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Make the environment protect you from disease: elevated CO$_2$ inhibits antagonists of the fungus-farming termite symbiosis

Suzanne Schmidt*, Nick Bos†, Robert Murphy¹, N’Golo A. Koné²,³, Kolotchelema S. Silué²,³, Nicolai V. Meyling⁴ and Michael Poulsen¹

Fungus-farming termite colonies maintain monoculture fungus combs in underground chambers without apparent problems with diseases. Multiple lines of defense contribute to the suppression or removal of antagonists of the symbiosis, but the role of the termite-manipulated environment within mounds has yet to be tested. Specifically, termite mounds have extremely high levels of CO$_2$ compared to atmospheric levels. We tested the effect of 5% CO$_2$ on the growth of fungal crops from *Macrotermes bellicosus* colonies, generalist fungi that could challenge the symbiosis, as well as a specialist stowaway fungus, *Pseudoxylaria*. For sporulating fungi, we also quantified the effects on conidia production. We found that elevated CO$_2$ significantly reduces mycelial growth and conidia production of the generalist fungi *Aspergillus* sp., *Beauveria bassiana*, and *Metarhizium brunneum*, whereas it overall had a net positive effect on the growth of the fungal crop *Termitomyces* and *Pseudoxylaria*; albeit, with variation between fungal strains within genera. Our findings point to elevated CO$_2$ being of adaptive significance to the fungus-farming termite symbiosis as an additional layer of defense that helps keep termite fungus gardens free from fungal infections. The mound-building activities that make termites ecosystem engineers may thus also generate environmental conditions that impact the fate of fungi inhabiting the extended phenotypes that massive termite mounds represent.

**KEYWORDS**

*Termitomyces*, *Macrotermes* bellicosus, extended phenotype, defense, *Pseudoxylaria*

**Introduction**

Colony constructs in social insects allow for radically different internal environments compared to the outside, allowing essential homeostasis despite fluctuating external conditions. The mounds built by fungus-farming termite species of the subfamily Macrotermitinae (Blattodea; Termitidae) are prominent features in African and Southeast Asian ecosystems (Figure 1A). Obligate mutualism with the fungal genus *Termitomyces* (Basidiomycota; Lyophyllaceae) allows these termites to obtain near-complete degradation of diverse plant
We predicted that Katariya et al., 2018; Moriya Kleineidam da Costa et al., 2019, the role of elevated CO$_2$ (Luescher, 1961). However, this leads to the accumulation of CO$_2$ (Matsumoto, 1978). This was found to be up to 5% in the central part of the mound in Macrotermes carbonarius (Matsumoto, 1978) and ~1.2% in the ventilation turrets of Macrotermes bellicosus (Korb and Linsenmair, 1999; Korb and Linsenmair, 2000; Turner, 2001). This is vastly higher than the ~0.04% CO$_2$ in the surrounding atmosphere and a product of limited gas exchange combined with high metabolism of termites and their fungal crop (Matsumoto, 1978), which are adapted to and benefit from these conditions (Luescher, 1961; Matsumoto, 1978; Korb, 2003). High CO$_2$ negatively affects the growth of most organisms, including negative effects on the respiration of the fungal crop of leaf-cutting ants (Kleineidam and Roces, 2000; Romer et al., 2017). However, Termitomyces does not appear to be affected (Katariya et al., 2018). Elevated CO$_2$ could thus aid in the selective suppression of competitor or pathogenic fungi, such as the specialist Pseudoxylaria. This was first proposed by Batra and Batra (1966). However, beyond recent investigations of the impact of hypoxia in low and high CO$_2$ conditions (Katariya et al., 2018), the role of elevated CO$_2$ has not been tested.

To test this hypothesis, we evaluated the effect of elevated CO$_2$ on the growth of Termitomyces, Pseudoxylaria, and other ecologically relevant fungi associated with fungus-farming termites. After confirming high CO$_2$ levels within Macrotermes bellicosus colonies, we subsequently quantified growth of 15 fungal strains from six genera under ambient (0.04%) and elevated (5.00%) CO$_2$ concentrations in vitro. We predicted that Termitomyces and Pseudoxylaria, which are specialized to life in these conditions, would appear to...
be unaffected or benefit from elevated CO₂, while opportunistic fungi should exhibit reduced growth or conidia production when exposed to mound CO₂ concentrations.

