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# FACULTATIVE ENVIRONMENTAL SEX DETERMINATION IN *CERATOPTERIS RICHARDII* GAMETOPHYTES: CHARACTERIZATION AND IMPLICATIONS FOR PLASTICITY OF RESOURCE ALLOCATION

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ABSTRACT

The fern species *Ceratopteris richardii* has long served as a model organism for the study of plant growth and development, owing to its short time to sexual maturity and ease of culture. Additionally, the free-living gametophytes of *C. richardii* are sexually undetermined at the time of spore germination and individuals can subsequently develop as either males, females, or hermaphrodites. Traditionally, sex determination in *C. richardii* has been explained mainly by population density and the ensuing concentration of the sex pheromone antheridiogen, secreted by developing gametophytes. More recently, the regulation of labile sex expression in *C. richardii* gametophytes has been examined within the theoretic framework of environmental sex determination (ESD). This perspective suggests abiotic variables like nutrient availability can potentially influence population sex ratios instead of accomplishing regulation by the antheridiogen response alone. When environments are heterogeneous with respect to required nutrients, and individual organisms have no control over the environment in which they develop, ESD can allow populations to adjust their sex ratio based on local conditions, resulting in increased individual fitness. Therefore, ESD is an adaptive trait and provides opportunities to understand the evolution of resource allocation strategies between and within genders in variable nutrient environments. This thesis emulated nutrient concentrations and stoichiometries that are likely to be experienced by plants in natural environments in order to test hypotheses about nutrient effects on sex expression and subsequent development in *C. richardii* gametophytes. I found that *C. richardii* gametophytes exhibit gender-dependent differences in relative nutrient content—a fundamental expectation underlying ESD theory. By manipulating the nutrient

environment, I also established that ESD could be turned on and off *in vitro* because ESD was only expressed under specific nutrient conditions. Finally, I demonstrated that nutrient limitation had significant effects on female reproductive allocation when ESD was absent, but the effect disappeared when ESD was present. Thus, ESD reduced the need for large allocation adjustments to female reproductive function because nutrient limitation caused some gametophytes to preemptively become the less nutrient-demanding sex. Thus ESD helped to match the resource demands of gender phenotypes to their local environment before the onset of developmental dimorphism by shifting a nutrient limitation response away from reallocation within an individual (phenotypic plasticity) to reallocation among individuals in the population. Opportunities to explicitly demonstrate such direct effects of ESD are rare because ESD itself is not a labile trait within a given species. The research reported in this thesis represents an important, general contribution for advancing the understanding of several conceptual areas in evolutionary and organismal biology, including resource and sex allocation theory, phenotypic plasticity, and ESD.

# CHAPTER 1:

# ABSOLUTE AND RELATIVE CONTENT OF CARBON AND NITROGEN DIFFER BY SEX IN *CERATOPTERIS RICHARDII* GAMETOPHYTES


## Abstract

When habitats are heterogeneous regarding key abiotic factors and individual organisms have no control over the environment in which they develop, labile sex expression can allow individuals to adjust their sex based on local environmental conditions, resulting in increased individual fitness. Sexual lability is found extensively in homosporous ferns, where sex expression is often regulated via the pheromone antheridiogen. Nutrient availability may provide additional signals for sex determination in fern gametophytes, particularly if nutrient demands required for sexual development differ by sex. The model fern *Ceratopteris richardii* has a well-characterized antheridiogen response and short time to sexual maturity. Although tests for nutrient effects on sex determination have been conducted in this fern, tests for differences in nutrient demands by sex have not. Elemental analysis demonstrated that 14 d-old ameristic male and meristic female or hermaphrodite gametophytes of *C. richardii* differ significantly in their relative carbon and nitrogen masses, resulting in significantly dissimilar C:N ratios between the sexes. Average gametophyte dry mass in ameristic males was approximately half that of meristic plants of the same age, and contained less N than meristic gametophytes in both relative and absolute terms. Those characteristic differences in elemental composition imply that variation in nutrient availability could potentially influence sex expression in *C. richardii* gametophyte populations, rather than regulation of sex determination by the antheridiogen system alone.

## Introduction

Sexual lability occurs when an organism's sex is not genetically fixed but is instead determined after growth begins (Korpelainen, 1998). Labile sex expression in sessile organisms can be adaptive because it allows each individual to adjust development into a male, female, or hermaphrodite based on prevailing environmental conditions as a means to improve reproductive success. Because the different sexes perform different reproductive functions, resource demands are likely to vary between males and females. In stressful environments, the most resource demanding sex is expected to occur less frequently, resulting in population sex ratios that diverge from a male-to-female ratio equal to one. (Ramadan et al., 1994; Stark et al., 2000).

In many species of homosporous pteridophytes, labile sex expression in the gametophyte phase is common, and can be a result of chemical signaling among individuals via the sex pheromone antheridiogen (Korpelainen, 1998). Antheridiogen is a biotic cue released into the environment by already developed female and hermaphrodite gametophytes, which increases the likelihood that undetermined local gametophytes will develop as males (Korpelainen, 1998; Yamane, 1998; Tanurdzic and Banks, 2004; Tanaka et al., 2014; Atallah and Banks, 2015; Ganger et al., 2014). This causes the sex ratio to be sensitive to population density (Näf et al., 1975; Dyer, 1979; Scott and Hickok, 1987; Banks et al., 1993). As plant number and antheridiogen concentration in the environment increase, the number of males also increases, although completely male populations are never observed (Scott and Hickok, 1987; Hickok et al., 1995).

Ferns with antheridiogen systems present an interesting problem in the evolution of labile sex expression because the potential presence of both biotic and abiotic cues for

determining sex may result in regulatory signals that may or may not be strictly aligned. In the gametophyte generation of homosporous ferns, spore dispersal density and abiotic habitat quality following spore dispersal cannot be predicted. In some instances, signals expected to provide information relevant for sex determination based on plant density and nutrient availability can be aligned and mutually reinforcing. Low-density populations in non-limiting nutrient environments both provide cues favoring the development of a higher frequency of the more expensive sex. In situations where signals are apparently at odds (e.g., low plant densities in nutrient-limiting habitats), it is unclear how the population sex ratio would respond (Fig. 1.1). Expectations about the regulation of sex determination under variable nutrient conditions depend in part on whether the sexes differ in their nutrient demands in absolute or relative terms (Charnov and Bull, 1977).

*Ceratopteris richardii* is a homosporous fern with a gametophyte generation that begins life as a single, sexually undetermined haploid cell; each viable spore eventually develops into a male or hermaphrodite plant, where hermaphrodites typically first pass through a female phase before any male gametangia are formed (Fig. 1.2; Scott and Hickok, 1987; Banks et al., 1993; Hickok et al., 1995; Chatterjee and Roux, 2000). Male gametophytes only form antheridia that release free-swimming sperm. Mature males are also 2· smaller in area on average than 14-day old female gametophytes, and lack an active lateral meristem (i.e., "ameristic"). Female gametophytes activate a lateral meristem (i.e., "meristic") from which archegonia (female gametangia) develop. Once fertilization occurs, an archegonium encloses a growing embryo (Scott and Hickok, 1987). Hermaphrodites are also meristic and resemble females developmentally and

phenotypically, but one or more antheridia are also produced which can result in self-fertilization (Tanurdzic and Banks, 2004).

A well-characterized antheridiogen system (*Ceratopteris* antheridiogen, or $A_{ce}$) and short time to sexual maturity has made *C. richardii* a useful model system to study effects of nutrient availability on sex determination (Ayrapetov and Ganger; 2009, Goodnoe et al., 2016; Chapter 2). The striking size dimorphism between sexes in *C. richardii* suggests *a priori* that male and female gametophytes differ in their absolute nutrient resource demands. But the complete absence of a meristem in determinate male plants and the strict requirement for the organization and continued development of a meristem to allow formation of female sexual organs means that differences in the relative demands for important nutrients might also underlie successful sex expression. In that case, plant sexual dimorphism would correspond to differences in the stoichiometry of important chemical elements required for successful sexual development. The current study aims to determine if any characteristic differences in macronutrient content exist between sexes in terms of total C and N mass per plant, and in terms of relative C and N content by sex in *C. richardii* gametophytes. Since the main developmental distinction between strictly male plants compared to plants with archegonia (i.e., females and hermaphrodites) depends on whether an active lateral meristem forms, the nutrient demands of ameristic and meristic plants are of keen interest.

*C. richardii* gametophytes have already been investigated to determine whether nutrients affect sex determination (Ayrapetov and Ganger, 2009; Goodnoe et al., 2016; Chapter 2). In this context, it appeared useful to also explicitly test the prediction that the different sexes of *C. richardii* differ in their nutrient demands. To my knowledge, such a

test has not previously been performed. This brief report does not test for nutrient effects on sex determination; direct tests for nutrient limitation effects on labile sex expression are reported elsewhere (Goodnoe et al, 2016; Chapter 2; T.T. Goodnoe and J.P. Hill, unpublished data; Chapter 3).

**Methods and Materials**

*Experimental Treatments*

*Ceratopteris richardii* gametophytes are expected to be adapted to elevated and potentially variable levels of $CO_2$ because they naturally live in the boundary layer at the soil-air or soil-water interface, where $CO_2$ levels may be as much as 10 times higher than ambient $CO_2$ concentrations due to microbial respiration and low rates of air mixing (Rau, 1978; Kimmerer, 2003). Although super-elevated $CO_2$ levels (>1,200 ppm) are expected to stimulate responses distinctive from those observed at moderately elevated levels (400-1,200 ppm) in angiosperm sporophytes (Kaplan et al., 2012), these same levels may be closer to what homosporous fern gametophytes normally experience. Growth in an ecologically realistic $CO_2$ environment could alter plant C:N stoichiometry (Verspagen et al., 2014). Therefore, culture dishes were located in a near-airtight acrylic chamber and $CO_2$ concentrations were elevated to 1,300 ppm.

Nutrient media with a C:N:P ratio of 5:16:1 was created in a background of 1/500 diluted basal salts media (BSM) recommended for culturing *C. richardii* gametophytes (C-Fern Web Manual, 2009). Glucose ($C_6H_{12}O_6$) served as the C source, with a concentration of 6 μM. The ammonium nitrate ($NH_4NO_3$) concentration was 58 μM and the potassium phosphate ($KH_2PO_4$) concentration was 7 μM. The C, N, and P levels were selected to emulate levels often found in natural environments, with the goal of

evaluating plant responses in an ecologically relevant resource context (Cleveland and Liptzin, 2007; Mathews and Chandramohanakumar, 2003; McGroddy et al., 2004; Sterner and Elser, 2002).

Dry, surface-sterilized spores of *C. richardii* were sown with a sterile cotton swab onto five 150 mm petri dishes containing the solid nutrient media. Levels of $CO_2$ were periodically measured with a wireless NODE $CO_2$ sensor (Variable Inc., Chattanooga, TN, USA). The near-airtight chamber was opened every 2-3 d to replace the NODE batteries and $CO_2$ was subsequently re-injected. The entire chamber was incubated inside an environmental chamber (Percival Scientific, model E-30B, Perry, Iowa, USA) (30º C, PAR ~30 µmol $m^{-2}$ $s^{-1}$, 12 h photoperiod) for 12 d after a 2 d induction under continuous light (30º C, PAR ~60 µmol $m^{-2}$ $s^{-1}$). Thus, plants were harvested a total of 14 d after sowing.

Because all females and hermaphrodites develop an active lateral meristem and males do not (Fig. 1.2), females and hermaphrodites were pooled and referred to collectively as meristic gametophytes, whereas males are referred to as ameristic gametophytes. Dry mass of ameristic and meristic gametophytes was estimated for each replicate by collecting 15 ameristic gametophytes and 15 meristic gametophytes from each dish, drying the gametophytes in an oven at 50° C for 48 h, and calculating individual plant averages from the total mass. All remaining gametophytes from each dish were separated into ameristic and meristic subgroups and dried in the same drying conditions. From each replicate, a 0.15 ± 0.004 mg sample of both ameristic and meristic gametophyte dry mass was collected and subsequently used for elemental analysis. Percent C and N in dry gametophyte tissue was estimated at the Idaho State University

Interdisciplinary Laboratory for Elemental and Isotopic Analysis (ILEIA) with a Costech ECS 4010 elemental analyzer interfaced to a Thermo Delta V Advantage continuous flow isotope ratio mass spectrometer. Samples were standardized with certified peach leaf samples (average %N = 2.94 ± 0.12). Average plant mass by sex and average percent C and percent N were used to calculate the average mass of C and N per ameristic and meristic plant.

*Statistical Analyses*

The differences in relative percent C and N between ameristic and meristic gametophytes analyzed with two-tailed, pooled-variance *t*-tests, based on the hypothesis that ameristic and meristic gametophytes differ in their relative percent C and N when grown in the same media. The variances for percent C and percent N for meristic and ameristic gametophytes were determined to be the same based on the Bartlett test for homogeneity of variances (P-value = 0.909 and 0.869, respectively; Bartlett, 1937). The difference in average ameristic and meristic gametophyte dry mass was tested with an upper-tailed, pooled-variance *t*-test, based on the hypothesis that meristic gametophytes weigh more than ameristic gametophytes. All statistical analyses were performed using the R computational environment (R Core Team, 2014) including the package asbio (Aho 2014).

