Canopy position has a profound effect on soybean seed composition

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Abstract

Although soybean seeds appear homogeneous, their composition (protein, oil and mineral concentrations) can vary significantly with the canopy position where they were produced. In studies with 10 cultivars grown over a 3-yr period, we found that seeds produced at the top of the canopy have higher concentrations of protein but less oil and lower concentrations of minerals such as Mg, Fe, and Cu compared to seeds produced at the bottom of the canopy. Among cultivars, mean protein concentration (average of different positions) correlated positively with mean concentrations of S, Zn and Fe, but not other minerals. Therefore, on a whole plant basis, the uptake and allocation of S, Zn and Fe to seeds correlated with the production and allocation of reduced N to seed protein; however, the reduced N and correlated minerals (S, Zn and Fe) showed different patterns of allocation among node positions. For example, while mean concentrations of protein and Fe correlated positively, the two parameters correlated negatively in terms of variation with canopy position. Altering the microenvironment within the soybean canopy by removing neighboring plants at flowering increased protein concentration in particular at lower node positions and thus altered the node-position gradient in protein (and oil) without altering the distribution of Mg, Fe and Cu, suggesting different underlying control mechanisms. Metabolomic analysis of developing seeds at different positions in the canopy suggests that availability of free asparagine may be a positive determinant of storage protein accumulation in seeds and may explain the increased protein accumulation in seeds produced at the top of the canopy. Our results establish node-position variation in seed constituents and provide a new experimental system to identify genes controlling key aspects of seed composition. In addition, our results provide an unexpected and simple approach to link agronomic practices to improve human nutrition and health in developing countries because food products produced from seeds at the bottom of the canopy contained higher Fe concentrations than products from the top of the canopy. Therefore, using seeds produced in the lower canopy for production of iron-rich soy foods for human consumption could be important when plants are the major source of protein and human diets can be chronically deficient in Fe and other minerals.

Introduction

Although soybean seeds from a given plant may appear physically homogeneous, it has long been known that seed produced at the top of the canopy can have higher protein and less oil compared to seeds from the bottom of the canopy (Collins & Cartter 1956). Subsequently it was demonstrated that positional effects are observed with determinate as well as indeterminate soybeans (Escalante & Wilcox 1993a) and in normal protein as well as high-protein breeding lines (Escalante & Wilcox 1993b). While these effects on protein and oil concentrations have been documented to occur, they are nonetheless not widely recognized today and there are no insights concerning possible physiological mechanisms that may underlie these positional effects. There are many other seed constituents, in particular minerals, but the impact of canopy position on many of these seed constituents is unknown. Because legumes like soybean can contribute not only protein to the human diet but also minerals like iron (Fe) and zinc (Zn), canopy position effects on the concentrations of essential minerals could be important, especially for the health and nutrition of children and women. According to the World Health Organization, Fe deficiency is currently the most widespread mineral deficiency affecting more than 30% of the world's population (http://www.who.int/nutrition/topics/ida/en/). One approach to control this problem is to increase Fe intake via dietary diversification with Fe-rich foods and it is possible that variation with canopy position could be exploited.

Several factors affect the development of seeds at the top of the plant differently than those at the bottom of the canopy and therefore could be responsible for differences in seed composition at maturity. First, flowering in the indeterminate soybean plants as used in the present study occurs first at lower nodes; thus, there is the potential for seeds lower in the canopy to develop over a longer period. However, while there is a lot of information about node position and flowering, there are few reports that have documented differences in duration of the seed fill period (SFP) as a function of node, as was demonstrated in cultivar 'Williams79' (Raboy & Dickinson 1987). A second factor is that seeds lower in the canopy also develop under altered environmental conditions in terms of temperature, irradiance, light quality and humidity, which are recognized to impact soybean seed composition (Carrera et al. 2009; Carrera et al. 2011; Wolf et al. 1982). Therefore, the role of canopy microenvironment on seed composition warrants consideration.

In the present study, we grew a core group of ten soybean lines in Urbana, IL, over a 3-yr period and monitored seed composition (protein, oil and mineral element concentration) at maturity as a function of node position. In general, there was a continuum in composition with seed that developed at the top of the canopy having more protein but less oil and reduced concentrations of minerals such as Mg, Fe, and Cu compared to seeds produced at the bottom of the canopy. Of particular note was the variation in Fe concentration, which was generally ~20% higher in seeds from the bottom of the canopy. The differences in mineral concentrations such as Fe could have direct impact on use of soybeans for human food in countries that primarily depend on plant protein sources for intake of minerals. We also tested several possible developmental and micro-environmental factors for their ability to influence the seed compositional gradients, and used metabolomic profiling of developing seeds to investigate biochemical determinants of the protein and oil gradients. Collectively, the results establish a new type of seed heteromorphism in soybean where seeds appear physically homogenous but differ in composition and provide new insights to some of the underlying factors that may be responsible for the gradients in composition from bottom to top of the canopy.

Results

Canopy position affects soybean seed protein, oil and mineral concentrations

We investigated positional effects with a core group of ten soybean lines (**Table S1**) grown in Urbana, IL, over a 3-year period. Main stems were harvested at maturity and divided into four canopy position quadrants (**Fig. 1**) and the seeds collected from each quadrant were analyzed separately for major storage products (protein and oil) and various minerals. Representative results obtained for one cultivar ('Chamberlain') are presented in **Fig. 2A**. As shown, protein concentration increased with node position at which seeds developed going from bottom to top of the mainstem while oil and iron (Fe) concentration decreased. For both protein and oil, which are the major seed constituents, there was variation in the absolute concentrations among the 3 years of study, but general trends were similar. Differences in absolute concentrations among years was most apparent for protein concentration with highest levels obtained in 2010 and lowest in 2011, presumably reflecting the impact of weather on seed development and composition. Another confounding source of variation for canopy position analysis is genotype,

Figure 1. Quadrants of a Soybean Plant. The mature plant is divided up into quadrants upon harvest and each quadrant is analyzed separately. Plat normalized data uses the average of all four quadrants to normalize year, plot and line affects.

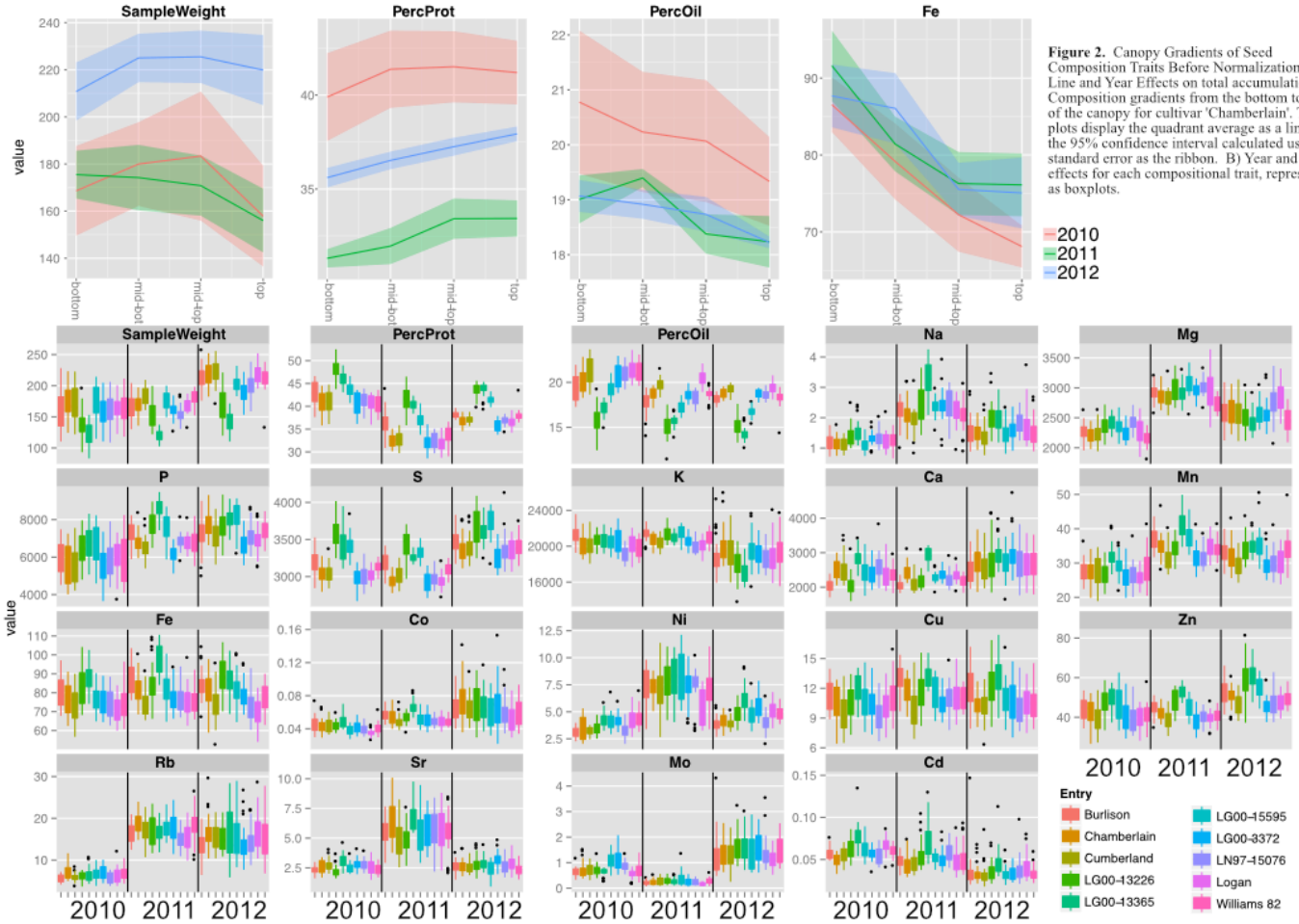


Figure 2. Canopy Gradients of Seed Composition Traits Before Normalization and Line and Year Effects on total accumulation. A) Composition gradients from the bottom to the top of the canopy for cultivar 'Chamberlain'. The plots display the quadrant average as a line with the 95% confidence interval calculated using standard error as the ribbon. B) Year and line effects for each compositional trait, represented as boxplots.

110 and **Fig. 2B** highlights the substantial variation in absolute concentrations of seed constituents
111 due to both genotype and year.
112 In order to compare positional effects for various parameters across genotypes and years
113 without the confounding effects of differences in absolute values, we normalized each canopy
114 gradient to a mean value of one and the values for each quadrant were then expressed relative to
115 the normalized mean. However, because the weather in each year of the study differed (**Table**
116 **S2**), the normalized results for each parameter are presented separately for each year. Across the
117 10 soybean lines, oil concentration decreased progressively from bottom to top of the canopy and
118 was associated with a reciprocal increase in protein concentration (**Fig. 3A**). Protein and oil
119 concentrations in soybean seeds are usually inversely related (Wilcox 1998) and this was
120 apparent with variation within the canopy as well. Single seed weight varied with canopy
121 position with seed produced in the middle portion tending to be slightly heavier than seeds
122 produced at either the bottom or top of the canopy; however, the storage product gradients were
123 independent of seed weight variation. Storage product gradients did not vary significantly across
124 the three years of the study; however, absolute protein and oil concentrations varied among the
125 three years of the study (**Figure S1**), This is perhaps a result of weather that varied substantially
126 in terms of temperature and precipitation among the three growing seasons (**Table S2**).
127 We also found that canopy position significantly affected the seed ionome, which comprises
128 all of the minerals and trace elements found in mature seeds (**Fig. 3B)**. While there have been
129 several studies of the soybean seed ionome (McGrath & Lobell 2013; Myers et al. 2014; Sha et
130 al. 2012; Ziegler et al. 2013), to our knowledge this is the first report demonstrating variation
131 with canopy position. **Figure 3B** shows normalized canopy gradient plots for elements where
132 there was a statistically significant ($p < 0.01$) variation in concentration with position. Several
133 groups of minerals exhibited common responses with canopy position. The elements Mg, Fe,
134 Cu, Cd and Zn were present at highest concentrations in seeds from the bottom of the canopy and
135 decreased progressively to the top of the canopy. Within this group, the profiles for Mg and Fe
136 were similar to one another in that variation was relatively low and the gradients were almost
137 identical across the three years; however, the relative changes in Fe concentration were much
138 greater in magnitude compared to changes in Mg concentration. Cu, Zn and Cd showed similar
139 patterns, but were more variable among years. The second group that was apparent included Ca
140 and Sr, where seeds from the middle of the canopy exhibited the lowest concentrations except in

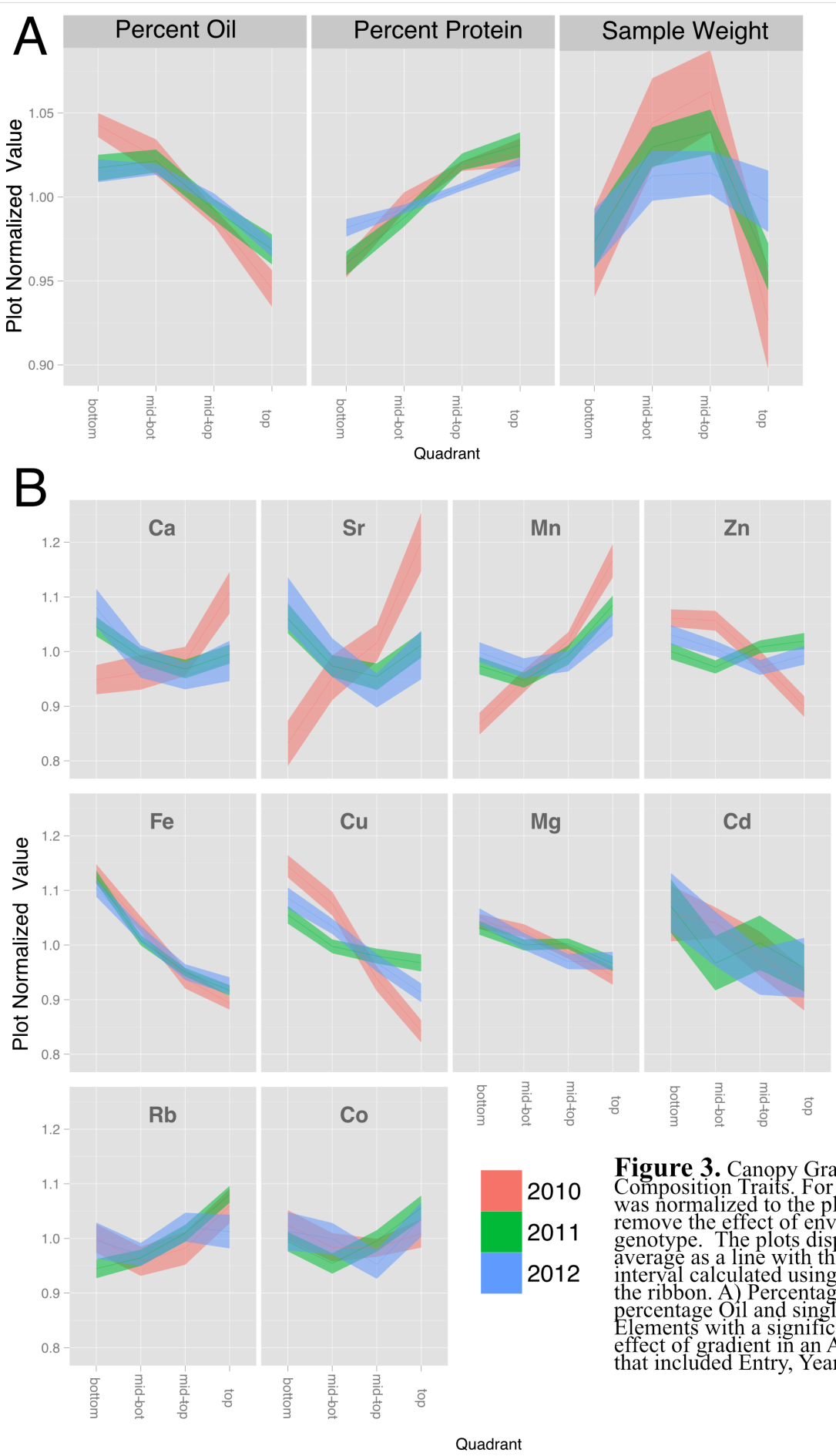


Figure 3. Canopy Gradients of Seed Composition Traits. For each trait, the data was normalized to the plot average to remove the effect of environment and genotype. The plots display the quadrant average as a line with the 95% confidence interval calculated using standard error as the ribbon. A) Percentage protein, percentage Oil and single seed weight. B) Elements with a significant ($p < 1 e^{-10}$) effect of gradient in an ANOVA analysis that included Entry, Year and Position.

141 2010, when concentrations of both Ca and Sr tended to increase going up the canopy. Finally,
142 Mn was alone in the third category that increased in concentration towards the top of the canopy
143 in all 3 years. Ca and Sr, and Cd and Zn, are chemically similar which may explain their parallel
144 profiles. It is interesting to note that while Rb is a chemical analog of K and the two are often
145 closely correlated (Baxter 2009), that was not the case for soybean seeds where significant
146 position effects on Rb were observed (**Fig. 3B**) but not for K (data not shown). It is also
147 noteworthy that 2010 was the one year where mineral profiles were often distinct from those in
148 2011 and 2012. All three years were above normal in terms of temperature, but 2010 was the
149 only year with above normal precipitation. Thus, water availability may be a major
150 environmental factor impacting positional effects on the seed ionome, and interestingly some
151 minerals were affected (Ca, Mn, Cu, Zn, Sr) while others (Mg, Fe, Co, Rb, Cd) were not. We
152 also measured other minerals (B, Na, Al, P, S, K, Ni, As, Se and Mo) that did not show
153 statistically significant variation with nodal position and are not presented in Fig. 3. **Figure S1**
154 shows non-normalized plots of minerals that identify differences among metals in absolute
155 abundance. As expected, absolute concentrations of Mg, S, K, P and Ca were highest (> 1000
156 ppm); Mn, Fe, Rb, and Zn were intermediate (10 to 100 ppm), and Na, Co, Ni, Cu, Sr, Mo, and
157 Cd were present at trace levels (< 10 ppm).
158 Another way to compare canopy profiles for the minerals measured is to do an overall
159 correlation matrix of quadrant variation normalized to plot averages. In this way, one can look
160 across the entire data set for parameters that are correlated based on variation with nodal
161 position. A strong positive correlation would indicate that both components changed not only in
162 the same direction but also to the same relative extent. As shown in **Fig. 4A**, only a few strong
163 correlations were apparent among the measured parameters. Variation in seed size did not
164 significantly correlate with positional variation of any of the measured elements or storage
165 products. Protein and oil concentrations were strongly negatively correlated, as expected. In
166 terms of minerals and storage products, the quadrant variation in protein concentration correlated
167 negatively with Fe and Cu, and positively with Mn. The reciprocal pattern was apparent with oil
168 concentration. Among the minerals, highly correlated element pairs included Fe-Cu, Ca-Sr, and
169 Zn-Cu, and between P and S, Zn, and Co. As noted earlier, Ca and Sr are chemical analogs and
170 frequently correlated (Baxter 2009), but surprisingly, other chemical analog pairs such as K-Rb
171 were not observed. Fe and Cu were positively paired and have been reported to be positively

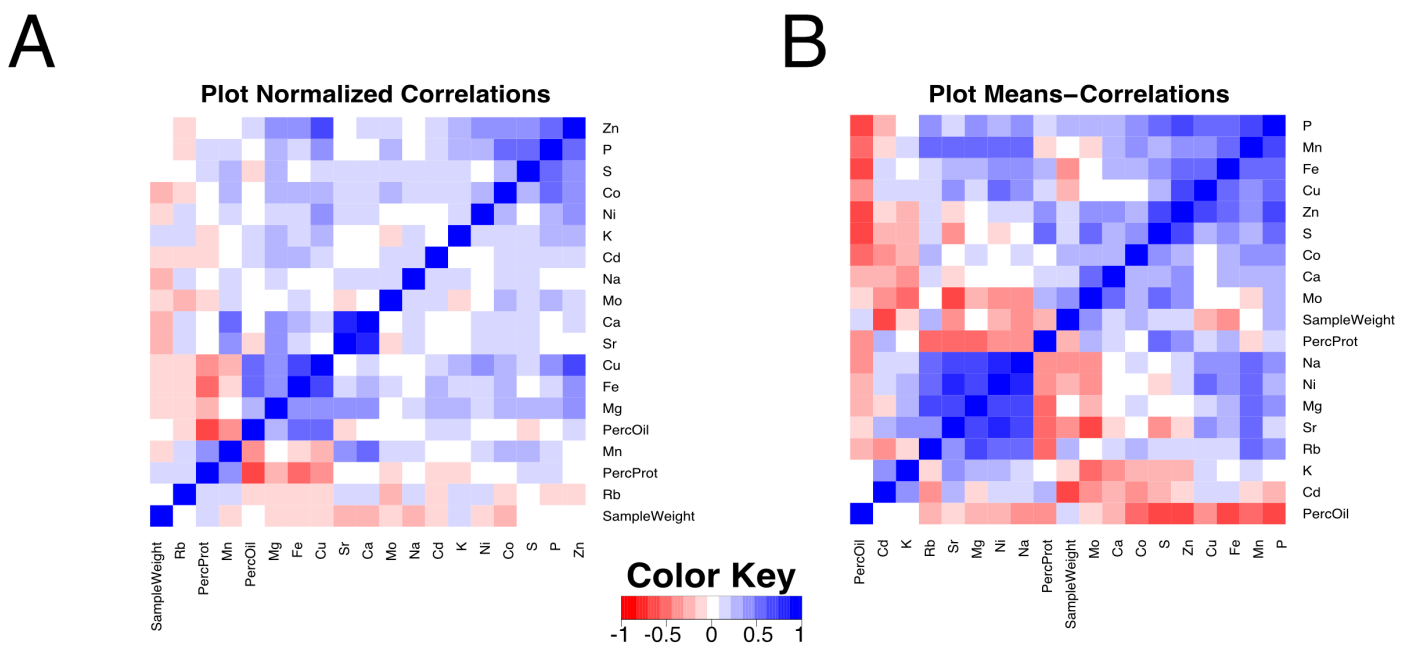


Figure 4. Correlation Plot among Composition Traits. Pearson correlation values between compositional traits. **A**) Correlation across 832 quadrants normalized to the plot average. **B**) Correlation across 208 plot means.

172 correlated in soybean seeds (Vasconcelos et al. 2014) but the basis for the pairing is unknown.
173 Correlations between P and minerals are often considered to reflect association of the mineral
174 with seed phytate, the principal form of P in seeds (Vreugdenhil et al. 2004).
175 In addition to comparing parameters based on quadrant variation, it is also worthwhile to
176 compare plot averages, which will reflect genetic and environmental effects on absolute values
177 of the parameters. **Figure 4B** shows a dynamic matrix plot of correlations between plot means.
178 Compared to the corresponding plot that focused on quadrant variation (**Fig. 4A**), many more
179 strong correlations were apparent when comparing plot means. For example, protein
180 concentration was positively correlated with S and Zn (and more weakly with Fe). The
181 correlation with S is expected as the total seed S has been shown to track closely with high
182 cysteine- and methionine- containing proteins in the soybean seed (Krishnan et al. 2012). The
183 correlations between protein content, Zn and Fe could be due to their primary role as cofactors of
184 metalloproteins. Accordingly, there was a significant negative correlation of Fe, S, and Zn with
185 oil concentration. Interestingly, there was also a strongly significant negative correlation of P
186 with oil, whereas the positive correlation of P with protein concentration was relatively weak.
187 The majority of mineral correlations were positive in nature, with a maxi-cluster of Rb, Mn, Sr,
188 Mg, Ni, and Na and a mini-cluster of Fe, Cu and Zn. The mini-cluster pairs of Fe-Cu and Cu-Zn
189 were noted in the plot of **Fig. 4A**, but several members of the maxi-cluster correlation were not
190 reported in the plot normalized correlation matrix. For example, Mn and Mg concentrations did
191 not relate to each other in terms of quadrant variation but were strongly positively correlated
192 based on plot means, indicating that mineral uptake and allocation among seeds in different
193 quadrants are controlled separately. Finally, P concentration exhibited a positive correlation with
194 Mn, Fe, Cu, Zn, S and Co. The link among P and Zn, S and Co concentrations with quadrant
195 variation was observed (**Fig. 4A**), but when analyzed in terms of plot means in **Fig. 4B** the
196 association of P with Mn, Fe, and Cu became apparent as well. It is worth noting that in terms of
197 plot means, there was no association between Ca and Sr suggesting that these chemical analogs
198 do not always behave similarly. There was one also a strong negative correlation between Mo
199 and Sr, perhaps suggesting a common component(s) of the uptake system.

200 Canopy microenvironment impacts seed composition

201 Our understanding of the environmental factors responsible for the positional effects on seed
202 composition is limited; however, many microclimatic factors vary from the top to the bottom of

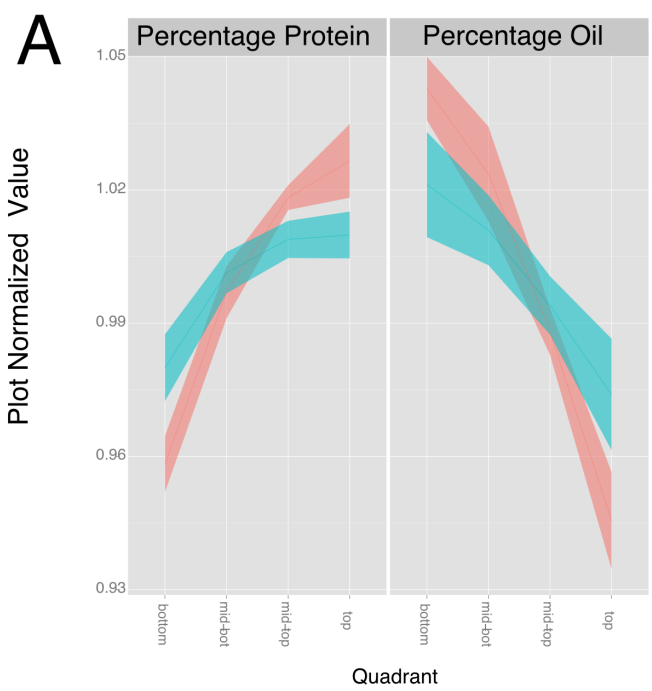
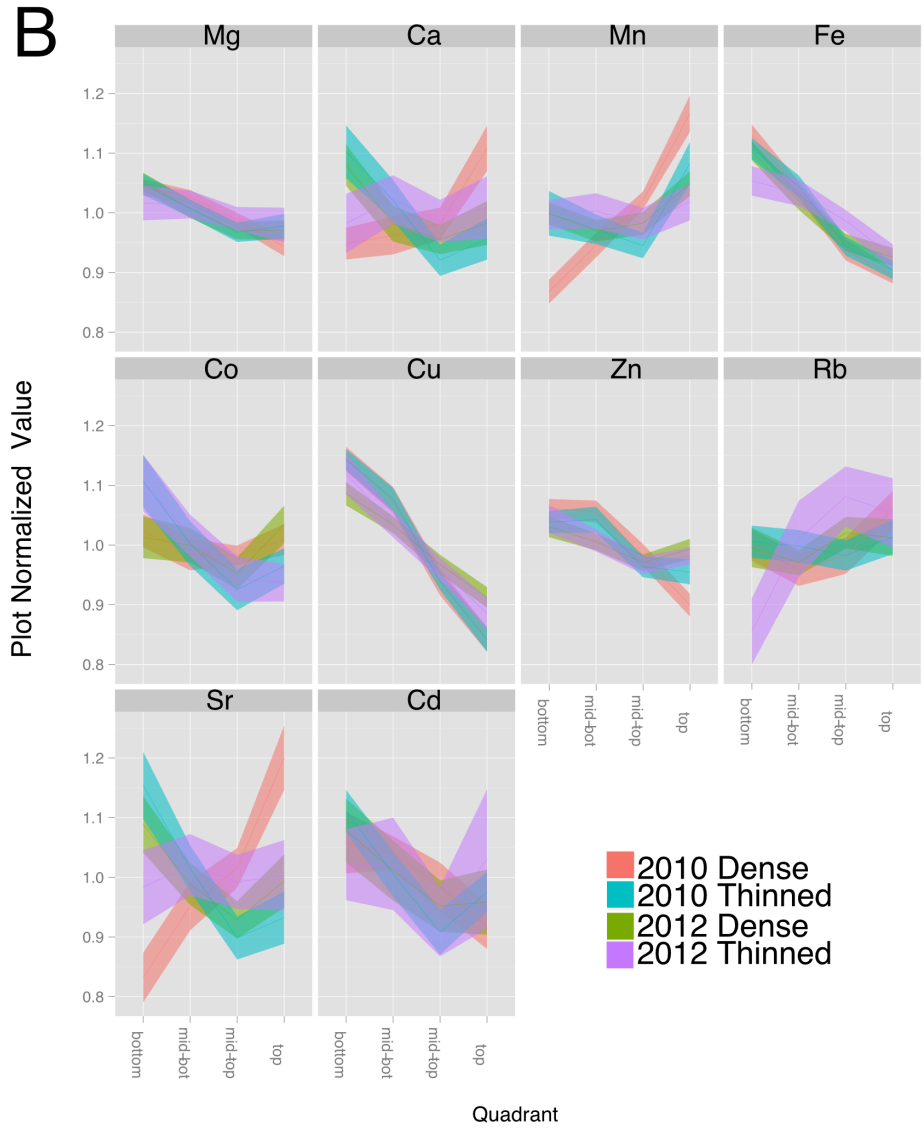


Figure 5. Effect of Thinning on Compositional Traits. For each trait, the data was normalized to the plot average to remove the effect of environment and genotype. The plots display the quadrant average as a line with the 95% confidence interval calculated using standard error as the ribbon. **A**) Percentage protein and percentage Oil in 2010. **B**) Elements (from 2010 and 2012) with a significant ($p < 1 e -10$) effect of gradient in an ANOVA analysis that included Entry, Year, Position and thinning.

