Elevated Carbon Dioxide (eCO_2) Reduces Pollen Protein Concentration in Three Floral Species Important for Pollinators

A Thesis Presented

by

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Abstract

Since the beginning of the industrial revolution, global CO_2 concentrations have risen by nearly 50% from a pre-industrial average near 280 ppm to modern day concentrations above 410 ppm.[1] Beyond the planet-warming effects of this buildup of greenhouse gas, elevated CO_2 (eCO_2) has been extensively shown to cause micronutrient and protein reductions in staple agricultural crops, from wheat to barley to rice. The consequences of nutrient reduction for human populations have similarly been documented — *Meyers et al.* (2015)[2] find that even marginal decreases in zinc, iron, and protein concentrations in human food sources may place at risk those communities already living at the cusp of nutrient deficiency.

However, the consequences of eCO_2 for non-human species remain relatively undocumented, in part because few studies have examined the effects of eCO_2 on non-human food sources. Pollen is of particular interest, as it serves as the principle protein source for pollinators like bees, upon which much of the human food system depends — bees pollinate one third of agricultural food crops[3] and the majority of all flowering plants globally.[4] However, bee populations are in rapid decline,[5,6] threatened by environmental stressors like toxic agrichemicals[7] and habitat loss. Now, recent research by *Ziska et al.* (2016) finds that eCO_2 may cause reductions floral species' protein content. If this is indeed the case, researchers worry that protein scarcity will exacerbate environmental stressors and could lead to further pollinator population collapse.

Ziska et al. (2016) have documented a strong negative correlation between rising CO_2 and pollen protein concentration for a single floral species, "*Solidago canadensis"* (goldenrod).[8] Goldenrod serves as an important source of pollen, but bees' dietary protein derives from a number sources, and it is

unknown whether CO_2-induced protein reductions occur in a broader array of floral species. To better understand how eCO_2 might affect a wider variety of floral species important to bees, we grew *C. tinctoria* (plains coreopsis), *H. annuus* (dwarf sunflower), and *L. inflata* (Indian tobacco) at two different CO_2 concentrations, one representing the modern atmosphere (400 ppm CO_2) and one simulating an enriched future CO_2 atmosphere (600 ppm CO_2, a concentration well within the Intergovernmental Panel on Climate Change's projections for atmospheric CO_2 levels reachable by the year 2100[9]). We then investigated these species' pollen protein concentrations using a colorimetric protein assay and elemental analysis.

Ziska et al. (2016) used an elemental analysis to detect nitrogen (N) content in *S. canadensis* across a range of CO_2 concentrations, using % N composition as a proxy for protein content. Because this technique requires the use of a proxy and a conversion factor, both of which may introduce uncertainty into protein calculations, we decided to verify the results of elemental analysis within our study with alternative methods. Due to historical precedence, we settled on an adapted Bradford microassay.

We developed a Bradford microassay protocol appropriate for detecting small concentrations of protein extracted from whole anthers and discuss the method's limitations. With elemental analysis, we observed that neither the pollen's C:N ratio nor its % C composition changed significantly across CO_2 treatments. However, we observed significant % N reductions for each species: 15.72% for *C. tinctoria,* 21.93% for *H. annuus,* and 29.37% for *L. inflata*, from flowers grown at 400 ppm to 600 ppm. These findings are consistent with *Ziska et al.'s* findings for *S. canadensis* in suggesting that CO_2 causes protein reductions and increased starch and carbohydrate content, although they raise questions about the mechanisms underlying CO_2-driven protein reduction. More broadly, our

findings highlight the importance of further investigation of the effects of eCO_2 on non-agricultural flora and their reverberating effects on the food system and consequences for bees.

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"let gardens grow, where beelines end,

sighing in roses, saffron blooms, buddleia;

where bees pray on their knees, sing, praise

in pear trees, plum trees; bees

are the batteries of orchards, gardens, guard them."

— from "Virgil's Bees" by Carol Ann Duffy

Chapter 1: Introduction

On many levels, bees are inextricably linked to the health of the planet and its human populations. This is mostly due to the extraordinary number of plants that they pollinate, including the majority of all flowering plants[4] and one third of the planet's agricultural food crops.[3] Through these "pollination services," bees promote ecological diversity, help feed billions of people, and make stable employment possible for millions of farmers around the world.[10] An analysis published in 2012 (*Lautenbach et al.)* found that pollinator species (of which bees are the most important) provide global economic benefits of up to $577 billion annually.[11] And even this astonishing value elides invaluable — but unquantifiable — benefits. For example, healthy bee populations support sustainable beekeeping practices (and broader agricultural methods) based on indigenous knowledge. Bee-pollinated crops may be used for medicinal purposes, and bees themselves have provided cultural and artistic inspiration for millennia.[10]

As the human population grows by an additional 4 billion people by the end of the century,[12] the need for a robust global bee population will only become more pressing, particularly in order to maintain our pollinator-dependent agricultural system. However, a number of environmental stressors like the increasing prevalence of agrochemicals and widespread habitat loss have spurred a rapid decline in populations of bees and other insects,[5,6] causing a global crisis of pollination. Meanwhile, our comprehension of the myriad ways these ongoing and future environmental changes will affect pollinators and the food system remains severely limited, inhibiting the possibility for a comprehensive and informed public policy response.

Rising global CO_2 concentrations underlie many of these uncertainties. Over the past 150 years, atmospheric CO_2 concentrations have continued to rise past 400 parts per million (ppm) and are predicted to continue increasing for the foreseeable future.[1] Even the most aggressive mitigatory measures reported by the Intergovernmental Panel on Climate Change (IPCC) project end-of-century CO_2 concentrations of up to 480 ppm, with the least aggressive measures predicting concentrations as high as 1000 ppm.[1] Such changes in atmospheric composition will have a number of impacts on plant life. For one, elevated CO_2 (eCO_2) is linked to nutrient declines in agricultural crops — it has been extensively documented that eCO_2 contributes to reduced protein concentrations in crops like wheat,[13] rice,[14] and barley,[15] and reduced zinc and iron concentrations across C3 grass species, in potatoes,[16] and in legumes.[17] However, these effects have not been widely studied in flowers, which are an important food source for pollinators like bees. We direct our attention to this uncertainty in this study.

1.1 Nutrient Reductions Impact Human Health

The mechanisms by which protein concentration and nutrient density decline in agricultural crops grown under eCO_2 remain unknown, although several hypotheses have been suggested and investigated. These hypotheses include protein dilution caused by the increased production of carbohydrates;[18–20] reduced transpiration and a reduction in mass flow of N and other mobile elements;[18,21,22] and a reduced need for Rubisco and a subsequent lowering of plant demand for N.[23] Regardless of the mechanism, nutrient declines have clear implications for human health,[17] especially for populations already living on the cusp of nutrient deficiency. Zinc and iron deficiencies already cause an estimated loss of 63 million life-years annually,[24] so even small reductions in the availability of these nutrients can put large populations at risk.

1.2 Bee Health is Tied to Human Health

Just as nutritional decline may jeopardize human populations, bee populations may be endangered by micronutrient and protein reductions in their food sources — specifically, in floral pollen. The apian diet is made of two main components: nectar and pollen. While nectar fulfills bees' energetic needs, pollen is their only source of dietary protein and is vital for larval growth and development.[25,26] Because only small quantities of pollen are stored within the hive at a given time, colonies are particularly sensitive to fluctuations in the availability of pollen,[27–29] and lower-protein pollen has been shown to reduce bees' longevity.[30–32]

For this reason, pollen has a unique significance in research on eCO_2's effects on floral physiology — if pollen protein concentrations are declining in flowers, bee health and nutrition could be directly impacted. Bees already face a number of environmental stressors including habitat loss, invasive pests, and agrochemicals (e.g., neonicotinoids).[6] Nutritional deficiency has the potential to exacerbate these environmental stressors by making it more difficult for bees to cope with them,[33,34] for example by decreasing their ability to detoxify pesticides.[35] In general, bees feeding on inadequate pollen have been shown to be less resilient to environmental stressors.[30]

Moreover, the interconnected nature of the food system and its dependence on insect pollination means that health consequences for bees could have a cascading effect up the food chain. Less abundant bee populations could reduce the availability of critically important, nutrient-dense food crops, further exacerbating human food insecurity and nutrient/protein deficiency. However, the effects of eCO_2 on pollinator food sources have not been widely studied, making it difficult to

understand and quantify these complex interactions between atmospheric composition, global food systems, and human health.