Materials and methods

In situ measurements of CO₂ concentration, humidity, and temperature

We identified two Macrotermes bellicosus colonies (IC0039 and IC0054) close to the Lamto Ecological Research Station1 in Côte d’Ivoire (Supplementary Table 1) and confirmed termite species with PCR amplification of the COXII gene (Zaman et al., 2022). We measured CO₂ concentrations using a 58 Miniature 5% CO₂ sensor.2 The sensor was placed in a falcon tube with holes to protect the probe from the termites while ensuring airflow. We placed the CO₂ probe as close to the central fungus chamber as possible via a small hole in the mound wall and avoided the probe touching anything that would agitate the termites (Figure 1B). We closed the hole with mound soil and left the termites to seal their mound properly and re-establish comb headspace conditions. The following day, we started CO₂ measurements in real-time in the morning (07:45–08:15), mid-day (13:30–14:00), and in the evening (19:30–20:00) by connecting the USB to the computer using Gaslab® (Figures 1B,C). We simultaneously measured temperature and humidity using iButtons (Hygrochron iButton®), which were placed in the falcon tube with the CO₂ sensor. For colony IC0039, it was possible to register CO₂ for four consecutive days; however, the termites in colony IC0054 covered the falcon tube within a few days, restricting the airflow to the CO₂ probe, which left us with only 2 days of measurements (Supplementary Table 2).

Fungal isolations and barcoding

We tested the impact of elevated CO₂ in vitro on 15 fungal strains from six genera (Supplementary Table 1). We obtained Termitomyces isolates from six colonies (hosting two Termitomyces species; see Results) in Côte d’Ivoire by asexually placing nodules (asexual fruiting structures) on potato dextrose agar (PDA; VWR, 39 g/L) and subculturing until pure. We also obtained strains from three species of the stow-away fungus Pseudoxylaria from South Africa (X802) and Côte d’Ivoire (IC0040-PS and IC0057-PS). These were isolated by leaving fungus comb in moist conditions for 1–3 days without termites present, transferring emerging Pseudoxylaria mycelium to PDA, and subculturing until pure. Strains from two locations were tested as Pseudoxylaria species appear to be generalists of fungus-farming termites (Visser et al., 2009). We also included three Aspergillus strains (three species) isolated from dead Macrotermes natalensis, M. bellicosus and Trinervitermes sp. termites in South Africa or Côte d’Ivoire two entomopathogenic fungi (Matarhizium brunneum and Beauveria bassiana) that were originally isolated from Cydia pomonella larvae (Lepidoptera: Tortricidae) in Austria and an adult of Anthocoris nemorum (Hemiptera: Anthocoridae) in Denmark (Meyling et al., 2009), respectively and finally a mycopathogen, Trichoderma harzianum T22, originally produced by the fusion of T. harzianum strains T12 and T95 (Ahmad and Baker, 1988; Sivan and Harman, 1991).

Strain genotyping

We verified species identities of the fungal strains by sequencing the Internal Transcribed Spacer (ITS) region of the nuclear ribosomal DNA (Schoch et al., 2012). For DNA isolation, we used a Chelex protocol as described in Conlon et al. (2022). We used either the basidiomycete-specific primers ITS1F and ITS4B (Gardes and Bruns, 1993) or the ascomycete-specific primers ITSF and ITS4B (White et al., 1990). PCR reactions were run with the following conditions: 94°C for 4 min followed by 35 cycles of 94°C for 30s, 58°C for 30s, and 72°C for 30s with a final extension step at 72°C for 4 min. PCR products were checked using agarose gel electrophoresis and purified using ExoSAP-IT™ (Affymetrix Inc., United States). Purified PCR products were sent to Eurofins MWG Operon (Ebersberg, Germany) for sanger sequencing. Sequences were identified using NCBI BLAST to estimate the number of species used for each fungal genus, with species delimitation based on a similarity threshold of >97% between ITS sequences, as this has been shown to be suitable for estimating the number of species within most fungal genera (Blaalid et al., 2013).

In vitro effects of colony CO₂ condition on fungal growth and sporulation

All fungi were grown on PDA supplemented with agar (10 g/L, VWR). First, plates of each fungus were grown until harvestable. Then, a ~2 cm² area of fungal biomass was scraped off the agar and deposited in a 150 mL solution of 0.05 g/L Tween 80 (MERCK) and 0.05 g/L Agar (Bie & Berntsen). After vortexing, a sterile inoculation needle was dipped in the solution, and then poked onto the middle of a 90 mm petri dish with PDA. We did 10 biological replicates for each fungal strain and each CO₂ treatment, considering a single plate per condition a replicate. This was repeated twice on separate dates, resulting in 20 replicates (with the exception of strain M17-03 (M. brunneum), for which we only measured growth once). Plates were incubated at 30°C and 70% relative humidity at either ambient CO₂ in a Digital incubator (INCU-Line®, VWR, Denmark) or at 5.00% CO₂ in a Midi 40 CO₂ Incubator (Thermo Scientific, Germany). Due to differences in growth rate, the strains were incubated for either 9 days (Termitomyces and Pseudoxylaria), 4 days (M. brunneum, B. bassiana and Aspergillus), or 2 days (T. harzianum). The difference in incubation time was set to ensure that the fungus would not overgrow the Petri dish and thus prevent correct measurements, while ensuring that there was enough growth to measure area. To compare growth across strains, we obtained the mycelial growth area by measuring the area in ImageJ (Rasband, 2023). If the colony was circular, we measured the diameter twice and used the average of the two measurements to calculate the growth area. If the colony was not circular, we obtained the growth area by manually drawing around the colony. Due to a systematic

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1 https://www.co2meter.com/collections/5-percent-co2-sensor
2 CO2Meter.com
effect of date, we standardized the measurements by subtracting the mean area of all replicates from the individual replicate area and then divided with the standard deviation of all replicates. We verified that CO$_2$ concentrations within Petri dishes were consistently at ambient or 5.00% using the S8 Miniature 5% CO$_2$ sensor. All individual measurements and calculations are available in Supplementary Table 3.

For Aspergillus strains N1IC19 and DT1W, and for M. brunneum M17-03, asexual spores (conidia) could be harvested after incubation, so we counted conidia production per plate after 4 days of growth for both CO$_2$ concentrations. We did this by depositing 10 mL 0.05% Triton-X (Sigma) on each fungal plate and rubbed the culture with a Drigalsky spatula to obtain a suspension of conidia, which was washed twice in 0.05% Triton-X by centrifugation and then discarding of the supernatant. We subsequently did a dilution series and counted conidia using a hemocytometer (Fuchs-Rosenthal). To test if prior exposure to elevated CO$_2$ affected sporulation even under ambient CO$_2$ levels, we pipetted 100 µL of a 1,000x dilution from plates grown at 0.04% or 5.00% CO$_2$ onto new PDA plates, spread conidia using a Drigalsky spatula, and counted the number of live (germinated) vs. dead (not germinated) conidia after overnight incubation. We did this for at least two replicates per strain. Neither Termitomyces or Pseudoxylaria produce conidia in our lab conditions, precluding testing of the impact of CO$_2$ on conidia production.

Statistical analyses

All analyses were performed in R 3.6.3 (RCoreTeam, 2021). The in situ CO$_2$ measurements of termite mounds were compared with a two-way ANOVA testing for colony and time of day effects. Subsequently we determined effect sizes by using Cohen’s $f$ and then conducted pairwise comparisons of time points with TukeyHSD post hoc testing. The effect of CO$_2$ level on fungal growth was analyzed using a linear model with the standardized growth area as the dependent variable and CO$_2$ treatment and replicate as fixed effects. Replicate was added as a fixed effect instead of a random effect, as there were fewer than five levels, which can make estimates of random effects unreliable. Post hoc pairwise comparisons between the two CO$_2$ conditions (0.04 and 5.00%) and fungal strains were conducted using the emmeans R package (Lenth et al., 2022). We tested the effect of CO$_2$ treatment on conidia production using a linear model on the log spore count as the dependent variable and CO$_2$ treatment and replicate as fixed effects. A two-way ANOVA of the regression model was used to test for significance of strain and CO$_2$ level. Conidia viability was tested by fitting a generalized linear model using a binomial family and a logit link function to assess the association between dead/alive spores and CO$_2$ concentration. A Pearson’s $\chi^2$-test was used to test if fungal strain affected the results, followed by a one-way ANOVA to test the significance of CO$_2$ concentration on spore viability.

Results

In situ measurements of CO$_2$ concentration in Côte d’Ivoire

Mound CO$_2$ concentrations were more than 100-fold higher (Mean ± SD: 4.15% ± 1.00%) than the external atmosphere (0.04±0.00%). CO$_2$ concentrations were highest in the morning (5.21 ± 0.74%), declined during the day (3.22 ± 0.47%), and increased again in the evening (3.82 ± 0.39%) (Figure 1E and Supplementary Table 2). The CO$_2$ sensor we used is designed to measure within the range of 0–5% CO$_2$ with a ±0.02% accuracy, implying that accuracy for the measurements exceeding 5% is not known. However, these represented only a few samples and, even if accuracy was lower than within the 0–5% range, it would not impact the overall pattern. Colonies were not significantly different in CO$_2$ level (two-way ANOVA; $F_1 = 0.7150, p = 0.4142$), while time of day did have an effect ($F_2 = 13.74, p = 0.0008$). Cohen’s $f$ indicated the effect size to be 1.51. Pairwise comparisons with TukeyHSD showed that CO$_2$ levels varied across different times of day: morning vs. mid-day: adj-$p = 0.0007$; morning vs. evening: adj-$p = 0.0122$, mid-day vs. evening: adj-$p = 0.2510$. The average temperature within mounds was 26.6°C (SD ± 0.98°C) and relative humidity was 98.2% (SD ± 1.14%). The temperature decreased approximately 0.5°C from 8:00–11:00 and again from 18:00–23:00.

Fungal growth in different CO$_2$ conditions

Combining the six Termitomyces strains, we found a significant positive effect of elevated CO$_2$ on growth (post hoc paired t-ratio test; $t = −2.339; df = 211; p = 0.0200$), with an overall medium effect of CO$_2$ (Cohen’s $f = 0.407$). However, when evaluated individually, three strains exhibited significantly increased growth, two were unaffected, and one strain exhibited significantly decreased growth when exposed to elevated CO$_2$ (Figure 2A and Table 1). The positive effect of growth was most significant on the Termitomyces species associated with colonies IC0010 and IC0034, whereas we observed more variable effects of elevated CO$_2$ on the species associated with IC0027 and IC0033 (unaffected), IC0032 (negative), and IC0031 (positive). The three Pseudoxylaria species were overall positively affected by elevated CO$_2$ ($t = 0.741; df = 89; p = 0.0318$) with an overall medium effect (Cohen’s $f = 0.397$), driven by a significant positive effect on one of the strains (Figure 2B and Table 1). Two of the three Aspergillus species were negatively affected by elevated CO$_2$ (N1IC19: $t = 2.806; df = 27; p = 0.0052$; GF10t1: $t = 3.459; df = 23; p = 0.0006$), as was B. bassiana ($t = 5.319; df = 43; p = 0.0001$) and M. brunneum ($t = 3.360; df = 17; p = 0.0008$), but not T. harzianum ($t = −0.5614; df = 63; p = 0.5749$) (Figure 2C and Table 1). The individual $p$-values and effect sizes are given in Table 1.

Conidia production and viability

Our evaluation of the impact of elevated CO$_2$ on conidia production and viability revealed that the two Aspergillus strains (N1IC19 and DT1W) produced fewer conidia after incubation at elevated CO$_2$ (post hoc paired t-ratio test; DT1W: $t = 2.967; df = 15; p = 0.0053$; N1IC19: $t = 13.45; df = 15; p < 0.0001$), but this was not the case for M. brunneum ($t = 1.457; df = 9; p = 0.1538$) (Figures 3A-B). Fungal strain did not significantly affect the number of viable conidia (Pearson $\chi^2 = 11.63; df = 6; p = 0.0709$). We therefore pooled all results, and a subsequent test of the pooled results did not reveal an impact on the proportion of live vs. dead conidia under subsequent CO$_2$ conditions of strains originally grown at ambient vs. elevated CO$_2$ concentrations (one-way ANOVA; $F_1 = 0.4652; p = 0.7074$) (Figure 3C).
We confirmed extraordinarily high CO$_2$ concentrations within the mounds of *Macrotermes bellicosus*, and found consistent patterns between colonies, which allowed us to set an appropriate CO$_2$ level for our *in vitro* studies. These measurement align with previous findings in conspecifics (Matsumoto, 1978) and underline how specialists of the symbioses are adapted to these relatively low CO$_2$ levels.
unique growth conditions. As expected, specialists of the symbiosis were generally either unaffected or positively affected by the high CO₂ levels, while generalists exhibited reduced growth and/or conidia production. Notably, we also observed substantial variation between replicates of the same fungal strains, which likely emerges from variation in inoculum size and from variation in the proportion of inocula that successfully establishes on plates. The CO₂ environment within mounds may thus not merely be a result of a trade-off between ventilation and protection from desiccation but also be of adaptive significance to the fungus-farming termite symbiosis as a potential additional layer of defense to help keep termite fungus gardens free from infections.

As expected for specialists of the symbiosis, five of the six Termitomyces strains and all Pseudoxylaria strains were either unaffected or exhibited increased growth with elevated CO₂, with some variability. The remaining strain of Termitomyces showed reduced growth under elevated CO₂, however only with a low to medium effect size. The mound microclimate may thus influence growth differently across species of specialists. This general overall effect underlines how these fungal genera are adapted to comb conditions and suggests that mound CO₂ conditions do not aid in the normal suppression of the stowaway fungus. In line with this, the absence of increased Pseudoxylaria growth at ambient (compared to elevated) CO₂ concentrations indicates that decreased CO₂ concentration when mound wall integrity is compromised is unlikely to be the signal that triggers rampant growth of Pseudoxylaria (Visser et al., 2011). Other defenses must therefore be more important to suppress this fungus, such as antifungals (Visser et al., 2012; Um et al., 2013; reviewed by Schmidt et al., 2022), termite weeding and grooming of the comb (Katariya et al., 2017; Murphy et al., 2023), or the competitive advantage that Termitomyces obtains through its dense inoculation during comb formation (Badertscher et al., 1983; Leuthold et al., 1989).

Elevated CO₂ suppressed the growth of several of the entomopathogens and generalist contaminants that may enter mounds with the plant substrates (Bos et al., 2021) and persist in trace amounts within fungus combs (Otani et al., 2019). Aspergillus spp. are ubiquitous fungi found in a variety of environments and substrates, such as soil, air, and decaying plant material, where they thrive as saprophytes (Mousavi et al., 2016). The Aspergillus strains exhibited reduced growth and conidia production for the two strains for which this could be quantified (Figures 2B, E). This implies that although the fungi may enter combs, they are unlikely to be adapted to the internal nest conditions. This suggests that tradeoffs exist that preclude adaptations to the environment within termite mounds. The two generalist entomopathogens also exhibited reduced growth, implying that elevated CO₂ may help prevent their spread between termite hosts within colonies. The discrepancy in the impact on conidia production on the fungal genera may imply that mechanisms affecting sporulation differ, which should be further explored, ideally across more strains and species per genus. In contrast to entomopathogens, the mycopathogen T. harzianum was unaffected by elevated CO₂. Trichoderma spp. are found in nearly all soil types worldwide, infect termite fungal combs (Mathew et al., 2012), and respond in species-specific ways to CO₂ conditions (Danielson and Davey, 1973). Since the mycopathogen was not impacted by elevated CO₂, it is likely critical that workers avoid plant material containing the fungus (Bodawatta et al., 2019), and that combinations of defenses collectively allow the termites to maintain robust defenses toward diverse fungi with different susceptibilities to environmental conditions.

Our findings do not determine the mechanism that causes growth suppression, but elevated CO₂ may reduce comb pH as CO₂ reacts with H₂O to form carbonic acid (Wang et al., 2016). This is consistent with comb pH levels being 4–5 in Macrotermes and Odontotermes spp. compared to 6–7 in the surrounding soil (Muwawa et al., 2014). Lower pH causes weak acid stress in Saccharomyces cerevisiae (Piper et al., 2001), while alkaline conditions have been shown to increase fungal virulence (Selvig and Alspaugh, 2011; Vykova, 2017). The elevated CO₂ inside mounds could thereby not only affect the growth but also the virulence of potential pathogens. Species of Metarhizium prefer alkaline conditions and can increase surrounding pH (Lovett and St. Leger, 2015) to optimize growth and conidia production (Gao et al., 2009).
Lowered pH could thus limit proliferation of certain fungi, but as many fungi inhabit mildly acidic environments, such as soils and plant and animal surfaces (Vylkova, 2017), low pH in itself is most likely not enough to rid unwanted fungi. Increased CO₂ concentration also stimulates the AMP/Protein kinase A pathway, which can regulate fungal growth, development, and conidia and secondary metabolite production (Arzam et al., 2010; Sun et al., 2022). We did not evaluate whether elevated CO₂ affected this pathway, but it could explain the observed effects. Irrespective of the causal effects of elevated CO₂ on growth and reproduction, our findings imply that comb CO₂ should impact fungal proliferation and ultimately reproduction, potentially in species-specific ways. The variation we observe implies that more elaborate comparisons are needed to establish how variable responses are to elevated CO₂ levels, and that different optima perhaps exist for specialists in the symbiosis vs. generalist pathogens and saprotrophs. Once established, a natural next step would be manipulation experiments to test the impacts of compromised CO₂ environments on colony health to establish the adaptive benefits of CO₂ in defense.

We guided our experiments based on CO₂ concentrations in the massive mounds of M. bellicosus, which were comparable to levels in another species that also builds large mounds, such as M. carbonarius (Matsumoto, 1978). CO₂ concentrations in species with comparatively large colonies and ventilation turrets, i.e., members of the genera Macrotermes and Odontotermes that represent ~33% of the known species within the Macrotermitinae (Bänki et al., 2022) are likely similar. However, ventilation and the propensity for CO₂ to play a role in defense is conceivably different in termite genera with different colony structures. For example, species of the genera Microtermes and Ancistrotermes build small interconnected fungal comb chambers within the soil (Jouquet et al., 2003; van de Peppel and Aanen, 2020). Insights into CO₂ levels here are absent, and future work should take a comparative approach to establish in detail the environment within fungal combs as well as the potential for (or limitations of) CO₂ to act in defense.

The extended phenotype of the termite mound is a cornerstone in termite ecology. Our findings point to an additional adaptive role emerging from generating a comb environment that aids in the suppression of alien fungi. This speaks to the need to view disease suppression in a more holistic view than previously considered. It also implies that other complementary parameters may play roles in defense, such as reduced oxygen availability or volatile compounds present within the comb headspace. Preliminary work on the latter suggests that this is indeed plausible (Kreuzenbeck et al., 2022, 2023). In addition, the ability to tolerate the extreme environment within mounds is likely an adaptive homeostatic trait that the termite can fine-tune to provide not only a defensive barrier but also optimal growth conditions for their specific crop species (and strains). Furthering our understanding of how termite farmers manage this complex environment for optimal farming of their fungal crop for food, while effectively suppressing antagonists, should thus integrate comb abiotic conditions.

### Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

### Author contributions

SS, NB, and MP: conceptualization and writing—original draft. SS, NB, KS, NK, and NM: data curation. SS, NB, MP, NM, and RM: methodology. SS, NB, and RM: formal analysis and visualization. SS, NB, RM, NM, KS, NK, and MP: writing—review and editing. MP and NK: supervision. MP: funding acquisition. All authors contributed to the article and approved the submitted version.

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### Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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### Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1134492/full#supplementary-material

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