Dense
Thinned



203 the closed soybean canopy (Baldocchi et al. 1983). Environment is well known to impact
204 soybean seed protein and oil composition (Rotundo & Westgate 2009). Therefore, we conducted
205 experiments to evaluate microclimatic differences within the canopy by thinning plants at
206 flowering to remove the influence of neighboring plants. Removal of neighboring plants
207 increased protein concentration at the expense of oil in seeds throughout the canopy of the
208 spaced plants but the changes were greatest in pods lower on the main stem. As a result, the
209 thinning treatment reduced the positional effect on protein and oil by 50-60% (**Fig. 5A**).
210 Increased light energy to drive photosynthesis at most leaf positions and increased temperature at
211 lower positions could both favor increased protein accumulation at lower nodes thereby reducing
212 the difference between top and bottom seeds; however, while thinning significantly altered the
213 main stem gradients in major storage products there was relatively little effect on minerals. As
214 shown in **Fig. 5B**, the canopy positional effect on Mg, Fe and Cu was unaltered by the thinning
215 treatment whereas Ca and Sr were similar to one another and showed a significant effect of
216 thinning but only in one of the two test years (2010). The general conclusion is that thinning
217 affects the canopy positional effect on some but not all minerals. This suggests that at least for
218 Mg, Fe and Cu, the transport and homeostasis mechanisms are generally independent of
219 instantaneous environmental factors and the transport of sucrose and amino acids into the
220 developing seeds is not the sole factor driving their movement into seeds.
221

222 Seed fill period and seed composition

223 Another factor that could contribute to canopy position effects on seed composition is the
224 duration of the seed-fill period (SFP), which is affected by genetic and environmental factors and
225 is one of the major determinants of yield potential in soybean (Evans et al. 1995). Soybeans
226 flower in response to photoperiod and the first flowers form lower in the canopy followed by
227 flowering at upper nodes. Pods then form in the same order and when fully elongated the
228 process of seed development is considered to begin when seeds are approximately 0.34
229 centimeter long (by visual inspection). In general, seeds lower in the canopy fill over a longer
230 period but at a lower rate compared to seeds at the top of the canopy (Raboy & Dickinson 1987)
231 so that at maturity, final seed size tends to be rather constant through the canopy rather than
232 increasing progressively from bottom to top of the canopy. We measured the SFPs with our core
233 group of ten lines and found substantial differences in SFPs at the bottom and top of the canopy

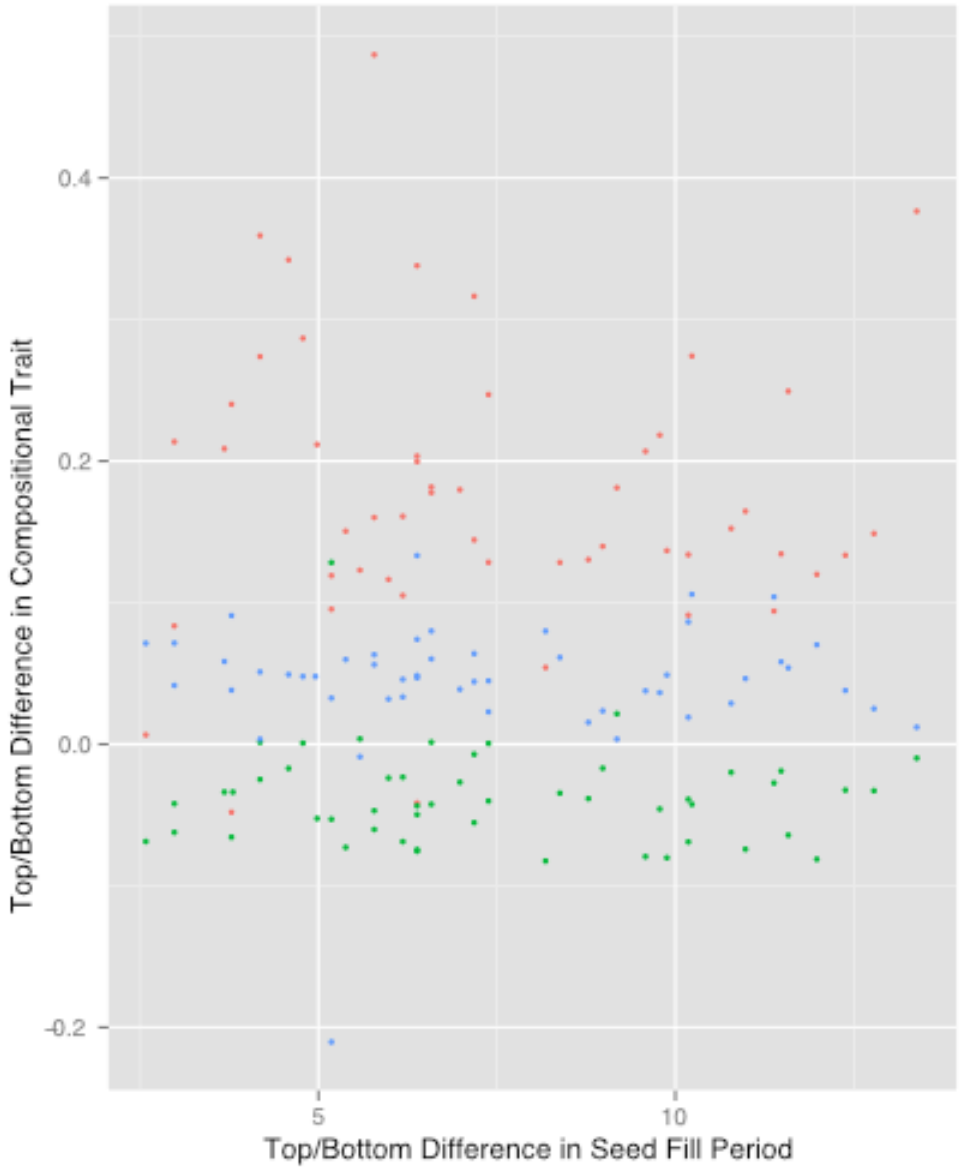


Figure 6. Difference in Top/Bottom composition traits is not correlated with Seed Fill Period. The difference in plot normalized composition between the top quad and the bottom quad for protein, oil and iron plotted versus the difference in seed fill period for 51 plots in 2012.

234 (**Table S3**). Top SFP was generally correlated with bottom SFP, as would be expected, but the
235 difference in SPF (bottom – top position) was not correlated with the canopy gradients of
236 protein, oil, or Fe (**Fig. 6**). Therefore, factors other than the duration of the SFP are responsible
237 for the documented variation in composition with nodal position.
238

239 Iron concentrations of soybean seed products

240 Our results raise the question of whether soy food products made from seed from different
241 portions of the canopy would vary in terms of their mineral concentrations. Three of the most
242 common and simplest products to make from soybean seeds are flour, milk and okara (the
243 particulate material remaining after preparation of milk). Because Fe is one of the most critical
244 minerals to human health and anemia is a global epidemic, we focused our initial analysis on the
245 Fe content of these soy food products. We prepared flour from seven lines, and milk and okara
246 from four lines and **Fig. 7** summarizes the results**.** With all three products, the concentration of
247 Fe was highest in products made from seeds produced at the bottom of the canopy and decreased
248 progressively with canopy position of the seeds used. Thus, as would be expected the
249 concentration of seed Fe affects the concentration of Fe in the flour, milk or okara produced from
250 those seeds. Although many questions remain, the public health implications of our findings are
251 apparent. Given that mineral content of seeds, especially Fe, is important our results uncover
252 another source of variation that can be directly exploited.
253

254 The vegetative soybean ionome

255 The canopy effect on seed mineral concentration prompted us to look at the distribution of
256 minerals in the shoots of vegetative plants. Minerals deposited in seeds are derived from
257 continued uptake from the soil or remobilization of previously accumulated minerals (Hocking &
258 Pate 1977; Waters & Grusak 2008), and therefore the leaf ionome of the vegetative plant is
259 relevant to studies of the mature seed ionome. Preliminary studies of the distribution of Fe in
260 stems, petioles and leaves of 'Cumberland' soybean plants indicated that the majority of Fe was
261 stored in leaves (data not shown). Consequently, we examined the leaf ionome as a function of
262 canopy position. As shown in **Fig. 8,** the concentrations of Mg, Al, Ca, Mn, Fe, Co, As, Se and
263 Sr were highest in leaves at the bottom of the canopy and decreased progressively to the top of
264 the canopy. Concentrations of P, S, K, Cu, Zn, Rb, and Mo increased from bottom to top leaves.