1.3 *Solidago canadensis* (Goldenrod)

To date, one of the only experiments of eCO_2 on floral pollen is a 2016 study conducted by *Ziska et al.* on goldenrod (*Solidago*), a small, yellow flower that grows widely in meadows and grasslands across the U.S. and is a key species for bees.[36] For bees that overwinter, the pollen collected from autumnal species like goldenrod provides enough protein for the colony to survive until spring. This is because, in temperate zones, bees begin rearing their young before springtime pollen becomes available — bee populations with greater pollen stores have a better chance of making it through the winter.[37] *Solidago canadensis,* the most widespread taxon of *Solidago*, was chosen in *Ziska et al.* (2016) as a test case to evaluate the impacts of eCO_2 on floral pollen's nutrient composition.

Results from *Ziska et al.'s* two-year *in situ* field trial of *S. canadensis* grown along a continuous CO_2 gradient from 250 to 500 ppm were compared with pressed floral collections of *S. canadensis* from the Smithsonian Museum of Natural History. Pressed samples from the museum spanned the period 1842-2014, corresponding to an approximate CO_2 increase from 280 to 398 ppm. This historical Smithsonian data indicated a strong, significant correlation between eCO_2 and reductions in pollen protein concentrations ($p < 0.001$). Experimental data reproduced this relationship, again linking decreasing protein concentration with rising concentrations of atmospheric CO_2. Together, these data implied a continuing decrease in pollen protein concentration for the near to intermediate term, i.e. as atmospheric CO_2 approaches 500 ppm.[8]

Because protein is volatile and quick to degrade, both the experimental and historical approaches in *Ziska et al.* (2016) used elemental analysis (C:H:N) to estimate protein concentration. N and C content of each sample was determined as a fraction of each sample's dry weight in a Perkin-Elmer 2400 CHN/O analyzer, and a conversion factor of 6.25 (N to protein) was applied to estimate pollen protein concentrations. This conversion factor had previously been used by Keller et al. (2005) to determine protein content of pollen, and there is generally a robust historical precedent for the use of this conversion value.[38,39]

Elemental analysis of the historical samples revealed pollen protein concentrations decreasing approximately one-third over the 170-year study period. Notably, *Ziska et al.* (2016) found that protein concentrations decreased most rapidly in the latter half of the twentieth century, during the rapid rise of atmospheric CO_2 concentrations from 315 to 398 ppm in the years 1960 to 2014. The researchers noted a parallel rapid rise in nitrogen dilution during this period: as eCO_2 promoted starchy growth of the goldenrod's shoots and leaves, it did not appear to cause a concomitant rise in N levels of the plant.

Although *Ziska et al.* (2016) determine a "clear and unequivocal link" between eCO_2 and declining pollen protein for *S. canadensis*, the authors call for further study into other species whose pollen may be an important food source for pollinators.

1.4 Methods for Protein Analysis

Elemental analysis has been used by many researchers to estimate pollen concentrations in plants. However, it requires the use of a proxy (nitrogen) and a conversion factor (in the form of a

nitrogen-to-protein ratio), all of which introduce uncertainty into protein calculations. For this reason, we decided that it may be helpful to verify the results of elemental analysis with alternative methods for measuring protein content. These alternatives included Biuret methods using protein-Cu chelation and secondary detection of reduced Cu (BCU Assay, Lowry Assay); fluorescent dye methods using detection of increased fluorescence associated with the bound dye (EZQ fluorescent assay, Qubit Protein Assay); or colorimetric methods using direct detection of a color change associated with protein binding to a dye (Bradford assay).[40,41] We opted to use the Bradford assay because it is comparatively rapid and easy — it requires no dilution or filtration and can be done at room temperature. Furthermore, there is historical precedent for using the Bradford assay for floral pollen protein analysis.[42]

The Bradford Assay binds Coomassie Brilliant Blue G-250 dye to protein samples, causing a color change that indicates the samples' protein concentrations. In this method, proteins exposed to an acidic reagent bind to the Coomassie dye, resulting in a spectral shift from the dye's brown form (with a maximum absorbance of 465 nm) to its blue form (with a maximum absorbance of 610 nm). The color shift is proportional to the amount of protein added. Dyed samples are then analyzed in a spectrophotometer and assigned protein concentration values based on the absorbances of a series of standards of known protein concentrations. Some researchers have suggested that, since different protein standards have different binding affinities, a pollen standard should be used when measuring pollen protein concentrations,[39] but most prior research has used bovine serum albumin (BSA) with no adverse consequences.[42–47]

We considered several drawbacks to this method before selecting it. For instance, the Bradford assay is sensitive to surfactants: if these surfactants are initially used to solubilize sample proteins, they

may subsequently cause the Bradford reagent to precipitate. Additionally, the reagent may react with detergents to cause a color shift even in the absence of protein. Finally, the Bradford assay produces higher levels of protein-to-protein variation than other protocols. However, despite these difficulties, the Bradford assay is overall highly convenient and is likely the most widely documented method used in previous pollen research.[43–47]

1.5 This Study: *C. tinctoria, L. inflata,* and *H. annuus*

Given the broad knowledge gaps alluded to in *Ziska et al.* (2016), we attempted to investigate the effects of eCO_2 on eight floral species whose pollen was determined to be an important source of nutrition for bees. We grew these species at ambient CO_2 concentrations (400 ppm) and at eCO_2 (600 ppm, a concentration that the IPCC has projected may be reached by the year 2100). We successfully collected pollen and anther samples from three species — *C. tinctoria* (plains coreopsis), *H. annuus* (dwarf sunflower), and *L. inflata* (Indian tobacco). We used colorimetric spectrophotometry and elemental analysis to investigate trends in C and N content, using N as a proxy for protein.

We were motivated by a number of questions regarding the effects of eCO_2 on plant life. Does pollen protein decline with CO_2 across all species, or at least in more than one? If so, does protein decline more in some species and less in others? The answers to these questions may have far-reaching consequences for pollinator populations. A global decline in pollen protein concentrations across many floral species could have dire consequences for pollinator population stability. But if protein losses vary across species, it may be possible to craft a mitigatory response that, among other

measures, prioritizes less affected flowers for planting in public spaces, home gardens, and elsewhere.

Chapter 2: Methods

2.1 Sample Collection

To investigate whether the protein-diluting effects of carbon dioxide extend to flora beyond *Solidago canadensis,* eight floral species known to be important sources of pollen for bees were selected: *Solidago flexicaulis* (zigzag goldenrod), *Lobelia inflata* (Indian tobacco), *Cirsium arvense* (Canada thistle), *Eupatorium perfoliatum* (common boneset), *Monarda bradburiana* (Bradbury's monarda), *Coreopsis tinctoria* (plains coreopsis), *Helianthus annuus* (dwarf sunflower), and *Papaver rhoeas* (red poppy). These species were grown from seed in controlled growth chambers at the USDA's Agricultural Research Service (ARS) in Beltsville, MD. Half of the seeds were grown at ambient CO_2 concentrations (roughly 400 ppm), while the other half were grown at elevated CO_2 (eCO_2, roughly 600 ppm). Table 1 shows the selected species, as well as seed counts for perennial species (annual seeds were scattered over the soil, rather than planted individually).

Genus	**Species**	**Common Name**	**Annual/ Perennial**	**# seeds planted at 400 ppm**	**# seeds planted at 600 ppm**
Solidago	*flexicaulis*	Zigzag goldenrod	perennial	29	30
Lobelia	*inflata*	Indian tobacco	perennial	28	29
Cirsium	*arvense*	Canada thistle	perennial	11	11
Eupatorium	*perfoliatum*	Common boneset	perennial	9	9
Monarda	*bradburiana*	Bradbury's monarda	perennial	9	5
Coreopsis	*tinctoria*	Plains coreopsis	annual	n/a	n/a
Helianthus	*annuus*	Dwarf sunflower	annual	n/a	n/a
Papaver	*rhoeas*	Red poppy	annual	n/a	n/a

Table 1: Eight floral species were grown from seed at ambient and elevated CO_2.

Following a growth period of several weeks, flowers were transplanted to eight outdoor plots at a USDA field site. Half of these plots were given a 600 ppm CO_2 treatment (the eCO_2 plots), while the other half were not (the ambient plots). Flowers that had been grown at eCO_2 in the growth chambers were transplanted to the eCO_2 plots, and ambient growth chamber flowers were planted in ambient plots. The eight plots were sorted into four sister pairs, with one plot of each pair exposed to ambient CO_2 concentrations and the other given the eCO_2 treatment. Although plots in each pairing were separated by the width of the field (~ 5 m), pairs were controlled for similar soil characteristics, previous tillage history, and grasshopper herbivory. Rye grass was planted around the perimeter of each plot as a natural plot border, and plots were labeled to reflect geographic location: 1N, 1S, 2N, 2S, 3N, 3S, 4N, and 4S, with 1-4 going east to west, and "N" and "S" standing for "north" or "south." Plot pairs are shown in Table 2:

Pairs	**eCO_2 (600 ppm)**	**Ambient (400 ppm)**
Pair 1	1S	1N
Pair 2	2N	2S
Pair 3	3S	4S
Pair 4	4N	3N

Table 2: Plots were paired to control for soil characteristics, previous tillage history, and grasshopper herbivory.

