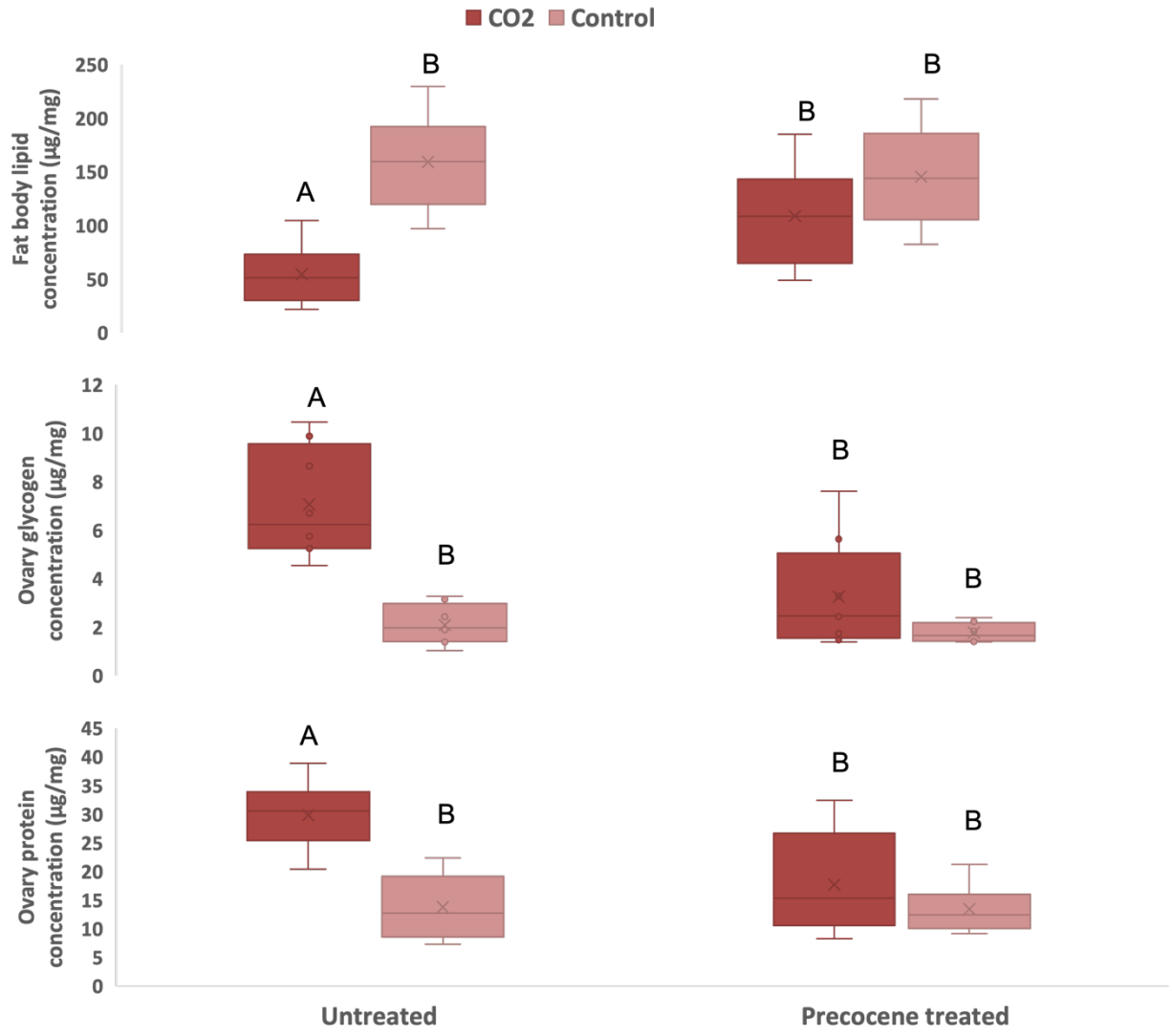

Figure 8: The effect of precocene and CO_2 treatments on ovary protein (a), ovary glycogen (b) and fat body lipid amounts (c). Gynes (n=32) were narcotized with CO_2 7 days post eclosion and sampled 10 days later. Half of the gynes were fed with precocene 1 day post eclosion until 17 days of age. Half of the gynes within each group were randomly selected for CO_2 narcosis on day 7. Different letters indicate statistical significances at α=0.05. Post hoc comparisons were preformed using Tukey's HDS.

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CHAPTER 3

The influence of the social environment on larval development and resulting caste in *Bombus impatiens*

Phenotypic plasticity, the ability of one genome to produce multiple variations of behavioral and morphologic forms is a fascinating phenomenon that is crucial for understanding how societies of social insects have evolved (Baird et al., 2008). Diversity of phenotypes such as workers of various sizes and morphologies and determination of female caste are critical for sustaining the social organization as they determine the female's life trajectory as reproductive or helper and its role within the colony.

Caste differences are the heart of social insect societies since they define the reproductive division of labor in the colony and have been documented in numerous species (Miura, 2005). While in many cases, differences between female castes are limited to body size (Alford, 1975; Plowright & Jay, 1968; Richards & Packer, 1996; E. D. Treanore, Derstine, & Amsalem, 2020; Trible & Kronauer, 2017; West-Eberhard, 1969), additional differences between queens and workers exist in other species (Gotoh, Billen, Hashim, & Ito, 2016; Gotoh, Ito, & Billen, 2013; Khila & Abouheif, 2010).Variation in worker body size and morphology has also been documented in numerous species and is associated with task allocation and reproductive roles. For example, in leaf-cutter ants, the smallest individuals specialize in tending the fungus garden and are more resistant to parasitic fungi (Poulsen, Hughes, & Boomsma, 2006; Wilson, 1980). Likewise, the guard caste of the stingless bee, *Tetragonisca angustula,* are generally heavier, have smaller heads and larger legs than forager bees (A. R. Smith, Wcislo, & O'Donnell, 2008). A morphologically-distinct solider caste responsible for nest defense is also common among termites where soldiers have a sclerotized head, enlarged mandibles, a stopper-like shape, or frontal glands which produce defensive secretions (Roisin, 2000).

