


Article

# Elevated CO<sub>2</sub> Levels Impact Fitness Traits of Vine Mealybug *Planococcus ficus* Signoret, but Not Its Parasitoid *Leptomastix dactylopii* Howard

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**Abstract:** Carbon dioxide (CO<sub>2</sub>) is one of the primary factors driving climate change impacts on plants, pests, and natural enemies. The present study reports the effects of different atmospheric CO<sub>2</sub> concentrations on the vine mealybug *Planococcus ficus* (Signoret) and its parasitoid wasp *Leptomastix dactylopii* (Howard). We investigated the life-history parameters of both species on grapevine *Vitis vinifera* (L.) plants grown under elevated (eCO<sub>2</sub>) and ambient (aCO<sub>2</sub>) CO<sub>2</sub> levels in a greenhouse and in a vineyard free-air carbon dioxide enrichment (FACE) facility. The greenhouse experiments with an eCO<sub>2</sub> level of around 800 ppm showed a significant increase in survival rates, a strong trend towards declining body size, and an increasing fecundity of female mealybugs, while fertility and development time did not change. However, none of these parameters were altered by different CO<sub>2</sub> concentrations in the VineyardFACE facility (eCO<sub>2</sub> level around 450 ppm). On the other hand, the parasitism success, development time and sex ratio of *L. dactylopii*, reared on *P. ficus* under eCO<sub>2</sub> or aCO<sub>2</sub>, varied neither in the greenhouse nor in the FACE facility. These results suggest that future CO<sub>2</sub> levels might cause small-scale changes in vine mealybug fitness; however, this is not necessarily reflected by parasitoid performance.

**Keywords:** climate change; elevated CO<sub>2</sub>; grapevine pest; mealybug; parasitoid; FACE

## 1. Introduction

Atmospheric CO<sub>2</sub> levels are on the rise, with the latest reports published by the Intergovernmental Panel on Climate change (IPCC) reporting an increase of 20 ppm per decade, resulting in an increase of over 35% since pre-industrial times. Current atmospheric CO<sub>2</sub> levels are close to 400 ppm, and this value is expected to double by the end of this century [1]. Plants react to elevated atmospheric CO<sub>2</sub> levels with a whole range of morphological and physiological adaptations. Most C3 plants increase their photosynthesis rates and primary production [2,3]. This also applies to grapevine plants (*Vitis vinifera* L.). For example, Bindi et al. [4] noted that atmospheric CO<sub>2</sub> enrichment stimulated grapevine growth and enhanced fruit and total biomass. Stimulated growth and yield, as well as enhanced stomatal conductance and transpiration, under elevated CO<sub>2</sub>, were also reported from a vineyard free-air carbon dioxide enrichment (FACE) facility in Geisenheim, Germany [5].

Alterations in temperature, precipitation, and other climatic factors are expected to have substantial direct impacts on grape insect pests and pathogens, as well as on the suitability of a grapevine as a host plant for a range of organisms [6]. The increased availability of carbon (C) vs. nitrogen (N) leads to an accumulation of carbohydrates in the foliage and consequently to a higher C:N ratio and a lower nutritional value for herbivores [2,3,7]. This has negative effects, especially on leaf-chewing herbivores,

while phloem-feeders seem to be less affected. Several studies report an improved aphid fitness under elevated CO<sub>2</sub> (eCO<sub>2</sub>) [3,8], although the mechanisms behind this are not yet fully understood. While aphids are a primary pest in many agricultural and horticultural systems, mealybugs, such as the vine mealybug *Planococcus ficus* Signoret (Hemiptera: Pseudococcidae), are a much bigger concern in grapevine production.

*Planococcus ficus* is an invasive phloem-feeding insect from the Mediterranean area, which has become a serious invasive pest in many grape-growing regions worldwide [9,10]. Mealybugs affect grapevines both directly and indirectly. By feeding on the phloem sap of all plant organs, mealybugs weaken the plants' vigour. Furthermore, the excreted honeydew promotes the growth of sooty mould on leaves and fruits, reducing photosynthesis, grape marketability and wine quality [9,11,12]. *Planococcus ficus* is also known to transmit grapevine leafroll-associated virus (GLRaV) and other diseases, which reduce the crop yield and wine quality [13,14]. Mealybug control was based on repeated applications of broad-spectrum insecticides, although with limited success, as mealybugs feed not only on the canopy, but also under the bark and in the root area [15], where they are inaccessible to contact-active pesticides. Additionally, the reiterated use of broad-spectrum insecticides is associated with negative effects on non-target organisms, including biological control agents, and the risk of future pesticide resistance [10,16,17]. Therefore, alternative methods for mealybug control include employing new pesticides, pheromone-based mating disruption, and biological control [18–22]. Models predict that under future climate change scenarios, *P. ficus* will thrive and continue to expand its range [23,24]. The only available study on the effects of eCO<sub>2</sub> on scale insects concludes that temperature, rather than CO<sub>2</sub>, alters the performance of the Madeira mealybug, *Phenacoccus madeirensis* (Green) [25].