All flowers were watered regularly as needed. For purposes unrelated to the outcome of this paper, each plot was enclosed with mesh after one week. One bumblebee colony was introduced into each enclosed plot, and bees were allowed to collect pollen freely.

Figure 1: Across all plots, samples were most robust in quality and quantity from *Coreopsis tinctoria* (plains coreopsis, left), *Lobelia inflata*, (Indian tobacco, center), and *Helianthus annuus* (dwarf sunflower, right).

From July to late August, floral samples were collected, with one or two plot pairs selected for collection on any given day. Samples included whole anthers, leaves, petals, sepals, stems, and, in the case of some *H. annuus* flowers, pure pollen. Not all sample types were collected across all species due to difficulties in collection (i.e., *S. flexicaulis* were too small for sepal or petal collection) or unavailability (i.e., *C. arvense* were not in bloom at the beginning of July). Samples were collected in Eppendorf tubes and frozen at -80º C within 1.5 h of collection. Table 3 shows samples collected by type and by species.

Relatively few samples were collected from plots 3S and 4S; these plots were overrun with rye grass and a large population of grasshoppers, which significantly damaged plant material. Across all plots, samples were most robust in quality and quantity from *Coreopsis tinctoria* (plains coreopsis), *Lobelia inflata*, (Indian tobacco), and *Helianthus annuus* (dwarf sunflower).

400 ppm	Flower part	Sample count	600 ppm	Flower part	Sample count
Coreopsis tinctoria	anther	34	***Coreopsis tinctoria***	anther	34
	leaf	29		leaf	30
	petal	34		petal	34
	sepal	34		sepal	34
	stem	34		stem	34
Lobelia inflata	petal	24	***Lobelia inflata***	petal	23
	anther	24		anther	23
	leaf	20		leaf	19
Eupatorium perfoliatum	leaf	27	***Eupatorium perfoliatum***	leaf	27
	bud	12		bud	16
	flower	11		flower	17
Cirsium arvense	flower	11	***Cirsium arvense***	flower	14
	leaf	26		leaf	29
Helianthus annuus	leaf	24	***Helianthus annuus***	leaf	24
	anther	21		anther	21
	petal	22		petal	21
	sepal	23		sepal	23
Papaver rhoeas	anther	2	***Papaver rhoeas***	anther	2
	petal	2		petal	3
	leaf	2		leaf	2
	stem	1		stem	2
Solidago flexicaulis	anther	3	***Solidago flexicaulis***	anther	3
	petal	3		petal	3
	leaf	1		leaf	1

Table 3: Anther, leaf, petal, sepal, and stem samples were collected from each of the eight floral species.

2.1.1 Anther collection and preparation

To collect anthers, we picked whole *C. tinctoria* and *L. inflata* flowers and brought them into a mobile, air-conditioned lab. We used forceps to remove anthers and place them in labeled Eppendorf tubes before transferring them to a -80 ºC freezer. For *H. annuus*, we used forceps to pluck anthers directly from the disk flowers without picking the plant. These anthers were placed

immediately into Eppendorf tubes, transported into the air-conditioned mobile lab, and stored at -80º C within 1.5 h.

Figure 2: Left: Collection of *L. inflata* in a field plot at the USDA. Right: The mobile lab.

2.2 Sample Sorting

Samples were shipped on dry ice from the USDA to Harvard's Biological Laboratories and returned to storage at -80 ºC to await processing. Samples were sorted into subsamples, destined for either a colorimetric protein assay (the Bradford assay), elemental analysis, or metabolomics (analysis of organic micro-compounds). Samples sorted for metabolomics were set aside for another study, the results of which are not presented in this paper. All samples were processed over dry ice and returned immediately to a -80º C freezer.

2.3 Colorimetric Analysis

2.3.1 Protein extraction

To extract and break down pollen in preparation for colorimetric analysis with the Bradford assay, we subjected samples to a multi-step process adapted from *Chang & Huang* (2017).[48] For this adapted process, samples first underwent a quick rinse: whole anthers were added to empty centrifuge tubes along with 1 mL of K-HEPES protein buffer (see recipe in Table 4). Tubes were vortexed at medium speed for 1 min each, then centrifuged at 400 x g for 2 min (4º C). The supernatant from each tube was removed using an auto-pipettor and discarded, leaving the anthers at the bottom of each tube. The following procedure was then used to create final protein samples:

1. Pulverize samples using a melted pipette tip and add 60 µL of K-HEPES protein buffer to each tube.
2. Chill tubes on ice for approximately 1 h.
3. Centrifuge samples at 18,000 x g (4º C) for 20 min.
4. Extract 50 µL of supernatant from each tube and collect it in new, clean sample tubes. These tubes will be the final protein samples.
5. Repeat steps 1-4 two more times until the final sample tubes have 150 µL of accumulated supernatant.

Protein Buffer Recipe
100 mL water
480 mg HEPES free acid
1080 mg K-acetate
410 mg MgCl2-6H2O
100 μL Tween-20
200 μL Triton X-100
1 mM phenylmethylsulphonyl fluoride (PMSF)*

Table 4: Our K-HEPES protein buffer recipe was adapted from *Chang & Huang* (2017).[48] *PMSF was prepared in advance in isopropyl alcohol and added to the K-HEPES solution immediately before use; PMSF is unstable in aqueous solution.

2.3.2 Pollen homogenization: optimization for the Bradford assay

The Bradford assay requires that protein samples be homogenized in solution — therefore, it was essential that step (1) of the above protocol reliably homogenize our pollen samples. *Chang & Huang* (2017) report using a melted pipette tip to homogenize pollen in their experiment on *Arabidopsis,* but we wanted to ensure this method led to optimal homogenization. To investigate, we performed a series of trials on stock pollen from *Zea mays* (maize), testing three alternate homogenization techniques for step (1), subjecting final samples to the Bradford assay, and comparing the final samples' absorbance.

One third of the experimental samples were homogenized using a bead beater (Biospec Products Mini Bead Beater); one third was homogenized with a melted pipette tip (inserted into Eppendorf tubes and used to physically grind pollen); and one third was homogenized by quantitatively removing pollen from tubes along with the supernatant, physically grinding using a Teflon pestle, and returning to the tube.

The Bradford assay was carried through to completion for all maize pollen samples, and we recorded absorbance at 595 nm. We averaged the absorbance for each homogenization technique, with results indicating little difference between the melted pipette method, the bead-beating protocol, and removal and grinding (here called "HT" for the Hungate tube into which the pollen was quantitatively transferred for grinding) (see Table 5). Given the similar absorbances produced by each method, the melted pipette technique was selected for processing future samples because of its relative ease and rapidity.

Samples	**Homogenization**	**Abs 595 nm**	**Average Absorbance**
A2	melted pipette tip	0.239	0.242
A3		0.238	
A9		0.249	
A14		0.253	
A15		0.231	
A7	Hungate tube	0.257	0.251
A8		0.267	
A12		0.233	
A13		0.245	
A1	bead beater	0.239	0.229
A4		0.236	
A5		0.217	
A6		0.227	
A10		0.224	

Table 5: Pollen homogenization by three different methods — pulverization with a melted pipette tip, quantitative removal into a Hungate tube and grinding with a Teflon pestle, and a bead beater — caused prepared samples of *Zea mays* pollen to yield similar absorbances in the Bradford assay.

2.3.3 Calibrating the calibration curve: refining the buffer

The Bradford assay protocol for this experiment was adapted from *He* (2011)[49] and a standard protocol published by Bio-Rad,[50] as well as Bradford's original publication.[42] With this protocol, a series of standard dilutions (in this case, of bovine serum albumin, BSA) is prepared and exposed to a Bradford reagent (Bio-Rad Laboratories, Quick-Start Bradford Protein Assay Kit). The reagent causes a color shift based on the amount of protein in the standard. By using a spectrophotometer to measure absorbance for each standard of known concentration, a linear response curve can be created and subsequently used to determine the protein concentration in an unknown sample.

Due to the small concentrations of protein expected in our samples, it was necessary to use a microassay protocol (to be able to measure concentrations within a range of 1-100 μg/mL of sample solution). However, we discovered that detergents in the *Chang & Huang* (2017) K-HEPES protein buffer were interfering with the Bradford reagent, causing a color shift even in the absence of protein. To find out which K-HEPES ingredient was causing the interference, alternative buffer solutions were prepared, with varying concentrations of Tween 20 and Triton X-100, and their effects on absorbance were recorded (see Figure 3 and Table 6).