Body size and caste in social insects are determined during early development and are influenced by the environment, genetics, or a combination of both (Schwander, Lo, Beekman, Oldroyd, & Keller, 2010). In some ant species, such as *Wasmannia auropunctata* and *Vollenhovia emeryi*,

caste is genetically determined with the worker caste being produced through sexual reproduction while queens are produced through parthenogenesis (Fournier et al., 2005; Kobayashi, Hasegawa, & Ohkawara, 2008; Ohkawara, Nakayama, Satoh, Trindl, & Heinze, 2006). In Meliponine bees, caste is influenced by a combination of genetic and environmental factors. There, genetic markers in larvae are associated with the queen phenotype but require adequate nutritional input to develop into gynes (future queen) (Hartfelder et al., 2006; Kerr, 1950). The same applies to the Florida harvester ant, *Pogonomyrmex badius*, where larval diet differs between castes but patrilineage also influences the resulting caste (C. Smith, Anderson, Tillberg, Gadau, & Suarez, 2008). In many other social species, caste and body size are determined solely by environmental factors such as diet, feeding regime, thermoregulation, and colony social conditions (Eyer, Dainat, Neumann, & Dietemann, 2017; Mao, Schuler, & Berenbaum, 2013; Mutti et al., 2011). In *Vespula maculifrons,* for example, both caste and body size are primarily determined by environmental factors, and no significant effect by the genetic patriline was found on either caste or body size (Goodisman, Kovacs, & Hoffman, 2007). Despite extensive study of phonotypic plasticity (Leimar, Hartfelder, Laubichler, & Page, 2012; Libbrecht et al., 2013; Miura, 2005; Simpson, Sword, & Lo, 2011; Weiner & Toth, 2012), our knowledge of the environmental factors determining development, size, and caste is limited to selected species, primarily ants and some termite, wasp, and bees, and is still mostly unknown in many other social insect species.

The social regulators of caste and body size are often mediated by the adults, who take care of the immobile young. Adults dictate larval diet, clean and thermoregulate the brood (Jandt, Suryanarayanan, Hermanson, Jeanne, & Toth, 2017; Molet, Péronnet, Couette, Canovas, & Doums, 2017; Yaguchi, Suzuki, Matsunami, Shigenobu, & Maekawa, 2019), and can manipulate not only the larval health but also their life trajectory. Differential feeding in the honey bee, for example, determines whether a female larva develops into a worker or a queen (Eyer et al., 2017; Mao et al., 2013; Mutti et al., 2011), and thermoregulation of brood in social insects has been shown to influence broods' metabolic rate, growth, caste determination, and health (Howard & Jeanne, 2004; Jones & Oldroyd, 2006; Kadochová & Frouz, 2014; Vogt, 1986). The amount of care adults provide is dependent on the resources available for them, which are driven by the colony size, age and seasonality (Chole, Woodard, & Bloch, 2019; DeGrandi-Hoffman et al.,

2018; Hoover, Higo, & Winston, 2006; Korb & Hartfelder, 2008; Molet et al., 2017; Quezada-Euán et al., 2011), but also by their reproductive interests, a theory known as "parental manipulation" (Alexander, 1974b), in which the caretaker limits the provisioning for the brood to generate smaller or submissive individuals. For example, worker larvae of *Polistine* wasps receive higher nutritional input compared to early-season larvae (Eberhard, 1969), and in the ant species *Aphaenogaster senilis,* larger colonies are more likely to rear new queens compared to smaller colonies (Villalta, Blight, Angulo, Cerdá, & Boulay, 2016). Parental manipulation by the queen was demonstrated in *Polistes fuscatus* wasps where queens influence larval physiology and caste with vibrational signals in combination with nutritional input (Jeanne & Suryanarayanan, 2011; Mignini & Lorenzi, 2015; Suryanarayanan, Hermanson, & Jeanne, 2011). Queens provide wasp larvae with fewer vibrational signals, resulting in larvae with a lower likelihood of developing into queens (Suryanarayanan et al., 2011). Similar work in *Apis mellifera* (Le Conte & Hefetz, 2008) *Bombus terrestris* (Jonathan Cnaani, Borst, Huang, Robinson, & Hefetz, 1997) and *Solenopsis invicta* (Fletcher & Blum, 1981) also showed that the presence of the queen inhibits the differentiation of larvae to gynes, presumably using a pheromone she produces. In some species of termites, queens are the only individuals in the colony able to provide the nutrients larvae require in order to develop as sexuals (Korb & Hartfelder, 2008; Yaguchi et al., 2019). Overall, the social environment is a strong regulator of larval development and caste in social insects.

Bumble bees are an excellent system for examining the social regulation of body size and caste because both factors were shown to be influenced by the social environment (Jonathan Cnaani, Gene E. Robinson, & Abraham Hefetz, 2000). Bumble bee colonies undergo several transitions during the colony cycle that may result in young receiving differential care. When a bumble bee queen founds a colony, she is the sole caretaker of the brood until the first workers emerge. Female brood in bumble bees is laid by the queen, whereas male brood can be laid by both queens and workers. *Bombus impatiens* brood development takes 24 days, on average, for males and workers, and 37 days, on average, for queens (J. Cnaani, Schmid-Hempel, & Schmidt, 2002). After being laid, eggs hatch into larvae within 5 days and go through four instars before pupating. The larval phase lasts 8, 9 and 18 days, and the pupa phase lasts 11, 10, and 13 days in males, workers, and queens, respectively (Jonathan Cnaani et al., 2000; J. Cnaani et al., 2002;

Tian & Hines, 2018). *B. impatiens* castes differ mostly in body size (Goulson, 2010; Michener & Michener, 1974), with queens being 3 times larger than workers, whereas males are slightly larger than workers (Goulson, 2010; Michener & Michener, 1974; Plowright & Jay, 1968). Bumble bee workers are highly variable in body size and workers within the same colony can vary up to tenfold in body mass (Couvillon & Dornhaus, 2010; Cumber, 1949). Size variation in workers is associated with task specialization. For instance, larger bees are more likely to be foragers (Holland, Nakayama, Porfiri, Nov, & Bloch, 2021; Jandt, Huang, & Dornhaus, 2009). The mechanisms determining caste and worker body size in *B. impatiens* are unknown.