The biological control of mealybugs with natural enemies forms an important part of sustainable pest management programs. *Planococcus ficus* can be parasitised by several encyrtid species, such as *Anagyrus pseudococci* (Girault), *Leptomastix dactylopii* (Howard), *Leptomastidea abnormis* (Girault), *Coccidoxenoides perminutus* Girault, and *Coccidoxenoides peregrinus* (Timberlake) [10]. *Leptomastix dactylopii* (Hymenoptera: Encyrtidae) is a solitary, arrhenotokous, koinobiont endoparasitoid, probably native to eastern Africa [26], which has been introduced into Europe, the United States, Pakistan, India, and Australia [27]. *Leptomastix dactylopii* has been used in biological control programs against the citrus mealybug, *Planococcus citri* (Risso) [28,29], but it has also been found in vineyards infested with *P. ficus* and other vineyard mealybugs in California (US), Iran, South Africa and Tunisia [9,30–32]. Laboratory trials showed that *P. ficus* is a suitable host for *L. dactylopii* and wasps might even have an innate preference for this host [26,33]. *Leptomastix dactylopii* is susceptible to low temperatures, and its geographical range might expand due to global warming, similar to other encyrtid wasps [24]. There are no studies available on the effects of eCO<sub>2</sub> on *L. dactylopii* or other Encyrtidae. Considering other related pest-natural enemy complexes, some studies investigated the knock-on effects of eCO<sub>2</sub> on aphid parasitoids, but results vary between neutral, beneficial, and detrimental effects [34,35]. If host quality decreases under eCO<sub>2</sub>, parasitoids will be adversely affected. If eCO<sub>2</sub> increases host quality, parasitoid performance may be maintained or increased. However, the number of available studies is too low to derive general patterns.

Besides host quality, parasitism success depends on the parasitoid's ability to locate possible hosts. When attacked by herbivores, plants release attack-specific volatiles which help natural enemies to locate these herbivores [36]. While chewing insects stimulate the jasmonic acid-signalling pathway, phloem-feeding insects trigger the salicylic acid signalling pathway [34]. Considering the reactions of grapevine plants to attacks by *P. ficus*, the transcriptional response of vine plants is rather weak [37]. However, elevated CO<sub>2</sub> stimulates the production of salicylic acid, which might favour parasitoid host location of phloem-feeders and improve parasitism success [38].

Systematic investigations on the consequences of changing temperature, precipitation or CO<sub>2</sub> concentration on grapevine diseases and pests are few in number [6,39] and multi-trophic interactions of grapevine pests and their natural enemies have never been studied under elevated CO<sub>2</sub> levels. The present study aims to investigate the effects of elevated CO<sub>2</sub> on the performance of *L. dactylopii* and its host, the vine mealybug, reared on grapevine plants in an FACE system and in the greenhouse.

We hypothesised that (I) vine mealybugs reared under eCO<sub>2</sub> would react like other phloem-feeders, as aphids, predicting that (II) life history parameters would not be altered or even improve from higher atmospheric CO<sub>2</sub> levels, and (III) the parasitoid *L. dactylopii* would either be unaffected or benefit from eCO<sub>2</sub>, depending on the mealybug performance.

## 2. Materials and Methods

Insects were obtained from the Department of Crop Protection at Geisenheim University, Germany. *Planococcus ficus* was reared on sprouted potatoes at 26 ± 1 °C and 60–70% relative humidity (RH) in darkness in an incubator. *Leptomastix dactylopii* cultures were reared on *P. ficus* on sprouted potatoes in transparent plastic containers (37 × 22 × 25 cm) at 26 °C, 60–70% RH, and a photoperiod of 16:8 (L:D) in an environmental chamber. Containers were also supplied with honey agar as an additional food for wasps.