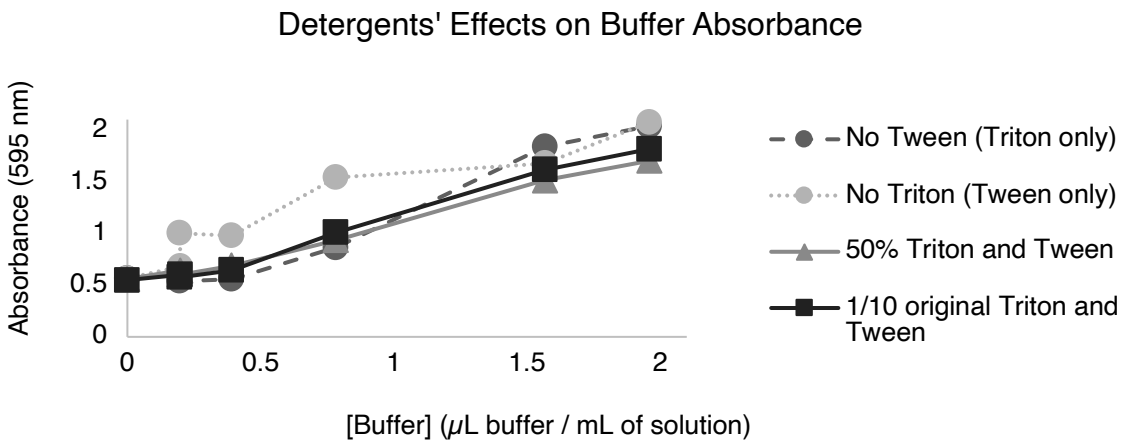


Figure 3: Detergents affect the absorbance of a buffer solution subjected to the Bradford assay. Here it is shown that Tween 20 causes greater interference with the Bradford reagent than does Triton X-100. Conclusions from this graph are described in Table 6.

Buffer Number	Buffer alteration	Effect on microassay
1	no Tween (Triton X-100 only)	interference beginning at approximately 0.8 μL buffer per mL water
2	no Triton X-100 (Tween only)	interference beginning at approximately 0.4 μL buffer per mL water
3	half concentrations of both Tween and Triton X-100	interference beginning at 0.4 μL buffer per mL water
4	1/10 concentrations of both Tween and Triton X-100	interference beginning between 0.5 and 0.8 μL buffer per mL water

Table 6: Tween 20 is shown to cause a problematic interference with the Bradford reagent, driving a color shift even in the absence of protein.

From these results, we identified Tween 20 as causing a problematic color shift when exposed to the Bradford reagent in the absence of protein. To investigate whether Tween 20's concentration should be reduced or if the detergent should be omitted altogether, we tested a series of three new K-HEPES protein buffer recipes with progressively less Tween 20 (see Table 7 and Figure 4). Based

on the results of this test, a recipe with one-tenth the original concentration of Tween 20 was selected for processing samples.

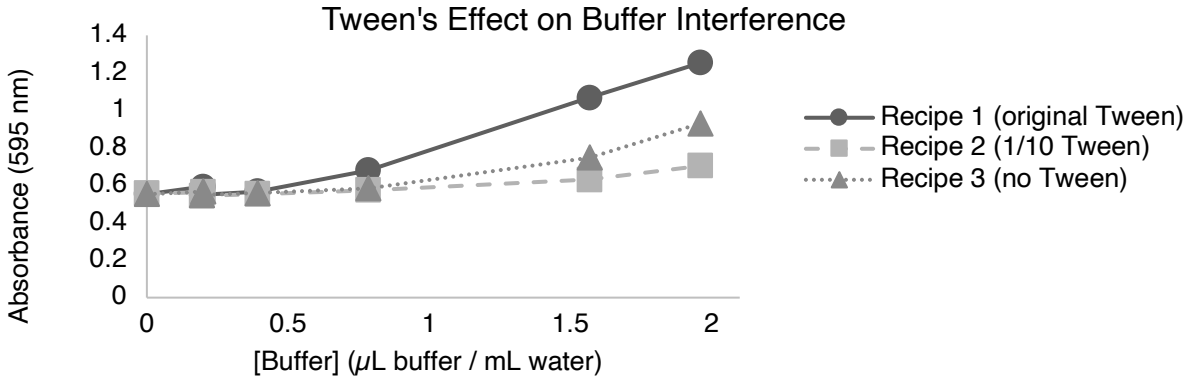


Figure 4: Tween 20 is shown to cause a problematic interference with the Bradford reagent, even in the absence of protein. This interference is resolved if the protein buffer uses only 0.01% Tween 20.

Buffer Number	Buffer alteration	Effect on microassay
1	original recipe (0.1% Tween)	interference beginning at approximately 0.8 μg/mL buffer (in water)
2	1/10 original [Tween] (0.01% Tween)	interference beginning between 1.0 and 1.5 μL buffer per mL water
3	no Tween	interference beginning between 1.0 and 1.5 μL buffer per mL water

Table 7: Further investigation of the effects of Tween 20 on absorbance in the Bradford assay show that reducing the concentration of Tween 20 to 0.01% resolves its problematic interference with the Bradford assay.

2.3.4 Weighing the options: sample weight versus anther count

Finally, we wanted to determine whether to quantify protein as a percentage of sample weight or by the number of anthers per sample. A trial with *H. annuus* anthers collected from a Harvard

greenhouse revealed that absorbance did not scale predictably with sample mass (greater sample mass should yield greater absorbance, since it causes more protein to end up in the supernatant). However, when calculated per anther, the fitted response curve was more accurate (Table 8 and Figure 5). For this reason, subsequent samples would not be weighed, but their protein content would be calculated per anther.

run number	tube number	pollen wt (g)	# anthers	abs 595 nm
1	11	0.00472	3	0.3
	12	0.02085	6	1.114
	13	0.02722	9	1.07
	14	0.04062	12	1.21
	15	0.04849	15	1.315
run number	**tube number**	**pollen wt (g)**	**# anthers**	**abs 595 nm**
2	16	0.00472	2	0.436
	17	0.02085	3	0.698
	18	0.02722	5	0.947
	19	0.04062	7	0.602
	20	0.04849	10	0.992
run number	**tube number**	**pollen wt (g)**	**# anthers**	**abs 595 nm**
3	21	0.0016	1	0.225
	22	0.00394	2	0.24
	23	0.00581	3	0.308

Table 8: *H. annuus* anthers were collected from a Harvard greenhouse. Different numbers of anthers were processed via the Bradford assay to determine whether anther count or sample weight provided a more precise relationship with absorbance (595 nm).

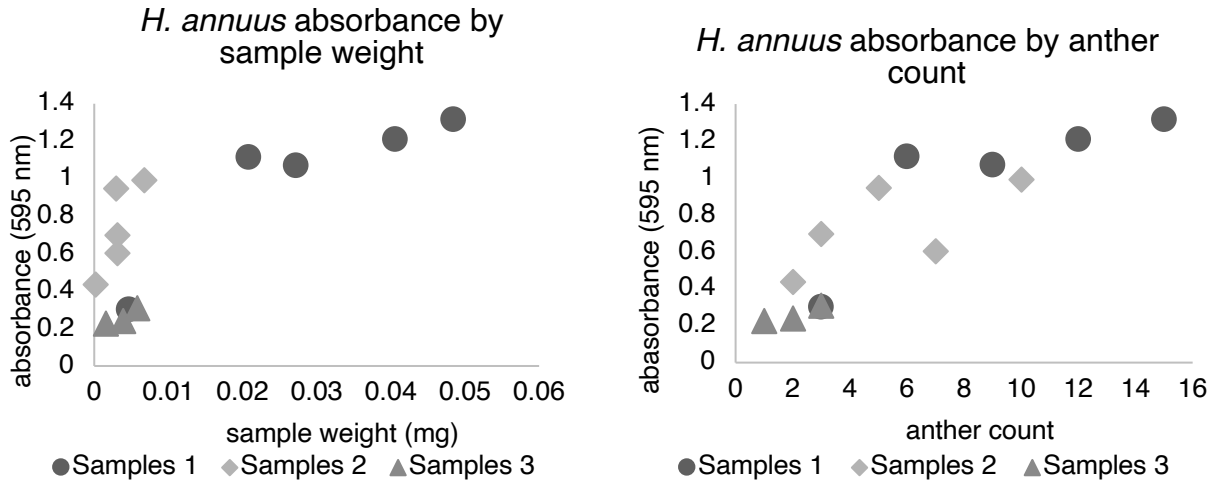


Figure 5: A graph of *H. annuus* absorbance as a function of increasing anther count shows a more linear relationship ($r = 0.758$) than the graph of absorbance by sample weight ($r = 0.608$).

Based on these results, five *H. annuus* anthers per sample would be processed in subsequent runs of the Bradford assay. For *C. tinctoria,* we undertook a similar project, preparing a series of 3, 5, and 7 anthers per sample for processing with the adapted Bradford microassay. When the linearized equation from the microassay's standard curve was used to calculate protein concentration based on each sample's absorbance, protein concentration increased with increasing number of anthers (Figure 6 and Table 9). Five was chosen as an appropriate number of anthers for further sample processing.

tube number	# anthers	abs 595 nm	µg/mL conc. from linear curve	µL supernatant	µg protein in the tube	µg protein / anther
B9a1	3	0.167	1.808	180	0.325355649	0.10845188
B9a2	5	0.191	2.310	180	0.415732218	0.08314644
B9a3	7	0.258	3.711	180	0.668033473	0.09543335
B10a1	3	0.173	1.933	180	0.347949791	0.11598326
B10a2	5	0.192	2.331	180	0.419497908	0.08389958
B10a3	7	0.252	3.586	180	0.645439331	0.09220562
B26a1	3	0.186	2.205	180	0.396903766	0.13230126
B26a2*	5	0.522	9.234	180	1.662175732	0.33243515
B26a3	7	0.313	4.862	180	0.875146444	0.12502092

Table 9: *C. tinctoria* anthers were subjected to the Bradford assay to calculate protein concentrations. *Sample B26a2 was removed from analysis due to anomalously high absorbance likely caused by a processing error.

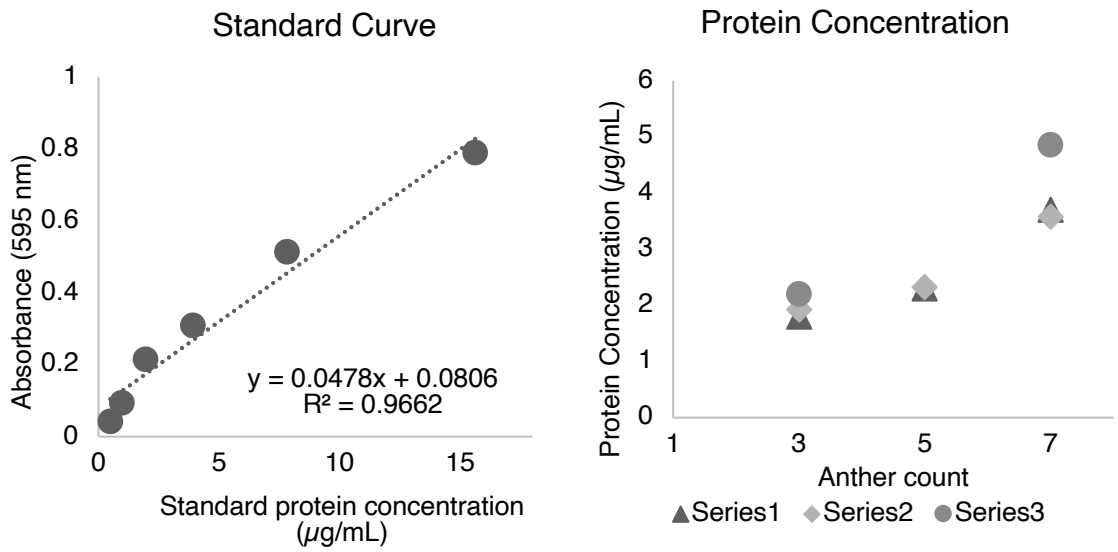


Figure 6: *C. tinctoria* protein concentration calculated as a function of anther count (based on the linearized equation from the BSA standard curve on the right).

2.3.5 The final, refined Bradford protocol

These refinements were all incorporated into a final Bradford protocol, which was then used to process all subsequent *H. annuus, C. tinctoria,* and *L. inflata* samples. Once samples had been prepared, standards were created by preparing dilutions of bovine serum albumin (BSA) (Table 10).

standard #	**protein concentration (μg / mL solution)**
1	62.5
2	31.25
3	15.625
4	7.8125
5	3.90625
6	1.953125
7	0.9765625
8	0.48828125
9	0

Table 10: Standard concentrations used to calculate protein concentration of unknown samples. Tube 9 was a blank containing only buffer.

150 μL of each standard and sample was pipetted into 2 mL cuvettes, along with 50 μL of water. 1000 μL of Bradford reagent (at room temperature) was added to each cuvette and samples were allowed to incubate at room temperature for at least 5 min and no longer than 1 h.

Cuvettes were then loaded into a spectrophotometer (Thermo Scientific GENESYS 20 Visible Spectrophotometer CAT 4001) set to 595 nm and zeroed using the Standard 9 (the 0 μg/mL standard with only buffer and Bradford reagent). Absorbance for standards 3 to 8 were plotted as a

function of protein concentration, and a linear curve was fitted to the plot (see Figure 7 for an example).

Once absorbance for sample cuvettes was recorded, the standard curve's linear equation was used to calculate protein concentration in the sample cuvettes. This number was used to determine the protein concentration in the total supernatant, then divided by the number of anthers per sample to arrive at a final protein concentration per anther number.

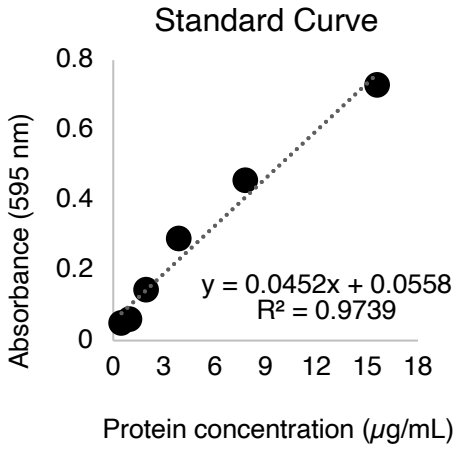


Figure 7: An example of a standard curve showing the absorbance of several dilutions of known protein concentration.

If, for example, sample absorbance was 0.45, using Figure 7 above, we would calculate that the protein concentration in the cuvette = (0.45 – 0.0558) / 0.0452 = 8.72 μg/mL. There was a total of 0.180 mL of solution collected from the original sample tubes, so 8.72 μg/mL * 0.18 mL = 1.57 μg total protein in the sample. Dividing by five anthers gives 1.57 μg / 5 anthers = 0.314 μg protein per anther. These calculations were performed for each tube and its associated standard curve.

2.4 Elemental Analysis

Subsamples of *C. tinctoria* (anthers), *H. annuus* (anthers and pure pollen), and *L. inflata* (anthers) were sent to the USDA for elemental analysis. These subsamples were from the same source samples set aside for the Bradford assay. Samples were analyzed using a Perkin-Elmer 2400 Series II Elemental Analyzer. They were encapsulated in aluminum and combusted in a pure oxygen environment, reducing the samples into the elemental gases CO_2, H_2O, N_2, and SO_2. These gases were then measured by a thermal conductivity detector as stepwise changes from the carrier gas baseline, returning data on % C and % N (by dry mass), as well as a C:N ratio. Sample weight was also recorded prior to combustion. A factor of 6.25 was used to convert N to protein for all samples: crude sample protein (% by weight) is estimated to be 6.25 times the nitrogen content by percent composition.[39] A constant multiplier was assumed for all species.[39]

Chapter 3: Results and Discussion

3.1 Overview

Using elemental analysis, we find a strong negative relationship between eCO_2 and N composition across all three species examined. Since % N is a proxy for protein content, we report a significant decrease of 15.71% in the protein content of *C. tinctoria,* 21.93% for *H. annuus,* and 29.37% for *L. inflata.*

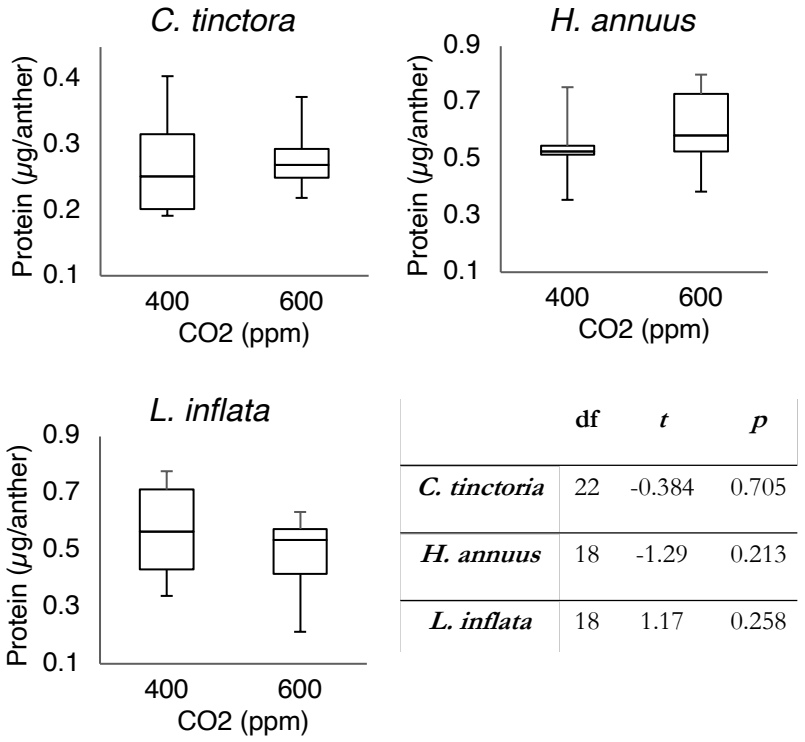
However, we find no such relationship between eCO_2 and protein content in the results from our Bradford assay data. We also note that protein concentrations given from this data far fall below the expected protein concentrations for pollen. This may be due to the extreme durability of pollen grains, and thus the difficulty in using solvents and/or physical grinding for protein extraction.[51] More research into this method may be needed to determine whether it is appropriate for measuring very small protein concentrations using limited sample material. For these reasons, we consider the elemental analysis data to be of higher quality and a better representation of the effect of eCO_2 on pollen protein concentration.

Within our results from elemental analysis, despite our finding that eCO_2 is significantly linked to protein declines, we find no significant relationship between eCO_2 and C:N ratio or % C. This suggests that, as pollen protein decreases, it may be offset by an increased composition of carbohydrates (see page 31 for a more detailed explanation). We report these findings at length in the following section.

3.2 Bradford Assay

We originally chose to analyze samples using the Bradford assay to provide a useful cross-verification against results from elemental analysis. Unlike elemental analysis, the Bradford assay measures protein through an induced color shift caused by the binding of an acidic reagent (Coomassie Brilliant Blue G-250) to protein samples. The change in color, measured as absorbance (in nm) using a spectrophotometer, is proportional to the concentration of protein in the sample. When compared to the absorbances of a series of standards of known concentrations, protein concentrations may be assigned to the samples. This method is highly convenient for its rapidity and simplicity, and it has been widely used in previous research on floral pollen.[39,43,44,46,51]

Calculated protein concentration values for each species showed relatively low protein concentrations in *C. tinctoria* (median near 0.25 μg/anther), with higher values in *H. annuus* and *L. inflata* (medians near 0.5 μg/anther in either case). Statistical analysis of results from the Bradford assay failed to show a significant difference in variance between samples, but revealed no significant relationship between protein content and CO_2 concentration ($p > 0.05$, see Figure 8), with pollen concentrations remaining relatively stable despite a 50% increase in CO_2 concentration.



	df	*t*	*p*
C. tinctoria	22	-0.384	0.705
H. annuus	18	-1.29	0.213
L. inflata	18	1.17	0.258

Figure 8: Bradford assay results did not show a significant relationship between CO_2 concentration and protein concentration per anther.

However, when absorbance results were used to determine protein values as a percentage of anther mass, this method yielded unrealistically small numbers (i.e., 6.08E-03 percent protein), suggesting that the Bradford microassay may not be useful in collecting absolute protein data. For this reason, we shift our attention for the rest of this section to the elemental analysis.

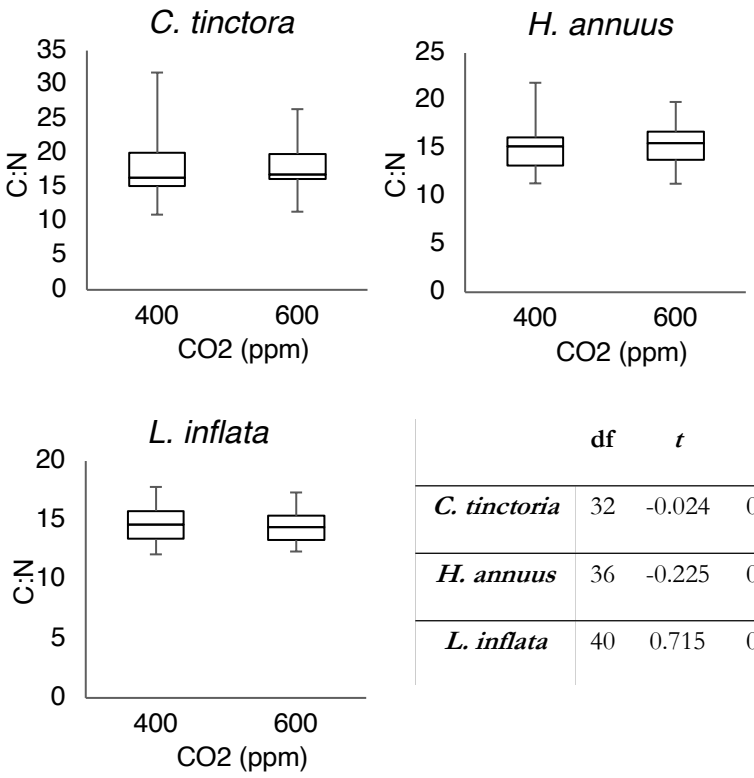
3.3 Elemental Analysis

Elemental analysis, by contrast with the Bradford assay, involves the combustion of sample materials in a pure oxygen environment and measuring the amount of resulting gases. This allows C, N, and sometimes O, H, and S to be reported as a dry mass percentage of the original sample. The analyzer

used in this study provided data on % C and % N by dry mass. Our results from elemental analysis provide more detailed insight than our results from the Bradford assay, for instance that there is a significant relationship between CO_2 and N content. We begin by looking at this method's reported C:N ratio.

3.3.1 C:N ratio

Previous literature has suggested that increasing CO_2 concentration may cause an increase in starch and sugar concentration while reducing the N concentration in plants.[23,52] Indeed, this inverse correlation between CO_2 concentration and C:N appeared in the *Ziska et al.* (2016) study on goldenrod[8] that inspired this project. However, elemental data received from the USDA showed no significant correlation between CO_2 concentration and C:N ratio when C and N content were determined as a percentage of dry mass ($p > 0.05$, see Figure 9).

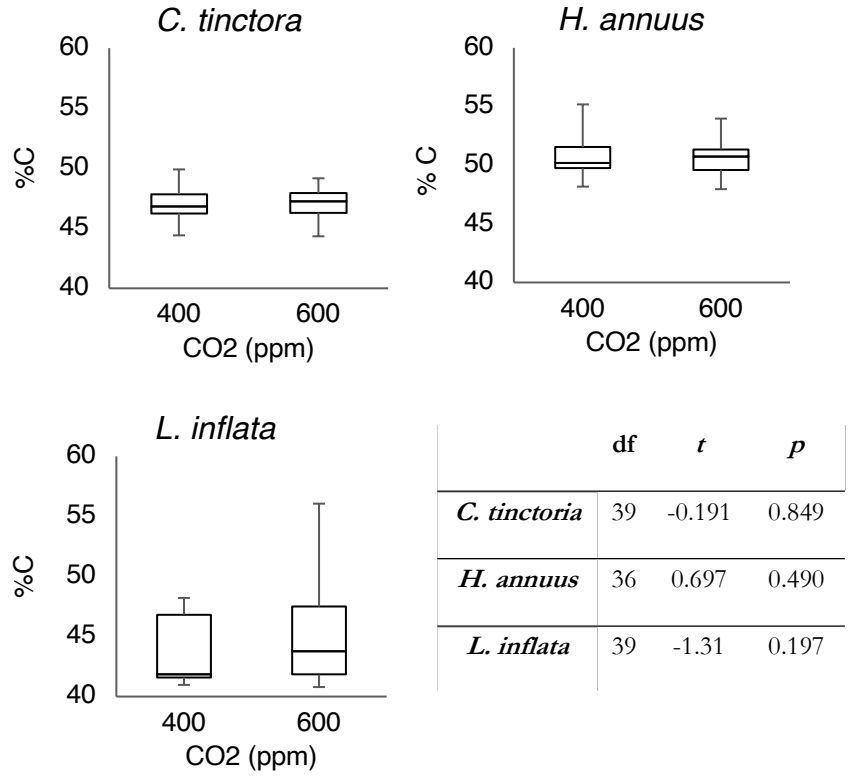


	df	*t*	*p*
C. tinctoria	32	-0.024	0.981
H. annuus	36	-0.225	0.823
L. inflata	40	0.715	0.479

Figure 9: C:N ratio by species showed no significant relationship between C:N and CO_2 concentration.