Other bumble bee species vary substantially in the mechanisms determining female caste. In *B. terrestris*, the species that was likely investigated the most, diet composition of provisions is not different for queens and workers (J. J. M. Pereboom, 2000). However, queens are fed more frequently than workers in the later stages of larval development (Ribeiro, Velthuis, Duchateau, & van der Tweel, 1999), although this has not been confirmed in a later study (J. J. M. Pereboom, Duchateau, & Velthuis, 2003). Furthermore, diploid eggs develop to gynes in the absence of the queen, as long as they were separated from the queen before the critical period for differentiation (approximately 5 days after larvae hatch) (Jonathan Cnaani et al., 1997; J. Cnaani, G. E. Robinson, & A. Hefetz, 2000). A similar critical period was found in *B. terricola* (Plowright & Pendrel, 1977; Röseler, 1970). Studies on *B. hypnorum*, *B. rufocinctus*, and *B. ternarius* suggest queen determination depends on food quantity and feeding regimes during development (Plowright & Jay, 1977; Plowright & Pendrel, 1977; Röseler, 1970). In these species, queens and workers have similar growth rates but queens are supplied additional food for longer and the critical period for caste determination is thought to be at the end of larval development (Roseler, 1976). Whether *B. impatiens* castes are regulated as in *B. terrestris* or as in more closely related bumble bee species has not been investigated.

Variation in worker body mass is also influenced by the amount and frequency of larval feeding in *B. terrestris* and *B. terricola* (Pendrel & Plowright, 1981; J. J. M. Pereboom et al., 2003). A study that manipulated the pollen intake of *B. terricola* colonies found that reduced food intake of colonies resulted in smaller-bodied workers (Sutcliffe & Plowright, 1988). Furthermore, larvae on the periphery of *B. impatiens* colonies are fed less frequently than larvae towards the

center of the colony, suggesting that within a colony, larvae may receive different care. The presence of the queen may also affect worker size. A previous study in *B. terrestris* showed that brood reared by a queen was significantly smaller than brood reared by a worker (Shpigler et al., 2013). A similar study in *B. impatiens* examined development in brood reared by five workers vs. one queen and found that the queen produced smaller individuals, although whether the effect is due to the identity of the caretaker or their number is not clear (Costa et al., 2021).

In this study, we examined how the social environment, specifically, the identity/caste and the number of the caretaking females affect larva body mass and duration of development and caste in the bumble bee *B. impatiens*. This was done by grouping female and male eggs with a queen or a varying number of workers (1, 3 and 10). We anticipated that the identity of the caregiver (i.e., queen or worker) would affect female caste, and the presence of the queen will prevent the production of new gynes. We also expected the number of the caretakers to positively correlate with body size and brood development. Finally, we hypothesized that a selective effect by the queen on female, but not on male body size and development, would indicate a parental manipulation aiming to control the resulting female caste and generate submissive and sterile workers.

Methods

Bumble bees rearing

Colonies of *B. impatiens* were obtained from Koppert USA, Inc. (Romulus, MI) and were used as a source for caretakers (workers and queens) and egg laying females producing male and female egg batches. Bumble bees lay eggs in batches, containing up to 10 eggs each and seal them with wax (Amsalem, Grozinger, Padilla, & Hefetz, 2015). In all experiments, groups containing 1 and 3 caretakers were kept in small plastic cages (11 cm diameter × 7 cm tall) whereas groups containing 10 caretakers were kept in larger plastic cages (19 x 16.5 x 14 cm) (i.e., to maintain similar density of worker across treatments). Group sizes were chosen based on a previous study showing that 10 worker groups behave similar to a colony, as opposed to smaller groups containing 3 and 5 individuals (Amsalem & Hefetz, 2011). All bees were kept in

a dark environmental chamber, at 28-30° C and 60% relative humidity and provided an unlimited supply of pollen and 60% sugar solution.

Social condition treatments

To examine how caretaker identity affects brood development, we set up 97 cages containing an egg-layer queen or a random-age worker together with a single 4-days-old egg batch of either females or males. To examine how the number of caretakers affect brood development, we set up 141 cages with 1, 3, and 10 random-age workers together with a 4-days-old egg batch of either female or male brood. Cages were sampled at different time points, covering the entire duration of brood development (egg to adult). In each cage, we measured larva weight, duration of development, sex, and caste. Nurse workers of random age were taken from young colonies which were not producing males or young queens. Caretaker queens were taken from colonies upon emergence. They were mated with unrelated males and treated with CO_2 to induce transition to egg laying as in (E. Treanore et al., 2021). They were then placed in individual cells until they confirmed to lay eggs. Female eggs were laid by mated queens while male eggs were laid by unmated queens or workers. Cages producing eggs were checked for eggs every 24 hours and were photographed and tagged to keep track of the date eggs were laid. Egg batches were then gently transferred to a treatment cage (i.e., with nurses) 4 days after they were laid, thus, supposedly before hatching and before the critical period for determination (5-6 days after larvae hatch) found in *B. terrestris* (Jonathan Cnaani et al., 2000). Workers may lay eggs in the absence of the queen (Amsalem et al., 2015), thus, new egg batches laid by the caretakers after the onset of the experiment were removed daily. Caretakers and egg batches were unrelated across both experiments, since brood care in *B. impatiens* is not affected by relatedness (Starkey, Brown, & Amsalem, 2019).