In order to answer the question of whether life-history parameters of *P. ficus* vary under different CO<sub>2</sub> concentrations, we conducted greenhouse experiments as well as field studies in the VineyardFACE facility (Geisenheim University, Geisenheim, Germany). The greenhouse experiment was performed with potted Riesling grapevine plants exposed to ambient and elevated CO<sub>2</sub> concentrations of 400 CO<sub>2</sub> and 800 ppm CO<sub>2</sub>, respectively (Table S1). At the onset of the experiment, plants were 12 weeks old and approximately 40 cm high. Grapevine plants used in the greenhouse experiments in this study were grown under ambient CO<sub>2</sub> conditions for 12 weeks before exposure to eCO<sub>2</sub> started. Each plant was infested with ca. 100 *P. ficus* nymphs using leaf disc transport [40]. Briefly, two to three ovisacs harvested from *P. ficus* females were transferred to 2 × 2 cm paper towel squares and placed on 5 cm vine leaf discs. Leaf disks were placed on water-soaked cotton wool and 5 cm filter papers to avoid leaf edges entangling in the cotton wool. After 48 h, paper towel squares were removed and the number of crawlers on the leaf disks was counted. Then, two to three leaf discs were placed on each experimental plant, applying a total number of approx. 100 crawlers per plant. Plants were covered with a fabric gardening bag (60 × 40 cm, 19 g/m<sup>2</sup>, Classic80, HECO Textilverlag, Memmingen, Germany), ensuring oxygen and light supply while preventing mealybugs from escaping and being attacked by natural enemies. A total of 40 plants were placed in a greenhouse chamber with elevated CO<sub>2</sub> concentrations, while another 40 plants served as the control in an ambient CO<sub>2</sub> greenhouse chamber. Half of the 40 plants in each cabin were used to study life history parameters of *P. ficus*, while the other 20 plants were used to raise adult *P. ficus* females for the parasitism experiments with *L. dactylopii*. During the course of the experiments (mid-July to late August 2016), an average temperature of 22 °C was reached in the greenhouse (average minimum temperature 16.0 °C, average maximum temperature 30 °C) (Table S2), and plants were watered twice per week with rainwater.

The VineyardFACE facility enhances open-air CO<sub>2</sub> concentrations using ring-like structures with a diameter of 12 m, which are placed over the rows of an actual vineyard. Three rings are sparged with CO<sub>2</sub> reaching eCO<sub>2</sub> levels of approx. 450 ppm, while the other three rings use air and serve as the aCO<sub>2</sub> (ca. 400 ppm) control. For a detailed description of the VineyardFACE design, see [5,41] and Supplementary Figure S1. Field experiments were conducted between mid-July and mid-September 2016. During the experiments described here, CO<sub>2</sub> concentrations were measured by using two LI-8100 analyser control units (Li-CorLI8100/8150 Multiplexer, Li-Cor Biosciences, Lincoln, NE, USA) installed at two heights (1.7 m and 0.75 m) in the grapevine canopy. Within aCO<sub>2</sub> rings, an average level of 401 ± 1.3 ppm was reached during the experimental periods, while in eCO<sub>2</sub> rings, air was enriched during daylight hours to approximately 12% to 18% above the ambient CO<sub>2</sub> (456 ± 16.1 ppm), which is the concentration predicted for the mid-21st century. Supplementary Figure S2 illustrates CO<sub>2</sub> concentrations in aCO<sub>2</sub> and eCO<sub>2</sub> rings during the first period of the experiments (mid-July to beginning of August 2016) described here. Data on weather conditions during the experimental periods are provided in the Supplementary Table S3. During the course of the experiments, an average temperature of 20.5 °C was reached in the field (average minimum temperature 14.0 °C, average maximum temperature 27 °C). Experiments were conducted on 10 five-year-old Riesling grapevine

plants per ring, resulting in three replicates, with 10 sub-replicates each. Similar to the greenhouse experiment, two canes per plant were inoculated with approximately 100 1st instar mealybug nymphs and covered with gardening fabric. Of the two infested canes per plant, one was used for the study on mealybug life-history parameters, while the other one served to provide adult mealybugs reared under eCO<sub>2</sub> as hosts for *L. dactylopii*.

In both the greenhouse and FACE experiments, the development time, body size, fecundity, fertility, and survival of *P. ficus* females were recorded. The developmental stages of mealybugs were determined according to Walton & Pringle [9]. Three weeks after infesting vines with mealybug nymphs, greenhouse and FACE plants were assessed for the presence of females reaching the oviposition stage. Plants were checked every other day and the first ten (greenhouse) or five (VineyardFACE) ovipositing females from every plant were collected and stored in ethanol until further analysis. The infestation and collection dates for each female were used to determine the development time. Body length (from head to anal lobes) of the collected *P. ficus* females was measured under a stereomicroscope. To determine the fecundity and fertility, one additional female at the onset of oviposition was sampled from each treated and untreated plant (Greenhouse:  $n = 20$ ; VineyardFACE:  $n = 3$ ). These females were transferred individually to a small piece of paper towel in a sealed Petri dish and incubated in the respective greenhouse chambers (eCO<sub>2</sub> and aCO<sub>2</sub>) for two weeks. Then, Petri dishes were frozen, and the number of crawlers and unhatched eggs was counted under a microscope. The sum of unhatched eggs and hatched crawlers accounted for fecundity, while fertility was determined by the percentage of hatched crawlers. Greenhouse experiments finished after six weeks, while the duration of the VineyardFACE experiments extended over 9 weeks, due to the slower mealybug development under field conditions. At the end of the experiments, the number of surviving adult females was recorded on each plant. In accordance with Ross et al. [42] and Cocco et al. [43], we assumed a baseline survival rate of 60% of females in our *P. ficus* culture. Hence, the survival of females was calculated as

$$\frac{(\text{Number of surviving female adults post-experiment} + \text{number of females collected for body size} + 1 \text{ female collected for the fertility})}{\text{Number of applied } P. ficus \text{ nymphs}} \times 0.6 \quad (1)$$