**Results**

Ameristic (Fig. 1.2A) and meristic (Fig. 1.2B, C) gametophytes differed significantly in percent N and C by total dry mass. Meristic gametophytes had a higher percentage of N than ameristic gametophytes ($t_8$ = -6.37*,* *P*-value = 0.0002), and a lower percentage of C than ameristic gametophytes ($t_8$ = 5.55, *P*-value = 0.0005). Ameristic gametophytes

contained 44.2 ± 1 percent C and 5.3 ± 0.3 percent N, whereas meristic gametophytes contained 40.9 ± 0.9 percent C and 6.5 ± 0.3 percent N.

Meristic gametophytes 14 d after sowing were heavier than ameristic gametophytes of the same age ($t_8$ = -6.38, *P*-value = 0.0001). Meristic gametophytes had an average dry mass of ($\overline{x} \pm SE_M$) 5 ± 0.4 μg, and were approximately twice as heavy as ameristic gametophytes, which had an average dry mass of 2.3 ± 0.8 μg. Meristic gametophytes each contained 3 ± 0.03 μg of N and 2 ± 0.2 μg of C. Ameristic gametophytes each contained 0.12 ± 0.05 μg N and 1 ± 0.4 μg of C. Therefore, meristic gametophytes had an internal C:N ratio of 6.7:1, whereas ameristic gametophytes had a C:N of 8.3:1.

**Discussion**

Environmental sex determination (ESD) is a form of labile sex expression in which abiotic environmental factors influence sex once development has begun (Bull, 1983; Korpelainen, 1998; Gilbert, 2000). Similar to labile sex expression, ESD is predicted to be an adaptive trait, specifically when (1.) the costs of reproduction differ between males and females, (2.) the environment is patchy with respect to key abiotic factors, and (3.) organisms cannot predict nor control which patch reproduction will occur in when they first originate as zygotes or meiospores (Charnov and Bull, 1977; Bull, 1983). Therefore, an *a priori* expectation of ESD theory is that the different sexes of a species (typically male and female) differ in their nutrient demands (Charnov and Bull, 1977; Bull, 1983). Finding such differences here lends support to the hypothesis that *Ceratopteris richardii* conforms to the expectations of nutrient-based sex determination, beyond a *Ceratopteris* antheridiogen ($A_{ce}$) response (Ayrapetov and Ganger, 2009; Goodnoe et al., 2016;

Chapter 2; T.T. Goodnoe and J.P. Hill, unpublished data; Chapter 3). Given the observed difference in C and N requirements by sex, the intriguing possibility that environmental signals based on nutrient availability might conflict with signals based on $A_{ce}$ during sex determination (Fig. 1.1) becomes plausible.

In bryophytes and ferns, sex is expressed in a free-living haploid gametophyte phase, and a sex ratio describes the relative number of male and female gametophytes (Shaw and Gaughan, 1993). In bryophytes with sex chromosomes, a male:female sex ratio equal to one is expected (Shaw and Gaughan, 1993), though the common occurrence of female-biased sex ratios suggests that males are the more costly sex (Stark et al., 2000). The opposite is true in the homosporous fern species *C. richardiii* (Hickok et al., 1995). A male-biased sex ratio in sexually labile populations of dimorphic *C. richardii* gametophytes implies that meristic females and hermaphrodites have higher nutrient requirements than ameristic males.

In the present study, 14 d-old meristic gametophytes were larger than ameristic gametophytes in absolute terms, containing on average twice as much total dry mass as ameristic gametophytes. Meristic *C. richardii* gametophytes also contained a higher percentage of N and a lower percentage of C than ameristic gametophytes, resulting in a lower C:N ratio in gametophytes with archegonia. Meristic gametophytes were therefore more invested in accumulating N and had more N than ameristic gametophytes in both absolute and relative terms. Although ameristic gametophytes had a higher percentage of C relative to meristic gametophytes, meristic gametophytes contained more total C due to their larger size at 14 days after sowing.

Differences in relative nutrient demands indicate disparities in ameristic and meristic gametophyte development, reproductive structures, and physiology. Vegetative cells are found predominantly on meristic gametophytes (Fig. 1.2B, C) while almost every cell of the ameristic gametophyte aside from rhizoids differentiates and forms an antheridium (Fig. 1.2A; Hickok et al., 1995). Meristic *C. richardii* gametophytes continue growth and development indefinitely until fertilization occurs, at which point the embryonic sporophyte begins development (Hickok et al., 1995; Banks, 1999). In general, N is needed to make all proteins, nucleic acids, and enzymes, so rapidly growing organisms commonly have relatively lower internal C:N and are considered more nutrient (here, meaning nitrogen) rich (Sterner and Elser, 2002; Elser et al., 2000). The differences in C and N content between determinate ameristic and indeterminate meristic gametophytes followed that pattern.

Because *C. richardii* gametophytes differ in C and N costs by sex, they conform to one of the theoretical expectations of ESD. The current results, therefore, validate tests for ESD in *C. richardii* gametophyte population due to variation in nutrient availability. Environmental stress (in the form of inadequate or imbalanced nutrient availability) often favors the less costly sex—in this case ameristic male gametophytes. Thus, changes in the percentage of ameristic plants as a result of variation in nutrient availability in previous studies (Goodnoe et al., 2016; Chapter 2; T.T. Goodnoe and J.P. Hill, unpublished data; Chapter 3) align with the predictions of ESD theory. Nevertheless, the low nutrient demands of haploid organisms (Mable and Otto, 1998) in combination with small absolute plant size may result in gametophytes rarely experiencing nutrient limitation in real ecological contexts, consequently reducing or eliminating a strong sex

determination response based on variation in nutrient availability. Understanding the ecological relevance of ESD remains a challenging open question for fern gametophytes.

In nutrient-rich environments, emerging gametophytes are only sensitive to $A_{ce}$ for a short period of time directly following spore coat opening and must be exposed to $A_{ce}$ for 48 h consecutively in order for male induction to occur (Banks et al., 1993; Ganger et al., 2014). The timing of gametophyte response to $A_{ce}$ could result in developmental commitment to a sex before plants can evaluate abiotic factors within their environment. Given the differences in plant nutrient content observed here, early commitment to being meristic or ameristic could result in gametophyte populations where many individuals develop a phenotype that is not well-suited for the local environment. That is especially true if nutrient availability would eventually limit growth and development of meristic gametophytes, and thus decrease the ability of those plants to harbor viable sporophyte embryos.

In summary, sexual lability does not necessitate the simultaneous occurrence of ESD. If sex determination does not vary in response to nutrient availability or some other abiotic factor, ESD is not occurring in the conventional sense. Ayrapetov and Ganger (2009) concluded that reduced nutrient availability did not increase the frequency of male gametophytes in *C. richardii*, though they acknowledged that the nutrient concentrations used might not have induced nutrient limitation. If *C. richardii* gametophytes do not exhibit ESD even under certain nutrient-limiting conditions, labile sex expression still allows them to respond to $A_{ce}$ and population density while ignoring variation in the quality of the abiotic nutrient environment. Nutrient availability may still influence reproductive allocation and functional gender in gametophytes after the initial round of

sex determination (Ayrapetov and Ganger, 2009; Goodnoe et al., 2016; Chapter 2; T.T. Goodnoe and J.P. Hill, unpublished data; Chapter 3). Meristic gametophyte size, including photosynthetic area and total mass, as well as the number of antheridia and archegonia present may be nutrient-sensitive.

**References**


Aho, K. 2014. Asbio: A collection of statistical tools for biologists. R package version 1.1-1, http://cran.r-project.org/web/packages/asbio/index.html.

Atallah, N. M., and Banks, J. 2015. Reproduction and the pheromonal regulation of sex type in fern gametophytes. Frontiers in Plant Science. **6**(100). doi: 10.3389/fpls.2015.00100.

Ayrapetov, A., and Ganger, M. T. 2009. Nutrient levels do not affect male gametophyte induction by antheridiogen in *Ceratopteris richardii*. American Fern Journal. **99**(4): 273-278. doi: http://dx.doi.org/10.1640/0002-8444-99.4.273.

Banks, J. A. 1999. Gametophyte development in ferns. Annual Review of Plant Biology, **50**(1): 163-186. doi: 10.1146/annurev.arplant.50.1.163.

Banks, J. A., Hickok, L., and Webb, M. A. 1993. The programming of sexual phenotype in the homosporous fern *Ceratopteris richardii*. International Journal of Plant Sciences. **154**(4): 522-534.

Bartlett, M. S. 1937. Properties of sufficiency and statistical tests. Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences, **160**(901): 268–282.

Bull, J. J. 1983. Evolution of sex determining mechanisms. The Benjamin/Cummings Publishing Company, Inc..

C-Fern Web Manual. 2009. http://www.magrinscience.com/images/biology/ferns/C-Fern_Manual.pdf.

Charnov, E. L., and Bull, J. 1977. When is sex environmentally determined?. Nature. **266**: 828 - 830. doi:10.1038/266828a0

Chatterjee, A., and Roux, S. J. 2000. *Ceratopteris richardii*: a productive model for revealing secrets of signaling and development. Journal of Plant Growth Regulation. **19**(3): 284-289.

Cleveland, C. C., and Liptzin, D. 2007. C: N: P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass?. Biogeochemistry. **85**(3): 235-252.

Dyer, A. F. 1979. The experimental biology of ferns. Transactions of the Botanical Society of Edinburgh. **43**(2): 75-90. Taylor & Francis Group. doi: 10.1080/03746607908685341.

Elser, J. J., Sterner, R. W., Gorokhova, E., Fagan, W. F., Markow, T. A., Cotner, J. B., Harrison,J.F., Hobbie, S.E., Odell, G.M., and Weider, L. W. 2000. Biological stoichiometry from genes to ecosystems. Ecology Letters. **3**(6): 540-550. doi: 10.1111/j.1461-0248.2000.00185.x.

Ganger, M. T., Girouard, J. A., Smith, H. M., Bahny, B. A., & Ewing, S. J. 2014. Antheridiogen and abscisic acid affect conversion and ANI1 expression in Ceratopteris richardii gametophytes. Botany. **93**(2), 109-116

Gilbert, S.F. 2000. Environmental Sex Determination. Developmental Biology: 6th edition. Sunderland (MA): Sinauer Associates.

Goodnoe, T. T., Hill, J. P., and Aho, K. 2016. Effects of Variation in Carbon, Nitrogen, and Phosphorus Molarity and Stoichiometry on Sex Determination in the Fern *Ceratopteris richardii.* Botany. doi: 10.1139/cjb-2015-0187.

Hickok, L. G., Warne, T. R., and Fribourg, R. S. 1995. The biology of the fern *Ceratopteris* and its use as a model system. International Journal of Plant Sciences. **156**(3): 332-345.

Kaplan, F., Zhao, W., Richards, J. T., Wheeler, R. M., Guy, C. L., and Levine, L. H. 2012. Transcriptional and metabolic insights into the differential physiological responses of *Arabidopsis* to optimal and supraoptimal atmospheric $CO_2$. PloS One. **7**(8): e43583. doi: 10.1371/journal.pone.0043583.

Kimmerer, R. W. 2003. Gathering moss. A Natural and Cultural History of Mosses. Oregon State University Press. Corvallis.

Korpelainen, H. 1998. Labile sex expression in plants. Biological Reviews. **73**(2): 157-180. doi: 10.1111/j.1469-185X.1997.tb00028.x.

Mable, B. K., and Otto, S. P. 1998. The evolution of life cycles with haploid and diploid phases. BioEssays. **20**(6): 453-462.

Mathews, L., and Chandramohanakumar, N. 2003. The ratios of carbon, nitrogen, and phosphorus in a wetland coastal ecosystem of Southern India. International Review of Hydrobiology. **88**(2): 179-186. doi: 10.1002/iroh.200390013.

McGroddy, M. E., Daufresne, T., and Hedin, L. O. 2004. Scaling of C: N: P stoichiometry in forests worldwide: implications of terrestrial Redfield-type ratios. Ecology. **85**(9): 2390-2401.

Näf, U., Nakanishi, K., and Endo, M. 1975. On the physiology and chemistry of fern antheridiogens. The Botanical Review. **41**(3): 315-359.

Ramadan, A. A., A. El-Keblawy, K. W. Shaltout, and J. Lovett-Doust. 1994. Sexual polymorphism, growth and reproductive effort in Egyptian *Thymelaea hirsuta* (Thymelaeaceae). American Journal of Botany. **81**(7): 847–857.

Rau, G. 1978. Carbon-13 depletion in a subalpine lake: carbon flow implications. Science. **201**(4359): 901-902. doi: 10.1126/science.201.4359.901.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. [Computer program]. Available from http://www.R-project.org/.

Scott, R. J., and Hickok, L. G. 1987. Genetic analysis of antheridiogen sensitivity in *Ceratopteris richardii*. American Journal of Botany. **74**(12): 1872-1877.

Shaw, A. J., and Gaughan, J. F. 1993. Control of sex ratios in haploid populations of the moss, *Ceratodon purpureus*. American Journal of Botany. **80**(5): 584-591.