Larval development duration, weight, and caste

Brood (eggs, larvae, pupae) or newly-emerged adults were collected daily between days 4 to 26 after the onset of egg laying. At least 10 individuals were collected for each day across all cages in the treatment groups and overall, 2001 brood and newly-emerged adults were collected in the study. The brood was frozen and removed from their wax cases. The developmental stage of the brood (eggs, larvae, pupae, or newly-emerged adults) was determined, individuals were weighed,

and the caste was determined for female individuals based on body mass. Adult females weighing above 500 mg were considered to be queens whereas adults weighing less than 400 mg were considered to be workers. Adult females between 400 to 500 mg are considered inter-castes.

Statistical analysis

Statistical analyses and data visualizations were performed using R studio (Version 1.3.959). Larval and pupal weights and the average day of pupation and eclosion were compared across treatments and between sexes using an ANOVA. The same test was also used to compare the number of queens produced in the different treatments. Post-hoc comparisons between treatment groups were conducted using the Tukey test. Growth rate of larvae was calculated using a regression line for larva mass over time during the feeding period. Larval growth rates across treatments and sexes were compared using ANCOVA. Weight was transformed using a square root transformation in all analyses. Data are presented as means ± S.E.M. Statistical significance was accepted at $\alpha=0.05$.

Results

All the brood in our study, of both females and males developed within 24-26 days. However, female and male brood exhibited small differences in the duration of the different developmental stages (described as follows using the minimal and maximal values observed in the study). In females, the duration of the egg phase lasted 6-7 days, slightly longer compared to previous reports in *B. impatiens* (J. Cnaani et al., 2002), although this could be explained by the method of counting (i.e., whether the first day eggs are found is day zero 0 or 1). The larval stage lasted from day 6 to day 18 and the pupa stage lasted from approximately day 14 to day 24. In males, the egg stage also lasted 6-7 days. However, the larval stage lasted from day 6 to day 16 and the pupal stage lasted from day 14 to day 25.

Effect of caretaker identity on development and caste

The identity of caretakers (worker/queen) did not significantly affect body mass of brood in either females or males, however workers reared slightly larger brood than queens (Figures 1A and 1B, respectively). In the female brood, the growth rate was not significantly influenced by caretaker identity, though the statistical result was marginal (ANCOVA, female: $F_{2,292} = 3.05$, p=0.08) (Figure 2A). Male growth rate was also not affected by caretaker identity (ANCOVA, male: $F_{2,217} = 0.038$, p=0.85) (Figure 2B). The growth rate was also not significantly different between males and females in either the queen or single worker caretaker treatment (ANCOVA, queen: $F_{1,554}= 0.083$, p=0.77, 1 worker: $F_{1,480}= 0.51$, p=0.48). Caretaker identity did not significantly affect emergence weight in males or females (ANOVA, male: $F_{1,22}= 1.27$, p=0.27; female: $F_{2,19}= 0.77$, p=0.4). Additionally, caretaker identity did not significantly affect emergence times in males or females (ANOVA, male: $F_{2,22}= 0.088$, p=0.77; female: $F_{2,19} = 0.008$, p=0.93). No gynes emerged from the female brood of either caretaker identity treatments (ANOVA, $F_{1,19}< 0.001$, p=1).

Effect of caretaker number on development and caste

The number of caretakers affected the brood weight in both females and males (Figures 1C and 1D, respectively). Growth rate was not significantly different between caretaker number for male brood but was significant for female brood (ANCOVA males: $F_{2,20}=0.268$, p=0.76, females: $F_{2,20}=3.41$, p=0.03) (Figure 3A and 3B, respectively). The 10-worker condition had a significantly higher growth rate than both the 3 and single worker conditions (p>0.001). The growth rate was not significantly different between males and female in any of the caretaker number treatments (ANCOVA, single worker: $F_{1,480,} = 0.51$, p=0.48, 3 workers: $F_{1,480}= 0.41$, p=0.38, 10 workers: $F_{1,481}=0.009$, p>0.001). Caretaker number significantly affected emergence weight in males and females (ANOVA, males: $F_{1,20}= 45.15$, p>0.001; females: $F_{2,32}= 27.5$, p>0.001). All three treatment groups were significantly different from one another in both male and female brood, with the 10-worker condition being the heaviest and the single worker condition weighing the least (p>0.001). However, caretaker number did not significantly affect emergence times in males or females (ANOVA, males: $F_{2,20} = 0.61$, p=0.7; females: $F_{2,32}= 0.55$, p=0.58). No gynes emerged from the female brood of any caretaker number treatments (ANOVA, $F_{2,32} <0.001$, p=1).

Discussion

This study aimed to investigate the influence of the social environment on the brood development of *B. impatiens*. We examined the effect of caretaker identity and caretaker number on brood developmental duration, weight, and caste determination. Overall, we found evidence for body size being influenced by the number of caretakers but not their identity. All female eggs in our study developed into workers, regardless of the identity or number of the caretakers.

The number of caretakers influenced brood development with growth rate and emergence weight increasing as the number of caretakers increased. These effects are likely mediated via an increase in feeding frequency, attributed to the number of caretakers. Increased growth rate and weight of emergent adults with colony size have been observed in many social insects (Korb & Hartfelder, 2008; Molet et al., 2017; Plowright & Jay, 1968; Plowright & Pendrel, 1977; Ramalho, Imperatriz-Fonseca, & Giannini, 1998). The growth rate of *B. terrestris* has been found to increase with the increase in feeding events (Shpigler et al., 2013). Interestingly, we observed no size difference between female and male brood. *B. terrestris* males were reported to be larger and fed more frequently than workers (Ribeiro et al., 1999; Shpigler et al., 2013). However, an analysis of *Bombus* species found a large overlap in the size range of males and workers, and although males are generally larger in *B. terrestris*, this is not the case in *B. impatiens* (Del Castillo & Fairbairn, 2012).

We found no effect of caretaker identity on the development time, growth rate, and weight at emergence, with the brood reared by a single worker being similar in all parameters to the brood reared by a queen. Based on the parental manipulation hypothesis, it was predicted that the queen would manipulate brood development to produce smaller workers in order to reduce competition with her offspring (Alexander, 1974a). Accordingly, we expected that queen-reared brood would develop quicker and be smaller than worker-reared brood. However, since the queen has no competition with males, we expected this effect to be specific to female eggs. In a previous study with *B. terrestris,* the presence of the queen decreased brood development time and size (Shpigler et al., 2013). Conversely, we found that queen-reared brood was not significantly smaller nor developed faster than worker-reared brood. The increased average body size of

individuals reared by workers in *B. terrestris* could be the result of *B. terrestris* rearing gynes in the absence of the queen. However, decreased development time in individuals reared by the queen was observed even when no gynes were produced, suggesting that in *B. terrestris,* the queen is able to manipulate worker size, an effect that was not observed in the current study with *B. impatiens*. In line with these findings, a previous study in *B. impatiens* also found no differences in the developmental time of female brood reared by a queen versus brood reared by five workers (Costa et al., 2021). However, this study did find that a single queen produced smaller individuals compared to the worker social condition, but this finding may be confounded by the number of caretakers (a single queen versus 5 workers) (Costa et al., 2021). Potentially, both queens and workers in *B. impatiens* may preferentially produce smaller individuals when there are few caretakers because it may be less costly and require less provisioning to produce smaller individuals (J. Cnaani & Hefetz, 2001; Couvillon & Dornhaus, 2010).

Regardless of caretaker identity or number, no gynes were produced in any of the social condition treatments. The absence of a queen has been shown to incite rearing of gynes in several social insects such as the ant species *Aphaenogaster senilis* and *Atta sexdens* and honey bees (Boulay, Cerdá, Fertin, Ichinose, & Lenoir, 2009; Tarpy, Hatch, & Fletcher, 2000; Winston, 1991). Additionally, in *B. terrestris,* female brood reared in the absence of the queen often result in gynes even in small groups of 10 workers (Shpigler et al., 2013), and increased colony size was suggested to impact the production of new gynes by (Bloch, 1999; J. J. Pereboom, Jordan, Sumner, Hammond, & Bourke, 2005). However, in this study, we found an increase in brood body mass with the increase in caretakers but did not observe gyne production even in 10 caretaker condition. That being said, ten workers may not be sufficient for inducing gyne production and an increase in the number of nurses combined with additional factors may have resulted in gyne production. A previous study in several bumble bee species has suggested a certain worker/larva ratio needs to be achieved for gyne production (Plowright & Jay, 1968). This ratio, however, may vary across bumble bee species. In some ant species it has been documented that the number of workers constrained reproductive decisions, and the production of queens was lower in small than in large groups. In the instances of low worker numbers, rearing queens may require an overhead that small worker groups cannot afford (Ruel, Cerdá, & Boulay, 2012).

Since we observed no difference in brood reared by one worker and one queen, our data do not indicate that there is an active manipulation of the queen on worker body mass or duration of development. The data further show that no gynes were produced in any of the worker cages, questioning whether the queen's presence is as important for gyne production in B. impatiens as it is in B. terrestris. Data in *B. impatiens* support a much simpler model in which worker size and maybe also caste are dependent upon the number of nurses, and consequently, the amounts of resources. Size variation in workers can be explained along the same lines with the production of relatively smaller individuals either early in the season (when a smaller number of nurses are available) or very late in the season when resources are no longer available (E. D. Treanore, Kiner, Kerner, & Amsalem, 2020). *B. impatiens* may have a similar caste determination mechanism to that seen in *Bombus hypnorum*, *Bombus rufocinctus*, and *Bombus ternarius*, where queen determination depends on food quantity and feeding regimes during development (Plowright and Jay 1977; Plowright and Pendrel 1977; Roseler, 1970; Roseler and Roseler 1974). However, the mechanisms determining gyne production in this species are yet to be explored, but our data suggest that the social conditions which regulate caste determination in *B. impatiens* are different than in *B. terrestris*.

Table 1: Developmental duration from egg to adult, body mass upon emergence, and growth rate of males and females under the different social condition treatments.

Sex/Treatment	Development duration (days) ± SDER, n	Average emergence weight (mg) ± SDER, n	Growth rate (mg/day), n
Female			
1 queen	25.3 ± 0.11, n=12	102.74 ± 4.03, n=11	21.97 mg/day
1 worker	24.97 ± 0.19, n=11	112.74 ± 6.29, n=13	24.81 mg/day
3 workers	24.75 ± 0.14, n=13	139.67 ± 5.96, n=11	26.45 mg/day
10 workers	24.83 ± 0.14, n=11	171.67 ± 5.48, n=12	28.45 mg/day
Male			
1 queen	24.86 ± 0.41, n=11	119.07 ± 4.1, n=12	23.55 mg/day
1 worker	25.07 ± 0.25 , n=15	115.23± 4.92, n=10	22.7 mg/day
3 workers	24.78 ± 0.31, n=10	151.24± 11.45, n=15	29.69 mg/day
10 workers	24.76 ± 0.28, n=12	194.7± 8.32, n=13	34.13 mg/day