A second experiment aimed to answer the question of whether the parasitism success, development time, or sex ratio of *L. dactylopii* vary under different CO<sub>2</sub> concentrations in the greenhouse or in the VineyardFACE facility. In the greenhouse, 20 potted cv. Riesling grapevine plants were inoculated with approximately 100 1st instar nymphs of *P. ficus* and placed in respective greenhouse chambers with ambient or elevated CO<sub>2</sub>. Likewise, in the VineyardFACE facility, one cane of each of the ten vine plants per FACE ring was infested with 100 1st instar *P. ficus* nymphs. When female mealybugs reached the 3rd instar to preoviposition stage, one leaf of each plant was placed in a small, water filled tube; glued to the ground of a round, transparent experimental arena (diameter 15 cm, height 15 cm); and covered with a detachable lid. Subsequently, five mealybugs, reared from the same plant from which the leaf was collected, were transferred to the leaf using a sable brush. Mealybugs were allowed to settle overnight; then, a male and a female individual of *L. dactylopii* were introduced into the arena for 24 h (greenhouse) or 48 h (VineyardFACE) and then removed. Experimental arenas were placed in their respective treatment greenhouse chambers or FACE ring (eCO<sub>2</sub> or aCO<sub>2</sub>). Water was refilled in the tubes, when necessary. After two weeks, arenas from the VineyardFACE facility were moved to the greenhouse and placed in the respective aCO<sub>2</sub> and eCO<sub>2</sub> treatments, since temperatures in the field decreased to unfavourable levels for *L. dactylopii* development. Subsequently, arenas were visually checked for parasitised mealybugs in order to determine parasitism success. In the following weeks, arenas were checked for *L. dactylopii* adult emergence every 24 h and wasps were removed. The date of emergence was recorded to determine the development time. Emerged individuals were transferred to glass vials and sexed. The experiment finished after 5 weeks when wasp emergence ended.

To detect the possible effects of eCO<sub>2</sub> on *P. ficus* and *L. dactylopii*, data of each measured parameter were tested for normality, followed by an unpaired t-test. Only *P. ficus* fertility data from the FACE

experiment did not follow a normal distribution and was analysed with a Mann-Whitney test for non-parametric data sets. Contingency tables and Fisher's exact test were used to detect possible differences in categorical variables, i.e., the sex ratio of newly emerged *L. dactylopii*. Pearson's correlation analysis was conducted to investigate the relation of female body size and development time (onset of oviposition) of female *P. ficus* mealybugs reared under elevated or ambient CO<sub>2</sub> conditions. All analyses were performed with Graphpad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA).

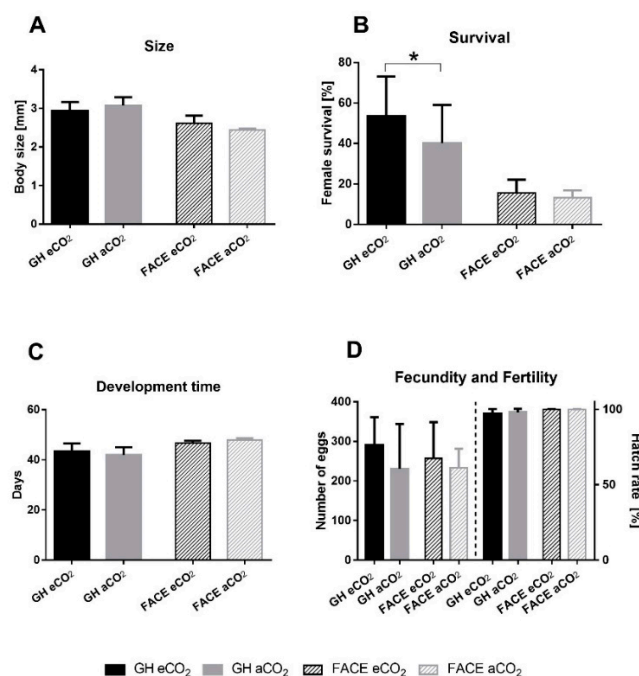
### 3. Results

#### 3.1. Life History Parameters of *P. ficus* Under Different CO<sub>2</sub> Levels

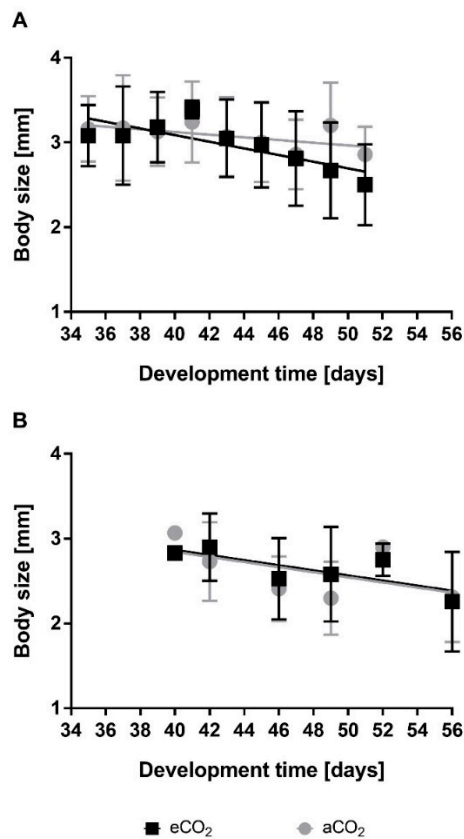
##### 3.1.1. Greenhouse Experiments

The mean body size of mealybugs reared on grapevine plants under aCO<sub>2</sub> was larger compared to those reared under eCO<sub>2</sub>. However, this difference was not significant, even though there was a tendency ( $p = 0.05$ ) towards smaller sized mealybugs under eCO<sub>2</sub> (Figure 1A; Tables S4 and S5). There was a significant negative correlation between the time of exposure and body size of ovipositing females in the eCO<sub>2</sub> treatment ( $R^2 = 0.63$ ,  $p = 0.01$ ), while such a correlation was not detected with aCO<sub>2</sub> (Figure 2).

The survival of adult females was significantly greater for mealybugs reared on grapevine plants under eCO<sub>2</sub> than for mealybugs under aCO<sub>2</sub> conditions (53.2% and 40.1% survival, respectively) ( $p = 0.04$ ) (Figure 1B; Tables S4 and S6). There was no difference between aCO<sub>2</sub> and eCO<sub>2</sub> in terms of the development time from the 1st instar to oviposition, neither for the mean development time (Figure 1C, Tables S4 and S5) nor considering the first ovipositing female of each plant. Fecundity, on the other hand, resulted in 230.9 (aCO<sub>2</sub>) and 290.1 (eCO<sub>2</sub>) mealybug eggs. The improved fecundity under eCO<sub>2</sub> was not significant, but might indicate a strong trend ( $p = 0.05$ ); while fertility, calculated as the percentage of 1st instar nymphs hatched from these eggs, was not different between the CO<sub>2</sub> treatments (Figure 1D; Tables S4 and S7).



**Figure 1.** Life history parameters of mealybug females reared under eCO<sub>2</sub> and aCO<sub>2</sub> in the greenhouse and in the VineyardFACE facility. Data displayed as means + SD. Statistically significant differences between CO<sub>2</sub> treatments are marked with \*. Solid columns display greenhouse data (GH), and hatched columns display data from the VineyardFACE experiment. (A) Body size of females at the onset of oviposition. (B) Survival of females. (C) Development time of females from 1st instar to oviposition. (D) Fecundity, shown as the total number of eggs laid per female, and fertility, shown as the percentage of hatched crawlers from these eggs.



**Figure 2.** Pearson's correlation analysis of female body size and sampling date of female *P. ficus* mealybugs at the onset of oviposition reared under elevated (eCO<sub>2</sub>, black squares) or ambient (aCO<sub>2</sub>, grey circles) CO<sub>2</sub> conditions. Data displayed as means + SD. **(A)** Greenhouse data: eCO<sub>2</sub>  $R^2 = 0.63$ ,  $p = 0.01$ ; aCO<sub>2</sub>  $R^2 = 0.37$ ,  $p = 0.08$ . **(B)** VineyardFACE data: eCO<sub>2</sub>  $R^2 = 0.60$ ,  $p = 0.07$ ; aCO<sub>2</sub>  $R^2 = 0.31$ ,  $p = 0.25$ .

### 3.1.2. VineyardFACE Experiments

Field data on mealybug survival, body size, development time from 1st instar to oviposition stage, fecundity, and fertility were compared between aCO<sub>2</sub> and eCO<sub>2</sub> VineyardFACE rings ( $n = 3$ ), but none of these parameters showed significant differences (Figure 1; Tables S8–S11). Generally, the field results followed similar trends to the results obtained in greenhouse experiments, but did not reach statistical significance. The relationship between body size and the onset of oviposition was not significant in the FACE experiment. (Figure 2B).