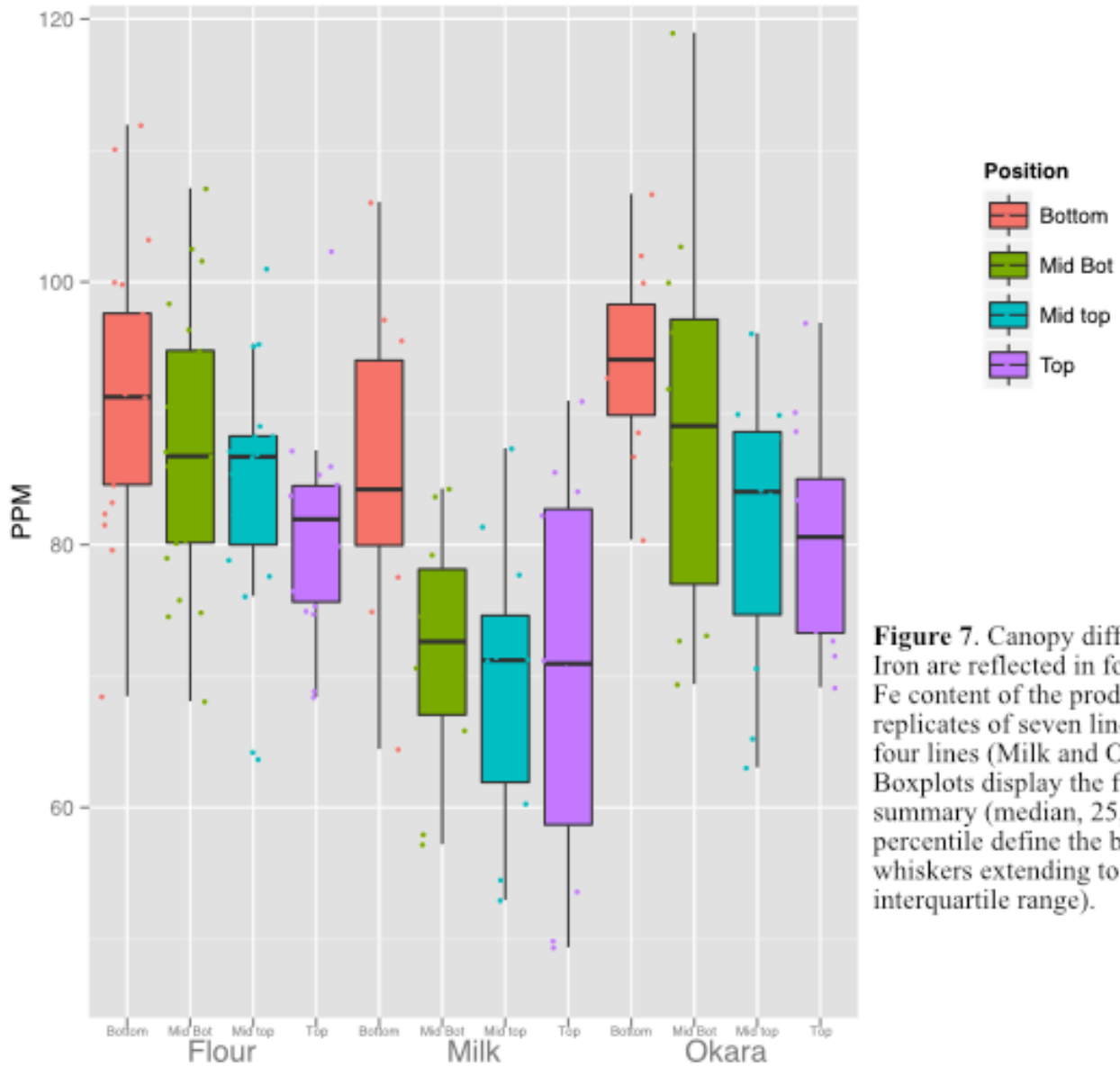


Figure 7. Canopy differences in Iron are reflected in food products. Fe content of the products from 3 replicates of seven lines (flour) and four lines (Milk and Okara). Boxplots display the five number summary (median, 25, and 75% percentile define the box, with whiskers extending to 1.5 x interquartile range).

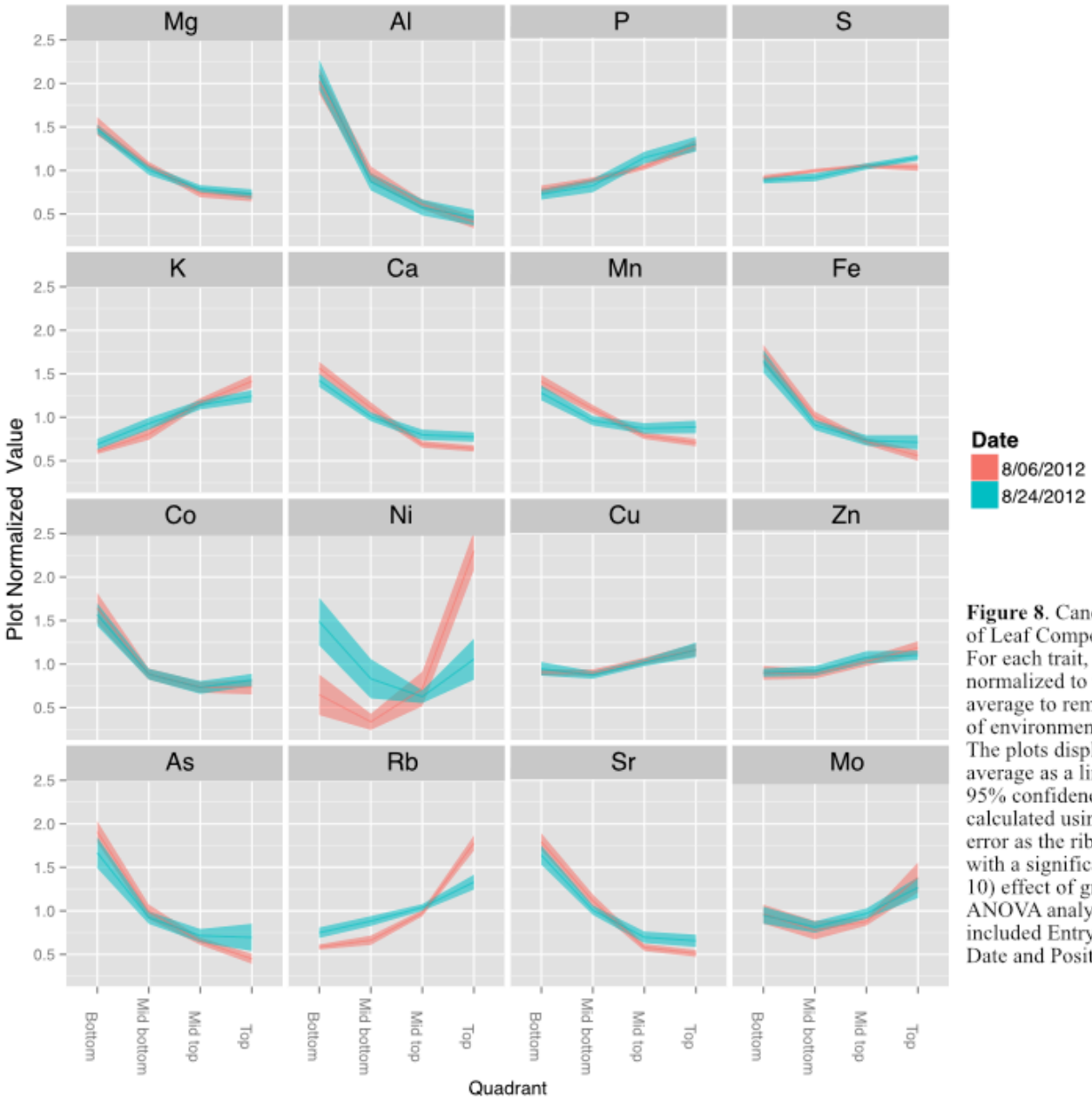


Figure 8. Canopy Gradients of Leaf Composition Traits. For each trait, the data was normalized to the plot average to remove the effect of environment and genotype. The plots display the quadrant average as a line with the 95% confidence interval calculated using standard error as the ribbon. Elements with a significant ($p < 1e-10$) effect of gradient in an ANOVA analysis that included Entry, Collection Date and Position.

265 Na and Ni were both present at low absolute concentrations and fluctuated but not in a
266 progressive pattern as for the other minerals. Although leaves at different positions are often
267 analyzed together (or as part of the 'shoot'), two previous studies with soybean also reported
268 differences in mineral concentrations of lower, middle and upper leaves (Drossopoulos et al.
269 1994) or young and old leaves (corresponding to different node positions) (Duke et al. 2012) that
270 are generally consistent with our results. The basis for differential accumulation of foliar
271 minerals at different positions within the canopy is not clear and will be important to address in
272 future studies. One possible explanation is that the greater phloem mobility of P and K facilitates
273 their enhanced remobilization to upper nodes whereas other less mobile elements (e.g. Fe, Ca,
274 and Mg) tend to remain at their point of initial deposition. This would not readily explain the
275 observed profiles for Cu, Zn and Mo, however, highlighting the complexities involved in metal
276 homeostasis and the significant variation with canopy position. Another working hypothesis
277 could be that K, P, Cu, Zn and Mo are mineral markers of metabolic activity and accumulate in
278 leaves at the top of the canopy that have highest rates of photosynthesis. Because minerals can be
279 remobilized from leaves to developing seeds (Drossopoulos et al. 1994; Jiménez et al. 1996;
280 Sankaran & Grusak 2014), it is tempting to speculate that canopy seed gradient in Fe and Mg
281 may be related to greater stores of both metals in leaves lower in the canopy. Opposite patterns
282 were observed for other minerals (Ca, Mn, K, and Cu) suggesting that remobilization is either
283 mineral specific or not quantitatively important in delivery of minerals to developing seeds.
284 A final point to note is that the potential exists for some soil particles to adhere to vegetative
285 plant parts, especially lower in the canopy, while seeds are protected from soil contamination by
286 the pods. Since some minerals exhibited opposite patterns, it seems that soil adhesion could not
287 be completely responsible for the patterns observed.
288

289 Node position and the developing seed metabolome

290 Developing seeds were analyzed to determine whether canopy position affected seed metabolism
291 sufficiently to explain the observed differences in protein and oil concentrations at maturity. To
292 do this, we collected developing seeds from the top and bottom of the canopy at several time
293 points over a 24-h period as illustrated in **Figure S2**. Because seeds at the top and bottom of the
294 canopy differed in size on the day of the experiment, seeds from the top of the canopy were also
295 collected 6 days later when they had reached the same size (fresh weight seed^{-1}) as the bottom

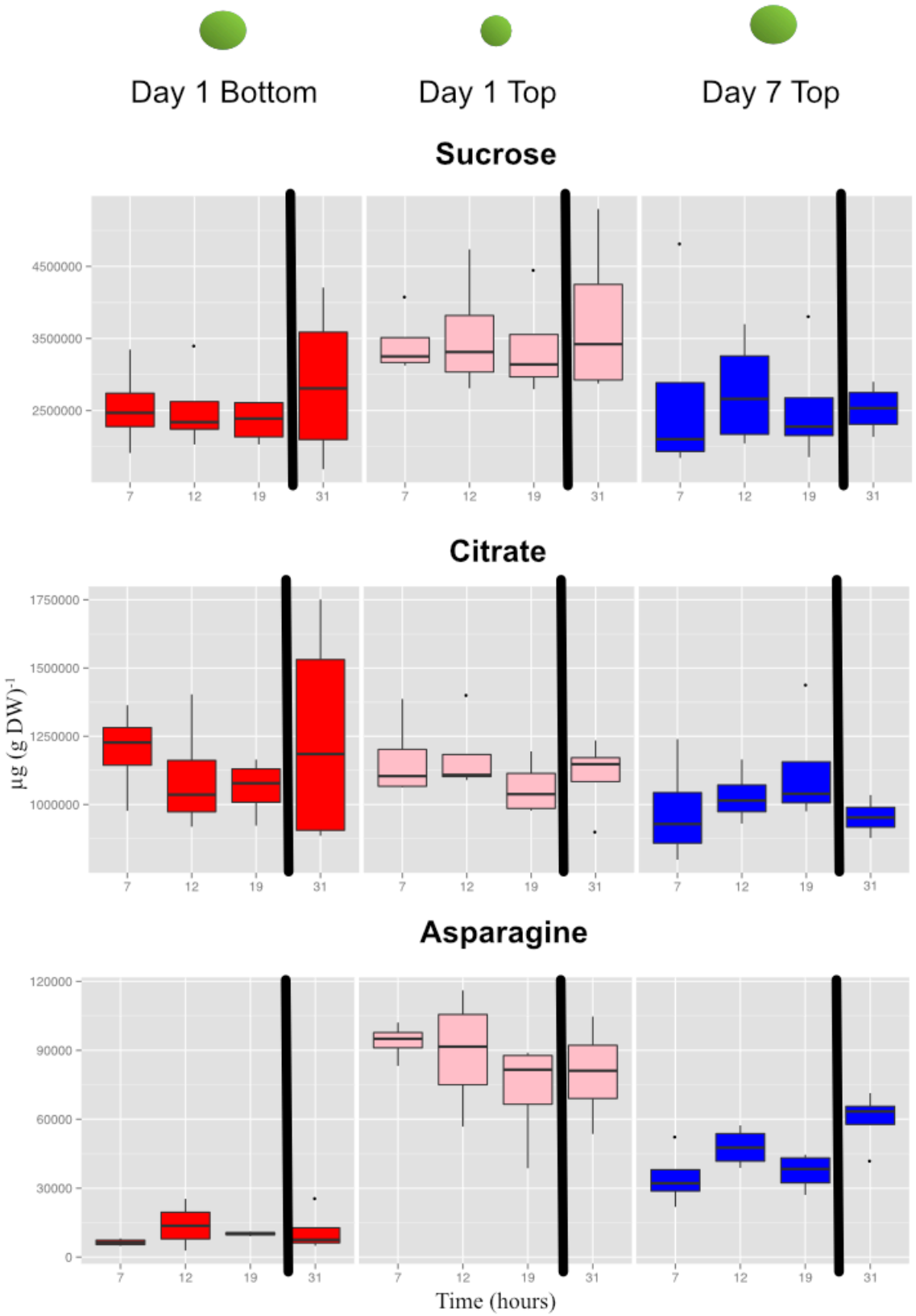


Figure 9. Concentrations of selected primary metabolites in developing seeds**. A**, Suc; **B**, citrate; and **C**, Asn. Boxplots display the five number summary (median, 25, and 75% percentile define the box, with whiskers extending to 1.5 x interquartile range) for three replicates at each sampling time: 7AM (7) , 12N (12) , 7PM (19) and the following morning at 7AM (31). The black vertical bars represent the intervening night period. Values are µg (g DW)$^{-1}$.

296 seeds on the first collection date. All seeds were at the stage of development where cell
297 expansion and accumulation of storage compounds (protein and oil) were the dominant
298 metabolic processes (Collakova et al. 2013). Untargeted metabolite profiling was conducted for
299 analysis of polar compounds, free amino acids, free fatty acids, and total fatty acids (**Fig. S3**).
300 In general, most metabolites did not show diurnal changes in concentration, but there were
301 differences in concentrations as a function of seed size and node position. The metabolite plots in
302 **Fig. 9** illustrate some of the different patterns observed. The concentration of sucrose (**Fig. 9A**)
303 in developing seeds did not vary diurnally and remained relatively constant but the concentration
304 was slightly higher in the smallest seeds (day 1, top seed) compared to the larger seeds sampled
305 at the bottom position on day 1 or top position on day 7. The decrease in sucrose concentration
306 comparing top seed on day 1 and day 7 likely reflects in part the dilution effect caused by storage
307 product accumulation as the seeds increased in size by roughly 2-fold. In contrast, the
308 concentration of citrate in developing seeds was roughly equal among the three samples (**Fig.**
309 **9B**). These results suggest that seeds actually accumulate sucrose and to a larger extent citrate as
310 they increase in dry matter during seed fill (thereby negating the dilution effect caused by seed
311 growth). This also indicates that developing seeds have ample sugars and organic acids
312 irrespective of size and node position and time of day. In marked contrast to sucrose and citrate
313 were the dramatic differences observed in free asparagine (Asn) concentration (**Fig. 9C**), which
314 was highest in top seed sampled on day 1 (Aug 20), and lowest in bottom seed sampled on the
315 same day; the difference was roughly 8-fold. Sampling top seed on day 7 (Aug 26), when seed
316 size was equivalent to that of bottom seed on day 1, still resulted in a ~4-fold elevation of free
317 Asn concentration. The roughly 2-fold decrease in Asn concentration in seeds at the top of the
318 canopy from day 1 to day 7 likely reflects the dilution effect of growth. The pattern for Asn
319 concentration is potentially of interest because free Asn concentration during seed development
320 correlates with protein concentration at maturity (Herman 2014b; Hernandez-Sebastia et al.
321 2005; Miller et al. 2008; Pandurangan et al. 2012). The results obtained in the present study
322 suggest that greater supply of Asn to developing seeds at the top of the canopy may contribute to
323 the observed greater accumulation of storage protein.
324 Importantly, Asn was also one of the important metabolites that distinguished the three sets
325 of seeds collected based on a global metabolite analysis (**Fig. S4**). Mean values for Asn, and
326 other protein amino acids are shown in **Fig. 10**. The concentrations of the free amino acids was

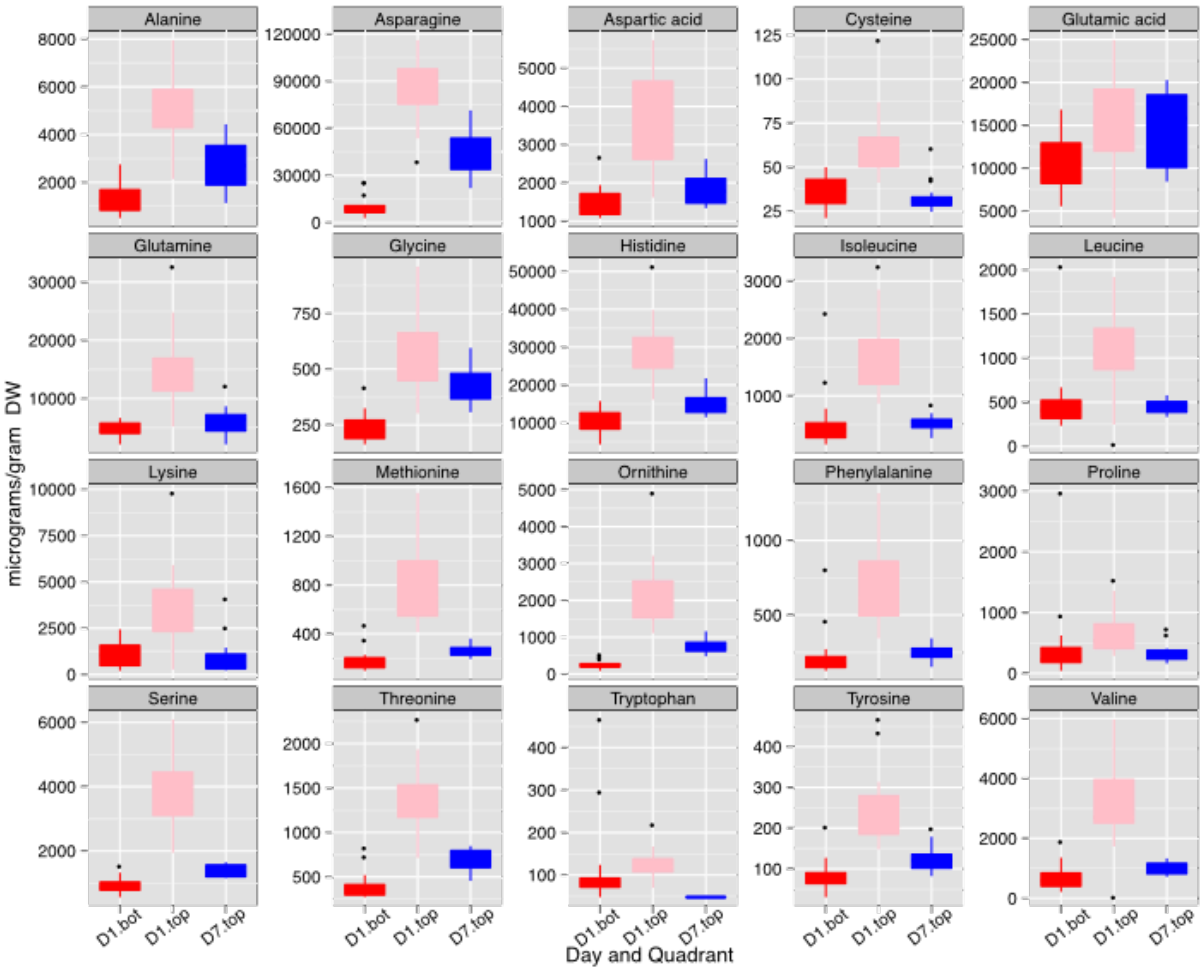


Figure 10. Concentrations of free amino acids in developing seeds. Boxplots display the five number summary (median, 25, and 75% percentile define the box, with whiskers extending to 1.5 x interquartile range) for values from each sampling interval (3 replicates and 4 time points are merged within each box) and nodal position. Ornithine levels reflect both ornithine and arginine as arginine is converted to ornithine during sample prep for GC-MS. D1.bot, D1.top and D7.top refer to the samples collected on day one top and bottom quadrants and the day seven top quadrant respectively.

327 highest in the small seed (top seed, day 1). Concentrations of Ala, Asn, Gly, and Thr were
328 substantially higher in top seed at day 7 relative to bottom seed at day 1 (when seed sizes were
329 similar). Of those amino acids, Asn was present at the highest absolute concentrations and may
330 contribute to the storage protein biosynthesis either by acting as a signal metabolite or providing
331 substrate for protein biosynthesis.
332
333
334 ## Discussion
335 The present study yields two major conclusions. First, the position along the main stem at which
336 soybean seeds develop has a profound impact on seed composition, affecting the concentrations
337 of protein, oil and certain minerals at maturity. Second, the canopy position effects on seed
338 mineral concentrations (in particular Fe) are sufficiently large that there may be direct
339 implications for human nutrition in countries where plants are the main source of protein and
340 soybeans are used for human food. These conclusions are discussed in more detail below.
341
342 **Positional effects on seed protein and oil concentration are broadly observed**
343 Results of the present study demonstrate that for 10 lines grown over a period of 3 years there
344 were remarkably consistent gradients in protein and oil concentrations in mature seeds as a
345 function of nodal position (**Figs. 2A and 3**). Increased concentration of oil in seeds from lower
346 nodes could result from the increased duration of the SFP documented for lower pods (**Table S3**)
347 because the accumulation of oil in seeds often starts earlier than protein (Rotundo & Westgate
348 2009; Saldivar et al. 2011). However, oil accumulation tends to plateau before protein
349 accumulation and therefore, percent oil will often decrease with increasing duration of the SFP
350 rather than increase. In the present study, the protein and oil concentration gradients from
351 bottom to top of the canopy were not correlated with the difference in SFP between the two
352 positions (**Fig. 6**) and thus it appears that SFP does determine the observed gradients in protein
353 and oil concentration. Micro-environment appears to be a factor controlling protein and oil
354 concentration gradients in the canopy because removal of neighboring plants at flowering
355 increased protein concentration at all positions and decreased the difference between top and
356 bottom nodes (**Fig. 5**). While it is not clear which micro-environmental factor(s) might actually
357 be involved, we suggest that increased light energy reaching lower leaves may be a contributing

358 factor. Metabolomic analysis of developing seeds that identified free Asn as one of the primary
359 metabolites distinguishing seeds at the bottom and top of the canopy supports this conclusion.
360 Asparagine is the major free amino acid in developing soybean seeds and differences in Asn
361 concentration during development are positively correlated with protein concentration at seed
362 maturity (Hernandez-Sebastia et al. 2005; Pandurangan et al. 2012). Furthermore, over-
363 expression of asparaginase in soybean, driven by an embryo-specific promoter, resulted in a
364 reduction in free Asn concentration during development and reduced protein concentration in
365 mature seed, measured by nitrogen analysis (Pandurangan et al. 2015). Collectively, these results
366 suggest that free Asn is a sensor or regulator of processes that determine protein accumulation in
367 soybean seeds (Herman 2014a). Our results are consistent with this hypothesis and suggest that
368 differences in free Asn concentration may explain the position effects on seed protein (and oil)
369 concentration. Nitrogen and carbon flux into pods is largely provided by nearest sources
370 (Seddigh & Jolliff 1986; Streeter & Jeffers 1979) including the nearest trifoliolate leaves. We
371 speculate that decreased light at lower positions in the closed canopy (i.e., with neighboring
372 plants) would reduce leaf metabolism as well as the xylem flux of ureides and/or nitrate from
373 roots to the lower leaves, thereby restricting the ability of those leaves to provide Asn (and Gln)
374 to developing seeds. In contrast, removal of neighboring plants (in the 'thinned' plant treatment)
375 would increase light at lower nodes thereby enhancing overall leaf metabolism and the flux of
376 reduced nitrogen to subtending pods resulting in increased protein (and reduced oil)
377 accumulation.
378
379 **Positional effects on seed mineral concentration are documented**
380 The concentration of minerals in seeds reflects the combined action of transport processes and
381 regulation at multiple steps starting with mobilization from the soil, uptake into the root, and
382 transport to the shoot for distribution among organs (Grusak et al. 1999; Waters & Grusak 2008).
383 Deposition of some minerals in seeds can also involve remobilization from leaves during seed
384 filling (Grusak et al. 1999; Hocking & Pate 1977), and it is interesting that different minerals
385 show fundamentally different profiles of accumulation in seeds as a function of canopy position
386 (**Fig. 3**). These differences could reflect alternate routes from the apoplast to the symplast or
387 differences in mobility in the phloem. Interestingly, minerals that tended to have highest
388 concentrations in seeds at the bottom of the canopy (e.g., Mg, Fe, and Cu) are considered to have

389 better phloem mobility compared to the minerals that tended to concentrate in the top of the
390 canopy (e.g. Mn and in some cases Ca) that are considered to have poor phloem mobility. These
391 results suggest that remobilization from leaves may be playing some role at least in the positional
392 effects on the mature seed ionome.
393 While multiple seed constituents exhibited canopy concentration gradients, it seems
394 unlikely that they are all caused by the same factors. Changing the microenvironment altered the
395 protein and oil gradients but did not affect observed gradients for most of the minerals (**Fig. 5**).
396 Furthermore, while the slope of many gradients changes across lines, treatment and year, the way
397 that they change is not well correlated between the different constituents, as illustrated in the plot
398 normalized correlation matrix (**Fig. 4A**), where relatively few strong correlations among the
399 various parameters were apparent. However, numerous correlations were apparent when mean
400 plot values were compared (**Fig. 4B**). Several minerals (e.g., P, Mn, Fe, Zn, S, and Co) had a
401 negative relationship with oil concentration and increased with protein concentration. Thus,
402 some coordination between seed storage product accumulation and mineral uptake into seeds is
403 evident. However, the results suggest that total uptake of a mineral and the allocation among
404 nodal positions are controlled by different mechanisms, and in general, canopy positional effects
405 on minerals and protein/oil appear to be controlled by distinct mechanisms. Future studies will
406 be required to sort out the mechanisms involved.
407
408 **Human nutrition implications for variation in seed composition**
409 Soybeans are valued for their protein and oil content, but when used for human nutrition the
410 content of minerals such as iron and zinc is also critically important. On a global scale, human
411 iron deficiency is one of the most prevalent nutritional disorders (McLean et al. 2009) especially
412 in countries where plant-based diets are prominent. As discussed above, nodal position affected
413 the concentration of several minerals such as Mg, Fe, and Cu that were present at higher
414 concentrations in seeds produced at the bottom of the canopy. Iron is of particular interest and
415 was generally 20% higher in seeds produced lower in the canopy relative to the top and as
416 expected, differences in seed iron concentrations affected the concentration of iron in soy food
417 products made from those seeds (**Fig. 7**). Soy flour preserved more Fe than did milk/okara.
418 Perhaps mineral retention improvement through product preparation is possible. An immediate
419 application of our results with respect to human nutrition would be to use seeds from the top and

420 bottom halves of the canopy for different purposes, with seeds produced in the lower half
421 reserved for production of iron-rich soy foods for human consumption. Thus, knowledge of
422 these canopy position effects provides an unexpected approach to link agronomic practices to
423 improve human nutrition and health.
424
425 **New type of seed heteromorphism and implications for climate change impacts**
426 Seed heteromorphism is well established (Matilla et al. 2005) but the seed heterogeneity
427 documented here establishes a new category where an individual plant produces a continuum of
428 seeds that differ in major aspects of their composition (protein, oil, and minerals) but are
429 morphologically very similar. Overall, our results raise a number of questions and directions for
430 future research. For example, it would be interesting to explore whether there are positional
431 effects on soybean seed functional traits such as seed vigor or seedling stress tolerance. Because
432 environment during reproductive development of plants is now recognized to broadly impact
433 seed properties, such as growth performance and stress tolerance of the progeny (Biodner et al.
434 2007; Tricker et al. 2013), it will be interesting to further explore similar properties of soybean
435 seed produced at the different parts of the canopy. Our results also raise the question of whether
436 similar effects occur in other species including non-domesticated plants where there might be
437 some ecological significance.
438 Another area that will be interesting to explore is the impact of elevated CO_2 on the
439 canopy positional effects described in the present study. It was recently reported (Loladze 2014;
440 Myers et al. 2014) that grain from many species, including soybean, have lower concentrations
441 of Zn and Fe when plants are grown at elevated CO_2 thereby uncovering a new climate change
442 challenge to global health. The meta-analysis established a ~5% reduction in soybean seed Fe
443 and Zn concentrations at high CO_2. It is relevant to note that variation in seed Fe concentration
444 with node position established in the present study is substantially larger (4-fold greater)
445 compared to the impact of climate change on mean seed Fe concentration. Therefore, our results
446 are likely to be meaningful from a quantitative standpoint and have important implications for
447 examining the impact of climate change on the seed ionome. For example, it will be interesting
448 to determine how this overall reduction in mean seed Fe concentration at elevated CO_2 is related
449 (if at all) to canopy position effects; is Fe reduced 5% in seeds at nodes throughout the canopy
450 or are certain positions affected to a greater degree than others? Identifying the molecular

451 mechanisms underlying canopy gradients in composition may provide new approaches to
452 controlling soybean seed quality for various uses, including food for human consumption under
453 conditions of global climate change.
454

455 ## Materials and Methods

456 **Plant growth and sampling**

457 Soybean lines were grown at the University of Illinois South Farm, Urbana, IL, in a
458 randomized complete block design with three replicates each year. Each plot consisted of three
459 rows 2.5 m long, with 0.75 m between rows and a planting density of roughly 30 seeds m^{-1}.
460 To produce the thinning treatment, all but three plants were removed from each row shortly after
461 flowering. Delaying thinning until after the reproductive period had begun minimized branching
462 on the remaining plants. Approximately 20 cm of plants were thinned from the ends of each row
463 and the third plant was left in the middle in the row. The remaining plants were spaced
464 approximately 1 m apart.

465 Plants were harvested at maturity. All plants were cut close to ground level and brought
466 into the laboratory. Each stem was divided into four quadrants and the stem fractions in each
467 quadrant were threshed together for each plot. Only normal-sized plants were included in the
468 analysis, and extremely small, wrinkled or off-color seeds were manually removed from all
469 samples before analysis.
470

471 **Soy products**

472 To produce flour, soybeans were blanched (boiled for ~25 minutes) and then baked
473 before grinding. To produce soymilk and okara (remaining solids), soybeans were blanched
474 (boiled for ~5 min) twice and then ground in water and cooled slightly. The soymilk (liquid
475 phase) and okara (solid phase) were separated using a cheesecloth and then dried separately and
476 reground before analysis.

477 **Seed storage product analysis**

478 Protein and oil were measured with an Infratech 1241 Grain Analyzer (FOSS Analytical
479 AB, Höganäs, Sweden), which is a true Near Infrared Transmission instrument that generates a
480 spectrum from 850 to 1050 nm via the monochrome light source and mobile grating system. A

481 50-ml seed sample was used that allowed for 10 subsample readings reported on a 13 % moisture
482 basis.
483
484 **Ionomic analysis**
485 Seed analysis was conducted as described in Ziegler et al. 2012. Briefly, single seeds
486 from each quadrant were weighed using a custom-built seed weighing robot and then digested in
487 concentrated nitric acid before loading onto an Elan ICP-MS. Internal standards were used to
488 control for differences in dilution and sample injection. Leaf and soy products were analyzed in
489 the same manner except that samples were added to digestion tubes by hand and weighed.
490 Custom scripts were used to correct for internal standards and correct for sample weight.
491
492 **Metabolomic analysis**
493 Metabolome analysis was done through Metabolomics Center, Roy J. Carver
494 Biotechnology Center, University of Illinois at Urbana-Champaign. Frozen seeds with attached
495 seed coats were homogenized in liquid nitrogen and about 25 mg FW was extracted at room
496 temperature with 1 mL of 50% methanol followed by addition of 800 µ1 of methanol:chloroform
497 (1:2) as outlined in **Fig. S3**. Each extraction was followed by centrifugation (5 min at 15,000 g),
498 and the supernatants were collected. With the exception of samples for analysis of coenzymes,
499 final extracts were evaporated under vacuum at -60 °C and subjected to GC/MS analysis.
500
501 Metabolic profiling: Dried extracts were derivatized with 100 µL methoxyamine hydrochloride
502 (40 mg/ml in pyridine) for 90 min at 50 °C, then with 100 µL MSTFA at 50 °C for 120 min, and
503 following 2-hour incubation at room temperature. 5 µL of the internal standard (hentriacontanoic
504 acid, 10 mg/mL) was added to each sample prior to derivatization. Metabolites were analyzed
505 using a GC-MS system (Agilent Inc, CA, USA) consisting of an Agilent 7890 gas
506 chromatograph, an Agilent 5975 mass selective detector and a HP 7683B autosampler. Gas
507 chromatography was performed on a ZB-5MS (60m×0.32mm I.D. and 0.25µm film thickness)
508 capillary column (Phenomenex, CA, USA). The inlet and MS interface temperatures were 250
509 °C, and the ion source temperature was adjusted to 230 °C. An aliquot of 1 µL was injected with
510 the split ratio of 10:1. The helium carrier gas was kept at a constant flow rate of 2 ml/min. The
511 temperature program was: 5-min isothermal heating at 70 °C, followed by an oven temperature

512 increase of 5 °C min^{-1} to 310 °C and a final 10 min at 310 °C. The mass spectrometer was
513 operated in positive electron impact mode (EI) at 69.9 eV ionization energy at m/z 30-800 scan
514 range.
515
516 Amino acid analysis: A 20 µl aliquot of the internal standard DL-chlorophenylalanine (1mg/ml
517 in 0.1M HCI) was added to the extracts, dried under vacuum, derivatized with 50 µl of neat N-
518 Methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA), and 50 µL of acetonitrile at 80
519 ^{0}C for 4 h, cooled to room temperature and centrifuged briefly to remove condensate from the
520 top of tube prior to injection of 1 µL at 5:1 split ratio into the GC/MS system, which consisted of
521 an Agilent 6890N (Agilent Inc, Palo Alto, CA, USA) gas chromatograph, an Agilent 5973 mass
522 selective detector and Agilent 7683B autosampler. Gas chromatography was performed on a 60
523 m ZB-5MS column with 0.32 mm inner diameter (I.D.) and 0.25 µm film thickness
524 (Phenomenex, CA, USA) with injection temperature and MSD transfer line of 230 °C both, and
525 the ion source adjusted to 230^{0}C. The helium carrier gas was set at a constant flow rate of 2 ml
526 min^{-1}. The temperature program was 5 min at 150 °C, followed by an oven temperature ramp of 5
527 °C min^{-1} to 315 °C for a final 3 min. The mass spectrometer was operated in positive electron
528 impact mode (EI) at 69.9 eV ionization energy in m/z 50-800 scan range. Acquired data were
529 normalized to the internal standard (DL-p-Chlorophenylalanine) and sample fresh weight.
530 Amino acid concentrations were calculated based on 2to 75 µgml^{-1} standard curves.
531
532 Free fatty acids, total fatty acids and coenzymes were also measured and values obtained used in
533 the global analysis, but specific results are not presented. Detailed methods for the analysis are
534 available on request.
535
536 The spectra of all chromatogram peaks were compared with electron impact mass spectrum
537 libraries NIST08 (NIST, MD, USA), W8N08 (Palisade Corporation, NY, USA), and a custom-
538 built database (460 unique metabolites). All known artificial peaks were identified and removed.
539 To allow comparison between samples, all data were normalized to the corresponding internal
540 standard and the sample fresh weight (FW). The spectra of all chromatogram peaks were
541 evaluated using the AMDIS 2.71 (NIST, MD, USA) program. Metabolite concentrations were
542 reported as concentrations relative to the internal standard (*i.e.*, target compound peak area

543 divided by peak area of internal standard: $N_I = X_i \cdot X^{-1}_{IS}$) per gram sample weight. The
544 instrument variability was within the standard acceptance limit (5%).
545
546 Metabolites with more than 50% of missing data were removed and for the rest of the
547 metabolites, any missing data was imputed with one-half of the minimum positive value in the
548 original data assuming their level was below the instrument detection limit. MVA and
549 visualization was performed with SIMCA-P+ 12.0 software (Umetrics AB, Umeå, Sweden) and
550 MetaboAnalyst (Xia & Wishart 2011) using log-transformed and autoscaled data and validated
551 by sevenfold Cross-Validation and permutation with 500 random. To address the problem of
552 multiple comparisons the False Discovery Rate (FDR) test was adopted. (Storey 2002).
553
554 **Data analysis**
555 Protein, oil, and elemental data were analyzed using R and the packages dplyr, ggplot2,
556 grid, reshape2, qtlcharts and gplots. All data and analysis scripts used in the analysis are
557 included as a supplemental file and are available on www.ionomicshub.org.
558
559

560

561 **Figure Legends:**

562 **(Figure 1.) Quadrants of a soybean plant.** The mature plant was divided into quadrants upon
563 harvest and seed collected from each quadrant was analyzed separately for protein, oil, and
564 mineral concentrations. Plot normalized data used the average of all four quadrants to normalize
565 year, plot and line effects.

566 **(Figure 2.) Canopy gradients of seed composition traits before normalization and line and**
567 **year effects on total accumulation.** (A) Composition gradients from the bottom to top of the
568 canopy for cultivar 'Cumberland.' The plots display the quadrant average as a line with the 95%
569 confidence interval calculated using standard error as the ribbon. (B) Year and line effects for
570 each compositional trait, represented as boxplots. In A and B, protein and oil are expressed on a
571 percent basis and minerals as $\mu g\ g^{-1}$ dry weight.

572 **(Figure 3.) Canopy gradients of seed composition traits**. For each trait, the data was
573 normalized to the plot average to remove the effect of environment and genotype. The plots
574 display the quadrant average as a line with the 95% confidence interval calculated using standard
575 error as the ribbon. (A) Percentage protein, percentage Oil and single seed weight**. (**B) Elements
576 with a significant ($p < 1\ e\ -10$) effect of gradient in an ANOVA analysis that included Entry,
577 Year and Position.

578

579 **(Figure 4**.) **Correlation plot among composition traits**. Pearson correlation values between
580 compositional traits. (A) Correlation across 832 quadrants normalized to the plot average. (B)
581 Correlation across 208 plot means.

582

583 **(Figure 5**.) **Effect of altering microenvironment by removal of neighboring plants on**
584 **compositional traits**. For each trait, the data was normalized to the plot average to remove the
585 effect of environment and genotype. The plots display the quadrant average as a line with the
586 95% confidence interval calculated using standard error as the ribbon. (A) Percentage protein
587 and percentage Oil in 2010. (B) Elements (from 2010 and 2012) with a significant ($p < 1$ e -10)
588 effect of gradient in an ANOVA analysis that included Entry, Year, Position and thinning.
589
590 **(Figure 6**.) **Difference in composition traits of seeds from top and bottom of the canopy is**
591 **not correlated with seed fill period**. The difference in plot normalized composition between
592 the top quad and the bottom quad for protein, oil and iron plotted versus the difference in seed
593 fill period for 51 plots in 2012.
594
595 **(Figure 7**.) **Canopy differences in seed iron concentration are reflected in food products**. Fe
596 content of the products from 3 replicates of seven lines (flour) and four lines (Milk and Okara).
597 Boxplots display the five number summary (median, 25, and 75% percentile define the box, with
598 whiskers extending to 1.5 x interquartile range).
599
600 **(Figure 8.) Canopy gradients of leaf composition traits**. For each trait, the data was
601 normalized to the plot average to remove the effect of environment and genotype. The plots
602 display the quadrant average as a line with the 95% confidence interval calculated using standard
603 error as the ribbon. Elements with a significant ($p < 1$ e -10) effect of gradient in an ANOVA
604 analysis that included Entry, Collection Date and Position.
605

606 **(Figure 9.) Concentrations of selected primary metabolites in developing seeds. (**A),
607 Sucrose; (B), citrate; and (C), asparagine. Boxplots display the five number summary (median,
608 25, and 75% percentile define the box, with whiskers extending to 1.5 x interquartile range) for
609 three replicates at each sampling time: 7AM, 12N, 7PM and the following morning at 7AM. The
610 grey vertical bars represent the intervening night period. Sucrose and citrate values are relative
611 concentrations, while asparagine is presented as μg (g DW)$^{-1}$.
612
613 **(Figure 10.) Concentrations of free amino acids in developing seeds.** Boxplots display the
614 five number summary (median, 25, and 75% percentile define the box, with whiskers extending
615 to 1.5 x interquartile range) for values from each sampling interval (3 replicates and 4 time
616 points are merged within each box) and nodal position. Ornithine levels reflect both ornithine
617 and arginine as arginine is converted to ornithine during sample prep for GC-MS. D1.bot, D1.top
618 and D7.top refer to the samples collected on day one top and bottom quadrants and the day seven
619 top quadrant respectively.
620

621

622 **Supplementary tables and figures**

623 Table S1. Indeterminate lines used in the present study and selected characteristics

624 Table S2. Weather summary (June 1– August 31) during the 2010 to 2012 growing seasons

625 Table S3. Genotype differences in Seed fill period (SFP) and the difference in SFP at two node

626 positions (bottom minus top; delSFP)

627

628 Figure S1. Absolute mineral concentrations in 3 years of study. The plots display the quadrant

629 average as a line with the 95% confidence interval calculated using standard error as the ribbon.

630 Figure S2. Experimental protocol for sampling developing soybean seeds for metabolomic

631 analysis.

632 Figure S3. Schematic representation of sample fractionation for global metabolite analysis.

633 Figure S4. Global analysis of metabolome of developing soybean seeds. (A) PLS-DA scores plot

634 (R^2 = 98.7%, Q2 = 81.1%, $P < 0.001$ by permutation test) of soybean seeds at different canopy

635 position and time of day. (B) Variable Importance in the Projection (VIP) for the first component

636 showing the fifteen most important compounds.

637

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