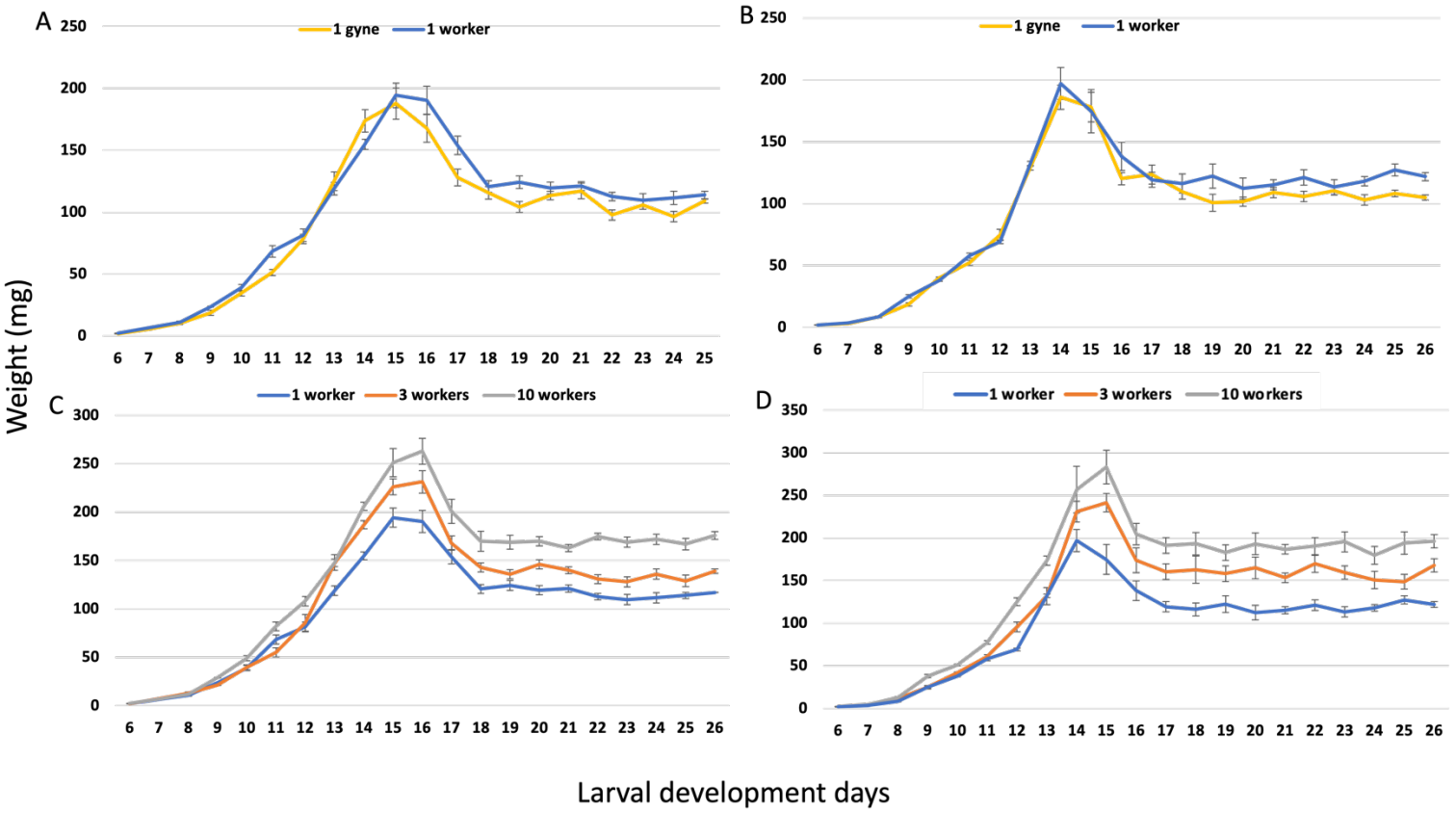

Figure 1: Body mass of female (A, C) and male (B, D) larvae and pupae across social condition treatments. Figures A and B compare body mass of females and males when reared by a single queen or a single worker. Figures C and D compare body mass of females and males when reared by varying number of caretakers (1, 3, and10 workers). Data of body mass are presented as means ± SE.

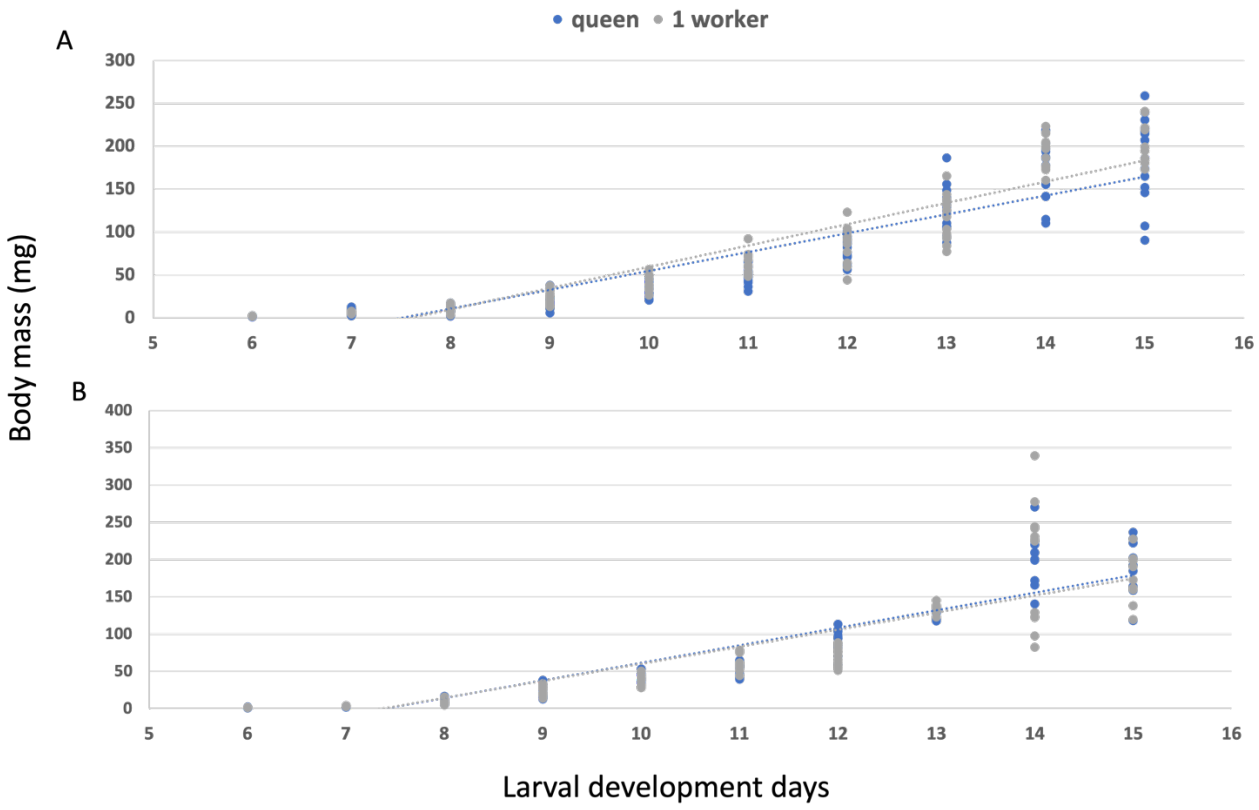

Figure 2: Growth rate of female (A) and male (B) larvae when reared with a single queen versus a single worker. Regression lines were generated using the body mass data during the larva development (7-15 days following emergence).

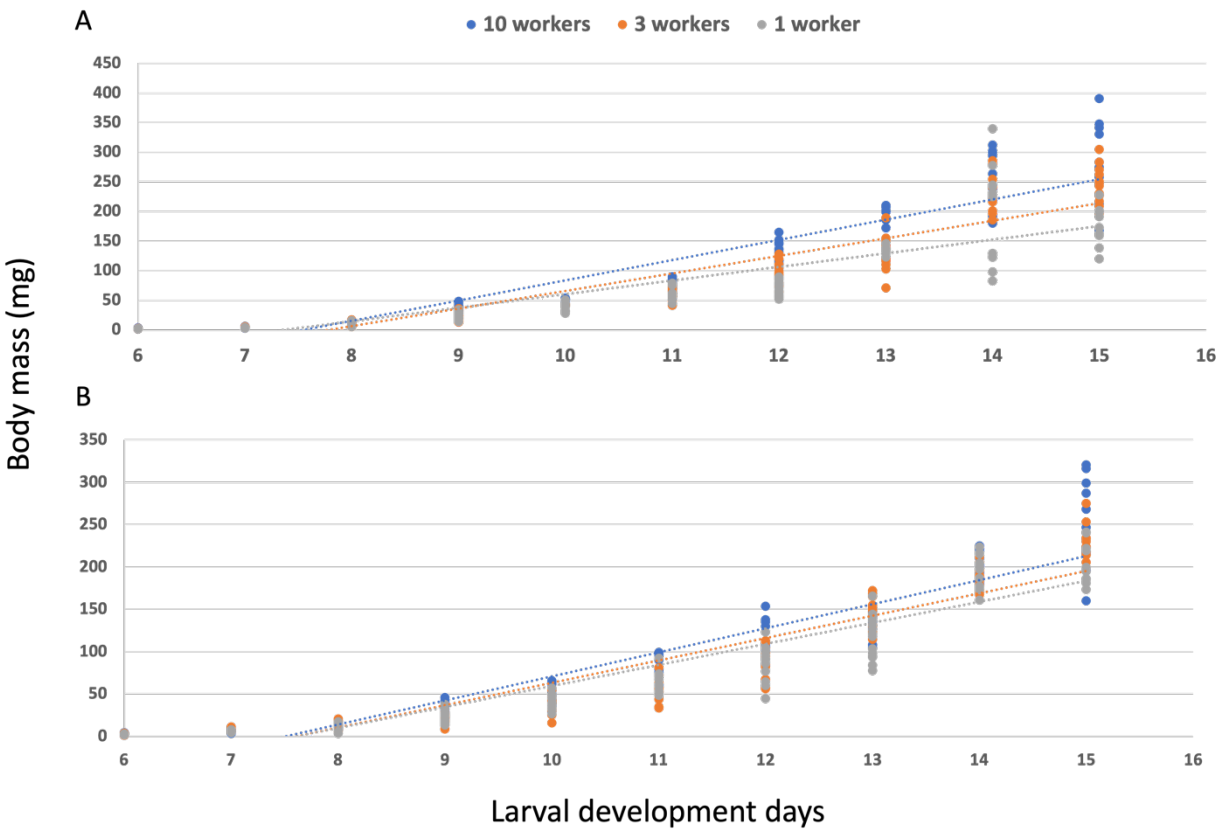

Figure 3: Growth rate of female (A) and male (B) larvae when reared with varying number of nurses. Regression lines were generated using the body mass data during the larva development (7-15 days following emergence).

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CHAPTER 4
Conclusions and future directions

Despite *Bombus impatiens* being widely used across North America for pollination and scientific research, our knowledge on brood development, queen production, and the physiological impact of common gyne rearing methods is still limited. This study examined the metabolic effects and the mechanism underlying CO_2 narcosis, a rearing practice used to initiate egg laying in bumble bee gynes. Additionally, I investigated the social environment's influence on brood development, worker body size, and gyne production. Both worker body size and gyne production are important in rearing colonies for pollination and research purposes (Jauker, Speckmann et al. 2016, Holland, Nakayama et al. 2021, Treanore, Barie et al. 2021).

Results from examining the physiological effects of CO_2 narcosis indicate that acute exposure to CO_2 alters gyne macronutrient allocation and triggers ovarian activation. Gynes treated with CO_2 had higher concentrations of protein and glycogen in the ovaries and lower concentrations of lipids in the fat body relative to untreated gynes. Additionally, our results suggest that the metabolic changes in response to CO_2 are not simply the byproduct of the transition to reproduction. In gynes with removed ovaries, lipid concentrations still decreased in the fat body when treated with CO_2, suggesting CO_2 results in metabolic changes that are independent of ovarian activation. My results also suggest that these metabolic and reproductive effects of CO_2 are mediated by juvenile hormone (JH).

Although JH has previously been shown to increase in response to CO_2 treatment in *B. impatiens* (Amsalem and Grozinger 2017), this is the first study providing a direct link between the reproductive and macronutrient allocation changes triggered by CO_2 and JH. How CO_2 treatment and JH are linked remains to be examined. Unfavorable environmental conditions, such as high CO_2 levels, may result in a neurohormonal stress response. In many insects, this stress response leads to changes in JH and biogenic amines levels (Hirashima, Sukhanova et al. 2000, Chentsova, Gruntenko et al. 2002). The CO_2 effect on JH levels may be mediated through the nervous system. CO_2 treatment has been shown to increase the levels of dopamine and octopamine and decreased serotonin levels, suggesting biogenic amines may play a role in

insects' response to CO_2 (Fuzeau-Braesch and Nicolas 1981, Puiroux, Moreau et al. 1990, Harris, Woodring et al. 1996).

The metabolic changes in response to CO_2 treatment can be used to improve bumble bee rearing, and the metabolic effects in response to CO_2 treatment should be considered when employing this methodology. Colonies headed by queens treated with CO_2 produced more gynes but fewer workers and had a reduced egg laying rate compared to colonies headed by queens following cold storage (Beekman and Van Stratum 2000, Gosterit and Gurel 2009). The depletion of lipids in the fat body following CO_2 narcosis may limit the nutrient stores available to gynes during egg laying and potentially explain the long-term effects seen in CO_2 treated gynes, particularly the reduced egg laying rate. Furthermore, CO_2 may also have detrimental effects when applied to bumble bee gynes in suboptimal nutritional condition. This may explain the variation in initiating colonies following CO_2 and maybe even the variation in the colony size and sex ratio across colonies (Duchateau 2004). Additional research needs to be conducted to fully understand how CO_2 affects colony development and queen longevity.

More broadly, this research illuminates the potential reasoning and mechanism underlying CO_2 pleiotropic effects seen across insect species. A reduction in lipid stores as a byproduct of CO_2 treatment may explain the reduced reproductive performance observed in other insects after CO_2 treatment. My results indicate that CO_2 caused a reduction in fat body lipids stores even in gynes that were ovariectomized, confirming that CO_2 primarily causes metabolic changes in *B. impatiens* gynes. Nutrient accumulation prior to and during reproduction is important for ovary activation and egg production and is highly influential over a insects' fitness (Ziegler and Van Antwerpen 2006). Other insects may experience a similar change to macronutrient stores in response to CO_2 and these metabolic changes may result in reduced reproductive output. A correlative link between CO_2 and metabolic changes exists in other insect species. For example, CO_2 treatment in the house cricket *Acheta domesticus* resulted in increased hemolymph lipids and carbohydrates, and reduced metabolic rate (Woodring, Clifford et al. 1978). CO_2 narcosis also decreased food conversion efficiency in crickets and cockroaches (Brooks 1957, Woodring, Clifford et al. 1978), Additional research is necessary to determine the metabolic changes

associated with CO_2 narcosis in other insect species and the impact of these metabolic changes on reproduction and overall fitness.

The examination of social factors affecting body size and caste determination show that body size is modulated by the social environment, however, gyne production is not affected by the caretaker identity or number when no more than 10 workers are used. My results demonstrate that increasing the number of caretakers leads to an increase in worker and male body size. Increasing the number of workers in a colony could be used to generate larger workers in commercial colonies and increase pollination services (Spaethe and Weidenmüller 2002, Willmer and Finlayson 2014, Jauker, Speckmann et al. 2016) My data, however, showed no significant effect of caretaker identity on male and female body size.

The lack of gyne production across all treatments in my study differs from previous research in *Bombus terrestris* which found queen production to be stimulated by the absence of the queen and by increasing the number of workers (Bloch 1999, Pereboom, Jordan et al. 2005, Shpigler, Tamarkin et al. 2013). There, gynes were produced in groups of 10 workers in the absence of the queen. These differences in gyne production in *Bombus impatiens* and *Bombus terrestris* suggest that the mechanism underlying caste determination in these two species may be different. Gyne production in *B. impatiens* may require different social conditions and may not be regulated solely by the social environment. *B. impatiens* may have a similar caste determination mechanism as *Bombus hypnorum*, *Bombus rufocinctus*, and *Bombus ternarius,* where queen determination depends on food quantity and feed regimes during development (Plowright and Jay 1977; Plowright and Pendrel 1977; Roseler, 1970; Roseler and Roseler 1974). Continued research is needed to examine additional variables potentially influencing *B. impatiens* gyne production and further explore differences in brood development between species. Gyne production may require a certain worker/larva ratio (Plowright and Jay 1977). An increased number of caretakers may be necessary for increased feeding frequency of larvae (Plowright and Jay 1968; Plowright and Pendrel 1977; Molet et al. 2017) or for reaching a critical colony size needed to stimulate gyne production (Ruel, Cerdá et al. 2012).

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