3.3.2 C composition

Just as our statistical analysis found no change in C:N across CO_2 regimes, we also find no significant change in % C by dry mass in any species ($p > 0.05$, see Figure 10). However, these result do not lead us to conclude that the concentration of starches and sugars within the pollen remained unchanged; rather, as we will show in the next section, our results suggest that carbohydrate concentration *increased* in all species.



	df	*t*	*p*
C. tinctoria	39	-0.191	0.849
H. annuus	36	0.697	0.490
L. inflata	39	-1.31	0.197

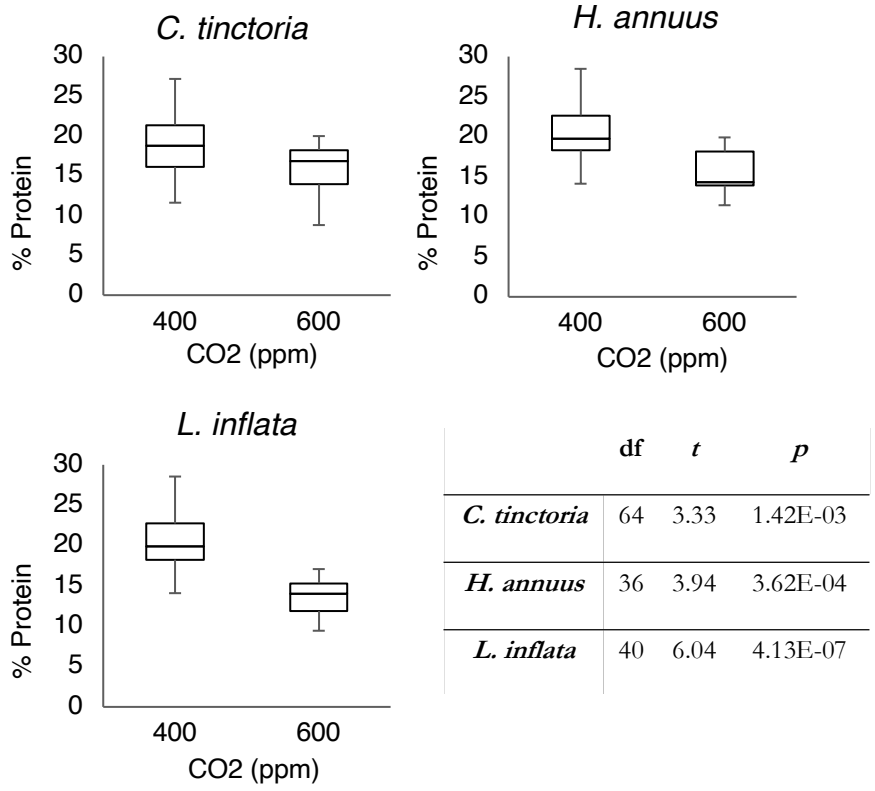
Figure 10: Elemental analysis did not show a significant relationship between % C and CO_2 concentration.

3.3.3 N composition

Estimated standard % N information (where % N is a percentage of dry mass) is not available for *C. tinctoria* and *L. inflata* (to the best of our knowledge), but such data on *H. annuus*, a more commonly studied floral species, does exist. Because % N is a proxy for protein (% N multiplied by 6.25 equals % protein by mass),[39] data on N composition allow us to analyze protein composition in our sample materials. Our results from elemental analysis are not in accordance with previously published data on this species, which finds *H. annuus* pollen to contain 30.6% (± 0.0) protein.[39] Our data, by

contrast, shows median values for *H. annuus* protein content between 15-18%. This could perhaps be attributed to different soil, temperature, or other growing conditions. It is also possible that our *H. annuus* samples were harvested at a different point in their development.

In our results we note that, despite apparently constant % C across CO_2 regimes, elemental results showed a significant decrease in % N by mass for each species tested ($p < 0.05$ for all cases, see Figure 11). From the 400 to the 600 ppm CO_2 regimes, we find a 15.72% decrease in protein content for *C. tinctoria,* a 21.93% decrease for *H. annuus,* and a 29.37% decrease for *L. inflata.* Protein composition of our samples is shown in Figure 11.



	df	*t*	*p*
C. tinctoria	64	3.33	1.42E-03
H. annuus	36	3.94	3.62E-04
L. inflata	40	6.04	4.13E-07

Figure 11: Protein concentration by species showed a significant relationship between % protein and CO_2 concentration for all species.

3.3.4 Declining protein, increasing carbohydrates

Moreover, the fact that % N appears to be decreasing while % C and C:N remain constant implies that protein reduction is being offset by another constituent of plant biomass. This is because we may assume a constant C:N ratio for plant protein. Therefore, N lost as protein carries with it a consequent loss in C. In order for the plant to maintain unchanging C concentrations, the lost C from protein loss must be replaced by another form of C-containing biomass.

Besides C and N, most of the rest of plant biomass comes in the form of O, H, and inorganic salts, with O comprising the vast majority.[53] Assuming constant molar ratios for carbohydrates (CH_2O), proteins ($C_5H_8O_2N$), and lipids ($C_8H_{16}O$) — the three constituents of plant biomass — it is possible to model a scenario in which relatively stable C:N ratios between 15-18 and constant % C between 42-50% are accompanied by significant protein reductions from roughly 20% to 15% (see Figure 12).

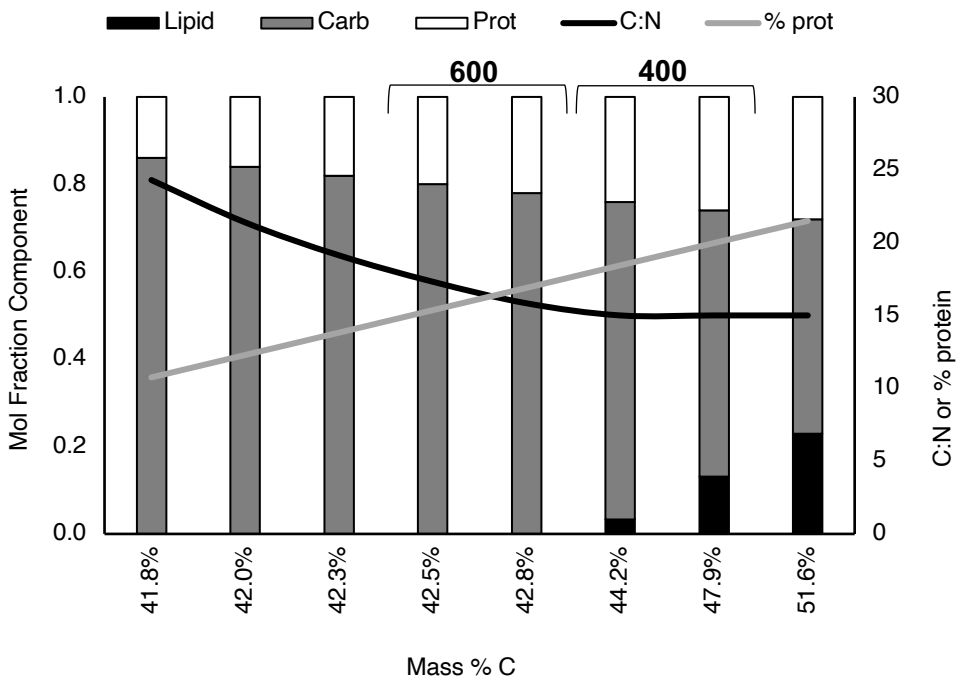
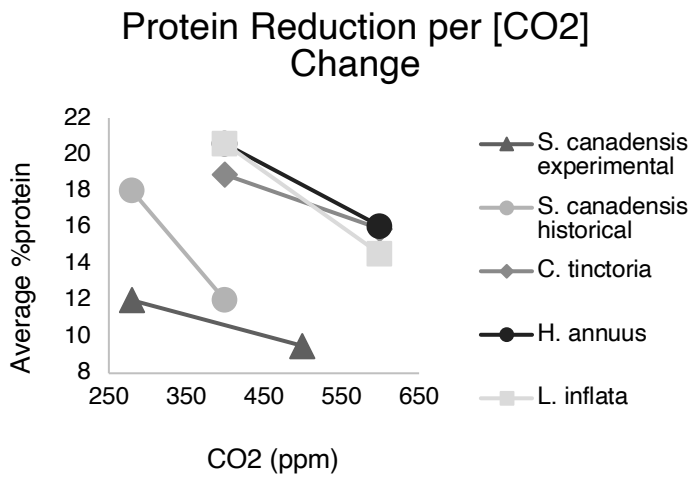


Figure 12: Protein reductions accompanied by a relatively stable C:N ratio and % C content are made possible by an increase in the sum of lipid and carbohydrate content. The two bars that mark the closest analogy to our 400 ppm and 600 ppm data are bracketed.

The model shows that in order for (1) protein reduction to occur, and (2) C:N and % C by mass to remain relatively stable, the C contribution from carbohydrates and lipids must increase. Therefore, although we do not find changes in C:N — in contrast to rising C:N ratios in *S. canadensis,* reported in *Ziska et al.* (2016) — our findings seem to be in alignment with *Ziska et al.* (2016) in showing that increases in carbohydrate content do occur concurrently with reductions in protein content for our three species. However, we still acknowledge that protein reduction may not be solely caused by carbon dilution; it has long been known that other mechanisms may account for the reduction of % N in plant material grown under eCO_2.[23]

3.3.5 Comparison to Ziska et al. (2016)

One question posed by *Ziska et al.* (2016) is whether the protein-reducing effects of eCO_2 have a saturation point (i.e., after a certain amount of CO_2 rise, does pollen protein concentration stop falling?). *Ziska et al.'s* historical and experimental analysis examined flowers grown from 280 to roughly 500 ppm CO_2, and this study further explored the effects of eCO_2 from 400 to 600 ppm, providing an opportunity to see how different CO_2 changes may affect the rate of protein reduction. It is particularly relevant to explore these greater CO_2 concentrations given the alarming likelihood that we will reach or exceed atmospheric CO_2 concentrations of 600 ppm or more by the end of this century.[1] We find that the slope of protein reductions observed in our study (average slope = -2.26 percent protein per 100 ppm, from 400 to 600 ppm) are similar to, but slightly greater in magnitude than, the slope observed in *Ziska et al.'s* experimental analysis (slope = -1.14 percent protein per 100 ppm, with a CO_2 range from 280 to 500 ppm) (Figure 13). Our experimental slopes were considerably less steep then *Ziska et al.'s* historical data (slope = -5.00 percent protein per 100 ppm, with a CO_2 range from 280 to 400 ppm, see Figure 13).



Experiment	Slope (% protein per 100 ppm CO_2)
S. canadensis experimental	-1.14
S. canadensis historical	-5.00
C. tinctoria	-1.48
H. annuus	-2.26
L. inflata	-3.02
Average slope (this study)	-0.023

Figure 13: Comparison of the size of the effect of eCO_2 on protein content between *Ziska et al.* (2016) and this study's results from elemental analysis. "*S. canadensis* experimental" and *"S. canadensis"* historical refer to results from *Ziska et al.* (2016).

Although there is a significant difference between the starting and ending protein concentrations, implying either different protein base levels for different species or inconsistencies with protein measurement, this graph suggests the absence of a saturation effect on protein reductions between 400 and 600 ppm.

3.4 Possible Mechanisms

This leads us to turn to the many alternative theories attempting to explain N reductions in plants exposed to eCO_2. Among the most plausible are hypotheses of N dilution by other compounds produced from photosynthesis and decreases in root uptake of N. *Taub & Wang* (2008) have synthesized these interacting mechanisms in a review published in the *Journal of Integrative Plant Biology*.[23] Citing observations from previous literature, they suggest that decreased root uptake of N is (1) caused by a synchronous increase in N use efficiency and the downregulation of photosynthetic,

N-heavy enzymes like RUBISCO,[54] and (2) exacerbated by the root system's diminished ability to draw N from the soil.[55] The latter effect may be explained by reduced transpiration — this reduction has been reliably documented in plants grown at eCO_2, and N uptake is facilitated by transpiration. Less transpiration means less N flow into the plant, and therefore less protein.[56]

It is beyond the scope of this project to explain which of these processes is driving % N down, but the fact that we may observe eCO_2-driven protein reduction and concurrent replacement by lipids and carbohydrates not only in *S. canadensis,* but also in *C. tinctoria*, *H. annuus*, and *L. inflata,* suggests the need for further research into the dilutive effects of eCO_2-driven increases in photosynthesis, as well as root responses to eCO_2.

Chapter 4: Conclusion

Two methods, a colorimetric protein assay and elemental analysis, were used to compare pollen protein concentrations in *C. tinctoria, H. annuus,* and *L. inflata* grown at 400 and 600 ppm CO_2, giving insight into the effects of eCO_2 on the nutritional composition of pollen and anthers from flowers that are important for pollinators like bees. With these methods, we investigated the relationship between C and N in an effort to better understand the underlying mechanisms driving % N reductions seen in many other plant species. We tested for the significance of trends across CO_2 regimes.

We developed a refined Bradford microassay protocol suitable for measuring small amounts of protein in small anther sample sizes. We find that detergents, and Tween 20 in particular, interfere with the Bio-Rad Bradford reagent used to induce a color shift in colorimetric protein analysis. To measure anther protein concentrations when few anthers are available, we find that a protein buffer solution containing roughly 10 μL Tween 20 per 100 mL (0.01% Tween 20) is appropriate so as to prevent interference with the binding of protein to the Bradford reagent. Our microassay used 150 μL of 0.01% Tween 20 solution with 50 μL of water and 1 mL of Bradford reagent.

Additionally, we find that when little sample material is available for Bradford microassay analysis, it is preferable to measure absorbance as a function of anther count rather than anther mass. In our protocol, a graph of absorbance as a function of sample mass produced a nonlinear response curve ($r = 0.608$), whereas our graph of absorbance as a function of anther count produced a better (although still not ideal) linear response curve ($r = 0.758$). We note that difficulties with weighing small amounts of anther material may have caused absorbance by anther count to yield a better fit.

We find that, while an adapted colorimetric assay was capable of directly measuring pollen protein at low concentrations, our Bradford protocol did not detect changes in protein composition that were apparent in elemental analysis. Bradford results showed no significance ($p > 0.05$) for correlation between CO_2 and protein concentration in any species. Moreover, although absorbance measurements were internally consistent within species, protein estimates based on this analysis fell far below the expected range of protein concentration for flowers, suggesting that this method may not be suitable for calculating absolute protein concentrations for small sample sizes. This may be because of incomplete protein extraction during preparation for the Bradford assay — pollen is extremely durable even when exposed to chemical solvents, making protein extraction difficult.[51] Future efforts may wish to experiment with alternative extraction protocols, including (1) different buffer recipes, (2) other methods for physically grinding pollen, and (3) longer incubation periods.

We find that elemental analysis does not show significant changes in the flowers' C composition or C:N ratio ($p > 0.05$ for all species). However, we find that elemental analysis reveals a strong negative correlation between CO_2 concentration and N composition ($p << 0.01$ for all species). Using % N as a proxy for protein composition, these results suggest that eCO_2 causes significant reductions in % protein across our three species, with protein reductions of 15.72% for *C. tinctoria*, 21.93% for *H. annuus,* and 29.37% for *L. inflata* between a 400 and 600 ppm CO_2 regime. These combined findings — reduced protein and stable C:N ratios and C compositions — suggest that as eCO_2 causes N reductions, protein loss is offset by an increase in net carbohydrates and lipids.

The decline in estimated pollen protein concentration under an eCO_2 regime observed in our elemental analysis is consistent with previous literature and meta-analyses showing that elevated

carbon dioxide concentrations systematically reduce N and protein concentration across a number of plant species.[8,18–22,52] Until recently, these observations were largely limited to agricultural crops, but *Ziska et al.* (2016) showed that CO_2-driven protein reductions occur in *S. canadensis* pollen, as well. Findings from our study suggest the same trend exists in *C. tinctoria*, *H. annuus,* and *L. inflata.*

This finding may be of special importance to the health of pollinators, who rely on these floral species and others as dependable sources of pollen and, consequently, protein. *C. tinctoria* and *L. inflata* are particularly notable for their ubiquity across North America and in Canada. Considering that bees are not believed to be able to preferentially forage for high-quality pollen,[39,57] the possibility that the protein content of these floral species and others may continue to fall augurs poorly for bees. Overall, it remains unknown precisely how declines in pollen protein will affect bees, but researchers warn that reduced dietary protein may act in combination with a number of other environmental stressors (habitat loss, neonicotinoids, etc.) to jeopardize population robustness.[8] There is also the possibility that decreased protein concentrations may be offset by absolute increases in protein availability — eCO_2 stimulates plant growth, after all[58] — but there is currently little direct evidence to support this conclusion.

In their 2016 paper, *Ziska et al.* wrote of an "urgent and compelling case" for quantifying pollen protein response to rising CO_2 in a greater number of floral species — our research begins to address this need for *C. tinctoria, H. annuus,* and *L. inflata.* However, additional research is needed to quantify the effects of eCO_2 on more species, as well as the mechanisms underlying these N reductions. Furthermore, we call for more research addressing the effects of changing nutrient composition on global pollinator populations and the food systems and biodiversity that depend on them.

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