### 3.2. Parasitism by *L. dactylopii*

The parasitism experiment with *L. dactylopii* did not reveal any differences between parasitism success, development time, or sex ratio under eCO<sub>2</sub> and aCO<sub>2</sub> treatments, neither in the greenhouse nor in the VineyardFACE facility (Table 1). In the greenhouse, the aCO<sub>2</sub> treatment resulted in a total of 15 parasitized *P. ficus* females, from which 14 wasps emerged: 10 females (69%) and 3 males, while 1 escaped before being sexed. Under eCO<sub>2</sub> greenhouse, 16 mealybug females were parasitized, all of which emerged: 12 females (80%) and 3 males, while 1 escaped before being sexed (Table 1, Table S12). In the field and under FACE eCO<sub>2</sub>, 40 mealybugs were parasitised and 36 wasps hatched (58% females) (Table 1, Table S13). In the FACE aCO<sub>2</sub> treatment, a total of 52 mealybugs were parasitized and 44 wasps hatched, 61% of which were female. The development time of *L. dactylopii* from egg to adult was approximately 24 days in both eCO<sub>2</sub> and aCO<sub>2</sub> greenhouse treatments, while wasps from the VineyardFACE facility needed 25 days (eCO<sub>2</sub>) and 26 days (aCO<sub>2</sub>) to complete their development.

Parasitism success was 15% in the aCO<sub>2</sub> treatment and 16% under eCO<sub>2</sub> conditions in the greenhouse, while in the field, more mealybugs were parasitised (35% under aCO<sub>2</sub>, and 29% under eCO<sub>2</sub>).

**Table 1.** Results from the parasitism experiment with *L. dactylopii* and *P. ficus* in a greenhouse and field (VineyardFACE) under ambient (aCO<sub>2</sub>) and elevated (eCO<sub>2</sub>) conditions. Values are expressed as means ± SD.

Site	Treatment	Parasitism Success (%)	Development Time (days)	Sex Ratio (% Females; % Males)
Greenhouse	aCO <sub>2</sub>	15 ± 17, n = 20	23.86 ± 1.35, n = 14	69.23; 30.77, n = 13
	eCO <sub>2</sub>	16 ± 22.1, n = 20	23.69 ± 0.79, n = 16	80; 20, n = 15
	<i>p</i>	0.87	0.67	0.67
VineyardFACE	aCO <sub>2</sub>	34.67 ± 34.01, n = 30	25.67 ± 3.34, n = 44	61.36; 38.63, n = 44
	eCO <sub>2</sub>	29.33 ± 27.66, n = 30	25.13 ± 2.54, n = 36	58.33; 41.67, n = 36
	<i>p</i>	0.51	0.41	0.82

#### 4. Discussion

Here, we report for the first time on the possible effects of eCO<sub>2</sub> in a grapevine-based pest-parasitoid system using the vine mealybug *P. ficus* and its parasitoid *L. dactylopii* as model organisms. Our greenhouse experiments with eCO<sub>2</sub> showed a significant increase in survival rates, and strong trends for a decreased body size and increased fecundity of *P. ficus* females, while fertility did not change under eCO<sub>2</sub> concentrations. Body size was negatively correlated with sampling date, as females that started oviposition later were of a smaller size. Body size is usually positively related to fecundity in mealybugs [42,43], but we did not find evidence for this in the present study. It is possible that the number of eggs suffered a decline over time, similar to mealybug size, but this was not evaluated. On the contrary, fecundity did increase under eCO<sub>2</sub>, although this trend was not significant.

The field experiment at the VineyardFACE site did not detect any differences between aCO<sub>2</sub> and eCO<sub>2</sub> treatment groups. There are several possible explanations for this. Firstly, field experiments take place in a more complex environment, including a whole series of uncontrolled variables, e.g., humidity, wind, and putatively other natural enemies. While this more realistic scenario is important to put laboratory results into context, it also makes it difficult to detect small-scale effects, as they might occur in the case of *P. ficus* under eCO<sub>2</sub>. Secondly, VineyardFACE eCO<sub>2</sub> concentrations were much lower (ca. 450 ppm) than in the greenhouse (ca. 800 ppm), due to the open-air nature of the facility. The differences detected under greenhouse eCO<sub>2</sub> were rather small; hence it is not surprising that the lower VineyardFACE eCO<sub>2</sub> levels did not achieve comparable results. Thirdly, despite its large scale and technical sophistication, the Geisenheim VineyardFACE facility only allows a limited number of replicates. There are only three independent test rings for each CO<sub>2</sub> treatment, which complicates the detection of subtle differences *per se*. To gain statistical power, we also analysed the ten biological replicates (subreplicates) per ring, see Supplement Tables S9–S11, S13). These subreplicate-based results are limited in their implications and no general conclusions should be drawn from them. Even so, none of the analysed life history parameters of *P. ficus* showed significant differences between aCO<sub>2</sub> and eCO<sub>2</sub>. This combined evidence suggests that VineyardFACE eCO<sub>2</sub> levels did not affect *P. ficus* and it appears unlikely that a higher number of independent treatments (i.e., FACE rings) would change this result.

Since the experimental conditions were substantially different, no statistical testing was done to compare field and greenhouse data. There are some differences which might be attributed to other environmental stress factors, such as temperature and precipitation amounts. Our field experiment yielded low survival rates, but they are comparable to those measured in a screenhouse experiment with *P. ficus* [43]. Female mealybugs in the field needed more time to reach the oviposition state and were slightly smaller than those from the greenhouse, especially in the aCO<sub>2</sub> treatment. Mealybug fecundity and fertility were similar at both sites. Interestingly, a negative correlation between size and development time was detected under eCO<sub>2</sub> at both experimental set-ups, although this relation was not significant (*p* = 0.07) in the VineyardFACE facility. It would be interesting to study these findings

in more detail. The overall difference between the obtained field (FACE) and greenhouse data might also be attributed to an overall plus of an average temperature of 2–3 °C in the greenhouse, which might have been more favourable for mealybug development.

Higher atmospheric levels of CO<sub>2</sub> have been shown to result in a higher biomass, increased yield, and lower nutritional values in grapevine and other plants [2,5,44]. However, plant tissues and fluids vary in their response to elevated CO<sub>2</sub> [2,3,45] and influence different feeding guilds of herbivores in different ways [3,7]. Leaf-chewing herbivores generally perform worse and phloem-feeders have been shown to be less affected by rising CO<sub>2</sub> concentrations [3,46]. Most literature on the effects of CO<sub>2</sub> and climate change on Hemiptera focuses on aphids. In the only study on mealybugs, Chong et al. [25] found that temperature rather than elevated CO<sub>2</sub> influenced the survival, development time and fecundity of *P. madeirensis*. Aphids can benefit from elevated CO<sub>2</sub>, showing increases in fecundity and survival, and decreases in development time [3,46]. However, several studies showed that the direction and size of the effect of eCO<sub>2</sub> depend on the specific combination of host plant and insect species [47–50]. Hughes & Bazzaz [47] tested five aphid species and their host plants under eCO<sub>2</sub>; one species was negatively affected, another positively affected, and no significant effects were found for the remaining three.

Cocco et al. [43] investigated the performance of female mealybugs reared on a grapevine with increasing levels of nitrogen (N) fertilisation. There are parallels between the results of the study by Cocco et al. [43] on *P. ficus* under elevated N and the results of our study on *P. ficus* under eCO<sub>2</sub>. In both studies, survival rates increased, and fecundity showed an increasing tendency, although this tendency was not significant in the present study. Fertility was not affected in both studies; body size augmented with increasing N levels, but decreased under eCO<sub>2</sub>. Cocco et al. [43] attributed their findings to a higher nutritional value of the phloem sap caused by fertilisation. It is known that the effects of eCO<sub>2</sub> can be mitigated by elevated temperatures, drought [25,46] or fertilisation [3]. Clearly, these three factors and eCO<sub>2</sub> interact in future agriculture, especially in perennial systems such as vineyards. A study by Sudderth et al. [8] investigated the effects of the interaction of fertilisation and eCO<sub>2</sub> on aphids by manipulating soil N and atmospheric CO<sub>2</sub> levels. At ambient CO<sub>2</sub> levels, high soil N increased the aphid population size, similar to the mealybugs in the study by Cocco et al. [43]. However, the aphid population size also increased in response to elevated CO<sub>2</sub> on plants grown under low soil N [8], which might relate to the higher survival rates of mealybugs in the present study. Apparently, elevated CO<sub>2</sub> and increased N fertilisation affect phloem-feeders in a similar manner, but this does not correspond to changes in the C:N ratios of the leaf tissue [8]. Studies on the nutritional quality of phloem sap suggest that nitrogen composition (predominantly free amino acids in phloem) is probably the most important determinant of aphid performance [45,51].

While the present study observed a decreasing mealybug body size with the duration of the eCO<sub>2</sub> treatment, we also found that elevated CO<sub>2</sub> levels were positively related to survival, but development time, fertility and fecundity were not significantly affected. Hence, we detected indicators for reduced fitness (body size), as well as factors that support the assumption that fitness was unaffected (development time, fertility and fecundity) or even improved (survival). These mixed results might also be caused by the experimental design. Grapevine plants used in the greenhouse experiments in this study were grown under ambient CO<sub>2</sub> conditions for 12 weeks before exposure to eCO<sub>2</sub> started. Elevated CO<sub>2</sub> levels affect sap-feeding insects mainly through changes in the host plant [3]. These changes do not happen instantly, hence more clear-cut results on their influence on life history parameters of *P. ficus* might take longer than the duration of the present experiment. It would be interesting to study the long-term effects of eCO<sub>2</sub> on *P. ficus* and *L. dactylopii* on grapevines grown under eCO<sub>2</sub> for a longer period than in the present study. Long-term studies could make changes of life-history of both species become more evident.

Host size is known to influence the sex ratio of parasitoid wasps, with females being more likely to emerge from bigger hosts according to de Jong & Alphen [28]. Despite the subsequent size decrease of *P. ficus* females at the onset of oviposition in the greenhouse experiments in our study, no



significant differences in the sex allocation of wasps were observed between eCO<sub>2</sub> and aCO<sub>2</sub> treatments. This might also be attributed to the fact that host size differences were rather small. In fact, none of the evaluated variables of *L. dactylopii* (parasitism success, development time, sex ratio) were altered by CO<sub>2</sub> levels, neither in the greenhouse nor in the VineyardFACE. Parasitism success was greater in the field than in the greenhouse, because wasps remained for 48 h in the VineyardFACE host arenas, compared to 24 h in the greenhouse. To the authors' best knowledge, there have been no studies investigating the performance of mealybug parasitoids under elevated CO<sub>2</sub> conditions. Studies on natural enemies of aphids showed mixed results [34,35]. Hymenopteran aphid parasitoids display the whole range of possible reactions: *Myzus persicae* (Sulzer) and *Brevicoryne brassicae* (L.) showed unaltered and improved life history parameters without changing the performance of the parasitoid wasp *Diaeretiella rapae* (MacInstosh). In a study by Klaiber et al. [52], however, the same parasitoid suffered a decrease in longevity and rates of parasitism on *B. brassicae*, which were lower quality hosts when reared under eCO<sub>2</sub>. On the other hand, the biocontrol efficiency of *Aphidius picipes* (Nees) against *Sitobion avenae* (Fabricius) was enhanced under elevated CO<sub>2</sub>, although elevated CO<sub>2</sub> had adverse effects on the growth and development of *A. picipes* [53].

Elevated atmospheric CO<sub>2</sub> levels affect parasitoids mainly through plant-mediated changes in host quality which cascade upwards [35]. Our results indicate a tendency towards a non-altered or even improved mealybug fitness under eCO<sub>2</sub>, which is, as hypothesised, in accordance with reports on other phloem-feeders reared in similar conditions under eCO<sub>2</sub>. The results of the present study also support the hypothesis that parasitoid performance seemed to be related to mealybug performance.

Climate change influences grapevine plants, their pests, and natural enemies today and in the future [6]. Rising temperatures affect grapevine pests as *P. ficus* directly, speeding up development and voltinism [10]. Gutierrez et al. [24] modelled the future distributions of *P. ficus* and its parasitoids in California based on weather data. Without taking into account elevated CO<sub>2</sub> concentrations, the model explains how mealybugs will boom at elevated temperatures. *Planococcus ficus* will seek cooler sites under the bark and in the root zone, to compensate for its rather narrow optimal temperature range. These refuges also function as a shelter from natural enemies and certain pesticides. Elevated CO<sub>2</sub> levels possibly benefit *P. ficus* and also increase the biomass in vine plants, which might offer more refuge sites and complicate host findings for parasitoids. It has been suggested that, in general, measured responses of manipulated systems to global change decrease over greater spatial and temporal experimental scales, as well as with the number of climate change drivers studied [35].

## 5. Conclusions

Rising carbon dioxide levels affect agricultural systems worldwide. Crop growers are especially interested in the possible effects of climate change on pest species and their respective antagonists used in biological control programs. This study is the first to test the effects of elevated CO<sub>2</sub> concentrations on a grapevine pest under field conditions and in the greenhouse. It is also the only study to investigate a mealybug-based pest-parasitoid complex under eCO<sub>2</sub> in a multitrophic approach. Our results suggest a trend towards a non-altered or improved fitness of *P. ficus* in the greenhouse with eCO<sub>2</sub> levels around 800 ppm, but not in the field, where eCO<sub>2</sub> concentrations reached approximately 450 ppm. Meanwhile, its parasitoid *L. dactylopii* did not seem to be affected in either of the scenarios tested. Further research should include prolonged eCO<sub>2</sub> exposure of greenhouse plants and investigate the response of grapevine plants to mealybug attack under different CO<sub>2</sub> levels. Additionally, climate change is characterised by a combination of multiple abiotic factors, including rising temperatures and CO<sub>2</sub> levels, as well as varying precipitation patterns. None of these factors act by themselves upon plants, pests and natural enemies and future studies should take this complexity into account. Future research should aim to integrate several trophic levels and environmental stress factors.

**Supplementary Materials:** The following can be found at <http://www.mdpi.com/2073-4395/9/6/326/s1>. Figure S1: (A) Aerial view of the FACE facility; (B) Schematic illustration of one FACE ring; Figure S2: FACE CO<sub>2</sub>; Table S1: Greenhouse CO<sub>2</sub>; Table S2: Greenhouse Temperature; Table S3: FACE climate data; Table S4: GreenhouseResults

(analysed); Table S5: Greenhouse size and develop; Table S6: Greenhouse Survival; Table S7: Greenhouse fertility fecund; Table S8 FACE results (analysed); Table S9: FACE size and development; Table S10: FACE Survival; Table S11: FACE Fertility Fecundity; Table S12: Greenhouse Leptomastix; Table S13: FACE Leptomastix.

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