Stark, L. R., Mishler, B. D., and McLetchie, D. N. 2000. The cost of realized sexual reproduction: assessing patterns of reproductive allocation and sporophyte abortion in a desert moss. American Journal of Botany. **87**(11): 1599-1608.

Sterner, R. W., and Elser, J. J. 2002. Ecological stoichiometry: the biology of elements from molecules to the biosphere. Princeton University Press.

Tanaka, J., Yano, K., Aya, K., Hirano, K., Takehara, S., Koketsu, E., Ordonio, R.L., Park, S.H., Nakajima, M., Ueguchi-Tanaka, M., and Matsuoka, M. 2014. Antheridiogen determines sex in ferns via a spatiotemporally split gibberellin synthesis pathway. Science. **346**(6208): 469-473. doi: 10.1126/science.1259923.

Tanurdzic, M., and Banks, J. A. 2004. Sex-determining mechanisms in land plants. The Plant Cell Online. **16**(suppl. 1): S61-S71. doi: http://dx.doi.org/10.1105/tpc.016667.

Verspagen, J. M., Waal, D. B., Finke, J. F., Visser, P. M., and Huisman, J. 2014. Contrasting effects of rising $CO_2$ on primary production and ecological stoichiometry at different nutrient levels. Ecology letters. **17**(8): 951-960. doi: 10.1111/ele.12298.

Yamane, H. 1998. Fern antheridiogens. International Review of Cytology. **184**: 1-32. doi: 10.1016/S0074-7696(08)62177-4

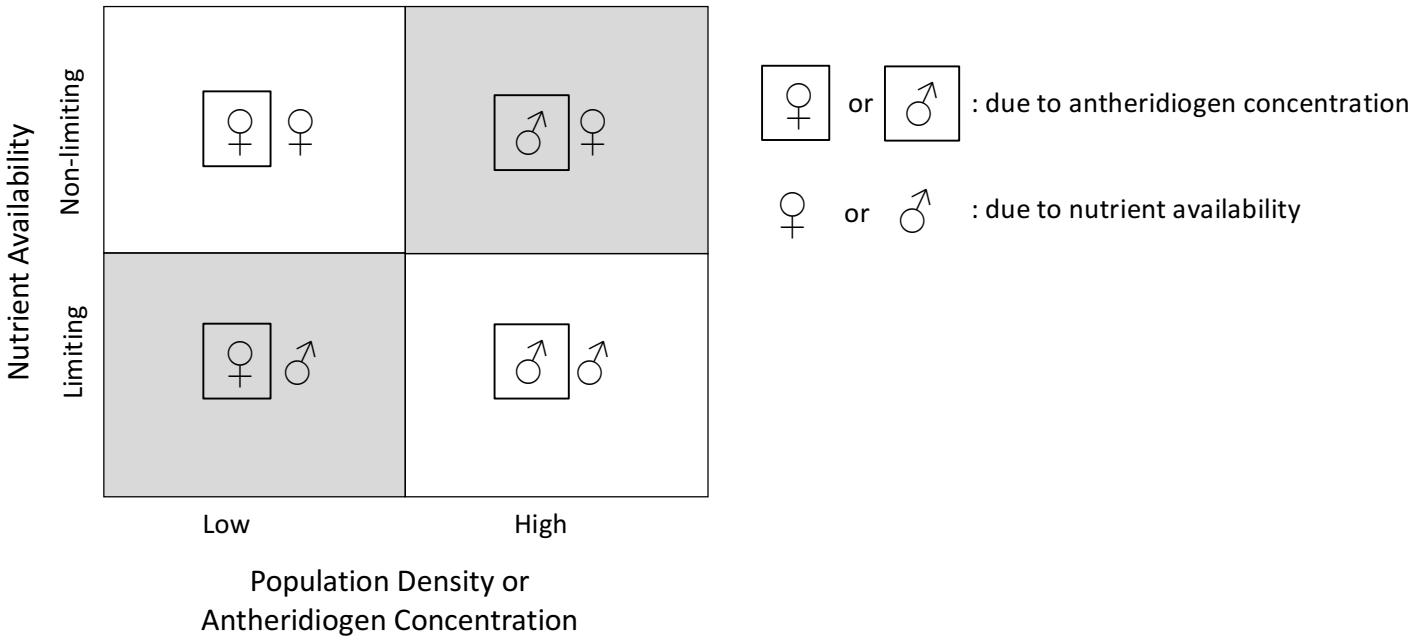



Figure 1.1- Hypothesis for how antheridiogen concentration and nutrient availability jointly influence determination as male or female (or hermaphrodite) in *Ceratopteris richardii* gametophytes when nutrient demands differ by sex. When grown in low antheridiogen concentrations and high nutrient availability, gametophytes are predicted to develop into females. When grown in high antheridiogen concentrations and low nutrient levels, more plants are expected to develop as males. However, when antheridiogen concentrations and nutrient availability are both high or are both low (gray cells), signals for sex expression are potentially in conflict.

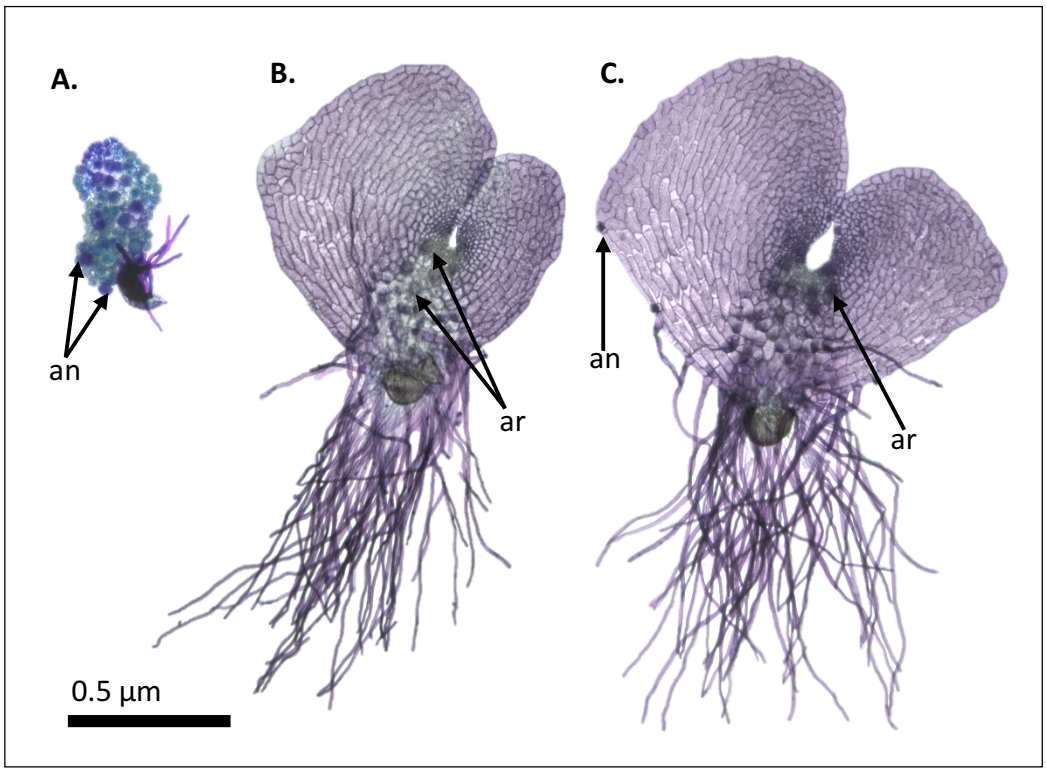



Figure 1.2- Representative (A.) male, (B.) female, and (C.) hermaphrodite gametophytes of *Ceratopteris richardii* cleared in 95% ethanol and stained with 0.01% aqueous toluidine blue. To become a female or hermaphrodite, the gametophyte must be meristic (i.e., possess a lateral meristem). Hermaphroditic gametophytes develop both archegonia (female gametangia; ar) and antheridia (male gametangia; an). Male gametophytes are ameristic (i.e., lack a meristem) and typically form many more antheridia than hermaphrodites. Scale bar = 0.5 µm.

# CHAPTER 2:

# EFFECTS OF VARIATION IN CARBON, NITROGEN, AND PHOSPHORUS MOLARITY AND STOICHIOMETRY ON SEX DETERMINATION IN THE FERN *CERATOPTERIS RICHARDII*


## Abstract

Carbon (C), nitrogen (N), and phosphorous (P) are needed by all organisms to perform basic biological processes. When an individual macronutrient is not accessible, nutrient limitation occurs. The balance between multiple nutrients and individual concentrations are both vital for normal growth and development. Labile sex expression in plants is a phenotypic trait predicted to be sensitive to local nutrient conditions because males and females differ in their nutritional demands. I applied concepts from ecological stoichiometry to assess effects of variation in individual nutrient concentration and multiple macronutrient stoichiometry on sexual development in the fern *Ceratopteris richardii*. Manipulation of N, P, and organic and inorganic C was expected to yield variation in the ratio of males to females, consistent with environmental sex determination theory. My results suggest nutrient stoichiometry, not strictly concentration, influences sex determination at ambient $CO_2$. However, an early response to population density preempted nutrient effects in elevated $CO_2$ environments with exogenous glucose, in which *C. richardii* gametophytes presumably grow naturally. Though sex determination is not nutrient dependent in the latter environment, C:N in dry mass of meristic gametophytes is influenced by the external nutrient context, suggesting sex determination takes place before abiotic environmental factors subsequently influence plant nutrient uptake.

## Introduction

The acquisition of macronutrients for growth, reproduction, and survival is a fundamental function of all organisms, including plants. The elements carbon (C), nitrogen (N), and phosphorous (P) typically make up the largest percentage of primary producer dry mass, and are required in characteristic stoichiometries for growth and development (Sterner and Elser, 2002; Austin and Vitousek, 2012; Sardans et al., 2012). Those proportions, however, are rarely in balance with the environment, because C:N:P ratios within organisms are tightly regulated through processes of differential uptake and release (Sterner and Elser, 2002; Hessen et al., 2013). Nevertheless, most organisms are not expected to be strictly homeostatic in terms of stoichiometry, allowing for adaptive responses to variable nutrient resource availability, like luxury consumption and storage for future use (Van Wijk et al., 2003; Wang et al., 2012). Additionally, rapidly growing organisms commonly have relatively low internal C:N and N:P ratios, due to an increased demand for P for ribosomal synthesis (Elser et al., 2007; Gibson et al., 2008). Smaller plants also have relatively lower N:P ratios (Elser et al., 2010).

A long-standing view is that plant fitness is limited by the quantity of the scarcest essential nutrient, as described by Liebig's Law of the Minimum (Danger et al., 2008). In that context, a nutrient-limited organism resumes growth once a limiting nutrient is reintroduced, until another nutrient becomes limiting (Bracken et al., 2015). Theoretical and empirical evidence suggest, however, that the balance of nutrients is often more influential than their absolute concentrations due to scarcity of multiple elements (Saito et al., 2008). When multiple nutrients become limiting in conjunction, co-limitation or multiple limitation results (Bloom et al., 1985; Harpole et al., 2011). Simultaneous co-

limitation is a special case of Liebig limitation (Harpole et al., 2011), occurring when the uptake of one nutrient is dependent on the availability of another (Saito et al., 2008). Consequently, the addition of a limiting nutrient will decrease the relative concentration of another co-limiting nutrient (Bracken et al., 2015). When access to all nutrients is limiting growth simultaneously, organisms will always be driven towards their optimum nutrient ratios (Knecht and Göransson, 2004; Ågren et al., 2012). When nutrients are at or above those optimal ratios, growth and development continue at rates determined by the chemical reactions intrinsic to metabolic pathways. As previously noted, optimal N:P ratios are not fixed, and often decline as growth rate increases (Elrifi and Turpin, 1985). At high growth rates organisms are more easily limited by P availability, but as growth rates slow N availability more often becomes limiting. Thus, the stoichiometric relationship between N and P is critical for regulating growth (Harpole et al., 2011).

The same principles of limitation that apply to N and P also apply to C, though the means of C acquisition by plants may complicate how such limitation affects growth and development. In the case of C-fixation in terrestrial vascular plants, $CO_2$ enters the intracellular leaf space through open stomata and moves through the cell walls of the mesophyll cells, to the sites of C fixation (Mooney, 1972). When N and P are available in excess, primary producers can become $CO_2$-limited, whereas increasing $CO_2$ concentrations could shift organisms from being C-limited to N- or P-limited (Verspagen et al., 2014). Additionally, many plants also possess the ability to consume sugars directly from their environment (Alongi et al., 2009; Graham et al., 2010; Yang et al., 2013), resulting in mixotrophic behavior (i.e., a mixed strategy of autotrophy and heterotrophy). The conditions that cause C-limitation may be different for mixotrophic

organisms, as mixotrophy may buffer against environmental variation in $CO_2$ availability (Katechakis et al., 2005).

Hypotheses about ecological stoichiometry and nutrient limitation at the whole-organism level can be readily tested empirically in plant populations with labile sex expression, because the sex ratio is predicted to be sensitive to the underlying ecological conditions. Environmental sex determination (ESD) theory predicts that environmental quality, including nutrient availability, influences determination as male or female once development has begun in species where gender is not genetically pre-determined (Bull, 1983; Korpelainen, 1998; Gilbert, 2000). Theoretical models predict that ESD is an adaptive trait when (1.) the costs of reproduction differ by gender, (2.) the environment is heterogeneous with respect to key abiotic factors, such as nutrients, and (3.) patches where reproduction occurs cannot be predicted by organisms when they first originate as zygotes or meiospores (Charnov and Bull, 1977; Bull, 1983). In the context of a finite nutrient budget, a certain fraction of resources is allocated to growth and another to reproduction (Zhang and Jiang, 2002), and this allocation differs between males and females (Charnov, 1982). A general expectation is that females commonly require higher nutrient levels to complete reproduction than male counterparts due to the elevated cost of post-zygotic investment in embryos by females (Haig and Westoby, 1988). Therefore, ESD can mitigate instances where female fecundity is likely to be environmentally limited due to resource availability by increasing the probability that more plants in a local environment will develop as males (Charnov and Bull, 1977; Stark et al., 2000).

Leptosporangiate fern gametophytes begin life as a single haploid cell that is sexually undetermined, eventually developing into a free-living female, hermaphrodite,

or male gametophyte (Klekowski, 1969; Raghavan, 2005). Sex determination in many fern gametophyte species is reliant on biotic interactions of local conspecifics, due to a response to the sex pheromone antheridiogen (Näf et al., 1975; Dyer, 1979; Scott and Hickok, 1987; Banks et al., 1993). Antheridiogen is a signaling molecule released by female and hermaphrodite gametophytes that contributes to mating system regulation as a function of population density by influencing undetermined individuals to develop as males (Korpelainen, 1998; Yamane, 1998; Banks, 1999; Tanurdzic and Banks, 2004; Tanaka et al., 2014; Atallah and Banks, 2015). A reaction to antheridiogen very early in gametophyte development may preempt any effects of environmental nutrient variation on sex determination. Tests for nutrient effects on fern gametophytes with antheridiogens have been conducted, providing support for nutrient-based ESD in some ferns (DeSoto et al., 2008), but not in others (Ayrapetov and Ganger, 2009). Additional research using ecological stoichiometric concepts and nutrient concentrations that emulate naturally occurring variation could help further demonstrate whether ferns generally exhibit adaptive responses based on the predictions made by ESD theory.

*Ceratopteris richardii* is a model homosporous fern species with a gametophyte phase that has a short time to sexual maturity and a well-characterized antheridiogen (Ceratopteris antheridiogen, or $A_{ce}$) system (Scott and Hickok, 1987; Hickok et al., 1995; Chatterjee and Roux, 2000). To date, the only test for nutrient effects on sex determination in *C. richardii* involved the moderate reduction in N and P availability, which had no effect on sex ratios (Ayrapetov and Ganger, 2009). This study extends that work, applying the concepts of ecological stoichiometry and the ideas of nutrient limitation to specifically address three questions with the *C. richardii* model system.

First, does variation in N and P molarity, N:P stoichiometry, or an interaction of the two across an ecologically relevant range elicit a sex determination response in a manner consistent with predictions made by ESD theory? Under that framework, an increased number of male gametophytes in the low or imbalanced nutrient treatments is expected. Second, does the same variation in N and P concentrations and N:P ratios alter sex determination under conditions with altered C availability? Environments with elevated $CO_2$ and/or added glucose may exhibit altered sex determination at all N:P ratios based on the principles of nutrient co-limitation. And third, does variation in C, N, and P concentration influence the estimated percent C or N of meristic gametophytes after sex determination has occurred? Meristic gametophytes grown in more nutrient-rich media would be expected to contain a relatively higher percentage of C and N than gametophytes grown in less nutrient-rich media. In general, I endeavored to study N, P, and C concentrations and balances likely to be experienced by gametophytes in nature, because those responses are expected to be shaped by selection as a result of reproductive success in natural environments.

**Materials and Methods**

*Experimental Design*

Ambient $CO_2$ Environment

Two 4x4 nutrient arrays were created consisting of 32 different nutrient combinations (Fig. 2.1A) in a background of 1/500 diluted basal salts medium (BSM) recommended for culturing *Ceratopteris richardii* gametophytes (C-Fern Web Manual, 2009). Each nutrient combination was replicated three times, for a total of 96 cultures. The range of C, N, and P levels was selected to emulate levels often found in natural environments with

the goal of evaluating plant responses in an ecologically relevant resource context (Cleveland and Liptzin, 2007; Mathews and Chandramohanakumar, 2003; McGroddy et al., 2004; Sterner and Elser, 2002). In one array, four levels each of N and P resulted in 16 molarities with 13 unique N:P stoichiometries. In a second array, 6 µM glucose ($C_6H_{12}O_6$) was added to the same N:P ratios used in the first array, resulting in 16 C:N:P resource medias. Ammonium nitrate ($NH_4NO_3$) concentrations were between 3.6 and 173.8 µM, and potassium phosphate ($KH_2PO_4$) concentrations were between 7.4 and 29.4 µM (Fig. 2.1A). The C:N:P and N:P combinations under study also allowed examination of molarity effects with and without changes in N:P stoichiometry, and link the work to the Redfield ratio (N:P = 16:1) described for aquatic systems (Redfield, 1958).

Dry, surface-sterilized spores of *C. richardii* were sown onto 60 mm petri dishes containing solid nutrient media with a sterile cotton swab, resulting in gametophyte populations of 9 to 36 individuals, and estimated densities between 0.2 and 0.6 plants/mm$^2$. Although there was no means to control individual plant spacing based on that sowing technique, any differences in plant densities varied randomly across the nutrient treatments, and total plant number was included as a concomitant variable in statistical analyses. Culture dishes were incubated in an environmental chamber (Percival Scientific, model E-30B, Boone, USA) for 12 d (30º C, photosynthetically active radiation (PAR) ~30 µmol m$^{-2}$ s$^{-1}$, 12 h photoperiod) after a 2 d induction under continuous light (30º C, PAR ~60 µmol m$^{-2}$ s$^{-1}$). Thus, plants were harvested a total of 14 d after sowing.

Each gametophyte was categorized as an ameristic male (lacking a lateral meristem and archegonia), a meristic female (the presence of a meristem and developing

archegonia) or a meristic hermaphrodite (archegonia and antheridia on a meristic plant) based on the Klekowski gender classification scheme (Klekowski, 1969). In the lowest concentration nutrient treatments, some gametophytes initiated development of a meristem, but insufficient nutrient availability halted the production of either archegonia or antheridia. Those plants were classified as meristic because of their commitment to meristem formation. The percentage of ameristic males was calculated for each dish.

Elevated $CO_2$ Environment

In the elevated $CO_2$ experiment, three levels of N and three levels of P were used to create five molarities with two unique N:P stoichiometries (N:P = 8 and 16; Fig. 2.1B). Five different N:P nutrient combinations and five C:N:P combinations (Fig. 2.1B) supplemented with 6 μM glucose were created in a background of 1/500 diluted basal salts media (BSM). Each nutrient combination was replicated six times, for a total of 60 cultures. The elevated $CO_2$ experiment was completed in two blocks. Non-significant block effects ($\alpha = 0.05$) prompted combining the two blocks in further analyses. Ammonium nitrate concentrations were between 57.9 and 173.8 μM, and potassium phosphate concentrations between 7.4 and 29.4 μM (Fig. 2.1B).

The same sowing technique used in the previous experiment was implemented again, resulting in culture populations between 14 and 64 individuals, and densities between 0.3 and 1.6 plants/$mm^2$. Culture dishes were incubated in the same manner as previously described. Plates were located in a near-airtight acrylic chamber environment with a $CO_2$ concentration of 1,300 ppm. $CO_2$ was increased to 1,300 ppm by injection of pure $CO_2$ gas, in order to reach super-elevated concentrations (>1,200 ppm) that are expected to induce responses distinctive from those observed at moderately elevated $CO_2$

concentrations (400-1,200 ppm) (Kaplan et al., 2012). Levels of $CO_2$ were periodically measured with a wireless NODE $CO_2$ sensor (Variable Inc., Chattanooga, TN, USA). The chamber was periodically opened to replace the NODE batteries, and $CO_2$ was re-injected. Phenotypic results were evaluated as previously described in the ambient $CO_2$ experiment.

All meristic gametophytes from each plate were harvested from media and dried in a drying oven at 50° C for 48 h. Gametophytes from each replicate of the same nutrient treatment were combined in order to increase the amount of dry mass to achieve a target weight of 0.16 mg, which was subsequently used for elemental analysis. Stable-isotope analysis of percent C and N in dry gametophyte tissue was analyzed at the Idaho State University Interdisciplinary Laboratory for Elemental and Isotopic Analysis (ILEIA) with a Costech ECS 4010 elemental analyzer interfaced to a Thermo Delta V Advantage continuous flow isotope ratio mass spectrometer. Samples were standardized with certified peach leaf samples (average %N = 2.94 ± 0.12).

*Statistical Analyses*

Nutrient Effects on Sex Determination

Contour plots graphically describing the change in percent ameristic males at each N:P treatment were created for the ambient $CO_2$ experiment. Contour plots could not be created for the elevated $CO_2$ experiment because only five N:P treatments were evaluated. To assess molarity effects, a "molarity score" for each culture was created by adding the relative number of N atoms to the number of P atoms in each N:P combination (e.g., a molarity score of 17 corresponded to an N:P ratio of 16:1). The ambient and elevated $CO_2$ experiments were analyzed separately. The effects of nutrient stoichiometry

and molarity on percent ameristic males were tested with an analysis of covariance (ANCOVA). Total plant number served as the concomitant variable to distinguish the effect of random differences in population size from the effect of (C):N:P resource treatments on percent ameristic males. A plot of percent ameristic males as a function of total plant number had a slightly positive slope (see Supplementary Material, Fig. S1). Because changes in stoichiometry are not independent of changes in molarity, the effect of the interaction of those factors on percent ameristic males was considered in a separate preliminary ANCOVA model. In addition to a concomitant variable defining gametophyte density, this model contained a categorical predictor that merged possible combinations of stoichiometry and molarity under consideration into a single interaction factor. Significance of that interaction at $\alpha = 0.05$ precluded consideration of molarity and stoichiometry main effects. Following this preliminary step, a specific model selection process was implemented to determine the most appropriate ANCOVA model (see Supplementary Material, Fig. S2). Assumptions of ANCOVA (e.g., equal slopes, and general linear model constraints) were met for all analyses (for diagnostic plots see Supplementary Material, Fig. S3, S4). Given significant interactions, comparisons of particular factor-level combinations of interest that were defined *post hoc* were performed. Specifically, because I expected a higher percentage of males in the less nutrient-rich media, upper-tailed, pooled variance *t*-tests were implemented to compare nutrient treatments with N:P stoichiometries 8 and 16, with significance at $\alpha = 0.05$. Results from those tests were adjusted for family-wise type I error using the Bonferroni correction method.

Effects on Gametophyte C and N

To analyze the effects of C, N, and P concentration on estimated percent C mass, an ANCOVA again was was used with sample size as the concomitant variable, in order to assure samples were uniform across all treatments. Akaike Information Criterion (AIC; Akaike, 1973) was used to identify the most parsimonious model for evaluating nutrient effects on estimated gametophyte percent N. The original model included C, N, and P concentrations, as well as sample weight. The optimal approximating model, according to AIC, included N and P concentration only.

All statistical analyses were performed using the R computational environment (R Core Team, 2014) with heavy reliance on the package asbio (Aho, 2014). Datasets examined here have been archived as SexDeterm, in the R-package asbio (https://cran.r-project.org/web/packages/asbio/index.html).

**Results**

*Ambient $CO_2$*

At ambient $CO_2$ in the absence of glucose the percentage of *Ceratopteris richardii* ameristic males was not affected by variation in the interaction between molarity and stoichiometry. In a subsequent analysis for main effects, N:P stoichiometry was statistically significant for the percentage of ameristic males at $\alpha = 0.1$, while nutrient molarity appeared unimportant (Table 2.1). Total plant number, the concomitant variable included in the analysis, strongly affected percent ameristic males (Table 2.1).

In the presence of glucose, stoichiometry-molarity interactions affected the percentage of amersistic males (Table 2.1), precluding examination of main effects in this context. The concomitant predictor, total plant number, was also significant (Table 2.1).

Upper-tailed, pooled variance *t*-tests indicated that the percentage of ameristic males in C:N:P treatment 5:16:1 was significantly higher than in treatments 5:16:2 ($t_4 = 3.83$, *P*-value = 0.009) and 5:32:2 ($t_4 = 3.58$, *P*-value = 0.0345) at $\alpha = 0.05$, and higher than 5:48:3 ($t_4 = 2.26$, *P*-value = 0.087) at $\alpha = 0.1$.

*Elevated $CO_2$*

At 1,300 ppm $CO_2$, the percentage of ameristic males was not influenced by the interaction of stoichiometry and molarity, either with or without elevated glucose, allowing consideration of main effects in both scenarios. In the absence of glucose N:P stoichiometry was statistically significant in determining percent ameristic males at $\alpha = 0.1$, whereas molarity did not influence the percentage of ameristic males (Table 2.1). When glucose was present, neither stoichiometry nor molarity affected the percentage of ameristic males (Table 2.1). Sex determination was not as strongly affected by the concomitant variable, total number of plants, with or without elevated glucose (Table 2.1).

Percent C in the dry mass of gametophytes cultured in elevated $CO_2$ was not influenced by N or P concentration, nor the presence or absence of glucose. Furthermore, variation in sample weight did not affect percent C estimates. Percent N in dry mass was only influenced by the concentration of N within the medium ($F_{1,17} = 8.1$, *P*-value = 0.011). As N concentration within the media increased, the estimated percent N in gametophyte biomass increased (slope = 0.022, $R^2 = 0.248$). Therefore, gametophyte C:N ratio decreased as a result of increases in media N concentration. The C:N ratio of the media did not affect percent C or N of gametophytes. The mean gametophyte C:N ratio was ( $\bar{x} \pm 1\ SEM$) 9.17 ± 0.76.

## Discussion

### *Effects of Macronutrients on Sex Determination*

N and P are among the most frequently limiting elements in both aquatic and terrestrial environments (Güsewell, 2004; Vitousek et al., 2010; Chapin et al., 2011; Harpole et al., 2011; Sardans et al., 2012), with limitation occurring regularly throughout the biosphere (Elser et al., 2007). In the present study, the percentage of ameristic *Ceratopteris richardii* male gametophytes did not increase with decreasing N concentration or P concentration at the levels tested in either ambient or elevated $CO_2$ environments in the absence of exogenous glucose. My results did, however, suggest a trend consistent with the hypothesis that stoichiometry can prompt environmental sex determination, but only when glucose is not present. An effect of stoichiometry on variation in the percentage of ameristic males suggests the balance of nutrients may influence sex determination more than individual nutrient concentrations when N and P are present in micromolar concentrations. Ayrapetov and Ganger (2009) concluded that nutrient limitation via serial dilution of standard C-fern media (where the least concentrated media was 62.5 times stronger than my least concentrated medium) did not affect the probability of male induction in *C. richardii*, although they acknowledged that the nutrient concentrations might have been too high to attain any limitation effects. Integrating those lines of experimental evidence suggests that gametophytes have fairly low requirements for exogenous nutrients in absolute terms, but ecologically austere nutrient environments can still elicit ESD consistent with theoretic predictions. Variation in macronutrient stoichiometry may be especially important at very low macronutrient nutrient molarities.

At ambient $CO_2$ with added glucose, variation in the interaction between nutrient molarity and stoichiometry influenced the percentage of males, indicating that the effects of absolute concentration and the balance of nutrients are interrelated. In addition, the percentage of ameristic males in treatment 5:16:1 was significantly higher than in 5:32:2, 5:48:3, and 5:16:2, suggesting P-limitation occurred in the 5:16:1 treatment. It is possible that simultaneous co-limitation was also occurring, due to the addition of both N and P in the more nutrient-rich medias, resulting in a synergistic response (Harpole et al., 2011). In that case, nutrient molarity and stoichiometry were both influencing determination as ameristic or meristic. Ghosh et al. (2012) and DeSoto et al. (2008) both concluded that nutrient availability influenced sex determination in other fern species, with nutrient poor environments resulting in a higher percentage of male gametophytes. However, effects of stoichiometric variation on sex determination in fern gametophytes with antheridiogen systems have not previously been directly assessed with the approach utilized here. Because macronutrients are needed in specific stoichiometries and multiple nutrients can become limiting concurrently, the examination of stoichiometric effects on development could be critical for establishing whether nutrients affect labile sexual systems in ferns.

In addition to antheridiogen sensitivity and nutrient availability, gametophyte sex determination may depend partly on variation in spore size (Verma and Selvan, 2001; Ganger and Sturey, 2012). In homosporous plants, a pool of nutrients provided to the gametophyte in the spore cytoplasm is available during germination. Spores of *C. richardii* are among the largest of homosporous ferns (Haig and Westoby, 1988), and are roughly 65% C and < 1% N (J.P. Hill, T.T. Goodnoe, and K. Boydstun, unpublished data). C availability in the spore may allow early gametophyte development to remain

initially insensitive to extracellular C concentrations, but the relatively small amount of N available inside the spore might mean that some of the N needed for growth and development must come from the environment. Though *C. richardii* is a homosporous fern species, variation in spore size is common (Ganger and Sturey, 2012). Significant variation in the size of the N pool in the cytoplasm could consequently be a key factor for sex determination in a species like *C. richardii* when nutrients are limiting. Small initial spore size may result in ameristic males or small meristic gametophytes owing to initial nutrient limitation, whereas gametophytes that germinate from large spores may escape this constraint. Variation in spore size did not predict gender when grown on full strength C-fern media (Ganger and Sturey; 2012), but the effects of spore size under more stringent nutrient-limiting conditions remain to be investigated.

*The Importance of Carbon: Glucose and Elevated $CO_2$*

The consumption of exogenous sugars from the environment may affect sex determination. Guillon and Raquin (2002) demonstrated that the addition of glucose and sucrose to the growth medium resulted in an increased number of male gametophytes in the non-antheridiogen genus *Equisetum*. However, much of that work used sugar concentrations in the millimolar range (Guillon and Raquin, 2002), far beyond what is reported in natural soils—generally < 50 μM (van Hees et al., 2005; Alongi et al., 2009; Hill et al., 2011). Quantitative comparison of the glucose treatments suggests that the addition of ecologically relevant concentrations of glucose in ambient $CO_2$ causes the interaction between molarity and N:P stoichiometry to alter $A_{ce}$-based sex determination in *C. richardii*.

It is not yet clear how the incorporation of exogenous carbon causes overall nutrient use to shift either $A_{ce}$ sensitivity or the rate of $A_{ce}$ secretion, though one possibility is that gametophytes may be C-limited in ambient $CO_2$ conditions. When given access to organic C in their environment at ambient $CO_2$ concentrations, plants may shift from being C-limited to being limited by a different nutrient, such as N or P, as explained by Liebig's law of the minimum (Hiddink and Kaiser, 2005). A key principle of ecological stoichiometry is that organisms incorporate nutrients in specific combinations, so the introduction of glucose may be influencing the amount of P required for growth and development. Glucose is also a well-known signaling molecule (Rolland et al., 2002; Moore et al., 2003; Yang et al., 2013), potentially altering growth and reproduction of gametophytes in ways not strictly related to Ace or nutritional requirements.

*C. richardii* gametophytes are sufficiently small to live directly in a gaseous boundary layer at the soil-air interface (or soil-water interface, if in an aquatic environment (Rau, 1978)), where $CO_2$ concentrations may commonly exceed the average atmospheric concentration by as much as 10 times owing to soil microbial respiration and low rates of air mixing (Kimmerer, 2003). Therefore, gametophytes may be adapted to elevated and potentially variable levels of $CO_2$, which could alter plant C:N:P stoichiometry (Verspagen et al., 2014). If the $CO_2$ concentrations normally encountered by gametophytes are actually higher than ambient atmospheric concentrations, ESD experiments done in vitro that neglect elevating $CO_2$ could be eliciting developmental responses caused by unnaturally low concentrations of inorganic carbon. If C availability

is limiting plant growth in a manner consistent with nutrient limitation theory, addition of $CO_2$ may allow gametophytes to more efficiently acquire and use available N and P. The natural gametophyte environment may also include micro-molar concentrations of sugars. In the present study, the experimental treatment that simultaneously supplemented both organic and inorganic C pools did not result in ESD as a result of nutrient variation in *C. richardii*. The addition of $CO_2$ and glucose in conjunction potentially decreased the constraint of C-limitation, consequently allowing gametophytes to escape macronutrient limitation effects. However, resultant meristic gametophytes from different treatments had different internal C:N ratios at 14 days after sowing. The ultimate differences in dry biomass stoichiometry were due to increased N uptake in treatments with higher exogenous N concentrations. The C:N ratios of gametophytes grown at elevated $CO_2$ in this study were much lower than C:N of gametophytes grown at ambient $CO_2$ (Goodnoe and Hill, unpublished data), indicating that N assimilation increased in high $CO_2$ environments. That response is unlike what has been observed in angiosperms (Conroy and Hocking, 1993; Cotrufo et al., 1998), where C:N ratios generally increase as a result of increased atmospheric $CO_2$ concentrations.

*Conclusions*

It is possible that the low nutrient demands of small haploid organisms (Mable and Otto, 1998), as well as adaptation to the boundary layer, allows *C. richardii* gametophytes to escape nutrient limitation and assimilate nutrients in excess of their immediate needs, consequently mitigating a sex determination response to variation in nutrient availability when grown in an elevated $CO_2$ environment. Although the N and P nutrient ratios and concentrations examined represent available levels commonly found in natural

environments, N and P did not appear to be limiting when $CO_2$ was elevated. Consequently, in order to further test the hypothesis that the nutrient environment causes ESD in *C. richardii*, macronutrient concentrations need to be reduced to growth-limiting levels. If determination as male or female in *C. richardii* gametophytes is not dependent on nutrient availability, even at levels that limit plant growth, sex determination is not truly environmentally regulated in the conventional sense. Nevertheless, those conclusions may not be applicable to all fern species with antheridiogen systems, because of the vast diversity in spore size, growth rate, timing of sex determination, and nutritional demands.

**References**

Aho, K. 2014. Asbio: A collection of statistical tools for biologists. R package version 1.1-1, http://cran.r-project.org/web/packages/asbio/index.html.

Akaike, H. 1973. Maximum likelihood identification of Gaussian autoregressive moving average models. Biometrika, **60**(2), 255-265.

Alongi, D. A., Hill, J. P., and Germino, M. J. 2009. Opportunistic heterotrophy in gametophytes of the homosporous fern *Ceratopteris richardii*. Botany. **87**(8): 799-806.

Atallah, N. M., and Banks, J. 2015. Reproduction and the pheromonal regulation of sex type in fern gametophytes. Frontiers in Plant Science. **6**(100). doi: 10.3389/fpls.2015.00100.

Austin, A. T., and Vitousek, P. M. 2012. Introduction to a Virtual Special Issue on ecological stoichiometry and global change. New Phytologist. **196**(3): 649-651. doi: 10.1111/j.1469-8137.2012.04376.x. PMID:23043585.

Ayrapetov, A., and Ganger, M. T. 2009. Nutrient levels do not affect male gametophyte induction by antheridiogen in *Ceratopteris richardii*. American Fern Journal. **99**(4): 273-278. doi: http://dx.doi.org/10.1640/0002-8444-99.4.273.

Banks, J. A. 1999. Gametophyte development in ferns. Annual review of plant biology, **50**(1): 163-186. doi: 10.1146/annurev.arplant.50.1.163.

Banks, J. A., Hickok, L., and Webb, M. A. 1993. The programming of sexual phenotype in the homosporous fern *Ceratopteris richardii*. International Journal of Plant Sciences. **154**(4): 522-534.

Bloom, A. J., Chapin, F. S., and Mooney, H. A. 1985. Resource limitation in plants--an economic analogy. Annual review of Ecology and Systematics. **16**: 363-392.

Bracken, M. E. S., Hillebrand, H., Borer, E. T., Seabloom, E. W., Cebrian, J., Cleland, E. E., Elser, J. J., Gruner, D. S., Harpole, W. S., Ngai, J. T. and Smith, J. E. 2015. Signatures of nutrient limitation and co-limitation: responses of autotroph internal nutrient concentrations to nitrogen and phosphorus additions. Oikos. **124**(2): 113–121. doi: 10.1111/oik.01215

Bull, J. J. 1983. Evolution of sex determining mechanisms. The Benjamin/Cummings Publishing Company, Inc..

C-Fern Web Manual. 2009. http://www.magrinscience.com/images/biology/ferns/C-Fern_Manual.pdf.

Chapin, F. S., III, Matson, P. A., and Vitousek, P. 2011. Principles of terrestrial ecosystem ecology. Springer Science & Business Media. doi: 10.1007/978-1-4419-9504-9.

Charnov, E.L. 1982. The Theory of Sex Allocation. *Princeton University Press*, Princeton.

Charnov, E. L., and Bull, J. 1977. When is sex environmentally determined?. Nature. **266**: 828 - 830. doi:10.1038/266828a0. PMID:865602.

Chatterjee, A., and Roux, S. J. 2000. *Ceratopteris richardii*: a productive model for revealing secrets of signaling and development. Journal of Plant Growth Regulation. **19**(3): 284-289.

Cleveland, C. C., and Liptzin, D. 2007. C: N: P stoichiometry in soil: is there a “Redfield ratio” for the microbial biomass?. Biogeochemistry. **85**(3): 235-252.

Conroy, J., and Hocking, P. 1993. Nitrogen nutrition of C3 plants at elevated atmospheric $CO_2$ concentrations. Physiologia Plantarum. **89**(3): 570-576. doi: 10.1111/j.1399-3054.1993.tb05215.x.

Cotrufo, M. F., Ineson, P., and Scott, A. 1998. Elevated $CO_2$ reduces the nitrogen concentration of plant tissues. Global Change Biology. **4**(1): 43-54.

Danger, M., Daufresne, T., Lucas, F., Pissard, S. and Lacroix, G. 2008, Does Liebig's law of the minimum scale up from species to communities?. Oikos. **117**(11): 1741–1751. doi: 10.1111/j.1600-0706.2008.16793.x

DeSoto, L., Quintanilla, L. G., and Méndez, M. 2008. Environmental sex determination in ferns: effects of nutrient availability and individual density in *Woodwardia radicans*. Journal of Ecology. **96**(6): 1319-1327. doi: 10.1111/j.1365-2745.2008.01425.x.

Dyer, A. F. 1979. The experimental biology of ferns. Transactions of the Botanical Society of Edinburgh. **43**(2): 75-90. Taylor & Francis Group. doi: 10.1080/03746607908685341.

Elrifi, I. R., and Turpin, D. H. 1985. Steady-state luxury consumption and the concept of optimum nutrient ratios: s study with phosphate and nitrate limited *Selenastrum minutum* (Chlorophyta). Journal of phycology. **21**(4): 592-602. doi: 10.1111/j.0022-3646.1985.00592.x.

Elser, J. J., Sterner, R. W., Gorokhova, E., Fagan, W. F., Markow, T. A., Cotner, J. B., Harrison,J.F., Hobbie, S.E., Odell, G.M., and Weider, L. W. 2000. Biological stoichiometry from genes to ecosystems. Ecology Letters. **3**(6): 540-550. doi: 10.1111/j.1461-0248.2000.00185.x.

Elser, J. J., Bracken, M. E., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., Ngai, J.T., Seabloom, E.W., Shurin, J.B., and Smith, J. E. 2007. Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. Ecology letters. **10**(12): 1135-1142. doi: 10.1111/j.1461-0248.2007.01113.x.

Elser, J.J., Fagan, W.F., Kerkhoff, A.J., Swenson, N.G., and Enquist, B.J. 2010. Biological stoichiometry of plant produc- tion: metabolism, scaling and ecological response to global change. New Phytol. 186(3): 593– 608. doi:10.1111/j.1469-8137. 2010.03214.x. PMID:20298486.

Ganger, M., and Sturey, T. 2012. Antheridiogen concentration and spore size predict gametophyte size in *Ceratopteris richardii*. Botany. **90**(3): 175-179.

Ghosh, L., Jiménez, A., and Quintanilla, L. 2012. Effect of Nutrients on Environmental Sex Determination and Size of Gametophytes in Culcita macrocarpa [online]. Journal of Life and Earth Science. **7**. doi: http://dx.doi.org/10.3329/jles.v7i0.20130.

Gibson, R. N., Atkinson, R. J. A., and Gordon, J. D. M. 2008. Use, abuse, misconceptions and insights from quota models—the droop cell quota model 40 years on. Oceanography and Marine Biology: An Annual Review. **46**: 1-23.

Gilbert, S.F. 2000. Environmental Sex Determination. Developmental Biology. 6th ed. Sinauer Associates, Sunderland, Mass.

Graham, L. E., Kim, E., Arancibia-Avila, P., Graham, J. M., and Wilcox, L. W. 2010. Evolutionary and ecophysiological significance of sugar utilization by the peat moss *Sphagnum compactum* (Sphagnaceae) and the common charophycean associates *Cylindrocystis brebissonii* and *Mougeotia* sp. (*Zygnemataceae*). American journal of botany. **97**(49): 1485-1491.

Guillon, J. M., and Raquin, C. 2002. Environmental sex determination in the genus Equisetum: Sugars induce male sex expression in cultured gametophytes. International journal of plant sciences. **163**(5): 825-830. doi: 10.1086/341229.

Güsewell, S. 2004. N: P ratios in terrestrial plants: variation and functional significance. New phytologist. **164**(2): 243-266.

Haig, D., and Westoby, M. 1988. A model for the origin of heterospory. Journal of Theoretical Biology. **134**(2): 257-272. doi: 10.1016/S0022-5193(88)80203-0.

Harpole, W.S., Ngai, J.T., Cleland, E.E., Seabloom, E.W., Borer, E.T., Bracken, M.E., Elser, J.J., Gruner, D.S., Hillebrand, H., Shurin, J.B., and Smith, J.E. 2011. Nutrient co-limitation of primary producer communities. Ecology Letter. **14**(9): 852-862. doi:10.1111/j.1461- 0248.2011.01651.x. PMID:21749598.

Hessen, D. O., Elser, J. J., Sterner, R. W., and Urabe, J. 2013. Ecological stoichiometry: An elementary approach using basic principles. Limnology and Oceanography. **58**(6): 2219-2236. doi: 10.4319/lo.2013.58.6.2219.

Hickok, L. G., Warne, T. R., and Fribourg, R. S. 1995. The biology of the fern *Ceratopteris* and its use as a model system. International Journal of Plant Sciences. **156**(3): 332-345.

Hiddink, J. G., and Kaiser, M. J. 2005. Implications of Liebig's law of the minimum for the use of ecological indicators based on abundance. Ecography. **28**(2): 264-271. doi: 10.1111/j.0906-7590.2005.04063.x.

Hill, J. P., Germino, M. J., and Alongi, D. A. 2011. Carbon-use efficiency in green sinks is increased when a blend of apoplastic fructose and glucose is available for uptake. Journal of Experimental Botany. **62**: 2013-2022. doi: 10.1093/jxb/erq407.

Kaplan, F., Zhao, W., Richards, J. T., Wheeler, R. M., Guy, C. L., and Levine, L. H. 2012. Transcriptional and metabolic insights into the differential physiological responses of *Arabidopsis* to optimal and supraoptimal atmospheric $CO_2$. PloS one. **7**(8): e43583. doi: 10.1371/journal.pone.0043583.

Katechakis, A., Haseneder, T., Kling, R., and Stibor, H. 2005. Mixotrophic versus photoautotrophic specialist algae as food for zooplankton: The light: nutrient hypothesis might not hold for mixotrophs. Limnology and oceanography. **50**(4): 1290-1299. doi: 10.4319/lo.2005.50.4.1290

Kimmerer, R. W. 2003. Gathering moss. A Natural and Cultural History of Mosses. Oregon State University Press. Corvallis.

Klekowski, E. J. 1969. Reproductive biology of the *Pteridophyta. II*. Theoretical considerations. Botanical Journal of the Linnean Society. **62**(3): 347-359. doi: 10.1111/j.1095-8339.1969.tb01972.x.

Knecht, M. F., and Göransson, A. 2004. Terrestrial plants require nutrients in similar proportions. Tree physiology. **24**(4): 447-460. doi: 10.1093/treephys/24.4.447.

Korpelainen, H. 1998. Labile sex expression in plants. Biological Reviews. **73**(2): 157-180. doi: 10.1111/j.1469-185X.1997.tb00028.x.

Mable, B. K., and Otto, S. P. 1998. The evolution of life cycles with haploid and diploid phases. BioEssays. **20**(6): 453-462.

Mathews, L., and Chandramohanakumar, N. 2003. The ratios of carbon, nitrogen, and phosphorus in a wetland coastal ecosystem of Southern India. International Review of Hydrobiology. **88**(2): 179-186. doi: 10.1002/iroh.200390013.

McGroddy, M. E., Daufresne, T., and Hedin, L. O. 2004. Scaling of C: N: P stoichiometry in forests worldwide: implications of terrestrial Redfield-type ratios. Ecology. **85**(9): 2390-2401.

Mooney, H. A. 1972. The carbon balance of plants. Annual review of ecology and systematics. **3**:315-346.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W. H., Liu, Y. X., Hwang, I., Jones, T., and Sheen, J. 2003. Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science. **300**(5617): 332-336. doi: 10.1126/science.1080585.

Näf, U., Nakanishi, K., and Endo, M. 1975. On the physiology and chemistry of fern antheridiogens. The botanical review. **41**(3): 315-359.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. [Computer program]. Available from http://www.R-project.org/.

Raghavan, V. 2005. Developmental biology of fern gametophytes. Cambridge University Press.

Rau, G. 1978. Carbon-13 depletion in a subalpine lake: carbon flow implications. Science. **201**(4359): 901-902. doi: 10.1126/science.201.4359.901.

Redfield, A. C. 1958. The biological control of chemical factors in the environment. American Scientist. **46**(3): 205-221.

Rolland, F., Moore, B., and Sheen, J. 2002. Sugar sensing and signaling in plants. The Plant Cell Online. **14**(supp1): S185-S205. doi: http://dx.doi.org/10.1105/tpc.010455.

Saito, M. A., Goepfert, T. J., and Ritt, J. T. 2008. Some thoughts on the concept of colimitation: three definitions and the importance of bioavailability. Limnology and Oceanography. **53**(1): 276-290.

Chicago.

Sardans, J., Rivas-Ubach, A., and Peñuelas, J. 2012. The elemental stoichiometry of aquatic and terrestrial ecosystems and its relationships with organismic lifestyle and ecosystem structure and function: a review and perspectives. Biogeochemistry. **111**(1-3): 1-39.

Scott, R. J., and Hickok, L. G. 1987. Genetic analysis of antheridiogen sensitivity in *Ceratopteris richardii*. American Journal of Botany. **74**(12): 1872-1877.

Stark, L. R., Mishler, B. D., and McLetchie, D. N. 2000. The cost of realized sexual reproduction: assessing patterns of reproductive allocation and sporophyte abortion in a desert moss. American Journal of Botany. **87**(11): 1599-1608.

Sterner, R. W., and Elser, J. J. 2002. Ecological stoichiometry: the biology of elements from molecules to the biosphere. Princeton University Press.

Tanaka, J., Yano, K., Aya, K., Hirano, K., Takehara, S., Koketsu, E., Ordonio, R.L., Park, S.H., Nakajima, M., Ueguchi-Tanaka, M., and Matsuoka, M. 2014. Antheridiogen determines sex in ferns via a spatiotemporally split gibberellin synthesis pathway. Science. **346**(6208): 469-473. doi: 10.1126/science.1259923.

Tanurdzic, M., and Banks, J. A. 2004. Sex-determining mechanisms in land plants. The Plant Cell Online. **16**(sup 1): S61-S71. doi: http://dx.doi.org/10.1105/tpc.016667.

van Hees, P. A., Jones, D. L., Finlay, R., Godbold, D. L., and Lundström, U. S. 2005. The carbon we do not see—the impact of low molecular weight compounds on carbon dynamics and respiration in forest soils: a review. Soil Biology and Biochemistry. **37**(1): 1-13. doi: 10.1016/j.soilbio.2004.06.010.

Van Wijk, M. T., Williams, M., Gough, L., Hobbie, S. E., and Shaver, G. R. 2003. Luxury consumption of soil nutrients: a possible competitive strategy in above-ground and below-ground biomass allocation and root morphology for slow-growing arctic vegetation?. Journal of Ecology. **91**(4): 664-676.

Verma, S. C., and Selvan, P. M. 2001. Intraspecific variation in spore-size of homosporous ferns and its implications on fern mating systems. Bionature. **21**: 1-9.

Verspagen, J. M., Waal, D. B., Finke, J. F., Visser, P. M., and Huisman, J. 2014. Contrasting effects of rising $CO_2$ on primary production and ecological stoichiometry at different nutrient levels. Ecology letters. **17**(8): 951-960. doi: 10.1111/ele.12298.

Vitousek, P. M., Porder, S., Houlton, B. Z., and Chadwick, O. A. 2010. Terrestrial phosphorus limitation: mechanisms, implications, and nitrogen-phosphorus interactions. Ecological applications. **20**(1): 5-15. doi: http://dx.doi.org/10.1890/08-0127.1.

Wang, H., Sterner, R. W., and Elser, J. J. 2012. On the “strict homeostasis” assumption in ecological stoichiometry. Ecological Modelling. **243**: 81-88. doi: 10.1016/j.ecolmodel.2012.06.003.

Yamane, H. 1998. Fern antheridiogens. International Review of Cytology. **184**: 1-32. doi: 10.1016/S0074-7696(08)62177-4.

Yang, L., Xu, M., Koo, Y., He, J., and Poethig, R. S. 2013. Sugar promotes vegetative phase change in *Arabidopsis thaliana* by repressing the expression of MIR156A and MIR156C. Elife. **2**: e00260. doi: http://dx.doi.org/10.7554/eLife.00260.

Zhang, D. Y., and Jiang, X. H. 2002. Size-dependent resource allocation and sex allocation in herbaceous perennial plants. Journal of Evolutionary Biology. **15**(1): 74-83. doi: 10.1046/j.1420-9101.2002.00369.x.

Ågren, G. I., Wetterstedt, J. Å., and Billberger, M. F. 2012. Nutrient limitation on terrestrial plant growth–modeling the interaction between nitrogen and phosphorus. New Phytologist. **194**(4): 953-960. doi: 10.1111/j.1469-8137.2012.04116.x.

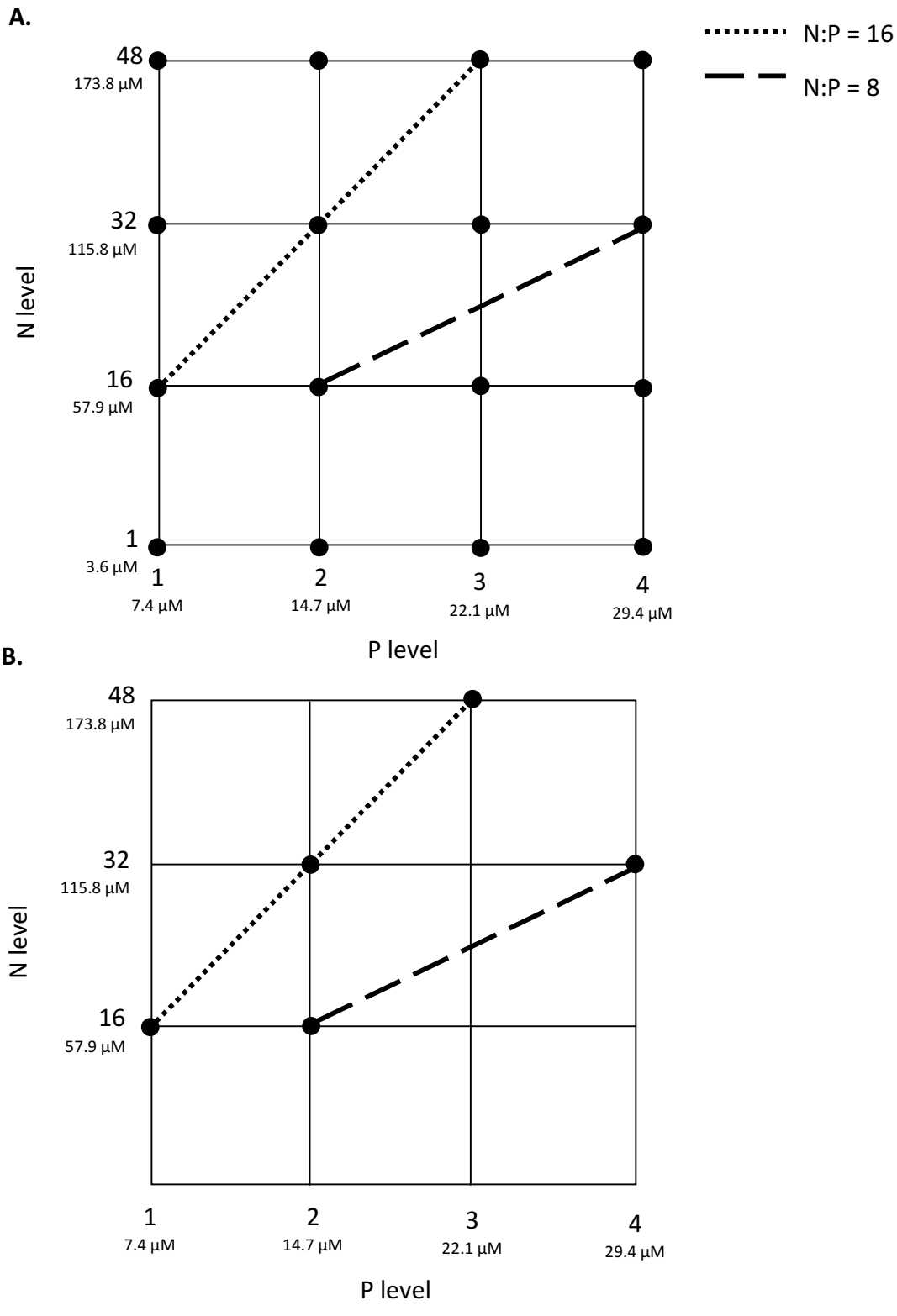

A.
N:P = 16
N:P = 8
48
173.8 µM
32
115.8 µM
16
57.9 µM
1
3.6 µM
N level
1
7.4 µM
2
14.7 µM
3
22.1 µM
4
29.4 µM
P level
B.
48
173.8 µM
32
115.8 µM
16
57.9 µM
N level
1
7.4 µM
2
14.7 µM
3
22.1 µM
4
29.4 µM
P level

Figure 2.1- Diagrams illustrating nutrient treatments used in both the (A.) ambient and (B.) elevated $CO_2$ experiments as indicated by the black dots. (A.) A 4x4 experimental design was implemented in the ambient $CO_2$ experiment. That array was employed twice; once with 6 μM glucose present and once without glucose. (B.) Five different N and P combinations comprised of 2 N:P stoichiometries were implemented in the elevated $CO_2$ experiment. Those same treatments were also supplemented with 6 μM glucose. The number of N or P atoms and nutrient source molarities (μM) are presented. The two cases where N:P ratio remains constant (8 or 16) while N and P molarities change are indicated by the black dashed and dotted lines, respectively.

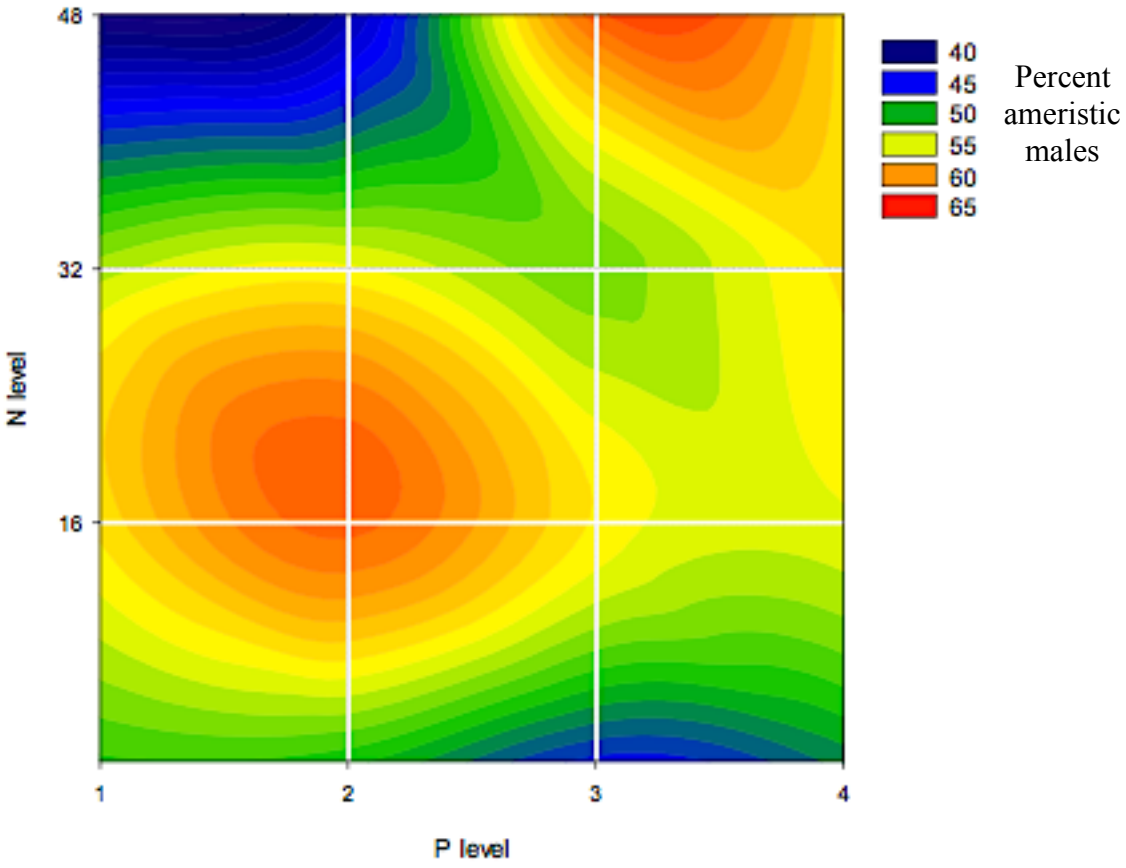



Figure 2.2- Contour plot indicating the percent of ameristic males at each of the 16 N:P treatments, in the absence of glucose at ambient $CO_2$. Numbers and their corresponding colors indicate percentage of ameristic males. Red indicates the highest percentage of ameristic males, while dark blue indicates the lowest percentage.

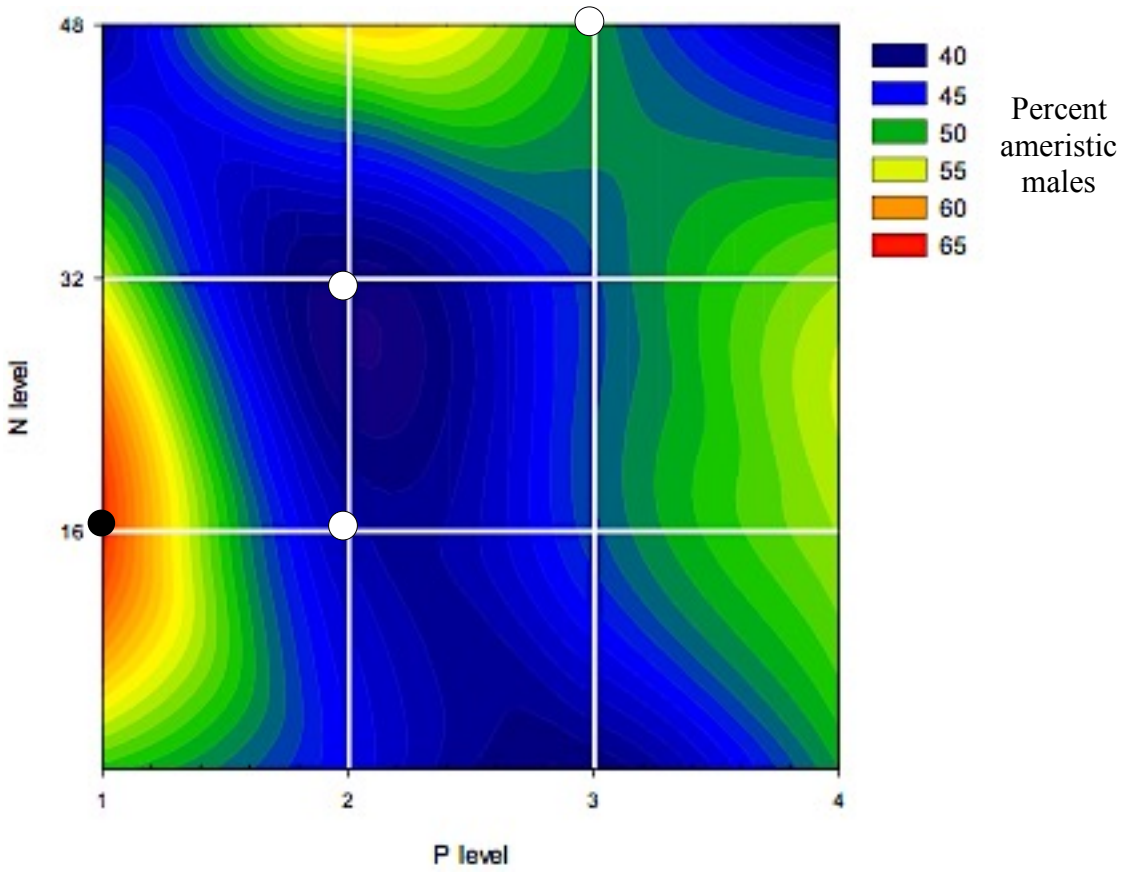



Figure 2.3- Contour plot indicating the percent of ameristic males at each of the 16 N:P treatments, in the presence of glucose at ambient $CO_2$. Numbers and their corresponding colors indicate percentage of ameristic males. Red indicates the highest percentage of ameristic males, while purple indicates the lowest percentage. Different colored circles denote significant differences in percentage of ameristic males. Treatment 5:16:1 had a significantly higher percentage of males than treatment 5:16:2 and 5:32:2 at $\alpha = 0.05$, and higher than treatment 5:48:3 at $\alpha = 0.1$, based on upper-tailed, pooled variance t-tests.

Table 2.1- Summary of ANCOVA results for the effect of nutrients on the percent of ameristic male *Ceratopteris richardii* gametophytes. Total plant number served as the concomitant variable in each analysis. A significant interaction at $\alpha = 0.05$ precluded consideration of stoichiometry and molarity main effects.

| Experiment | Glucose | Effect in Model | Type II Sums of Squares | d.f. | Mean Square | F | P-value |
|---|---|---|---|---|---|---|---|
| Ambient $CO_2$ | - | N:P ratio | 303.3 | 1 | 303.30 | 3.481 | 0.069* |
| | | Molarity | 36.3 | 1 | 36.30 | 0.417 | 0.522 |
| | | Total | 670.1 | 1 | 670.10 | 7.691 | 0.008** |
| | | Residuals | 3833.4 | 44 | 87.12 | | |
| | + | Interaction | 2428.02 | 15 | 161.87 | 2.158 | 0.034** |
| | | Total | 398.25 | 1 | 398.25 | 5.309 | 0.028** |
| | | Residuals | 2325.63 | 31 | 75.02 | | |
| Elevated $CO_2$ | - | N:P ratio | 541.1 | 1 | 541.10 | 3.423 | 0.076* |
| | | Molarity | 56.4 | 1 | 56.40 | 0.357 | 0.556 |
| | | Total | 445.4 | 1 | 445.40 | 2.817 | 0.105 |
| | | Residuals | 4110.3 | 26 | 158.09 | | |
| | + | Interaction | 242.92 | 4 | 60.73 | 0.773 | 0.554 |
| | | Total | 213.58 | 1 | 213.58 | 2.718 | 0.112 |
| | | Residuals | 1886 | 24 | 78.58 | | |

Note: *P-*value* < *0.1,* **P-*value* < *0.05.*

# CHAPTER 3:

# PLASTICITY OF REPRODUCTIVE RESOURCE ALLOCATION TO FEMALE SEXUAL FUNCTION DEPENDS ON THE PRESENCE OR ABSENCE OF PRIOR ENVIRONMENTAL SEX DETERMINATION IN *CERATOPTERIS RICHARDII*


## Abstract

Resource allocation plasticity enables individuals to alter patterns of nutrient use between reproductive and vegetative output to better fit their current environment. In sexually labile plant species, abiotic environmental factors can influence expression of dimorphic gender, resulting in environmental sex determination (ESD), which potentially reduces plasticity of resource allocation by pre-emptively matching an individual's future resource demands to its location. *Ceratopteris richardii* exhibits gender-dependent differences in relative carbon and nitrogen content, and ESD in certain nutrient environments. This study examined if prior ESD in *C. richardii* populations reduced subsequent plasticity of reproductive allocation compared to instances where no ESD occurred, resulting from $CO_2$, N, and P Liebig limitation. All three nutrient-limited environments resulted in decreased size of egg-bearing (meristic) gametophytes compared to non-limited environments, but gametophytes failed to respond to N- and $CO_2$-limitation at the time of sex determination, resulting in no ESD. N-limitation resulted in a predictable allometric re-allocation, based on small gametophyte size, whereas $CO_2$-limitation caused a change in reproductive output consistent with true plasticity. Withholding exogenous P caused ESD, but had no effect on relative reproductive output of resultant meristic gametophytes because the size decrease was minor. Under P-limitation, ESD helped match the resource demands of gender

phenotypes to their environment before the onset of developmental dimorphism, reducing the need for large allocation adjustments after sex determination.

## Introduction

Nitrogen (N) and phosphorous (P) in combination with carbon (C) are required in specific concentrations and stoichiometries for growth and development by all organisms. However, those environmental resources are often limiting. According to Liebig's law of the minimum, organisms feeding on a finite pool of required nutrients will become limited by whichever resource is least abundant compared to their needs (Liebig, 1842; Sterner and Elser, 2002; Hiddink and Kaiser, 2005). That resource will continue to limit growth until its abundance increases, or until another resource becomes more limiting. When the availability of one nutrient begins to limit growth, consumption of another nutrient above what is immediately required for growth can occur, resulting in luxury consumption, which can lead to a disparity between growth rate, net nutrient uptake, and nutrient content at any point in time (Sterner and Elser, 2002). Collectively, those integrated processes influence the strategies individuals use to allocate resources (Bazzaz and Grace, 1997; Bennett at al., 2011).

Sessile plants cannot escape from unfavorable nutrient environments, but plasticity, strict genetic adaptation, or a combination of both can enable growth and development across a range of edaphic environmental conditions (Bradshaw, 1965; Dorken and Barrett, 2004; Weiner, 2004; Nunney, 2016). In order to facilitate persistence in a heterogeneous or changing environment, individual plants alter allocation of resources to different structures based on both abiotic factors and resource availability (Weiner, 2004; DeBiasse and Kelly, 2016). Because plastic responses are evoked within a single generation and are not expected to result in changes in successive generations, the buffer provided by plasticity is immediate, yet potentially limiting to genetic change

within populations (Via and Lande, 1985; DeBiasse and Kelly, 2016). Environmentally responsive phenotypes play a vital role in the progress of an organism's life history and their underlying ontogenies are shaped by natural selection to optimize fecundity and overall individual fitness (Bazzaz and Grace, 1997; Bennett et al., 2011).

Specifically, plant responses to environmental variation and potential limitation in required nutrients require the strategic allocation of resources based on trade-offs between important functions. For example, sex allocation theory addresses plasticity of resource partitioning to male and female functions in hermaphrodites (Delph, 2003; Charlesworth and Charlesworth, 1981). Additionally, strategic allocation of biomass to any reproductive structures, regardless of gender, must be balanced in relation to allocation to vegetative structures (Reekie and Bazzaz, 1987a; Mandák and Pyšek, 1999; Reekie and Avila-Sakar, 2005).

Before reproduction can begin in either the diploid (sporophytic) or haploid (gametophytic) phase of the plant life cycle, individuals must grow to a specific minimum size, confirming there are costs and trade-offs between growth and reproduction (Van Noordwijk and de Jong, 1986; Reekie and Bazzaz, 1987a; Reznick, 1985; Roff, 1992; Obeso, 2002). Because reproduction expends nutrients provided by vegetative tissues (Delerue et al., 2013), a negative correlation between vegetative growth and production of reproductive structures often results (Obeso, 2002). Furthermore, plants frequently display characteristic species-specific relationships between reproductive output (R) and vegetative biomass (V) (i.e., an R-V relationship; Klinkhamer et al. 1992; Delerue et al., 2013). When individuals of the same species are grown in different patches of a heterogeneous environment, they display plasticity of

allocation to reproductive structures, which implies a change in the R-V relationship, not simply a change in growth rate (Weiner, 2004; Delerue et al., 2013). When resource availability is limited, allocation to reproduction may impede vegetative growth or *vice versa*, and increased investment in reproduction may be detrimental to the individual's fitness (Obeso, 2002). Those strategic adjustments to resource allocation are accomplished through plasticity.

Labile sex expression—sex determination plasticity—occurs in plants species where sex is not genetically determined and provides the opportunity for environmental factors to influence nutrient allocation strategies by partitioning individuals within a population into separate sexes (Korpelainen, 1998). The ability to regulate sex based on environmental conditions is considered adaptive when the environment is heterogeneous with respect to key abiotic factors and individual organisms cannot predict where in the environment they will end up at origination (Charnov and Bull, 1977; Bull, 1983; Janzen and Phillips, 2006). In a resource-limited environment where an individual can either become a below average female or an above average male, predictable disparities in future nutrient demands between genders may result in environmental sex determination (ESD) favoring populations with a higher probability of individuals becoming male because the likelihood of genetic transmission through male gametes is higher (Charnov and Bull, 1977). Therefore, ESD provides a means to adjust the sex ratio and improve individual fitness in a given nutrient patch (Charnov and Bull, 1977). When ESD is absent, an individual's gender has not been preferentially matched to the local environment and plasticity of reproductive allocation at the individual level should be the main mechanism that organisms can use to maintain individual fitness. Alternatively,

when ESD is present, plasticity of resource allocation may be of less importance because many individuals have already established their gender in response to cues about the prevailing environmental conditions. Thus, nutrient limitation is expected to evoke different allocation strategies depending on whether prior ESD has occurred or not (Fig. 3.1).

In the pteridophyte species *Ceratopteris richardii*, gametophyte sex determination is labile (Scott and Hickok, 1987; Banks, 1999). Macronutrients have recently been shown under certain $CO_2$ and glucose regimes to influence the probability of meristem development (Goodnoe et al., 2016; Chapter 2). Meristems are the site of female gametangial development and a prerequisite for expression of female (meristic) sexual functions (Hickok et al., 1987; Tanurdzic and Banks, 2004). Gametophytes lacking meristem formation (ameristic) develop as males. Furthermore, *C. richardii* gametophytes differ in their nutrient demands by sex (Goodnoe and Hill, 2016; Chapter 1). Therefore, it appears possible to turn ESD on or off *in vitro* based on the nutrient context in which gametophytes are grown in a species where future nutrient demands depend predictably on sex.

By inducing Liebig limitation via variation in $CO_2$, and variation or elimination of N and P concentrations, I created environments where ESD was present and environments where it is absent. Because plasticity of allocation may be influenced by the extent to which an individual's gender is previously matched to the environment, I studied female reproductive output in order to evaluate resource allocation strategies of meristic *C. richardii* gametophytes once gender had been determined, in settings with and without ESD. Furthermore, by inducing nutrient limitation, changes in R were evaluated

expressly as a function of experimental decreases in allocation to V. To examine plasticity of resource allocation (Hendry, 2016), genetically identical individuals from an inbred line (Hickok et al., 1995) were assessed under different nutrient conditions. Using the number of female eggs per unit area as a proxy for R and total meristic gametophyte area as a proxy for V, I predicted that populations of meristic gametophytes grown in environments where ESD occurs would subsequently exhibit relatively less variation in R than those grown in environments where ESD did not occur.

**Materials and Methods**

*Experimental Treatments*

P Limitation Experiment

Two C:N:P combinations supplemented with 6 μM glucose ($C_6H_{12}O_6$) were created. One media included P at 7.4 μM, whereas the other media lacked an exogenous P source. Each nutrient treatment was replicated six times. The N concentration was held at 57.9 μM. Both media were created in a background of 1/500 altered-basal salts media (BSM). To withhold the P normally provided in BSM as potassium phosphate ($KH_2PO_4$) without simultaneously depleting potassium (K), ammonium chloride ($NH_4Cl$) and potassium nitrate ($KNO_3$) were substituted for ammonium nitrate ($NH_4NO_3$), and disodium phosphate ($Na_2HPO_4$) was substituted for potassium phosphate ($KH_2PO_4$) in order avoid changing the concentration and stoichiometry of other macro- and micronutrients. A 1/500 BSM treatment with N:P adjusted to 16:1 served as a control media.

Dry, surface-sterilized spores of *C. richardii* were sown onto 60 mm petri dishes containing solid nutrient media with a sterile cotton swab, resulting in culture populations between 13 and 43 individuals, and population densities between 1.6 and 6.4 plants/$mm^2$.

Although there was no means to precisely control individual plant spacing based on this sowing technique, total plant number was included as a concomitant variable in statistical analyses. Plates exposed to elevated $CO_2$ were placed in a near-airtight acrylic chamber and the $CO_2$ concentration was increased to 1,300 ppm in order to reach super-elevated concentrations (>1,200 ppm), which are expected to induce responses distinctive from those observed at moderately elevated levels (400-1,200 ppm) (Kaplan et al., 2012). Levels of $CO_2$ were periodically measured with a wireless NODE $CO_2$ sensor (Variable Inc., Chattanooga, TN, USA). The chamber was periodically opened to replace the NODE batteries, and $CO_2$ was re-injected. The acrylic chamber was incubated in an environmental chamber (Percival Scientific, model E-30B, Boone, USA) (30º C, photosynthetically active radiation (PAR) ~30 µmol $m^{-2}$ $s^{-1}$, 12 h photoperiod) for 12 d after a 2 d induction under continuous light (30º C, PAR ~60 µmol $m^{-2}$ $s^{-1}$).

N Limitation Experiment

Five different C:N:P combinations supplemented with 6 µM glucose ($C_6H_{12}O_6$) were created in a background of 1/500 diluted basal salts media (BSM) (C-Fern Web Manual, 2009). Each nutrient treatment was replicated six times. The range of N molarities was selected to emulate nutrient concentrations that may be limiting to plant growth or development. Ammonium nitrate concentrations were between 0 and 57.9 µM, while the potassium phosphate concentration was held constant at 7.4 µM.

The sowing technique described in the P limitation experiment was implemented again, resulting in culture populations between 13 and 68 individuals, and densities between 6.8 and 34.3 plants/$mm^2$. Plates were located in a near-airtight acrylic chamber environment with a $CO_2$ concentration of 1,300 ppm to ensure plants were not C-limited.

Concentrations of $CO_2$ were monitored in the same manner as previously described. Light conditions were also the same as previously described.

C-Limitation Experiment

The C:N:P combination 5:48:3, supplemented with 6 µM glucose, was created in a background of 1/500 diluted basal salts media (BSM) and replicated three times at both ambient and elevated $CO_2$ concentrations. The elevated $CO_2$ experiment was completed twice. The C, N, and P levels were selected to emulate levels often found in natural environments with the goal of evaluating plant responses in an ecologically relevant resource context (Cleveland and Liptzin, 2007; Mathews and Chandramohanakumar, 2003; McGroddy et al., 2004; Sterner and Elser, 2002). The ammonium nitrate concentration was 173.8 µM, and the potassium phosphate concentration was 22.1 µM.

Spores were sown as previously described. At ambient $CO_2$, resulting culture populations were between 11 and 20 individuals, and densities were between 2.5 and 5 plants/$mm^2$. In the two elevated $CO_2$ trials, populations were between 14 and 41 individuals, and densities were between 3.2 and 9 plants/$mm^2$. Plates subjected to ambient $CO_2$ were incubated in an environmental chamber (Percival Scientific, model E-30B, Boone, USA). Plates exposed to elevated $CO_2$ were placed in a near-airtight acrylic chamber, with $CO_2$ concentration increased to 1,300 ppm. Concentrations of $CO_2$ were monitored in the same manner as previously described. