Exploring the impact of sub-ambient CO_2 concentrations on *Oxalis* growth: Implications for food sustainability among Pleistocene human foragers in the Greater Cape Floristic Region

THESIS

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Abstract

Humans inhabiting the glacial refugia of the Cape Floristic Region during the Middle Pleistocene are believed to have relied on underground storage organs (USOs) as a crucial carbohydrate source. While previous research has highlighted the nutritional potential of these plants for early human gatherers, such studies are typically conducted under modern climatic conditions and do not account for the reduced plant productivity and USO yields associated with the lower atmospheric CO_2 concentrations characteristic of Pleistocene glacial periods.

To evaluate the impact of CO_2 availability on plant productivity, I cultivated two *Oxalis* species—*O. pes-caprae* and *O. punctata*, both known to have been harvested by early foragers—under CO_2 concentrations ranging from 180 ppm to 400 ppm. Results showed that glacial-level CO_2 concentrations significantly reduced bulb production in *O. pes-caprae* and photosynthetic rates in both species. Using these findings to model Pleistocene foraging behaviour, I estimate that human foragers would have needed to spend an additional 8 hours gathering food to meet their daily dietary requirements of 2000 calories, suggesting the need for alternative food sources to supplement their diet.

These findings emphasise the importance of considering historical plant growth conditions, particularly atmospheric CO_2 levels, when reconstructing past human diets. Failure to account for these factors may represent a significant gap in our understanding of human subsistence strategies during glacial periods.

Chapter 1: Introduction

1.1 Background

The genesis of anatomically modern *Homo sapiens* from hunter-gatherers is associated with dietary shifts, environmental adaptations, and climatic alterations. The evolution of modern humans has attracted significant scholarly attention, resulting in extensive research on the emergence and evolution of *Homo sapiens*. Archaeological remnants, such as plant and animal deposits in caves, offer invaluable insights into the dietary habits (De Vynck et al., 2016; Kyriacou et al., 2014; Singels et al., 2016), social behaviour (Hill and Hurtado, 1989), and habitats of early humans (Ambrose, 1998; Laden and Wrangham, 2005; Marlowe, 2005).

The Middle Pleistocene epoch was characterized by significant climatic changes, notably the Marine Isotope Stage 6 (MIS 6) glacial phase (195 – 123 kya, thousand years ago). This phase coincides with the emergence of modern humans (200-195 kya) (Hetherington, 2012) and would have resulted in extremely cold and dry conditions, seasonal rainfall, and reduced sea levels (Compton, 2011). At this time, atmospheric carbon dioxide concentrations ([CO_2]) dropped to ~200 parts per million (ppm), in stark contrast to the current concentration of approximately 400 ppm (Khon et al., 2014) (*Fig 1-1*).

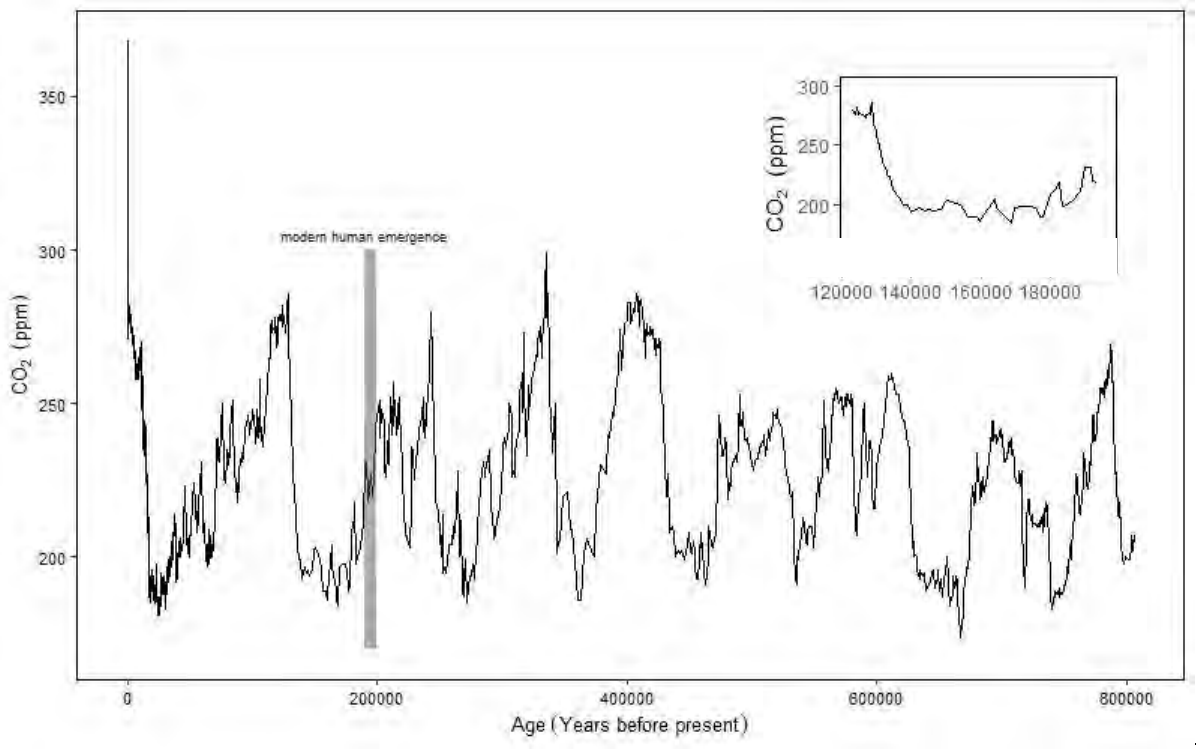

Figure 1-1: Atmospheric CO$_2$ concentrations for the last 800 000 years with the period of modern human emergence highlighted. The plot was created from records from Bereiter *et al*., 2015 and Luthi *et al*., 2008.

The Greater Cape Floristic Region (GCFR) on South Africa's west coast has been a focal point in the study of modern human evolution due to its role as a Pleistocene refuge for hunter-gatherers. The GCFR is characterized by winter rainfall and nutrient-poor soils, and boasts over 9000 plant species, approximately 67% of which are endemic (Helme and Trinder-Smith, 2006). The region also features carbohydrate-dense geophytes and a coastline abundant with marine resources, providing a diverse food source during the Pleistocene (De Vynck et al., 2016; Deacon, 1993; Dominy et al., 2008; Procheş et al., 2006).

Numerous studies have focused on the geophyte species harvested by hunter-gatherers and the time spent foraging daily (De Vynck et al., 2016; Singels et al., 2016; Singels et al., 2016; Vincent, 1985). Evidence indicates that certain species were easy to harvest or provided a high caloric value and that the effort required to meet daily nutritional requirements was minimal. However, models predicting hunter-gatherer harvesting patterns are based on present-day populations and current climatic conditions. Consequently, predictions regarding food availability and sustainability are contingent on the prevailing temperatures and CO_2 concentrations, which significantly influence photosynthesis, plant growth, and nutrient levels.

This thesis aims to enrich the existing body of research on hunter-gatherer harvesting patterns, taking into account climatic changes between the present and the Pleistocene era. The study specifically examines the effect of CO_2 and nutrient concentrations on plant growth, photosynthetic rates, and anatomy. Plants were cultivated under sub-ambient concentrations to assess their response, and the findings were incorporated into harvesting models to simulate harvesting under glacial CO_2 concentrations. The working hypothesis is that low CO2 levels and nutrient scarcity would restrict overall plant growth, reflecting a limitation in food availability and increased harvesting efforts for hunter-gatherer communities.

1.2 Thesis outline

Chapter 2: Growth responses to glacial [CO_2]

This chapter focused on the effect of sub-ambient [CO_2] and nutrient limitation on geophyte growth. Bulb and total plant biomass, and reproduction are reported.

Chapter 3: Physiological responses to glacial [CO_2]

This chapter reports the effect of sub-ambient [CO_2] on the photosynthetic rates of *O.pes-caprae* and *O. punctata.* RUBISCO activity and limitations to photosynthesis are included, as well as carbon and nitrogen concentrations of the plants.

Chapter 4: Plant anatomy

This chapter reports on the effects of [CO_2] on *Oxalis* leaf anatomical traits. Leaf thickness, stomata and epidermal traits are reported.

Chapter 5: Modelling

Data from current studies on harvesting patterns was used to estimate the amount of time required for harvesting during the Pleistocene as well as the percentage of foraging trips achieving the required calories per day.

Chapter 6: Conclusion

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Chapter 2: Growth responses of Oxalis punctata and O. pes-caprae at sub-ambient CO_2 concentrations and with nutrient limitations

2.1. Introduction

Climate change is a complex and far-reaching phenomenon that significantly influences plant growth, thereby intricately affecting the availability of food resources vital for human sustenance. The consequences of changing climatic conditions extend beyond ecological concerns, posing a direct threat to global food sustainability and potentially shaping the trajectory of human development. As such, climate change has become a central and necessary theme in contemporary studies exploring the complexities of human evolution and development.

Approximately 200 thousand years ago, the emergence of anatomically modern humans in Africa coincided with a significant cold event, Marine Isotope Stages 6 (MIS 6, occurring around 195 thousand years ago) (Hetherington, 2012).

For most of Africa, the cold events during MIS 6 introduced reduced rainfall, temperature decreases of up to 10°C below present values, and atmospheric CO_2 concentrations as low as 180 ppm (Khon et al., 2014; Marean, 2010). These environmental conditions significantly impacted the accessibility of food resources for emerging human populations, prompting a need for adaptive strategies and alternative habitats or refugia during maximum glacial conditions (Henshilwood and Marean, 2003).

This study focuses on investigating one such potential refugium, the Greater Cape Floristic Region (CFR) located on the south coast of South Africa. The CFR, characterized by its diverse shoreline and abundant flora, is hypothesized to have provided early human populations with crucial carbohydrate-dense geophytes, including corms, bulbs, rhizomes, and tubers, alongside other dietary staples such as fruit and shellfish (Kyriacou et al., 2014; Marean, 2010a; Marean et al., 2014).

Despite the wealth of data derived from excavations and models predicting human populations in Africa during glacial conditions, existing models of human forager diets and Pleistocene conditions have primarily been developed under the assumption of current climatic conditions.

This oversight raises questions about the accuracy of these models in capturing the interplay between climate variations and their impact on food availability and nutritional value.

This research aims to bridge this gap by specifically investigating the effects of CO_2 concentrations on plant growth, with a particular emphasis on underground storage organs (USOs), using species from the family Oxalidaceae. By incorporating varying nutrient levels, the study seeks to unravel the interactions between CO_2 concentrations and environmental factors, especially soil nutrient quality (De Vynck et al., 2016; Singels et al., 2016). Through this detailed examination, the research contributes to a more comprehensive understanding of how climate change, specifically variations in CO_2 concentrations, may have influenced the availability of plant resources, thereby playing a pivotal role in shaping the course of human evolution and development. I hypothesized that sub-ambient CO_2 and nutrient limitation would limit plant growth, resulting in smaller plants.

2.2. Methods

Oxalis pes-caprae L. and *Oxalis punctata* L.f. were used as model species to investigate the effects of CO_2 on USO growth. *Oxalis* is one of the species used by contemporary Khoe-San descendants in South Africa and it is considered one of the top ranked edible species (De Vynck et al., 2016) and would have been harvested by human foragers during the Pleistocene. The effect of CO_2 and nutrient concentrations on *Oxalis* growth was studied by performing experiments investigating the effect of CO_2 and nutrient supply, and the interaction of both nutrient and CO_2 supply.

2.2.1 Experiment 1: CO_2 x Nutrients

To study the combined effects of CO_2 and nutrients, twenty *O. punctata* plants harvested from the Rhodes University Botany department garden, South Africa were grown at three CO_2 concentrations and at two nutrient levels. Plants were grown from bulbs and were transplanted into custom-made CO_2 chambers once the leaves had started to sprout. An average bulb weight of (0.43 ± 0.18g) was used as a starting point to standardise the initial weights of the plants.

Custom made open-top chambers (OTCs) had a volume of ~1L and were constructed from 1.12 mm polycarbonate sheeting and consisted of a cylinder (110 x 110 mm) extended a further 30 mm by a frustum with a 75 mm opening at the top end. A transparent petri-dish with five 13 mm holes was used to cover the opening of the open-top chambers. The chambers were inserted over open ends of 104 x 300 mm cylindrical pots constructed from PVC piping and closed at one end with a stop-end. The chambers were held in position by a ring such that they overlapped the pots by 10 mm and fitted snugly onto the outer diameter of the pots *(Fig. 2-1)*. Pots were divided into two halves with a longitudinal division running the entire length of the pot and glued at the pot diameter and across the stop-end to form two completely isolated compartments. Stop-ends were perforated with several 5 mm hole to prevent water logging. Plants were grown on either side of the division.

Figure 2:1: Custom-made mini open top chambers used to grow *Oxalis pes-caprae* and *O. punctata* to study the effects of glacial CO_2 concentrations.

Air was introduced into OTCs via a circular perforated diffuser 25 mm above the soil into the chambers at a flow rate of 900 ml min^{-1}. To obtain the desired sub-atmospheric CO_2, a portion of the air stream was passed through a soda-lime column using Accu-Air aquarium valves (J.W. Pet Company, USA). The CO_2 concentration in the chambers was monitored and measured hourly. A solenoid-valve system controlled via a CR-1000 logger and SDM-CD 16AC relay driver (Campbell Scientific, USA) sequentially diverted air to a Li-820 infra-red gas analyser (Licor Biosciences, Nebraska, USA). The analyser was zeroed and calibrated hourly.

The experiment was carried out in a poly-tunnel with day/night average (± SD) temperatures within the chambers at 28.4 ± 3.7 and 15.3 ± 0.9°C respectively. The plants received 11 hours of daylight at an average daytime Photosynthetic Photon Flux Density (PPFD) or 490 µmol m^{-2} s^{-1} and daily maximum values in excess of 1500 µmol m^{-2} s^{-1}.

Three treatments of CO_2 (180, 280, 400ppm) were applied with 50 ml Long Ashton nutrient solution (50% for low nutrient and 100% for high nutrient) added weekly. The nutrient treatments were applied in the experiment to simulate pH levels and nutrient soils in the Cape region of South Africa (Dakora, 2012; Cramer, 2010). In addition to the Long Ashton solution, plants were watered with tap water twice a week. CO_2 concentrations in the chambers were monitored and average ± sd values in each chamber, and the percentage of values within the standard deviation, 50 ppm and 100 ppm of the target CO_2 values is presented in *Fig. 2-2* and *Table 2-1*. High and low nutrient treatments were added to each pot, each on one side of the central division. The bulbs were grown in a mixture of building sand and river sand as the growth medium. The leaves were clipped off at the start of the experiment so that only the bulbs remained.

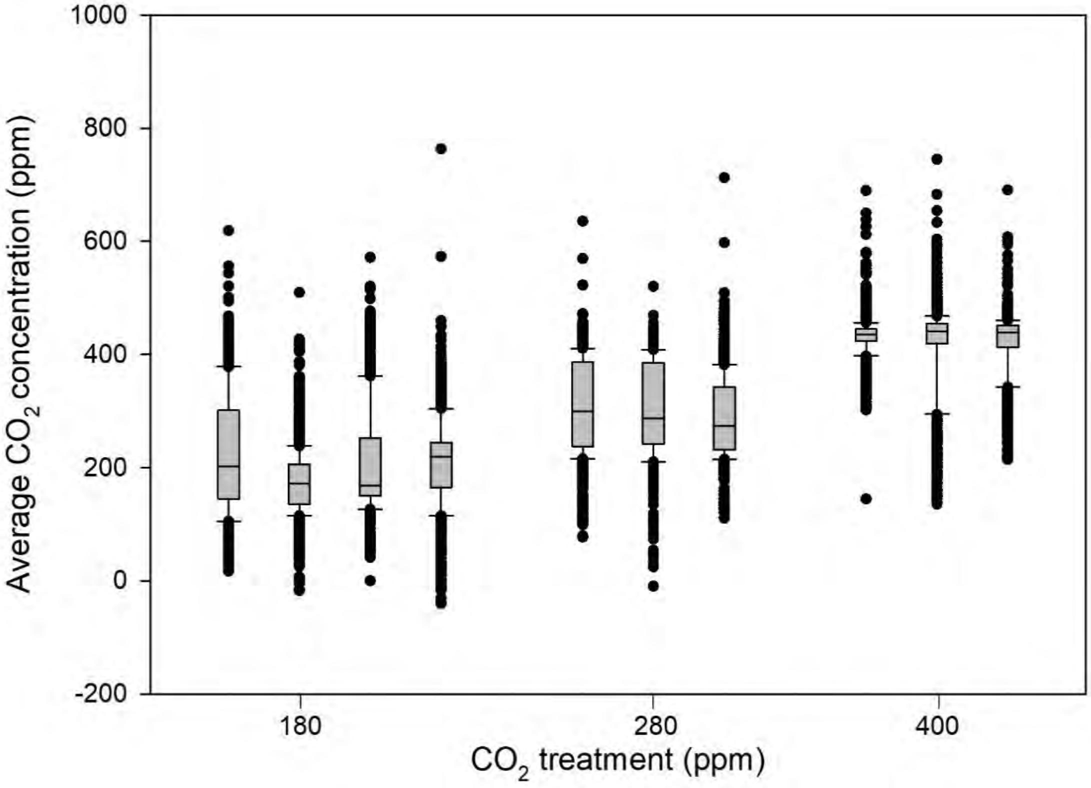

Figure 2-2: Average values for CO_2 concentrations supplied to *O. punctata* for 8 weeks. Number of columns in boxplots represents the number of chambers used for each CO_2 x nutrient treatment.

Table 2-1: Mean ± sd [CO_2] in each open-top chamber supplied to *O. punctata* for 12 weeks with the percentage of CO_2 values that fell within the sd, 50ppm or 100 ppm of the target [CO_2].

Target [CO_2] (ppm)	Chamber	Mean (ppm) ± sd	% of points within sd	% of points within 50 ppm	% of points within 100 ppm
180	7	221.4 ± 106.5	60.53	40.02	59.05
180	8	175 ± 57.09	72.68	63.11	92.94
180	9	205.6 ± 88.8	75.81	59.91	78.76
180	10	209.65 ± 79.9	73.45	45.85	79.1
280	4	302 ± 77.4	68.87	47.82	70.35
280	5	304.27 ± 83.5	52.98	41.07	67.53
280	6	286.76 ± 72.21	73.36	45.49	86.19
400	1	432.13 ± 32.91	36.16	80.36	97.97
400	2	415.98 ± 76.16	36.16	50.83	87.35
400	3	420.98 ± 53.71	68.69	60.96	93.19

2.2.2 Experiment 2: CO_2

Experiment 1 was replicated with *O. pes-caprae* L. at 100% Long Ashton to study the individual effects of CO_2 under no nutrient modification. Twenty *O. pes-caprae* plants were grown from bulbs (0.32 ± 0.07g; n=5) and four CO_2 concentrations (180, 240, 300, 400ppm) were applied with increased replication from experiment 1 (n=5). Bulbs were obtained from Devon Valley Road in Stellenbosch, South Africa. Carbon dioxide concentrations in the chambers were monitored and average ± sd values in each chamber, and the percentage of values within the standard deviation, 50 ppm and 100 ppm of the target CO_2 values are presented in *Fig. 2-3* and *Table 2-2*. The same open-top chambers were used, except in this instance pots were not permanently split down the middle.

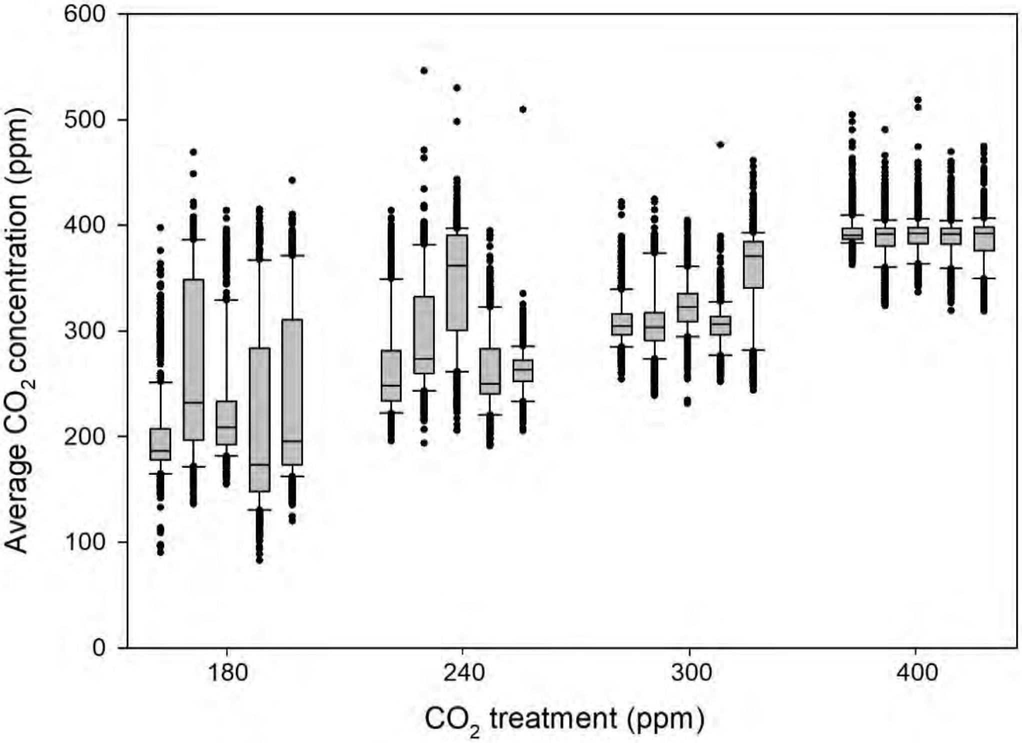

Figure 2-3: Average values for CO_2 concentrations supplied to *O. pes-caprae* for 12 weeks. Number of columns in boxplots represents the number of chambers used for each CO_2 treatment.

Table 2-2: Mean ± sd [CO_2] in each open-top chamber supplied to *O. pes-caprae* for 12 weeks with the percentage of CO_2 values that fell within the sd, 50ppm or 100 ppm of the target [CO_2].

Target [CO_2] (ppm)	Chamber	Mean (ppm) ± sd	% of points within sd	% of points within 50 ppm	% of points within 100 ppm
180	1	199 ± 42.1	82.3	85.4	92.3
180	5	262.7± 81.4	58.8	49.2	62.5
180	9	227.1 ± 56.3	76.7	73.8	84.7
180	13	213.7 ± 88.6	72.1	59.1	74.3
180	17	234.4 ± 80.2	67.9	64.5	69.3
240	2	264.8 ± 46.5	77	75.8	84.2
240	6	295.7 ± 51.5	59.9	59.6	78.5
240	10	344.5 ± 54.3	22.6	21.3	39.4
240	14	262.3 ± 38.6	71.5	77.6	95.2
240	18	262.1 ± 21.6	44.4	92.9	100
300	3	309 ± 23.7	78	93.3	99.6
300	7	309.7 ± 32.7	76.2	86.4	99.5
300	11	324 ± 26	56.9	86.2	99.9
300	15	305.1 ± 20.7	73.5	98	100
300	19	356.8 ± 41.6	23.1	31.4	94.7
400	4	394.3 ± 15	78.1	98.7	100
400	8	388.2 ± 20.2	72.2	93.1	100
400	12	390.4 ± 18.9	72.2	97.1	99.9
400	16	388.4 ± 19.7	72.1	94.1	100
400	20	386.7 ± 23.9	70.4	88.7	100

2.2.3 Experiment 3: Nutrients

To study the individual effects of nutrients, twenty-one *O. pes-caprae* bulbs (0.29 ± 0.02g) were planted in sand to a depth of 5 cm under ambient CO_2 concentration. Bulbs were obtained from Devon Valley Road in Stellenbosch, South Africa. Three nutrient concentrations were applied (50%, 70%, 100% Long Ashton growth solution). Plants were grown under controlled greenhouse conditions in standard garden plastic pots and 50 ml of Long Ashton solution was applied weekly.

For all 3 experiments plants were harvested, dried and weighed, and divided into shoots, bulbs, tubers and roots. Bulbs were defined as short fleshly underground stems with overlapping protective swollen scales and are involved in vegetative reproduction or the survival of the plant during seasons of dormancy (Dominy et al., 2008). Tubers were defined as underground horizontal swollen stems aiding in the storage of nutrients (Dominy et al., 2008; Mauseth, 2003). These descriptions were used to identify different plant components at harvest.

2.2.4 Statistical analysis

Since shoots did not emerge at the same time, harvest biomass was corrected for date of emergence to account for different lengths of time plants were under treatment. The following formula was used to calculate corrected biomass accumulated during the experiment.

$$\frac{harvest\ biomass * days\ after\ emergence}{total\ length\ of\ experiment}$$

To test the effects of CO_2 and nutrient concentrations on biomass, a Generalised Linear Model (GLM) was used in R. In cases where there was a significant effect of CO_2 or nutrient concentrations, a Tukey post-hoc test was conducted to test for differences between CO_2 and nutrient concentrations. Differences in the number of flowers produced between species were examined using a GLM with Poisson distribution.

2.3. Results

2.3.1. Total and above-ground responses

As hypothesized, when well supplied with nutrients (100% Long-Ashton), *Oxalis pes-caprae* total biomass decreased with lower CO2 concentrations. CO2 had a significant effect on total biomass ($F_{1,17} = 21.2, p < 0.01$), and there were significant differences between CO2 treatments (*Fig. 2-4*). When grown with limited nutrient supply (50% Long-Ashton), *O. punctata* total biomass showed a similar trend to the plants grown with 100% Long-Ashton, and the response was now significant ($F_{2,7} = 5.24, p = 0.041$). The above-ground components of *O. punctata* growth had no significant changes between CO2 treatments (*Fig. 2-4*).

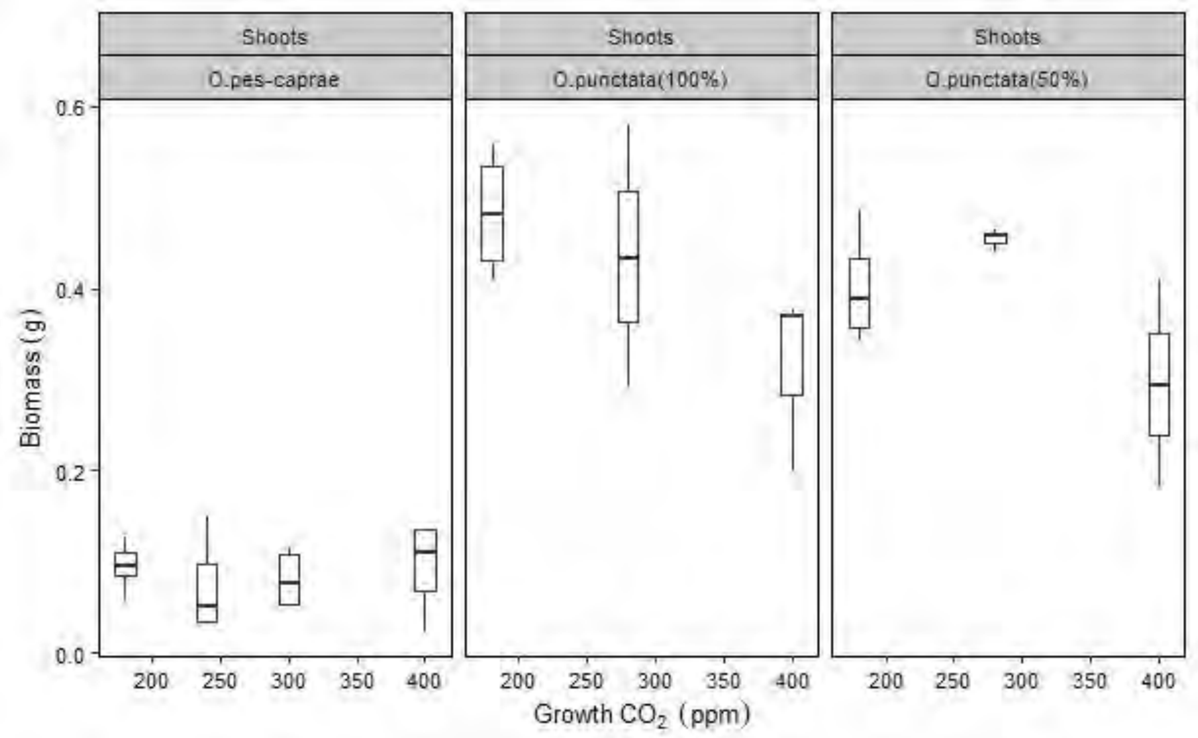

Figure 2-4: Total and above-ground responses of *Oxalis pes-caprae* grown under four CO2 concentrations (180, 240, 300, 400ppm) and 100% Long Ashton (n = 5 for each treatment); and *O. punctata* grown under three CO2 concentrations (180, 280, 400 ppm) and two nutrient concentrations (100% and 50% Long Ashton) (n = 3 for each treatment, except at 180 ppm where n = 4).

2.3.2. Below-ground responses

Below-ground responses were similar to above-ground responses, and *O. pes-caprae* showed a negative response to CO2 deprivation. *O. pes-caprae* developed bulbs (*Fig. 2-5A*), and the number of bulbs produced remained the same across the CO2 gradient. Bulb biomass was significantly affected by CO2 ($F_{2,19} = 4.3, p = 0.006$), increasing by up to 80% from 180 to 400 ppm. On the other hand, *O. punctata* bulb biomass did not respond in the same way to CO_2 enrichment. The bulbs that were planted at the beginning of the experiment were absent at harvest, and a new fleshy tap root (referred to as a "tuber") and associated fibrous roots had developed (*Fig. 2-5B*). There was an unexpected increase in root and tuber biomass with decreasing CO_2 (*Fig. 2-6*), suggesting biomass reallocation during the first month of growth.

Figure 2-5: Images of *O. pes-caprae* grown under four CO_2 concentrations for 12 weeks (A) and *O. punctata* grown at three CO_2 concentrations and two nutrient concentrations (B) at the point of harvest. Arrows on images represent bulbs (b), roots (r) and the tuber (t).

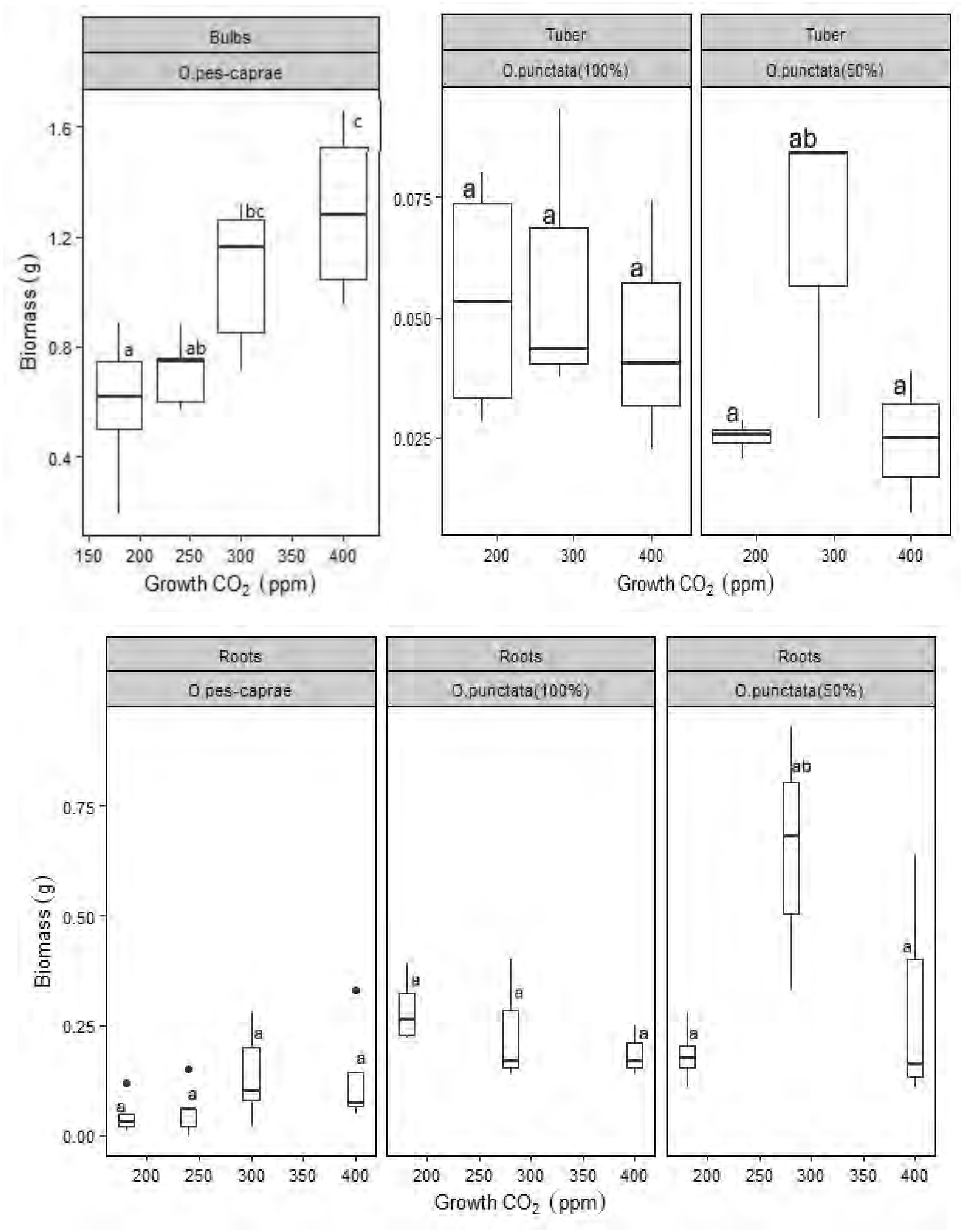

Figure 2-6: Below-ground responses of *Oxalis pes-caprae* grown under four CO$_2$ concentrations (180, 240, 300, 400ppm) and 100% Long Ashton (n = 5 for each treatment); and *O. punctata* grown under three CO$_2$ concentrations (180, 280, 400ppm) and two

nutrient concentrations (100% and 50% Long Ashton) (n = 3 for each treatment except at 180 ppm where n = 4). Significant differences at $p < 0.05$ between treatment groups are denoted by different letters.

2.3.3. Reproduction

Oxalis species can reproduce both vegetatively and sexually. The effect of CO_2 on reproduction was measured by observing the frequency of flowering versus the number of bulbs produced. When comparing reproduction between the two species, I found that *O. pes-caprae* allocated resources to vegetative reproduction, with only 8 (out of 15) of the plants flowering from 180 to 300 ppm and 2 (out of 5) at 400 ppm (*Fig. 2-7A*). In contrast, *O. punctata* allocated resources to flowering. All plants flowered throughout the growing season, and no bulbs were produced at the time of harvest. The timing of flowering was consistent across the CO_2 gradient. On average, flower production was higher under low CO_2 and nutrient-limited conditions, but there was no significant effect of individual or combined treatments (*Fig. 2-7B*). The differences observed in reproductive patterns may be species-specific, with *O. pes-caprae* predisposed to vegetative reproduction and *O. punctata* predisposed to sexual reproduction. Statistical analysis showed that species type had a significant effect on the number of flowers produced ($Z = 5.82, p < 0.001, df = 38$). However, the increase in flowering at glacial CO_2 levels could suggest a preference for sexual reproduction when CO_2 is limiting.

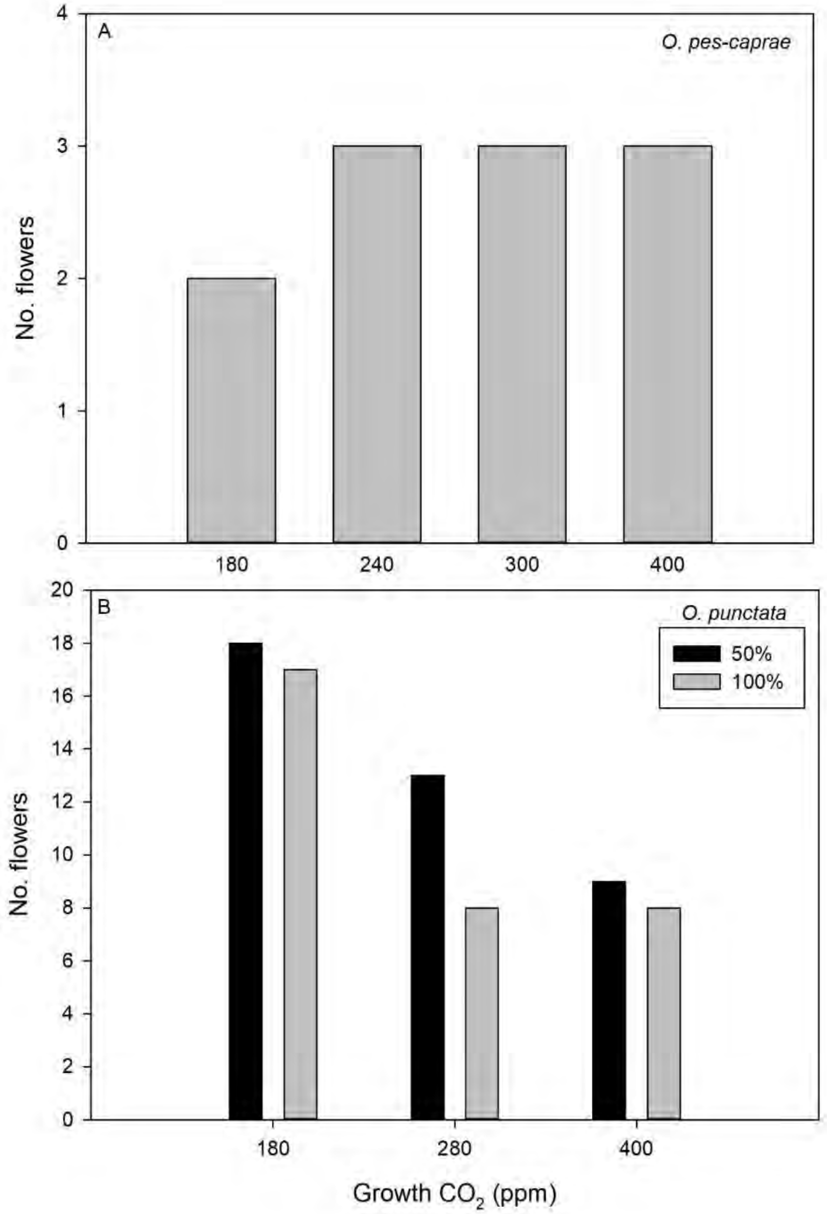

Figure 2-7: Number of flowers produced in *O. pes-caprae* grown for 12 weeks under varying CO_2 concentrations in one growing season (A), and of *O. punctata* grown at three CO_2 concentrations within a period of 8 weeks (B). *O. punctata* plants were grown at three concentrations of CO_2 (180, 280, 400 ppm) and two nutrient concentrations (100% and 50% Long Ashton). N=3, except for 180ppm where n=4.

2.3.4. Nutrient effects on **Oxalis pes-caprae**

Increasing the available nutrients in the soil was expected to increase plant growth, and this was observed after applying three nutrient treatments to *Oxalis pes-caprae* at ambient CO_2. There was a significant effect of nutrients on bulb biomass ($F_{2,18} = 5.77, p = 0.01$), and in all plant components, biomass increased in a similar manner from 50% to 100% nutrient strength (*Fig.2-8*). In considering reproduction, plants mostly produced bulbs and there was no emphasis on flowering. However, plants grown at 100% nutrient strength did produce flowers throughout the growing season in addition to bulbs

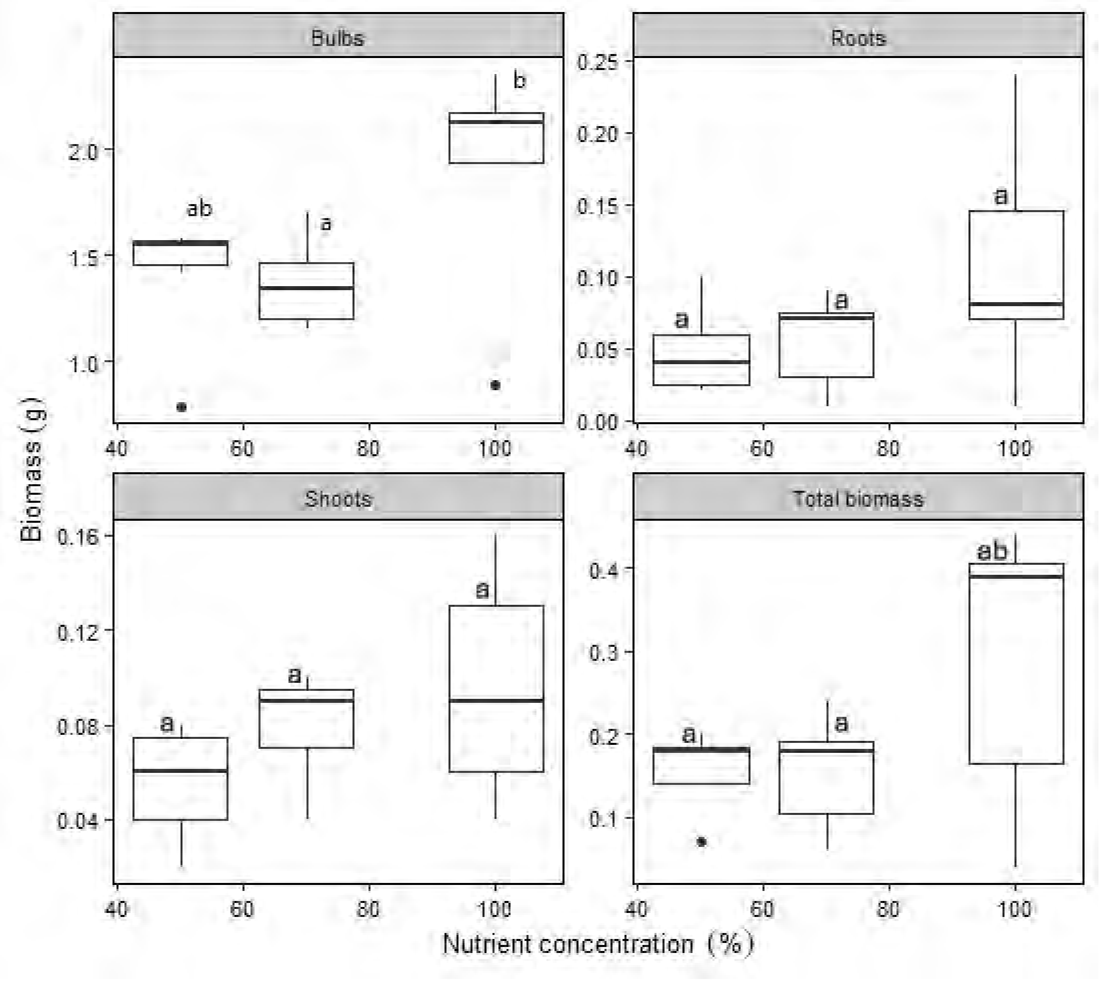

Figure 2-8*: Oxalis pes-caprae* responses to three nutrient concentrations (50%, 70% and 100% Long Ashton) and ambient CO_2 (N = 7 for each treatment). Significant differences at $p < 0.05$ between treatment groups are denoted by different letters.

2.4. Discussion

The present study explored the responses of two *Oxalis* species to diverse carbon dioxide concentrations. The initial hypothesis was that both species would exhibit a decrease in biomass under low [CO_2] circumstances. This hypothesis was supported in the case of *O. pes-caprae*, while the biomass of *O. punctata* remained remarkably constant despite changes in the CO_2 supply.

At the point of harvest, *O. punctata* demonstrated no indications of vegetative reproduction and produced only flowers. Existing literature indicates that this genus largely relies on sexual reproduction, resorting to vegetative reproduction solely under adverse conditions (Rossa et al., 2011). The responses displayed by *O. punctata* can be attributed to the reallocation of carbon resources from the initial bulbs utilized in the experiment. As the bulbs were consumed, new tap roots, aboveground shoots, and flowers developed in the plants. Preliminary observations indicate that the carbon redistribution could fully compensate for the carbon constraint when photosynthesis took place in a CO_2 deprived atmosphere. A more extensive experiment might elucidate the impact of CO_2 on this species' sexual and asexual reproduction. Conversely, *O. pes-caprae* yielded fewer flowers and showed a preference for asexual reproduction, with bulb mass significantly diminished at glacial CO_2 concentrations.

The responses of *O. pes-caprae* align with the recorded responses of C3 species to low CO_2 conditions. Flowering in these species is frequently delayed or halted at low atmospheric CO_2 concentrations, resulting in a biomass redistribution from below-ground to shoots and an overall decrease in plant biomass. This is a typical response for C3 species grown under low CO_2 concentrations (Dippery et al., 1995; Campbell et al., 2005). Although a reduction in growth is anticipated under low CO_2 concentrations, in this study only *O. pes-caprae* was affected by decreased CO_2 concentrations. An increase in carbon allocation to roots and sexual reproduction in *O. punctata* could have potentially compromised vegetative reproduction. The duration of the study was not sufficient to fully interpret how plants might grow and allocate carbon across multiple seasons at low CO_2 concentrations. Prolonged studies would offer a more comprehensive understanding of species responses, particularly with respect to potential reproductive strategies and bulb formation.

A limited supply of nutrients can have detrimental effects on plant growth and may amplify the effects of low CO_2 growth conditions (Sage & Cowling, 2001). Nutrient limitations were instrumental in determining plant biomass in *O. pes-caprae* and likely influenced growth responses to altered CO_2 supply. This interaction was not investigated for *O. pes-caprae*, but it was for *O. punctata*, without observing any impact on biomass production. This hints that nutrient and carbon reallocation from bulbs could mitigate treatment effects across a single growth season.

The study demonstrated that growth at glacial CO_2 concentrations would have significantly influenced USO production in *O. pes-caprae*, but not in *O. punctata*. This underscores the need for long-term experiments, growth from seeds, and the exploration of more species vital for human foragers.

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Chapter 3: Physiological response of Oxalis to sub-ambient CO2 concentrations during growth

3.1. Introduction

Plant productivity is linked to the atmospheric concentration of carbon dioxide (CO_2), exerting profound influences on growth dynamics, reproductive processes, and resource allocation within plant systems. The essential role of CO_2 in facilitating photosynthesis, the process through which plants generate crucial carbohydrates for growth, maintenance and reproduction, emphasizes its significance in shaping plant development. Elevated CO_2 concentrations have been associated with enhanced photosynthesis, leading to increased plant growth and overall size.

The impact of CO_2 on plant systems is widespread, manifesting across various scales, from the biochemical intricacies within individual plants to broader ecosystem dynamics (Ziska and Bunce, 2006). This understanding assumes particular importance in the context of studying plant growth during the Pleistocene. Altered CO_2 levels can induce physiological responses, including changes in photosynthetic rates, stomatal behaviour, and carbon allocation within plants. These responses play a pivotal role in determining the potential size of plants under sub-ambient CO_2 conditions and the rates at which photosynthesis occurs.

Central to the process of carbon fixation is the Calvin cycle, wherein CO_2 is converted into carbon skeletons critical for synthesizing plant organic molecules (Quick and Neuhaus, 1997). Initiated by the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO), the Calvin cycle transforms ribulose-1,5-bisphosphate (RuBP) and CO_2 into 3-phosphoglycerate (3-PGA). The ensuing production of triose phosphates, essential for the synthesis of sucrose and starch, along with the regeneration of RuBP, perpetuates the cycle (Raines, 2003). While the Calvin cycle encompasses 13 reactions catalysed by 11 enzymes, this study focuses on RUBISCO, given its primary role in determining the rate of CO_2 fixation.

RUBISCO, despite its abundance, (accounting for up to 50% of the total soluble protein in C_3 plant leaves (Phillips and Milo, 2009; Sharkey et al., 2012)), is characterized by inefficiency and a slow catalytic rate compared to other enzymes. Catalysing only three reactions per second with a turnover frequency of 2 to 10 carbon molecules per second (Erb and Zarzycki, 2018), RUBISCO's multifunctionality also includes an affinity for oxygen (O_2), inhibiting its carboxylation activity. The binding of O_2 triggers photorespiration, resulting in the release of fixed CO_2 back into the atmosphere and the dissipation of approximately 30% of photosynthetic energy in plants (Walker et al., 2016; Zhu et al., 2010). This 30% limitation due to photorespiration is anticipated at current CO_2 concentrations.

The evolutionary history of RUBISCO, shaped during a period of significantly lower atmospheric O_2 and higher CO_2 levels (Buick, 2008; Tabita et al., 2007), contributes to its inability to discriminate between the two gases. In low CO_2 atmospheres, RUBISCO favours O_2 as the substrate, limiting photosynthetic rates and increasing the occurrence of photorespiration.

The rate of photosynthesis isn't only limited by RUBISCO, but by the efficiency of CO_2 absorption from the atmosphere. Stomata, pivotal structures regulating CO_2 uptake from the atmosphere, respond to changes in CO_2 concentration by adjusting their opening and closing. In low CO_2 environments, stomatal opening intensifies to maximize CO_2 absorption (Pinto et al., 2014), concurrently escalating water loss through transpiration (Sharkey et al., 2012).

While the concentration of CO_2 is a critical determinant of plant growth rates, its effects are often modulated or intensified through interactions with other environmental factors such as soil nutrient concentrations and water availability. Drought conditions, for instance, magnify the effects of CO_2, as stomatal closure to conserve water restricts CO_2 absorption (Sage, 1995; Sage and Cowling, 1999). Conversely, high nutrient concentrations have been shown to enhance photosynthetic rates (Evans, 1989).

This chapter seeks to unravel the effects of CO_2 on plant physiology, employing species from the family Oxalidaceae. The study investigates changes in photosynthetic rates, stomatal conductance, and leaf carbon and nitrogen content under sub-ambient and ambient CO_2 levels. By examining the individual and interactive effects of CO_2 and nutrient concentrations, this research aims to contribute to the understanding of the relationship between environmental factors and plant physiological responses and explain the observations in biomass presented in Chapter 2. The expectation was that under sub-ambient CO_2, photosynthetic efficiency and stomatal conductance would decrease.

3.2. Methods

3.2.1 Plant growth conditions

Plants from Experiment 2 and 3 (as described in Chapter 2) were used in making the following measurements.

3.2.2 Gas exchange

Photosynthetic responses to CO_2 were measured on mature *O. punctata* and *O. pes-caprae* leaves with a LI-COR 6400 portable photosynthesis system (Li-Cor Inc. Lincoln, Nebraska, USA). Measurements were made at least 8 weeks after bulbs were planted. For each measurement, plants were removed from the greenhouse and measurements were made in the laboratory. Photosynthetic rates were measured at 11 points across a range of reference CO_2 concentrations from 50 ppm to 900 ppm.

Curves of photosynthetic rate vs intercellular [CO_2] (*A-Ci* curves) were plotted from the resulting measurements to illustrate the biochemistry of the leaf in response to changing [CO_2]. Individual *A-Ci* curves were fitted into the model of Farquhar et al. (1980) for each growth CO_2 concentration. From the curves, the carboxylation efficiency of RUBISCO was estimated from the initial rate of change of photosynthesis as *Ci* increases from a minimum (V_{cmax}). This is the photosynthetic rate when RUBISCO is limiting. The maximum rate of electron transport for RuBP regeneration (J_{max}) was estimated from the inflection point of the curves with a low rate of change of photosynthesis. All analyses were done in R (version 4.2.2) using the plantecophys package (version 1.4-6).

Maximum photosynthetic rates at each of the growth [CO_2] were measured, as well as the limitation on those rates presented by stomata. Stomatal limitation and stomatal conductance were calculated to estimate the limitation that stomatal opening impose on photosynthetic rates. Stomatal conductance was modelled using the 'BBOpti' model as described by (Medlyn et al., 2011) using the plantecophys package in R, and stomatal limitation was calculated as

$$L_s = \frac{(A0 - A)}{A} * 100$$

where A is the net CO_2 assimilation rate and A_0 is the net CO_2 assimilation rate if stomatal resistance was equal to zero.

3.2.3 Carbon and nitrogen analysis

After harvest leaves were dried in an oven at 80°C and ground into a fine powder to determine the percentage of carbon and nitrogen in the leaves. The samples were analysed using mass spectrometry at the University of Pretoria Stable Isotope Laboratory, Mammal Research Institute, South Africa. Aliquots of approximately 1.2 to 1.3 mg of the homogenized plant samples were weighed into tin capsules that have been pre-cleaned in toluene and several samples were done in duplicate. Isotopic analysis was done on a Flash EA 1112 Series coupled to a Delta V Plus stable light isotope ratio mass spectrometer via a ConFlo IV system (equipment supplied by Thermo Fischer, Bremen, Germany). A blank and two standards (Merck Gel :$\delta^{13}C$ = -20.26‰, $\delta^{15}N$=7.89‰, C%=41.28, N%=15.29 and DL-Valine : $\delta^{13}C$ = -10.57‰, $\delta^{15}N$=-6.15‰, C%=55.50, N%=11.86) were run after every 11 samples. All results were referenced to Vienna Pee-Dee Belemnite for carbon isotope values and to air for nitrogen isotope values. The results are presented as percentage carbon, percentage nitrogen and carbon to nitrogen ratios.

3.2.4 Statistical analysis

To test the effects of CO_2 and nutrient concentrations on biomass a Generalised Linear Model (GLM) was used in R (version 4.2.2). The individual effects of CO_2 and nutrients were tested, as well as their interactive effect. In cases where there was a significant effect of CO_2 or nutrient concentrations, a Tukey post-hoc test was conducted to test for differences between CO_2 and nutrient concentrations.

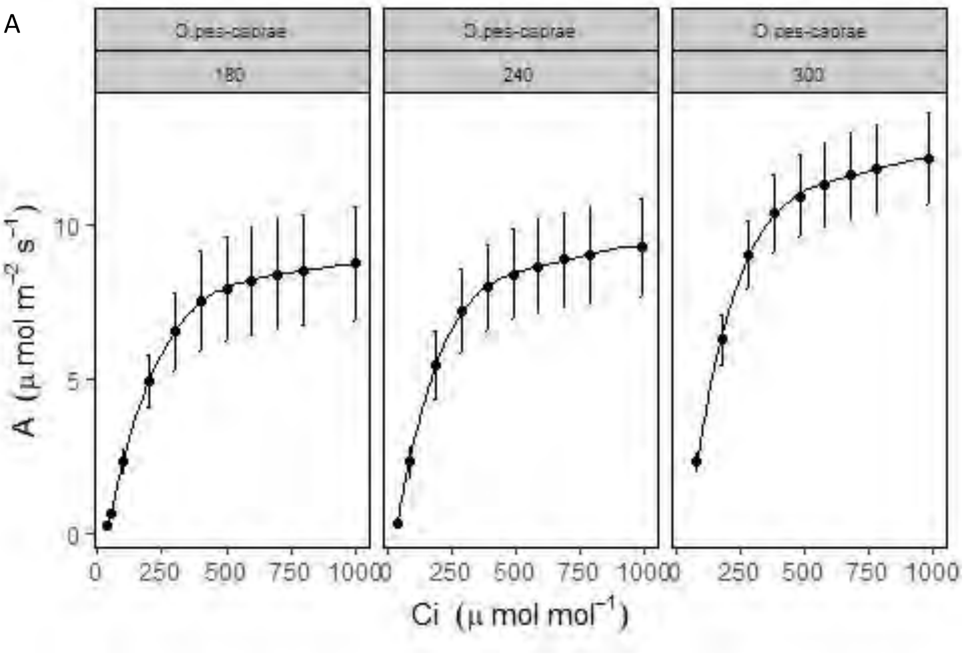
3.3. Results

3.3.1. Photosynthetic responses

A-Ci curves can be used to investigate the phenomenon of down regulation (*Fig 3-1*). It is common for the initial slopes of the *A-Ci* curves (V_{cmax}) and the CO_2 saturated rates of photosynthesis (J_{max}) to decline as plants are grown at higher CO_2 concentrations. The results of my experiments with *O. pes-caprae* and *O. punctata* also follow this pattern. The *O. pes-caprae* curves for plants grown at 180 and 240 ppm were similar (*Fig 3-1A*). However, the response of plants grown at 180 ppm and 400 ppm was not consistent across both nutrient levels. Photosynthetic rates at 180 ppm were higher under high nutrient concentrations, while plants grown at 400 ppm had higher photosynthetic rates under low nutrient concentrations (*Fig. 3-1 B & C*).

No distinct pattern was observed between CO_2 and the maximum rate of carboxylation (V_{cmax}) of both species (*Fig. 3-2*), which is consistent with the presented *A-Ci* curves. The same was observed for the maximum rate of electron transport (J_{max}), with all CO_2 levels having similar values (*Fig. 3-2*). There was no significant treatment effect on both parameters.

A


O.pes-caprae
180
O.pes-caprae
240
O.pes-caprae
300
A (μ mol m^{-2} s^{-1})
0
5
10
0
250
500
750
1000
250
500
750
1000
250
500
750
1000
Ci (μ mol mol^{-1})

B

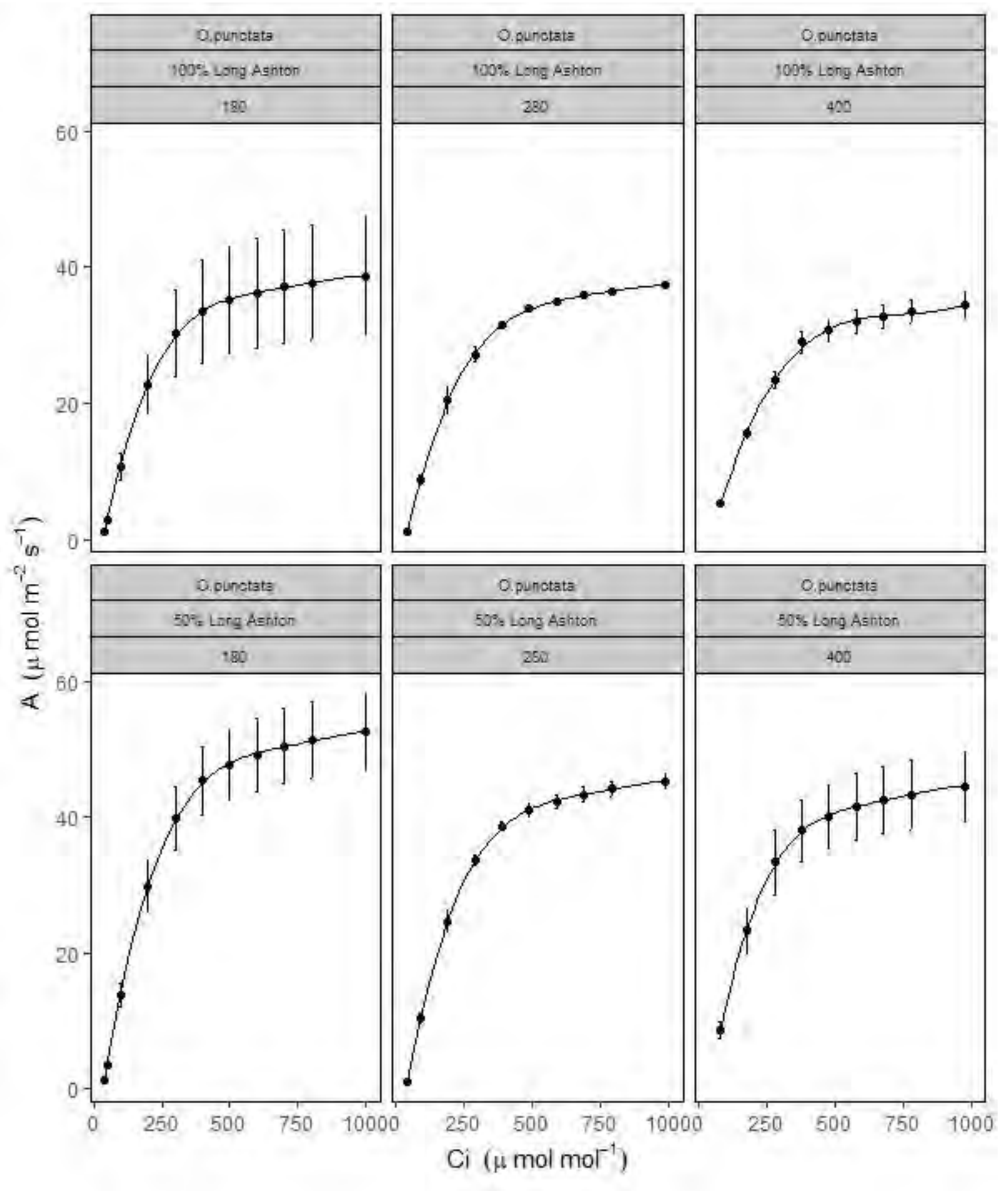


Figure 3-1: Photosynthetic responses of *Oxalis pes-caprae* (A) and *Oxalis punctata* (B) to internal CO_2 concentrations. *O. pes-caprae* plants were grown under four CO_2 concentrations (180, 240, 300) and 100% Long Ashton (n = 5 for each treatment) and *O. punctata* plants were grown under three CO_2 concentrations (180, 280, 400 ppm) and two nutrient concentrations (100% and 50% Long Ashton), n = 3 for each treatment except at 180 ppm where n = 4. Measurements were made with a LI-6400. Values plotted are mean ± SE for each treatment.

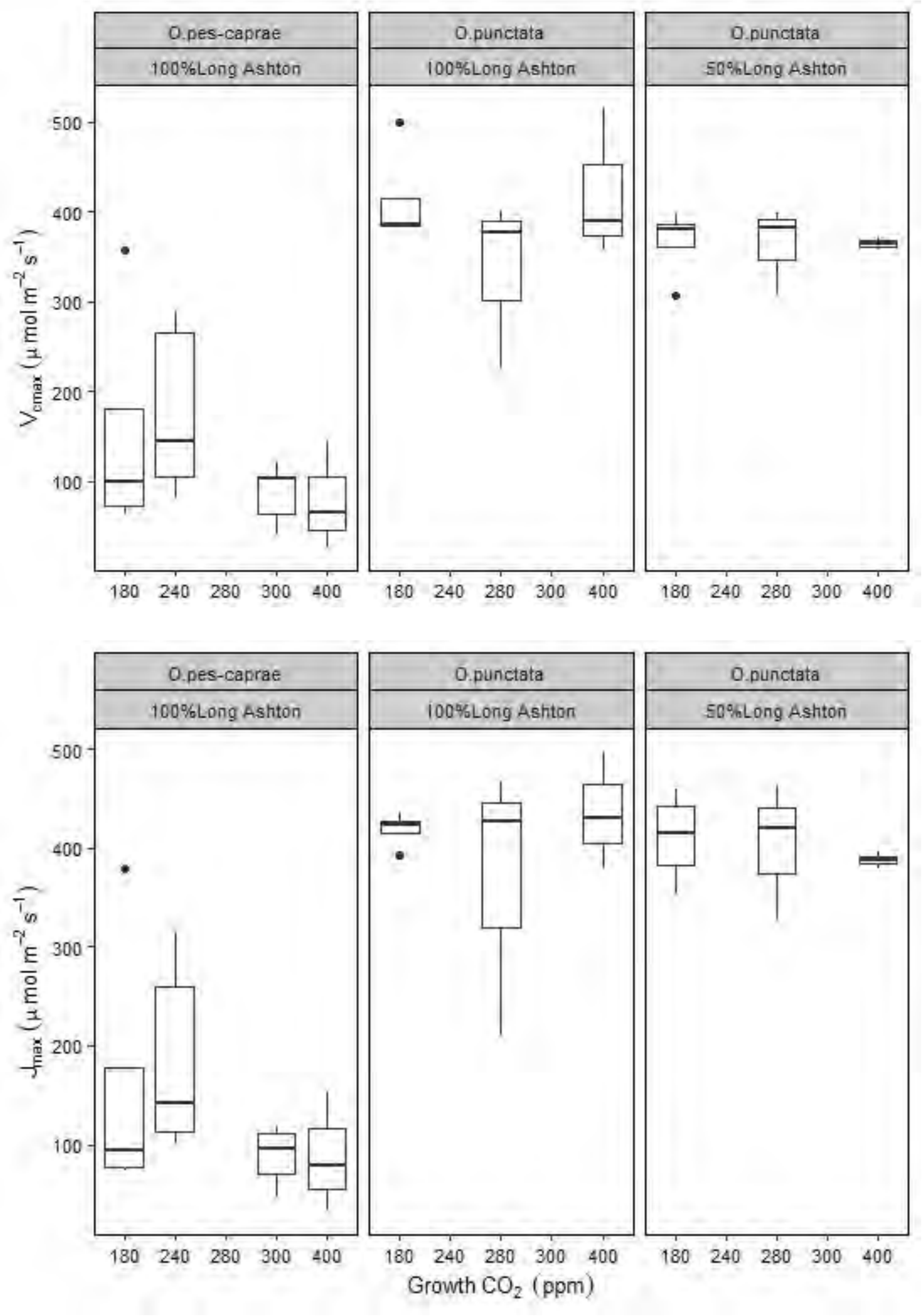


Figure 3-2: Carboxylation efficiency of RUBISCO (V_{cmax}) and maximum rate of electron transport for RuBP regeneration (J_{max}) for *Oxalis pes-caprae* and *O. punctata* in response to CO_2 concentrations. *O. pes-caprae* plants were grown under four CO_2 concentrations (180, 240, 300, 400 ppm) and 100% Long Ashton (n = 5 for each treatment), and *O. punctata* plants were grown

under three CO_2 concentrations (180, 280, 400 ppm) and two nutrient concentrations (100% and 50% Long Ashton), n = 3 except at 180 ppm where n = 4. Measurements were made with a LI-6400.

Photosynthetic rates measured at the growth CO_2 concentration provide a comparative value for the rates that would drive carbon assimilation and plant growth. *Oxalis pes-caprae* photosynthetic rates measured at the growth CO_2 concentrations responded as expected across the CO_2 gradient (*Fig 3-3*). From sub-ambient to ambient CO_2, photosynthetic rates increased for both species. A significant CO_2 effect was observed in *O. pes-caprae* ($F_{3,27} = 9.06$) and in *O. punctata* ($F_{2,15} = 32.81, p < 0.001$), and its interactive effect with nutrient concentrations was also significant ($F_{5,12} = 10.5, p < 0.001$). Significant differences between CO_2 levels are indicated with letters. Nutrients did not appear to have a significant impact on *O. punctata* photosynthesis, as the differences observed were uniform across both nutrient concentrations applied.

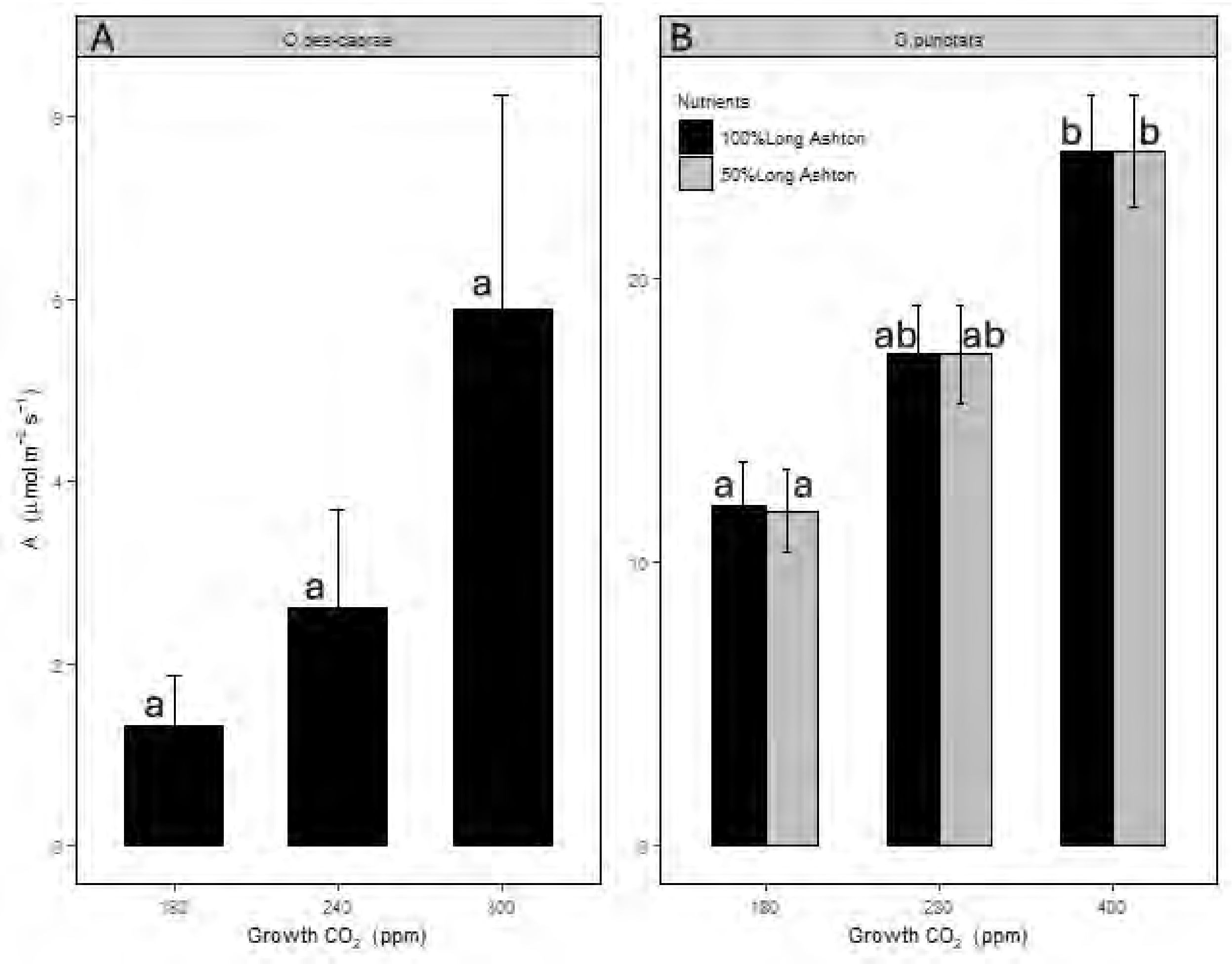


Figure 3-3: Photosynthetic rates of *O. pes-caprae* (A) and *Oxalis punctata* (B) in response to CO_2 concentrations. *O. pes-caprae* plants were grown under four CO_2 concentrations (180, 240, 300, 400 ppm) and 100% Long Ashton, (n = 5 for each treatment),

and *O. punctata* plants were grown under three CO_2 concentrations (180, 280, 400 ppm) and two nutrient concentrations (100% and 50% Long Ashton), n = 3 for each treatment except at 180 ppm where n = 4. Measurements were made with a LI-6400. Values were calculated using the method of Farquhar et al (1980). Significant differences at $p < 0.05$ between treatment groups are denoted by different letters. Values are mean ± SE

In *O. pes-caprae* there was no clear pattern of stomatal limitation among the CO_2 treatments (*Fig. 3-4A*). There was no significant treatment effect and no significant differences between treatments. In *O. punctata* the pattern was distinct from 180 to 400 ppm (*Fig. 3-4B*). Plants grown under glacial CO_2 concentrations were more limited by stomata compared to those grown at 400 ppm, and the differences were significant for all CO_2 levels ($F_{2,15} = 14.75, p < 0.01$). The effect of both nutrients and CO_2 was significant ($F_{5,12} = 4.75$) at the 5% level of significance ($P = 0.01$).

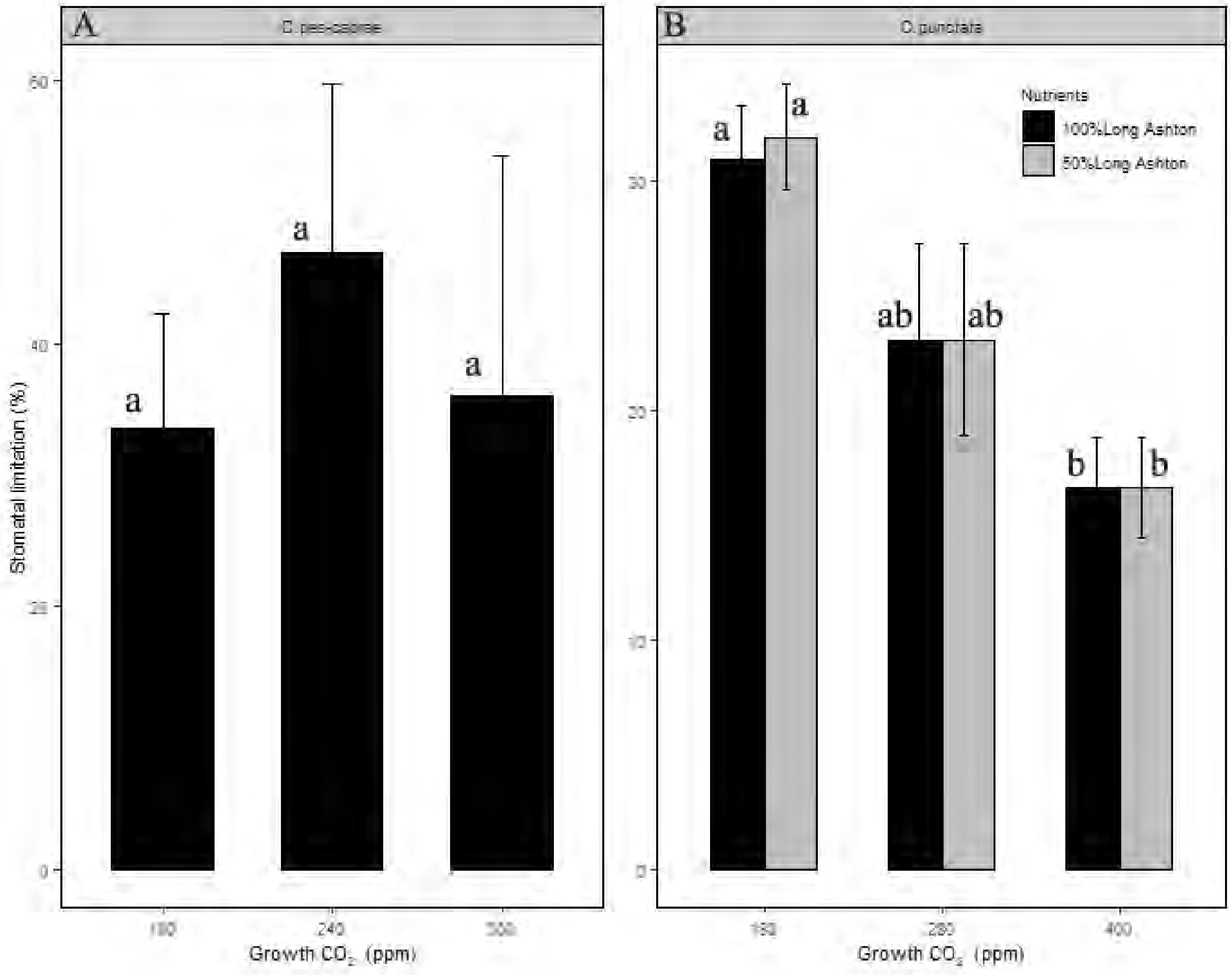


Figure 3-4: Percentage limitation imposed by stomata on photosynthesis in *Oxalis pes-caprae* (A) and *O. punctata* (B) in response to CO_2 concentrations. *O. pes-caprae* plants were grown under four CO_2 concentrations (180, 240, 300, 400 ppm) and 100% Long Ashton (n = 5 for each treatment), and *O. punctata* plants were grown under three CO_2 concentrations (180, 280, 400 ppm) and

two nutrient concentrations (100% and 50% Long Ashton), n = 3 for each treatment except at 180 ppm where n = 4. Measurements were made with a LI-6400. Values were calculated using the method of (Farquhar et al., 1980). Significant differences at $p < 0.05$ between treatment groups are denoted by different letters. Values are mean ± SE

3.3.2. Stomatal response

Stomatal conductance (g_s) remained unchanged in both species because of lowered [CO_2]. None of the differences presented in *Fig. 3-5* were significant for both species, and concentrations of CO_2 did not have a significant effect on either species.

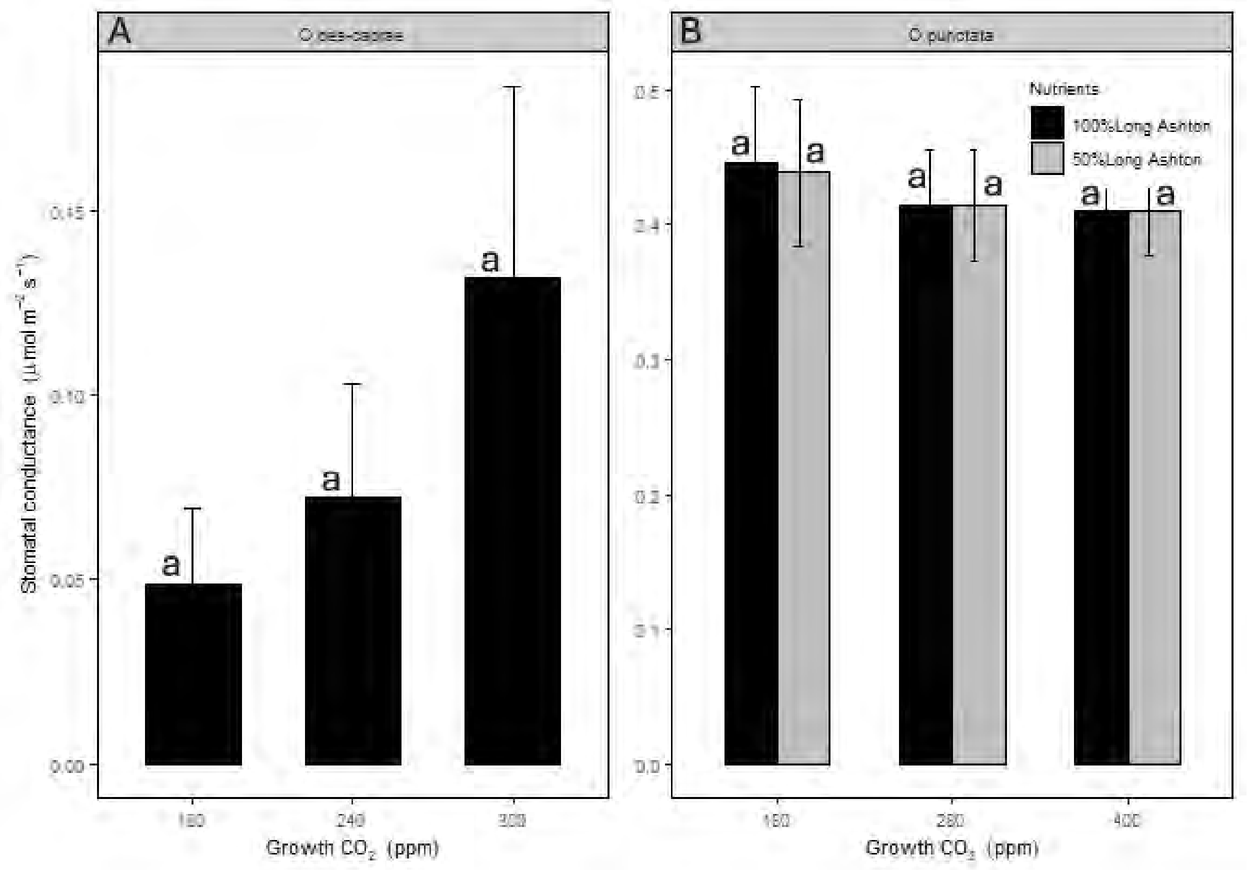
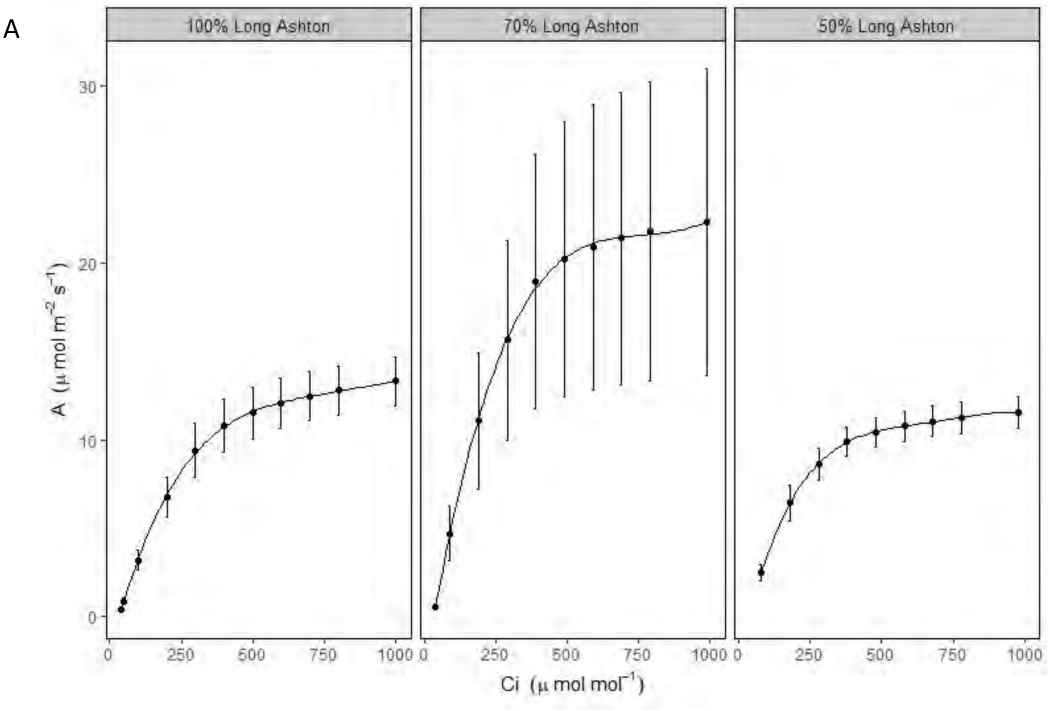


Figure 3-5: Stomatal conductance for *O. pes-caprae* (A) and *O. punctata* (A). *O. pes-caprae* plants were grown under four CO_2 concentrations (180, 240, 300, 400 ppm) and 100% Long Ashton (n = 5 for each treatment), and *O. punctata* plants were grown under three CO_2 concentrations (180, 280, 400 ppm) and two nutrient concentrations (100% and 50% Long Ashton), n = 3 for each treatment except at 180 ppm where n = 4. Values are mean ± SE

3.3.3. Nutrient effect on photosynthesis

When analysing photosynthetic responses at ambient [CO_2], plants grown under higher nutrient concentrations had higher photosynthetic rates compared to those grown under low nutrient concentrations (*Fig. 3-6 A & B*). The observed stomatal limitation also followed the same pattern and plants grown at higher nutrient concentrations had a higher stomatal limitation with nutrient concentrations having no significant effect on this (*Fig. 3-6 C*).



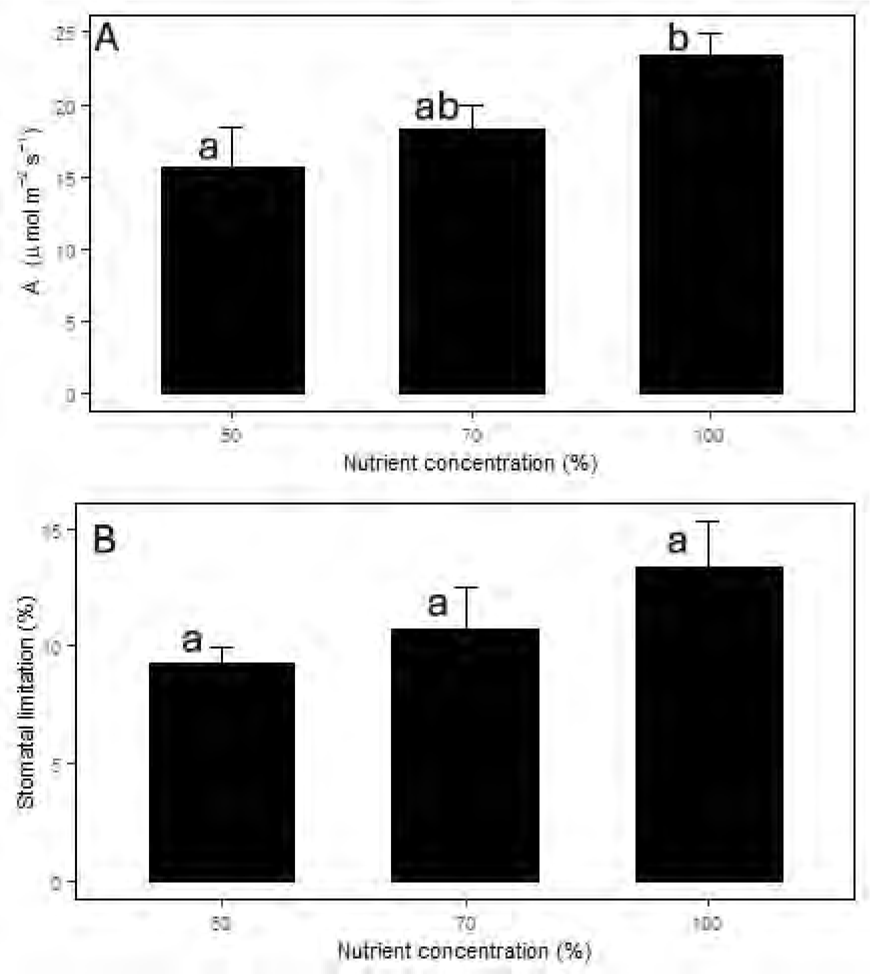


Figure 3-6: Photosynthetic (A) and stomatal (B) responses of *Oxalis pes-caprae* grown under ambient CO_2 and three nutrient treatments (50%, 70%, 100% Long Ashton) to CO_2 concentrations, n = 7. Measurements were made with a LI-6400. Significant differences at $p < 0.05$ between treatment groups are denoted by different letters. Values are mean ± SE

Values for J_{max} and V_{cmax} both increased with nutrient concentrations (*Fig. 3-7*). Nutrients had a significant effect on J_{max} ($F_{2,10} = 4.6$, $p = 0.038$) and no effect on V_{cmax}. Even though an increase in V_{cmax} values is evident, there were no significant differences between nutrient concentrations, unlike J_{max} where values reported for 50% Long Ashton were significantly different to those at 100% Long Ashton.

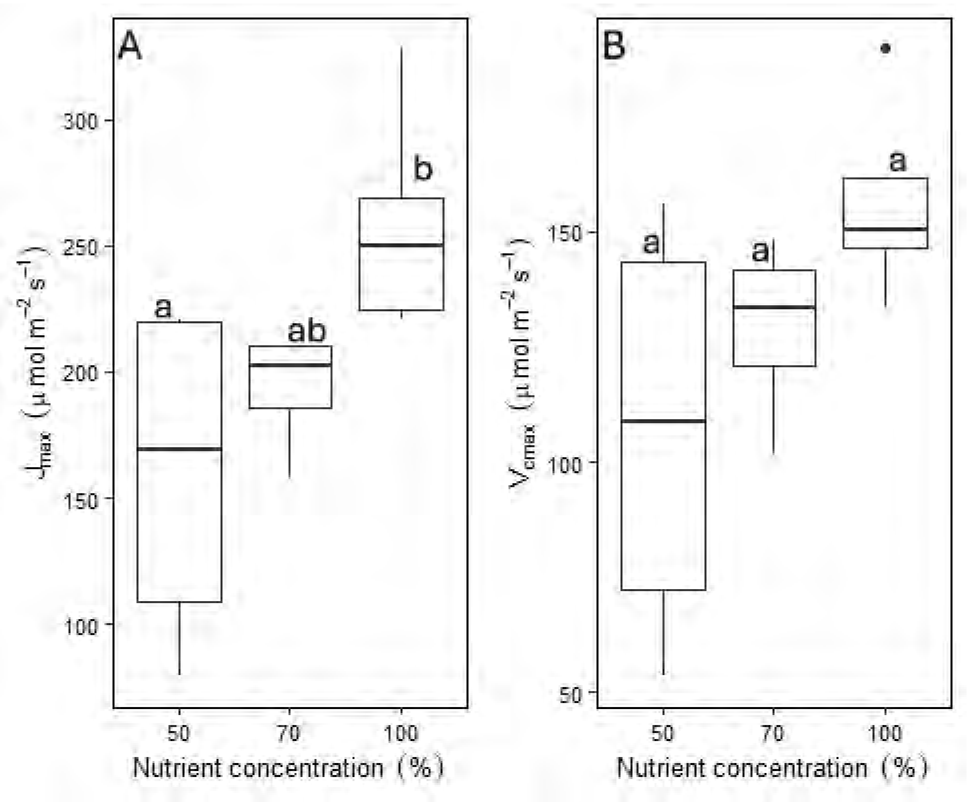


Figure 3-7: Carboxylation efficiency of RUBISCO (V_{cmax}) and maximum rate of electron transport for RuBP regeneration (J_{max}) for *Oxalis pes-caprae* in response to varied nutrient concentrations. Plants were grown at ambient CO_2 and 3 nutrient concentrations (50%, 70% and 100% Long Ashton), n=7. Measurements were made with a LI-6400. Significant differences at $p < 0.05$ between treatment groups are denoted by different letters.

3.3.4. Leaf carbon and nitrogen

A comparison of the carbon and nitrogen content showed no changes in leaf carbon concentration across the gradient of growth [CO_2], and a slight but not significant, decrease in nitrogen concentration from glacial to current ambient CO_2 (*Fig. 3-8*). This response is evident in both species. The C: N ratio decreased as CO_2 concentrations decreased but the differences between treatments were not statistically significant and there was no significant treatment effect.

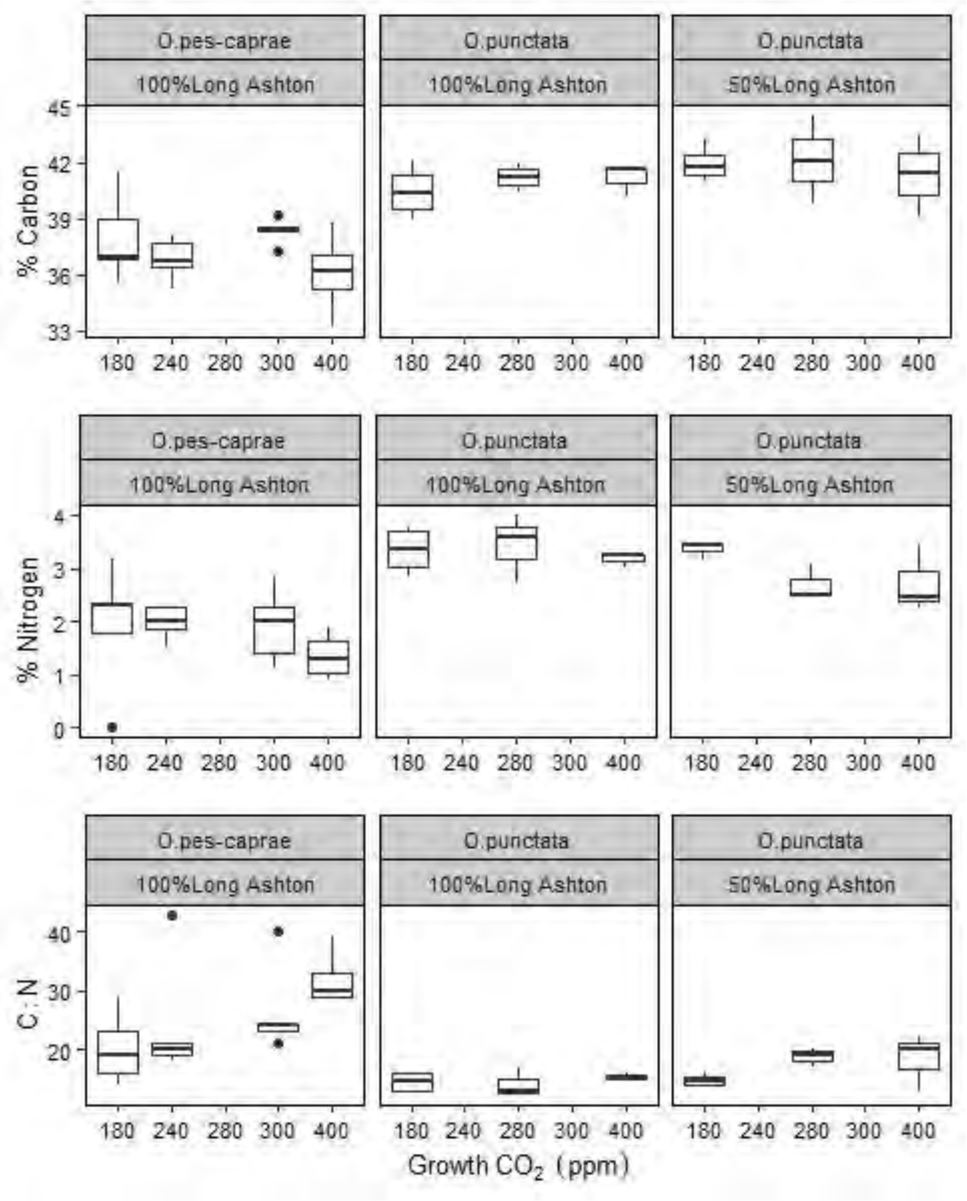


Figure 3-8: Nitrogen and carbon content of *O. pes-caprae* and *O. punctata* leaves. Percentage carbon, percentage nitrogen and their ratio are presented. *O. pes-caprae* plants were grown under four CO_2 concentrations (180, 240, 300, 400 ppm) and 100% Long Ashton (n = 5 for each treatment), and *O. punctata* plants were grown under three CO_2 concentrations (180, 280, 400 ppm) and two nutrient concentrations (100% and 50% Long Ashton), n = 5 for each treatment except at 180 ppm where n = 4.

3.4 Discussion

The overarching objective of this chapter was to investigate the effects of glacial carbon dioxide (CO_2) concentrations on plant physiology, with a specific focus on the enzyme Ribulose-1,5-bisphosphate (RUBISCO) and its implications for photosynthetic rates. Under conditions of low CO_2 availability, the carboxylation function of RUBISCO is hindered, resulting in decreased efficiency and, consequently, a diminished photosynthetic rate in plants. This investigation extended to two distinct species, *O. punctata* and *O. pes-caprae*, and encompasses varying nutrient concentrations to capture an understanding of the relationship between CO_2 levels and plant responses.

Photosynthetic rates of *O. punctata* were decreased with decreasing CO_2 supply, but as was evident in Chapter 2, this did not translate into differences in biomass. I assume this was the result of the reallocation of resources from the original bulbs, but further experimentation would be necessary to confirm this. *O pes-caprae* had much lower photosynthetic rates and although there was the predicted trend in response to growth CO_2 concentration, differences were not significant and thus cannot directly support the observed CO_2 effects on biomass. These photosynthetic rates represent only one point in time, whereas the biomass is an integrated measure across the entire experimental period, and this may account for the misalignment of results.

The activity of RUBISCO of both *Oxalis* species was unaltered by CO_2 supply, indicating no photosynthetic up-regulation at glacial CO_2 levels. This is in contrasts to various previous studies that have shown up-regulation of V_{cmax} rates with decreasing CO_2 levels, reflecting a compensatory mechanism by plants to counteract the diminished availability of CO_2 for photosynthesis (Anderson et al., 2001; Tissue and Lewis, 2010). My results indicate that any observed changes in photosynthetic rates were the consequence of altered CO_2 concentration at the sites of RUBISCO carboxylation within the leaf and not due to changes in enzyme activity. A reduction in substrate availability for RUBISCO at low growth CO_2 concentrations would promote photorespiration rates, a process catalysed by the binding of O_2 to the RUBISCO active site. Photorespiration, accounting for approximately 30% of the reduction in net photosynthesis in C3 species when CO_2 levels drop from 380 to 180 ppm, amplifies the limitations posed by CO_2 scarcity (Sharkey, 1988; Ward and Gerhart, 2010).

Stomatal conductance and limitations contributed to observed differences in photosynthetic rates. A decrease in CO_2 concentration triggers increased stomatal conductance, as plants compensate for reduced CO_2 availability by widening stomatal apertures (Franks and Beerling, 2009). This response was less evident for *O. pes-caprae* than *O. punctata* and would help to reduce the effect of glacial CO_2 concentrations on photosynthetic rate. However, increased stomatal conductance would have had important implications for plant water use and would increase water loss, as the pathway for CO_2 uptake and water evaporation is shared (Sharkey et al., 2012). This could be ecologically important if glacial precipitation had limited soil water availability and could limit plant growth and therefore the usefulness of these species as a food source.

Growth at different CO_2 concentration also influences the C: N of plant material (Kunz et al., 1995) and hence its calorific and nutrient value. However, my results showed no change in the C: N under glacial CO_2 conditions and implies that both the calorific and nutrient supply per unit bulb mass to hunter-gatherers would have been unchanged. The major effect, at least for *O. pes-caprae* would have been the reduction in overall bulb mass and hence limiting returns on the gathering of bulbs (explored in Chapter 5). The lack of a change in N concentration was aligned with the absence of changes in either V_{cmax} and J_{max}, demonstrating no photosynthetic acclimation to the growth CO_2 concentrations.

As predicted, nutrient supply had a large impact on the photosynthesis and stomatal conductance of *O. pes-caprae.* This effect was not evident for *O. punctata* and again I suggest that this was related to the reallocation of nutrients from the original bulbs. The impact on photosynthesis of *O pes-caprae* were underpinned by change to V_{cmax} and J_{max} and would hence likely affect the interaction of nutrient supply and growth CO_2 concentration. Low nutrient supply would likely diminish any CO_2 effects although this was not directly investigated. Ecological implication would likely mean reduced USO production in nutrient poor soils that would be further compromised at glacial CO_2 concentration, diminishing the value of USO to hunter gatherers.

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Chapter 4: Response of Oxalis punctata leaf traits to glacial CO_2 concentrations

4.1. Introduction

The intrinsic role of leaves in the process of photosynthesis is substantial, with their functioning intricately linked to alterations in anatomy. Stomata, microscopic pores dotting the leaf surface, emerge as key conduits in the exchange of CO_2 and water vapor with the atmosphere. The orchestrated functioning of guard cells, specialized cells enveloping stomata, plays a pivotal role in regulating the aperture of these pores in response to a spectrum of internal and external stimuli. This sensitivity of stomatal behaviour to shifts in atmospheric CO_2 concentration emphasizes the relationship between plant physiology and environmental variables (Hetherington and Woodward, 2003; Lawson and Blatt, 2014; Lawson and Matthews, 2020).

Throughout the history of land plants, the fundamental structure of stomata has largely remained unchanged, with advancements observed in the functionality of guard cells to regulate gas exchange processes (Franks and Farquhar, 2007). Stomatal behaviour significantly impacts both plant water loss and CO_2 uptake. During stressful periods, stomata are inclined to remain closed to mitigate water loss, limiting CO_2 uptake. Guard cells undergo swelling to expose the stomatal pore, facilitating CO_2 absorption from the atmosphere, and regulate the extent of stomatal opening (Roelfsema and Hedrich, 2005). Besides serving as the conduit for CO_2 absorption, stomata also mediate water loss through transpiration, necessitating a balance between maximizing CO_2 uptake and minimizing water loss during pore opening (Lawson and Blatt, 2014; Lawson and Matthews, 2020).

The extent of stomatal opening is not the sole determinant of gas exchange; rather, stomatal density and size carry significant implications for this process. Stomatal density, defined as the number of stomata per unit leaf area, along with size, interact to influence stomatal conductance (Franks and Farquhar, 2001). Like the modulation of stomatal opening by CO_2, changes in atmospheric [CO_2] also affect stomatal density and size. Research aimed at clarifying the relationship between atmospheric [CO_2] and stomatal traits reveals an inverse correlation with density, where reduced [CO_2] result in higher stomatal densities (Van de Water et al., 1994; Woodward and Kelly, 1995), and a direct correlation with size (Beerling et al., 1993; Franks et al., 2012). Plants cultivated in low CO_2 environments tend to possess smaller stomata, likely as a mechanism to allocate space on the leaf epidermis for other cell types (Franks and Beerling, 2009).

Internally, the structural complexities of leaves influence the movement of CO_2 absorbed through stomata and the penetration of light. The journey of CO_2, post-absorption, involves diffusion through the mesophyll to the sites of carboxylation—a process acutely influenced by mesophyll conductance. The complex balance of airspaces in the mesophyll regulates CO_2 movement, with restricted airspaces potentially affecting the efficacy of CO_2 transport (Gu et al., 2010). Additionally, the manipulation of palisade thickness, a constituent of leaf anatomy, assumes a pivotal role in governing the distribution of light within the leaf while optimizing airspaces. The link between palisade and spongy mesophyll ensures an efficient absorption of light essential for the process of photosynthesis (Vogelmann et al., 1996).

The crux of this chapter lies in an examination of the response exhibited by *Oxalis punctata* leaf anatomy to CO_2 and nutrient supply. At the time of harvest, *O. pes-caprae* leaves exhibited wilting, rendering them unsuitable for the experiment as their altered physiological state could compromise result accuracy. I hypothesised that low CO_2 conditions are likely to result in high densities of small stomata, coupled with decreased stomatal index and thinner leaves.

4.2. Methods

4.2.1 Plant growth conditions

Plants from Experiment 1 (as described in Chapter 2) were used in studying the leaf traits below.

4.2.2 Wax embedding

Fresh leaflets were removed from each *O. punctata* plant at harvest and preserved in formalin-acetic-acid (FAA). Three plants were sampled at each [CO_2], except for 180 ppm where 4 plants were sampled. The leaf material was gradually dehydrated in an alcohol series and embedded in paraffin wax (Johansen, 1940). Transverse sections ranging from 10 to 12 µm in thickness were cut with a rotary microtome and stained using Safranin-Alcian-Blue. To preserve the sections to permanent slides, DPX glue was used to mount them to glass slides.

4.2.3 Leaf impressions

Clear nail polish was applied to a new batch of fresh *O. punctata* leaflets to create epidermal impressions. The nail polish impressions were peeled off with clear tape and pasted on microscope slides for photography (*Fig 4-1 A*).

4.2.4 Epidermal measurements

Photographs of the leaf impressions were analysed to determine the stomatal density (SD) and stomatal index (SI). SD was defined as the number of stomata per unit area, and was calculated with the following formula:

$$\frac{No. stomata}{Area}$$

Epidermal cell expansion and stomatal initiation are a source of variation in SD calculations, and to compensate for this SI is usually the preferred method of reporting changes in stomata. Stomatal index was defined as the number of 'stomata calculated as the percentage of the total epidermal cells per unit area' (Poole and Kurschner, 1999) and was calculated with the following formula:

$$\frac{No.stomata}{No.stomata + No.epidermal\ cells} * 100$$

As a proxy for stomatal size, guard cell length was measured instead of guard cell area because stomata are dynamic, and their guard cell width is not always constant (Beaulieu et al., 2008).

In addition to measuring and reporting on stomatal dimensions and density on the leaf surface, the relationship between stomatal behaviour and stomatal conductance was explored. Stomatal conductance was modelled using the ‘BBOpti’ model as described by (Medlyn et al., 2011) using the plantecophys package (version 1.4-6) in R. (version 4.2.2).

4.2.5 Leaf traits

Transverse sections cut from wax embedded leaf material were analysed with a light microscope and photographed. From the photographs five epidermal traits were measured: leaf thickness (LT), upper epidermal thickness (UET), palisade mesophyll thickness (PMT), spongy mesophyll thickness (SMT) and lower epidermal thickness (LET) (*Fig 4-1 B*). For each photograph, measurements were taken at three different points on the leaf and the values were averaged. Measurements were taken using ImageJ software (Schneider et al., 2012).

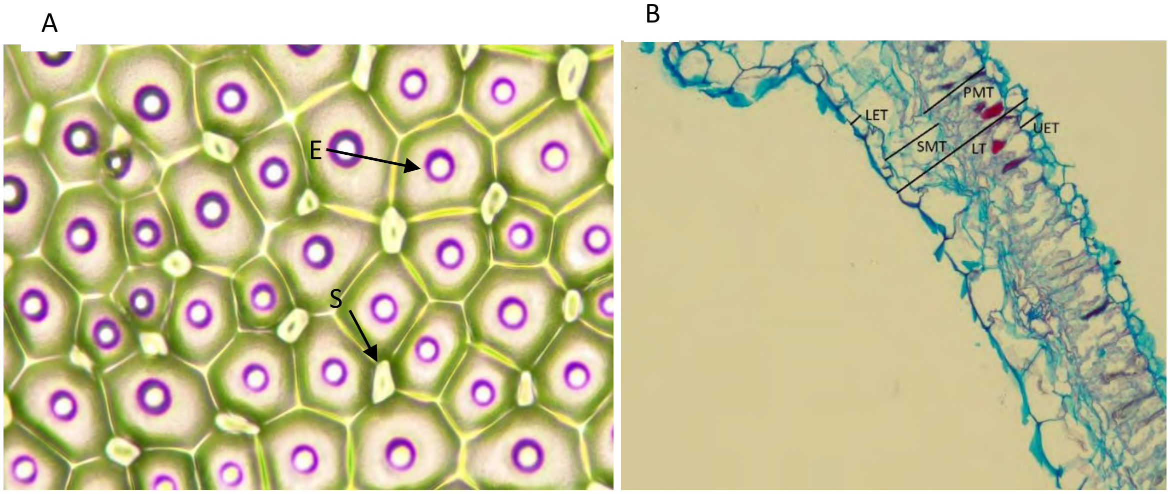


Figure 4-1: Nail polish impression illustrating epidermal cells and stomata on the surface of an *Oxalis punctata* leaf (A), and transverse section illustrating the anatomical traits measured (B). *UET*: upper epidermal thickness, *PMT*: palisade mesophyll thickness, *SMT*: spongy mesophyll thickness, *LET*: lower epidermal thickness, *LT*: leaf thickness, *S*: stomata, *E*: nucleus.

4.2.6 Statistical analysis

To test the effects of CO_2 and nutrient concentrations on biomass a Generalised Linear Model (GLM) was used in R. The individual effects of CO_2 and nutrients were tested, as well as their interactive effect. In cases where there was a significant effect of CO_2 or nutrient concentrations, a Tukey post-hoc test was conducted to test for differences between CO_2 and nutrient concentrations.

4.3. Results

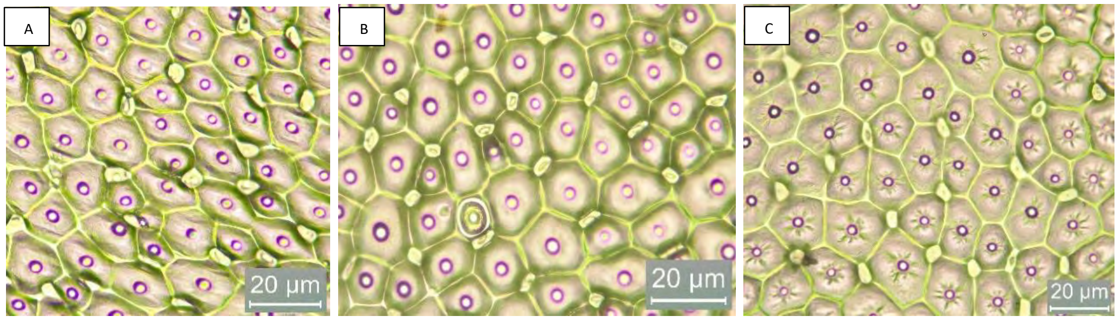


Figure 4-2: Leaf impressions of *Oxalis punctata* leaves showing epidermal cells and stomata (A – 180ppm, B – 280ppm, C - 400ppm).

At low nutrient concentrations, no discernible pattern was observed in stomatal size across the CO_2 gradient. At high nutrient concentrations, however, stomatal size had a positive relationship with CO_2 and plants grown at sub-ambient CO_2 had smaller stomata while those grown at ambient CO_2 had larger stomata (*Fig 4-3A*). The response of stomatal density (number per square mm) was the opposite, and there were more stomata per unit area at glacial CO_2. The effect of CO_2 was significant ($F_{5,12} = 5.53$, $p < 0.01$) while nutrient concentrations did not have a significant effect (*Fig 4-3B)*. No significant effect of CO_2 was observed on stomatal index, but there was a significant nutrient effect ($F_{1,16} = 10.89, p < 0.01$).

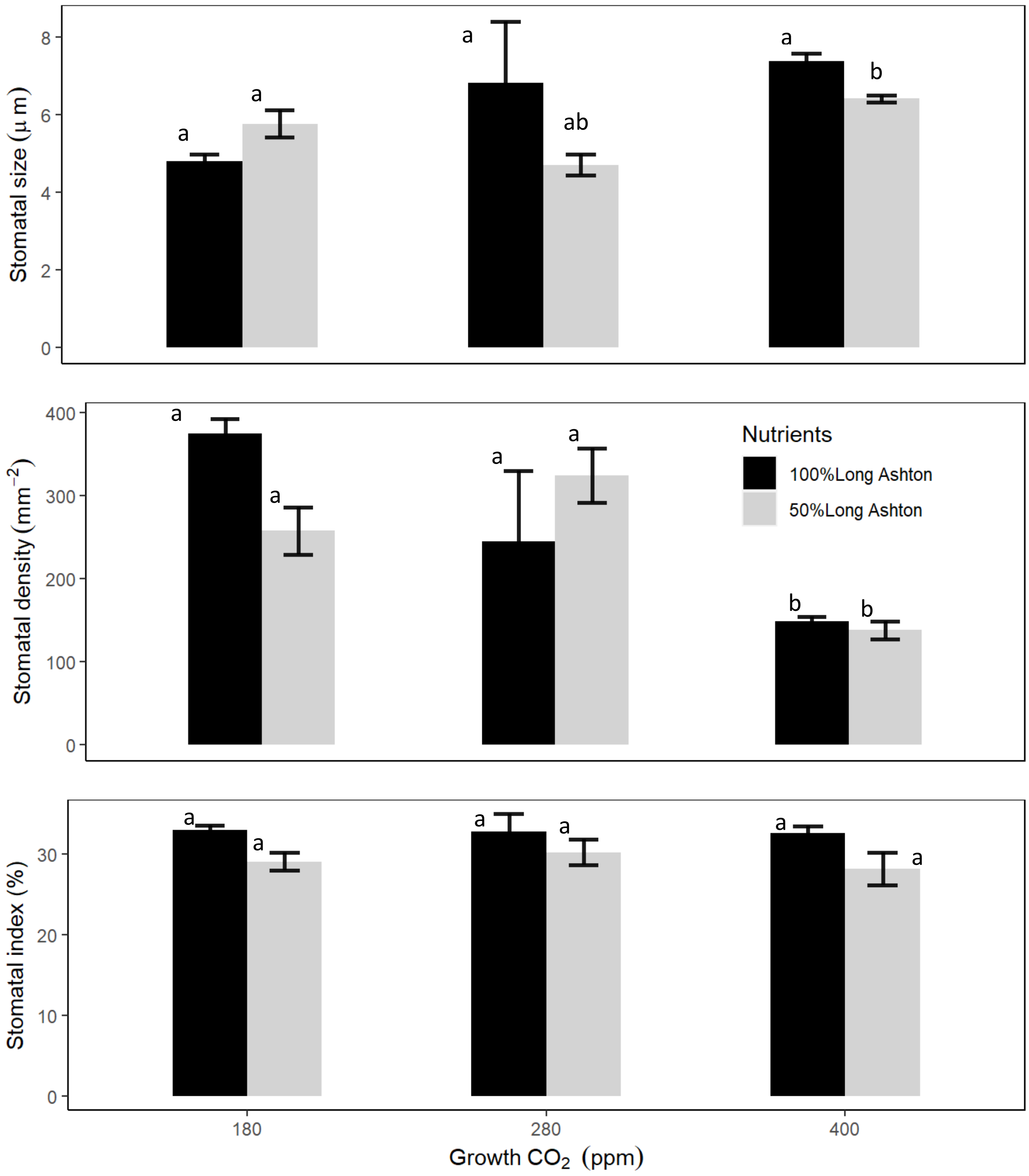


Figure 4-3: Stomatal size, density and index for *Oxalis punctata* plants grown at 3 CO_2 concentrations (180, 240, 400 ppm) and 2 nutrient concentrations (50% and 100% Long Ashton). Values are mean ± SE.

The relationship between stomatal size and density was negative, and smaller stomata appeared in high densities on the leaf surface. Smaller stomata were associated with lower conductance as measured in Chapter 3 (*Fig 4-4*).

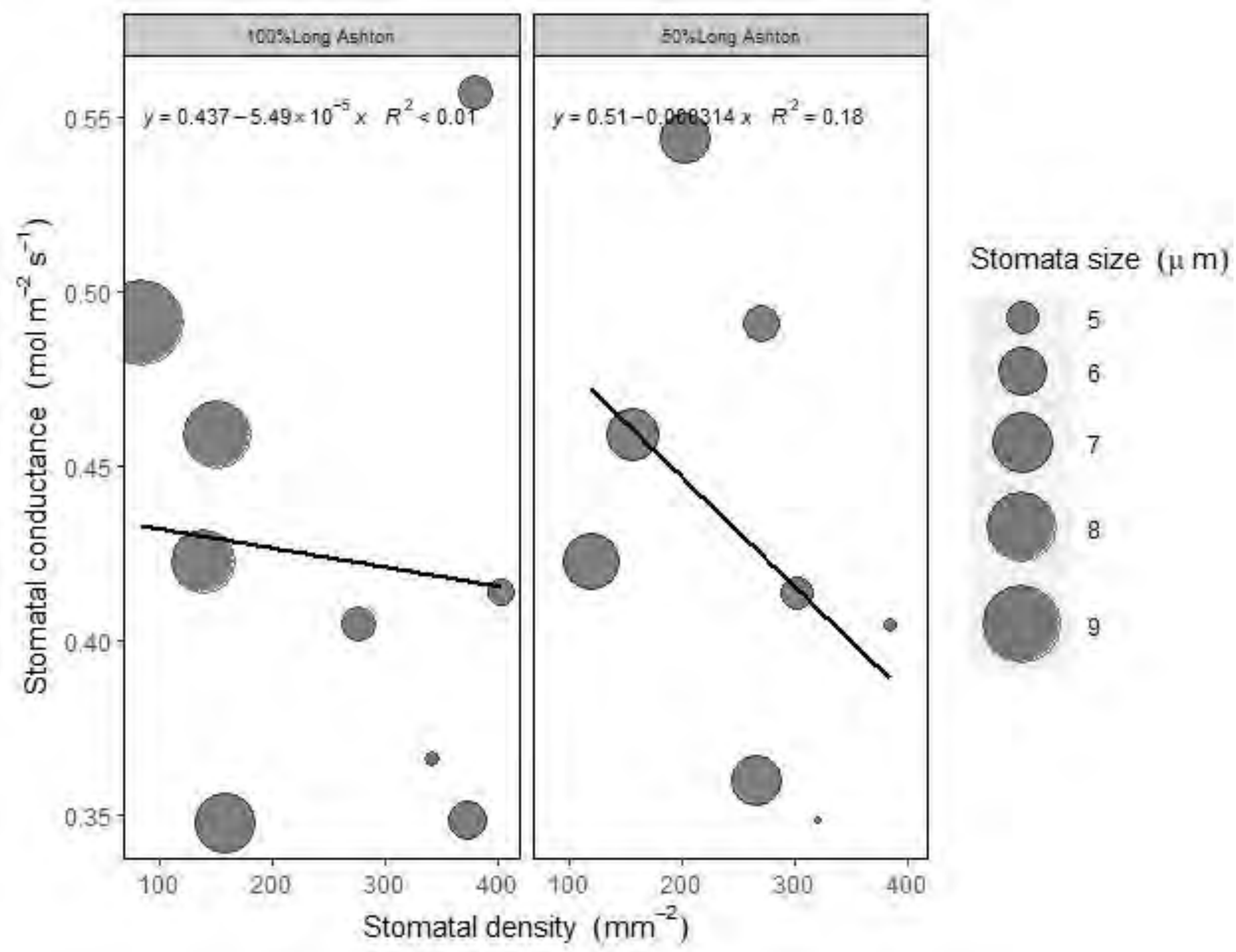
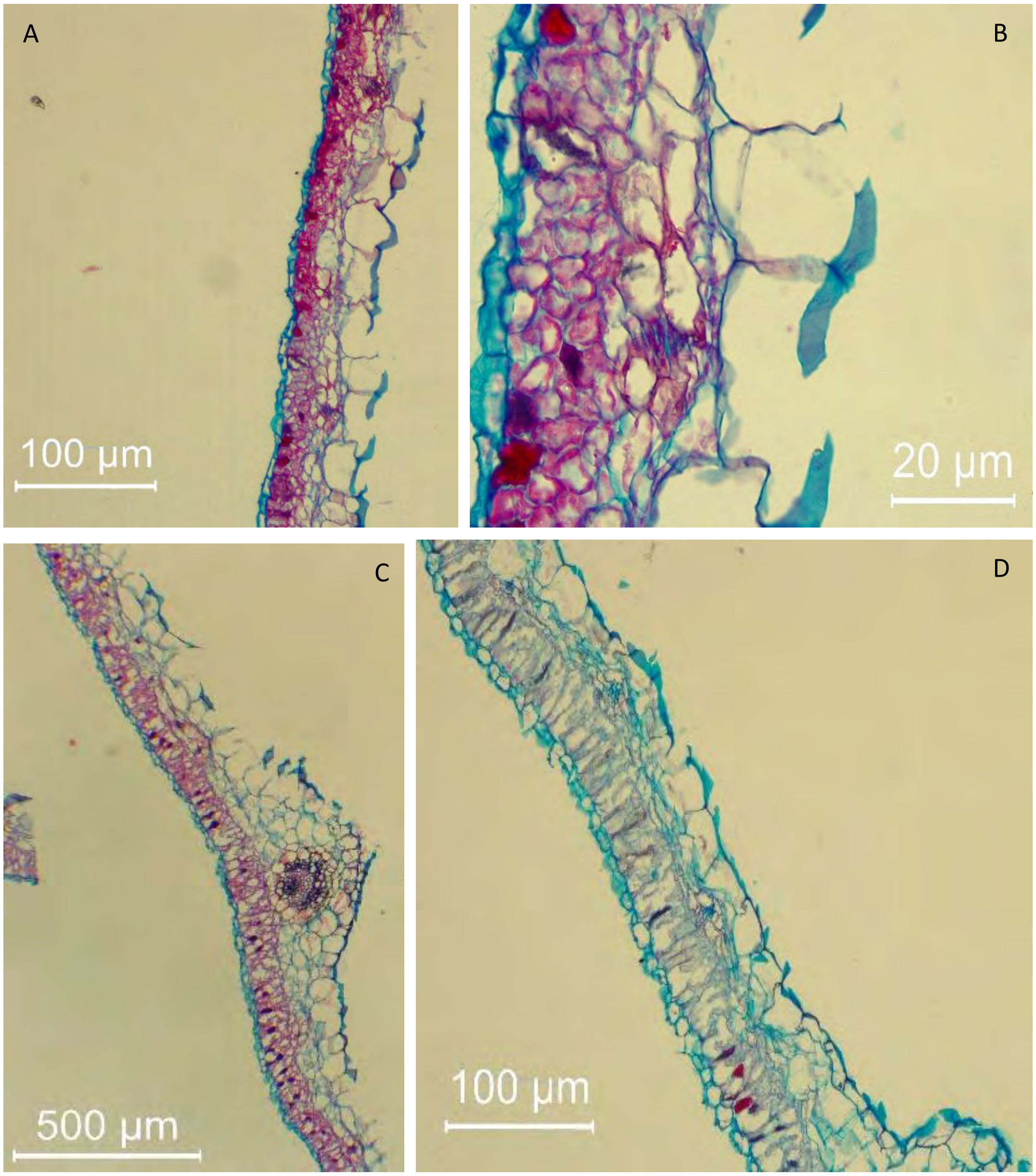


Figure 4-4: Relationship between stomatal density, size and conductance in *Oxalis punctata* leaves. The size of the bubbles represents stomatal size. Plants were grown under 3 CO_2 concentrations (180, 280, 400 ppm) and 2 nutrient concentrations (100% and 50% Long Ashton), n = 3 for each treatment except at 180 ppm where n = 4.

Transverse sections of the leaf material sectioned showed that leaves had a bifacial mesophyll with four layers: the upper epidermis, palisade mesophyll, spongy mesophyll and lower epidermis in that order from the adaxial to the abaxial side (*Fig 4-5*). The palisade mesophyll layer is found on the adaxial side of the leaf (upper side) and consists of elongated cells populated by large amounts of chloroplasts, while the spongy mesophyll is found on the abaxial side of the leaf (lower side) and consists of cells that are irregular in shape. The sections showed a decrease in palisade and mesophyll length with decreasing [CO_2].


A
100 µm
B
20 µm
C
500 µm
D
100 µm

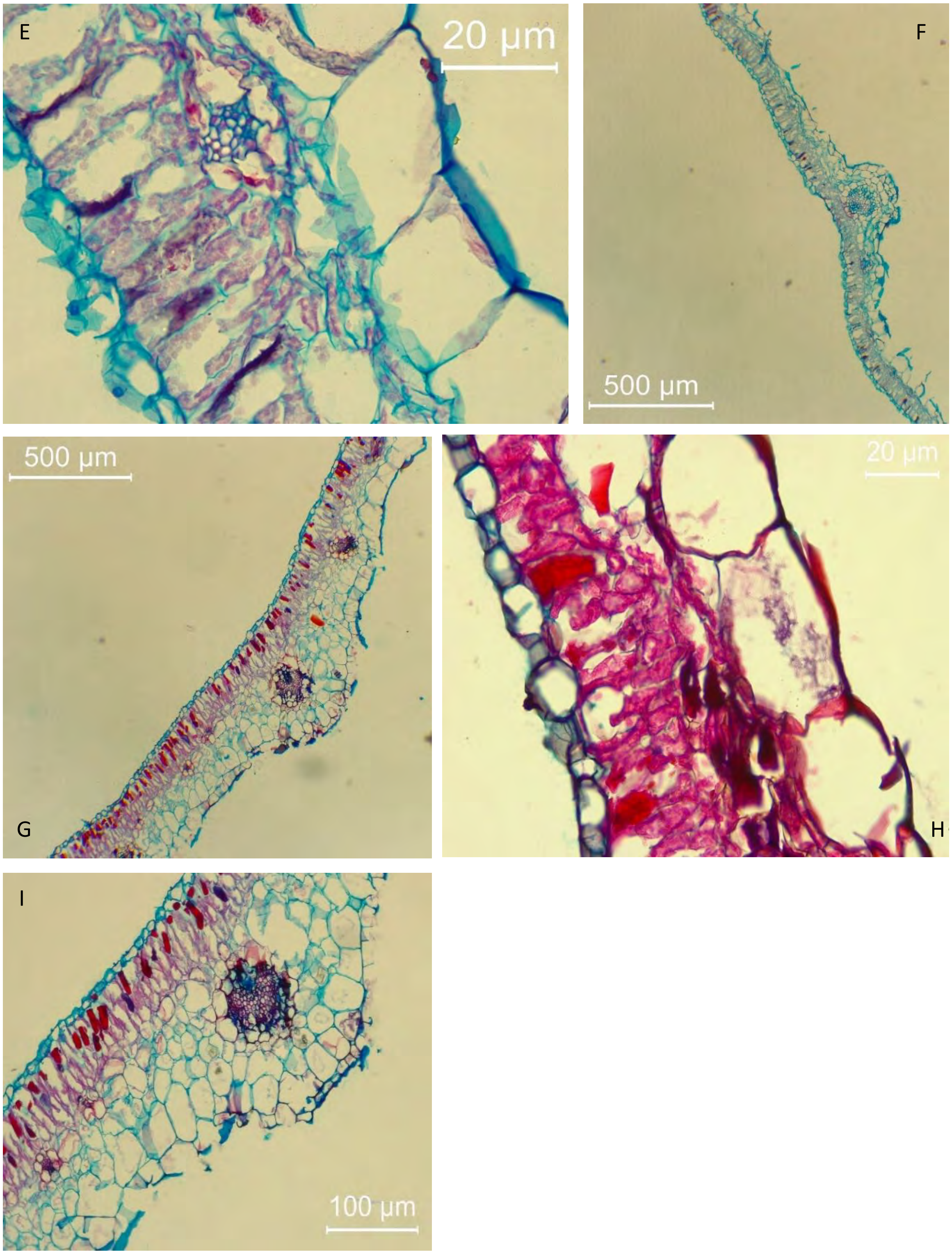


Figure 4-5: Transverse sections of *Oxalis punctata* leaves arranged with the top side on the left and the bottom side on the right (A-C: 180ppm, D-F: 280ppm, G-I: 400ppm).

The size of the lower epidermis, palisade mesophyll, upper epidermal length and spongy mesophyll cells were unchaged by [CO_2]. Comparisons of the total leaf thickness also showed no significant changes as a result of changes in [CO_2] (*Fig 4-6*). Carbon dioxide and nutrient concentrations had no effect on leaf traits.

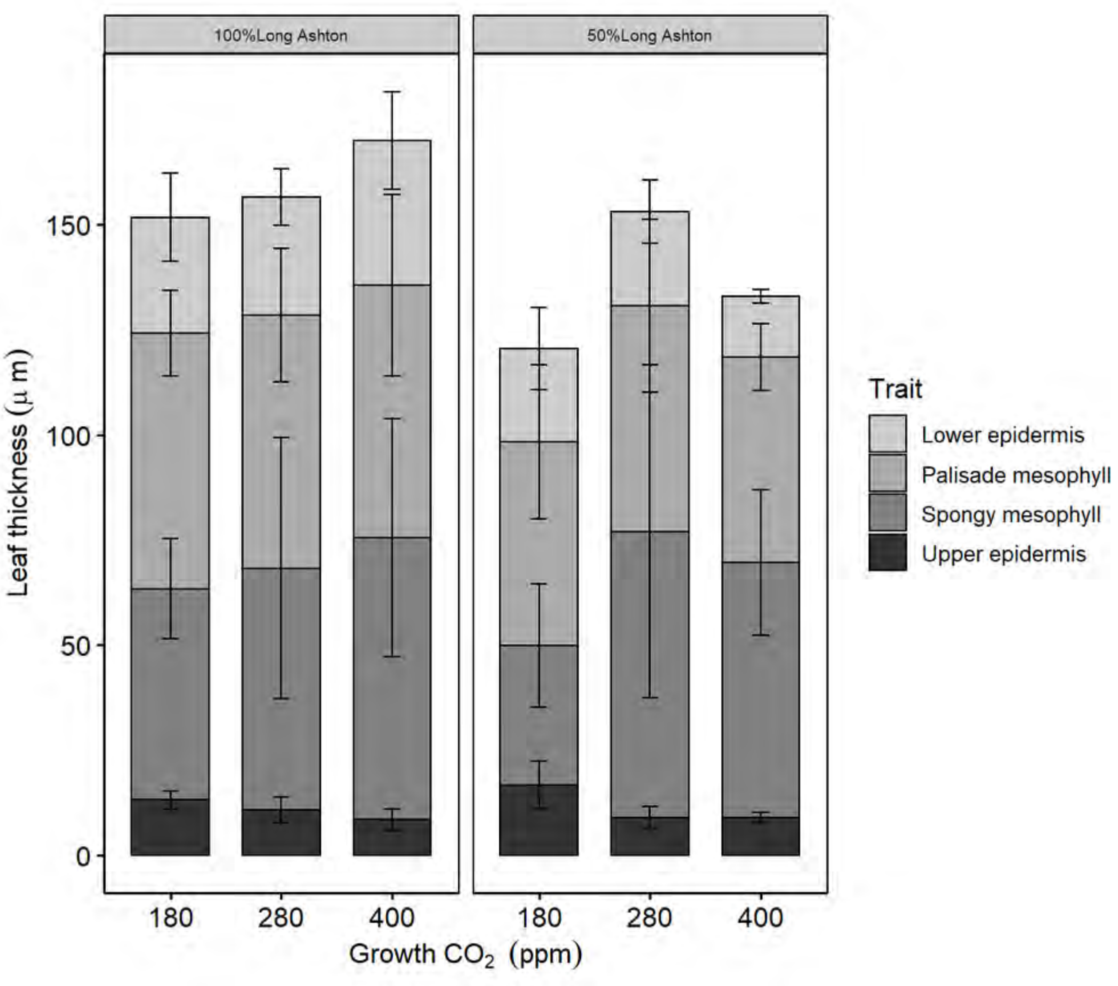


Figure 4-6: Stacked bar graph of *Oxalis punctata* anatomical traits: lower and upper epidermis, spongy mesophyll and palisade mesophyll cells. Total bar lengths add up to total leaf thickness. All values are mean ± SE.

4.4. Discussion

This chapter investigated the influence of three varying carbon dioxide (CO_2) concentrations on the anatomical and functional attributes of stomata. The objective was to gain a deeper understanding of modifications in photosynthetic activity and plant productivity, as outlined in preceding chapters. Consistent with extensive previous research, I observed that a restricted CO_2 supply increased the density of stomata, and diminished stomatal size, but this was solely under conditions of high nutrient availability. Interestingly, no alterations were noted in stomatal index or leaf epidermal characteristics.

Smaller stomata demonstrate a heightened adaptability to environmental changes, particularly in environments characterized by low CO_2 concentrations (Hetherington and Woodward, 2003). In such conditions, smaller stomata exhibit a rapid response, facilitating an efficient absorption of atmospheric CO_2 while concurrently minimizing water loss (Drake et al., 2013). However, it is paramount to acknowledge the inherent limitations associated with smaller stomata, particularly in terms of their CO_2 absorption capacity. A compensatory mechanism, characterized by an increase in stomatal density across the leaf surface, can mitigate this limitation - an observation that is aligned with the findings from previous studies focused on plants cultivated within cold CO_2 environments (Beerling et al., 1993; Royer, 2001). Leaves featuring a higher density of smaller stomata tend to exhibit enhanced stomatal conductance, attributable to the shorter diffusion path facilitated by smaller pores (Franks and Beerling, 2009). However, the findings presented within this chapter deviate from this general expectation. I observed no significant alteration in stomatal size, and only stomatal density was notably reduced by low [CO_2]. As delineated in Chapter 3, stomatal conductance remained unaffected by a reduction in [CO_2], exhibiting only a weak correlation with stomatal density.

The development of stomata and mesophyll within leaves is a coordinated process, facilitating the continual movement of gases and optimizing gas exchange (Baillie and Fleming, 2020). Under conditions of CO_2 deprivation, it is theoretically anticipated that leaves would become thinner, thus creating a more efficient pathway for CO_2 diffusion through the mesophyll (Gu et al., 2010). This adaptation aligns with the broader strategy of enhancing photosynthetic efficiency under conditions of resource limitation. However, in the present study, CO_2 deprivation did not induce alterations in leaf thickness, and the characteristics of mesophyll and epidermal layers remained consistent across all CO_2 treatments.

The results presented in this chapter substantiate the responses of *Oxalis* to sub-ambient [CO_2] as discussed in previous chapters. In this context, the adjustment in stomatal density alone was likely enough to offset the effects of CO_2 deprivation, without altering stomatal size or epidermal traits. It is important to note, however, that the experiment conducted here only spanned one growing season, so it would be insightful to observe the long-term response of stomatal size and leaf traits to glacial CO_2 as long term exposure to [CO_2] has been shown to alter stomatal development leading to significant changes in both density and size (Royer, 2001).

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Chapter 5: Modelling geophyte biomass and return rates under glacial CO_2 concentrations

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5.1. Introduction

The main objective of this thesis was to extensively investigate and analyse the geophytic plants that were utilized by hunter-gatherers during the Pleistocene era in the Greater Cape Floristic Region (GCFR), focusing particularly on the potential impact of [CO_2] on their growth. The study encompassed comprehensive evaluations of biomass, physiology, and anatomy. To establish a contextual framework regarding the dietary habits and harvesting practices of hunter-gatherers, the findings were utilised to construct models that could potentially simulate and anticipate the patterns of harvesting during the Pleistocene.

Extensive research on contemporary hunter-gatherer communities has demonstrated that the geophytes gathered in the GCFR by inexperienced foragers are plentiful enough to adequately sustain the dietary needs of hunter-gatherers. In fact, it was found that the required calories, amounting to 2000 calories per day, along with a protein intake of 93 grams per day, could be harvested in under 2 hours (Singels et al., 2016; Botha et al., 2020). Consequently, the evidence garnered from this research suggests that the hunter-gatherer populations residing in the GCFR had access to sufficient food resources that met their dietary requirements, which could be obtained with minimal effort (Hill and Hurtado, 1989).

The existing models that were developed to predict the harvesting patterns of hunter-gatherers are primarily based on contemporary populations that currently harvest geophytes under the prevailing climatic conditions. Therefore, the predictions made regarding food availability and sustainability are primarily founded upon present-day CO_2 concentrations, which are approximately 400 ppm. However, previous chapters of this thesis have indicated that CO_2 levels can significantly influence plant growth and photosynthesis. It follows logically that these harvesting patterns would also be impacted.

To accurately estimate the availability of food resources during the Pleistocene and to determine if hunter-gatherers would have had adequate sustenance, data from *Oxalis pes-caprae* bulb grown under varying [CO_2] levels was utilised, as well as foraging data from Singels et al., (2016) to construct models that simulate foraging efforts under glacial [CO_2] conditions. The hypothesis that I tested in this study speculated that at low [CO_2] levels, foraging yields would be significantly diminished, necessitating increased efforts to attain the desired 2000 calories per day or alternative food sources.

5.2. Methods

Oxalis pes-caprae bulbs were used to model foraging patterns under glacial [CO_2] as they showed the most significant reduction in mass under glacial CO_2. *O. punctata* did not produce bulbs in my experiment, but rather utilised planted bulbs to regrow and produced a tap root and was therefore not useful in testing my hypothesises about bulb production in the Pleistocene.

The response of *O.pes-caprae* bulb mass to growth [CO_2] was fitted with a polynomial equation (bulb mass = $-1.145 + 0.0114*[CO_2] - 0.0000088*[CO_2]^2$) in R (R Development Core Team, 2018). This equation was used to calculate bulb masses at 400, 300, 280 and 180 ppm. Bulb masses were then normalised by the mass calculated at 400 ppm (2.01 g) to give a proportional response of bulb mass to growth [CO_2]. To assess how [CO_2] induced changes in USO biomass would potentially affect availability of USO resources to early human foragers, it was assumed that all USO species assessed in Singels et al. (2016) would respond to growth [CO_2] in a similar way to *O. pes-caprae*. These authors used local women from the Cape region of South Africa to assess how long it took to find and excavate identified USOs, using a digging tool in the form of a hardened stick with a digging stone. They also assessed the number of calories obtained by the women on each harvesting event. On each of 26 harvesting events, a single forager was tasked with harvesting an individual target species (six species in total, in different vegetation types and seasons). The species selected was based on its presence in each habitat. The dry biomass of harvested USOs was converted to calorie estimates using species' mean nutritional conversion values from Vincent (1985). The harvesting times and calorific estimates were used to calculate the harvested calories min^{-1} and the time required to harvest 2000 calories, which is considered the minimum threshold required to support hunter-gatherer people (Hill and Hurtado, 1989).

To calculate the time required to reach the 2000 calorie threshold, data for each of the 26 harvesting events reported in Singels et al., (2016) was used, after bulb biomass had been corrected for growth [CO_2] according to proportional correction based on data for *O. pes-caprae*. These corrections were performed for the concentrations 400, 300, 240 and 180 ppm. These calculations were performed for each individual harvest, which included 3, 4, 7, 5, 4 and 3 harvesting events when the target species were *Chasmanthe aethiopica, Cyanella lutea, Cyphia digitata, Ferraria crispa,* and *Watsonia meriana*, respectively. Data from these calculations was fitted with a second order polynomial equation and 95% prediction intervals in R. Data were also averaged across harvest occasions and species to present single values for each [CO_2]. The calculated threshold (minutes to reach 2000 calories) data were also analysed to determine the percentage of harvesting occasions that would have yielded 2000 calories, within 1 or 2 hours of harvesting.

5.3. Results

Decreasing the concentration of CO_2 significantly decreased plant biomass ($F_{3,18} = 5.49, P = 0.012$) and bulb yield ($F_{3,18} = 4.3, P = 0.006$) of *O. pes-caprae* plant growth for three months at a range of [CO_2] values from current to glacial (*Fig. 1 A & D*). At glacial [CO_2] bulb biomass decreased by up to 80% compared to current ambient concentrations, while total plant biomass showed a two-fold decrease. Shoot and root biomass were not significantly affected by growth [CO_2].

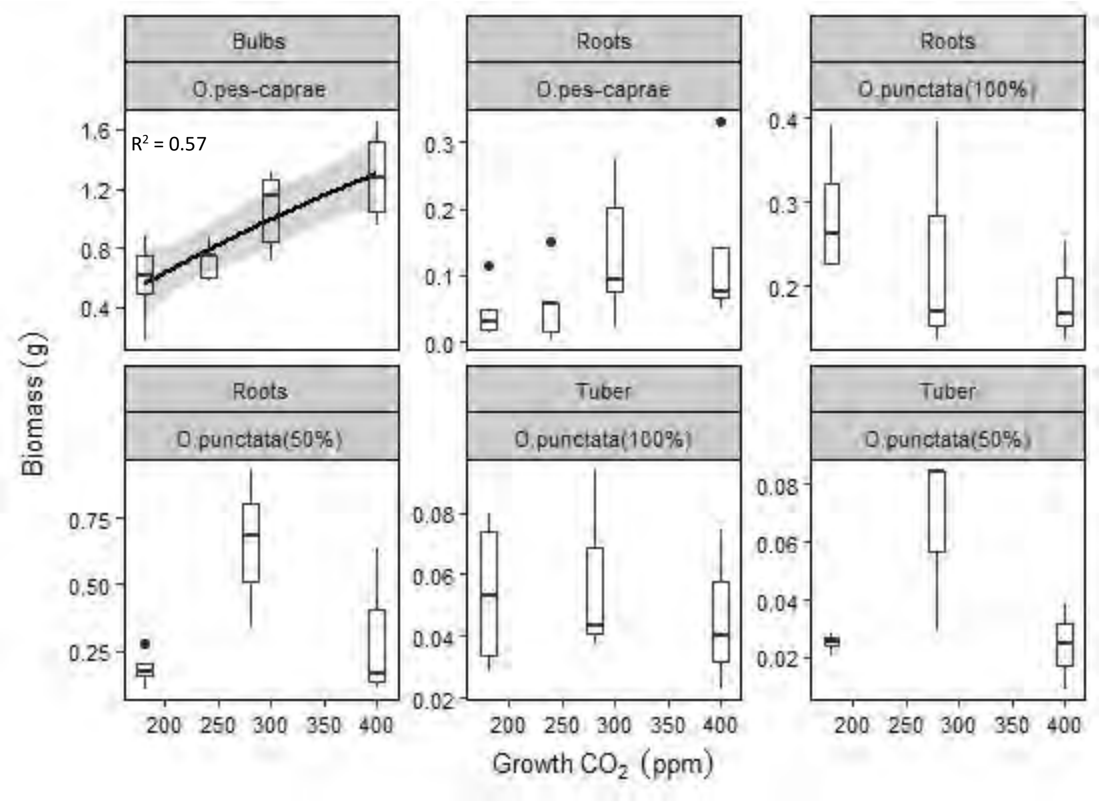

Figure 5-1: *Oxalis pes-caprae* responses to growth in a range of sub-ambient CO_2 concentrations (n = 5 for each treatment). Significant differences at $p < 0.05$ between treatments groups are denoted by different letters.

The modelled time to collect 2000 calories at glacial CO_2 shows that the time required to reach the threshold would have doubled at 180 ppm compared to 400 ppm (*Fig 5-2*). The average time to reach the satisfactory number of calories per day at 400 ppm is approximately 6 hours, and at 180 ppm it increases to approximately 14 hours.

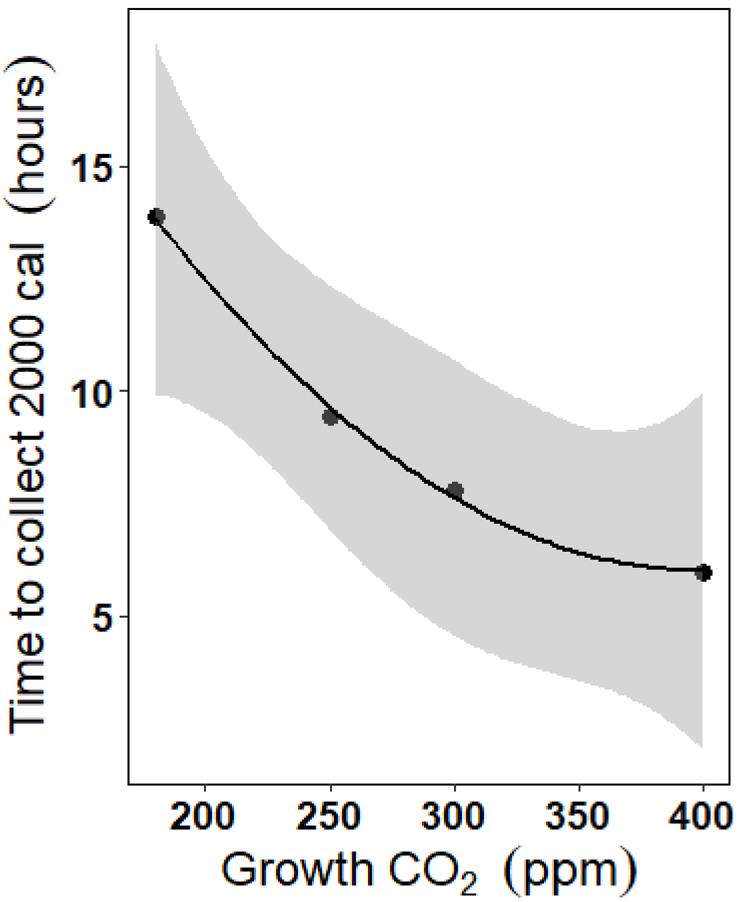

Figure 5-2: Modelled time taken to collect bulbs with calories reaching 2000 in a single harvesting trip under 4 CO_2 concentrations (180, 240, 300 and 400 ppm). The time taken to harvest bulbs at 180, 240 and 300 ppm was modelled from the results of Singels et al., (2016) where USOs were harvested by local women from the Cape region of South Africa under current [CO_2].

The percentage of foraging trips achieving the 2000 calorie threshold decreased with [CO_2], with less than 10% of trips achieving it in 1 hour, and approximately 20% achieving it in 2 hours (*Fig 5-3*).

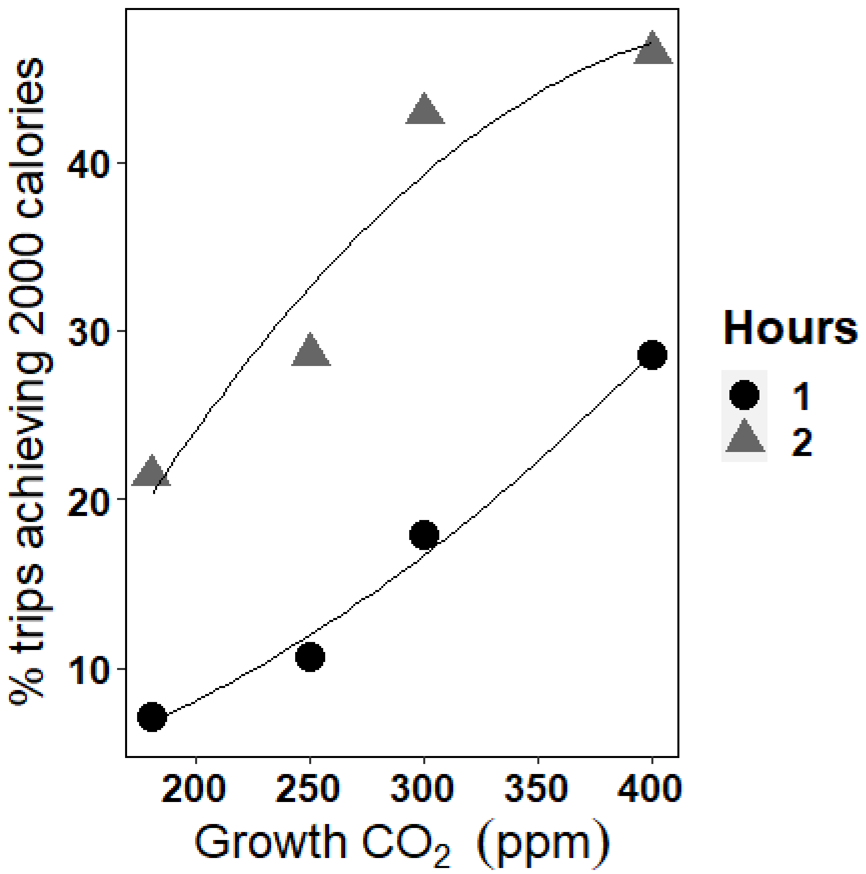

Figure 5-3: Percentage of foraging trips achieving 2000 calories in 1 hour or 2 hours. Percentages for plants grown at 180, 240 and 300 ppm were modelled from the results of Singels et al., (2016).

5.4. Discussion

During the Pleistocene era, hunter-gatherer communities in refugia within the Greater Cape Floristic Region (GCFR) sustained themselves by relying on the harvesting of geophytes and the extraction of their underground storage organs (USOs) for sustenance. Dietary studies conducted on these communities have identified certain geophyte species and specific locations that would have been more favourable for higher yields. The physical activity and smaller stature of these hunter-gatherers necessitated a recommended daily caloric intake ranging from 1900 to 2000 calories, requiring approximately 6 to 10 hours of daily foraging (Botha et al., 2020; Singels et al., 2016).

The findings presented in this study indicate that during glacial periods characterized by lower carbon dioxide (CO_2) concentrations, hunter-gatherers likely had access to less food due to the reduced size of the geophyte USOs. For communities heavily dependent on geophytes for nutrition, this implies an increased effort to fulfil their daily caloric requirements. The model employed in this study, predicting forager return rates at glacial CO_2 levels, assumed a baseline of 2000 calories per day, necessitating efforts double those required under present-day CO_2 levels. This heightened effort encompasses the time spent locating and extracting USOs, potentially extending to the distances travelled by foragers to access food sources.

It is plausible that hunter-gatherers, intimately acquainted with their habitat and the target species for harvest, would naturally gravitate towards locations or species maximizing their return rates (Charnov, 1976; Hawkes et al., 1982). Botha et al. (2020) discovered that the majority of sampled foraging sites yielded fewer calories than optimal, with only a few select sites offering the potential to easily achieve the 2000 calorie threshold. Even when modelling yields at glacial CO_2 levels for recognized hotspots, an increased effort would still be required for plant harvesting.

Chapter 2 of this study reports changes in biomass in response to CO_2 levels, specifically noting significantly smaller bulbs in *Oxalis pes-caprae* in response to sub-ambient CO_2. Consequently, the modelled time required for food collection aligns with the decreased biomass, indicating that human foragers would have needed to invest more time in foraging to meet their dietary needs. In scenarios where less food is available, and fewer foraging trips meet daily caloric requirements, it is reasonable to allocate more time to foraging and exploring new potential hotspots.

An alternative hypothesis suggests that under low CO_2 levels, with escalating geophyte foraging efforts and diminishing return rates, hunter-gatherers would have diversified their diet by incorporating alternative food sources instead of solely relying on geophytes. Various studies (Kyriacou et al., 2014; Marean et al., 2007; De Vynck et al., 2016) report the consumption of marine resources, fruits, and seeds alongside geophytes in the GCFR. Archaeological sites and return rate estimations emphasize that these hunter-gatherers preferred a variety of resources offering higher nutritional yields.

The model presented in this study presupposes uniform return rates across all vegetation types and USO growth forms. However, Botha et al. (2020) demonstrated that the effort required for daily food harvest varied based on vegetation type and its characteristics. By observing inexperienced foragers, Botha et al. (2020) revealed that vegetation types with softer soils yielded higher returns. A crucial refinement for more precise predictions of return rates at glacial CO_2 levels would involve categorising results by vegetation type and USO growth form, acknowledging the variations inherent in different ecological settings. This refinement would contribute to a better understanding of the intricacies associated with forager behaviours and subsistence strategies during the Pleistocene era in the GCFR.

5.5. References

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Chapter 6: Thesis conclusion

The evolutionary predecessors of anatomically modern humans find historical roots in diverse locations across Africa, notably within the Greater Cape Floristic Region (GCFR) situated on the southern coast of South Africa. Throughout the climatic variations of the Pleistocene era, refugia within the GCFR served as pivotal enclaves, offering sustenance and refuge for our predecessors. The connection between environmental dynamics, dietary practices, and the evolutionary trajectory of anatomically modern humans constitutes a compelling narrative that frames the core inquiry of this thesis. This research endeavours to investigate the complexities of plant-based sustenance available in these refugia, casting light upon the efforts demanded of Pleistocene hunter-gatherers to fulfil their daily dietary requirements. The investigation scrutinized the influence of carbon dioxide (CO_2) on *Oxalis* biomass, physiology, and anatomy, with the ensuing findings providing the framework for modelling geophyte harvesting under low [CO_2]. In its essence, this study augments the existing body of knowledge pertaining to contemporary hunter-gatherer communities within the GCFR.

Initial chapters show how glacial [CO_2] levels determine *Oxalis* biomass and photosynthetic rates. This provided the necessary inputs to model foraging rates in Pleistocene environments and depict a scenario wherein the effort required by hunter-gatherers to secure sustenance and meet their daily caloric needs would have undergone a twofold increase. This prompts a re-evaluation of Pleistocene foraging strategies and suggests adaptability to alternate food sources that must have allowed these ancestral communities to survive the challenges of climate change.

Unlike *O. pes-caprae*, *O. punctata* showed little response to growth CO_2 mainly because of differences in allocation strategy where bulb reserves were reallocated to support growth even at lowered atmospheric CO_2. This emphasises that experiments of limited duration may hamper ecological interpretation and point to the necessity of longer experiments and experimentation with a greater variety of USO species. The GCFR boasts an impressive array of approximately 2000 geophytic species, many of which have underground storage organs (USOs) with the potential of providing nutritional value (Procheş et al., 2006). Hence there are many options that could be explored with this sort of experimentation.

The study of hunter-gatherer foraging behaviour, when extrapolated beyond the sphere of USO growth, introduces a complex interplay of factors, ranging from the distances covered during a foraging expedition and the time invested in locating suitable species to the foragers' familiarity with their environment and their preference for certain species (Botha et al., 2020; Pontzer et al., 2015). While this study did not address these factors, it emphasizes the importance of gaining insights to how atmospheric CO_2 needs to be considered when studying the nature of hunter-gatherer behaviours and their dietary patterns.

Geophytes, acknowledged as a pivotal component of the proposed hunter-gatherer diet, coexist with a rich spectrum of dietary elements within the GCFR. Archaeological evidence points to the consumption of shellfish (Marean, 2010), large mammal fauna (Marean et al., 2014), and plant components beyond USOs (De Vynck et al., 2016). Expanding the scope of the foraging model to encompass multiple dietary elements holds the potential to unravel a more comprehensive picture of the resources likely harvested during the Pleistocene, taking into account their varying availabilities and the dynamic interplay of human-environment interactions. This multidimensional approach aligns with the complexity inherent in Pleistocene subsistence strategies and provides a perspective on the dietary choices made by hunter-gatherers.

In conclusion, this thesis represents a step in understanding Pleistocene foraging practices within the GCFR. The findings contribute not only to our understanding of past human-environment interactions but also serve as a platform for future research endeavours. The adaptive strategies and dietary choices made by Pleistocene hunter-gatherers offer a lens through which we can explore the resilience and resourcefulness of our ancestors in the face of environmental challenges. This exploration of the Pleistocene not only sheds light on our evolutionary past but also has implications for contemporary discussions on biodiversity, subsistence strategies, and their consequences for humans and their environment.

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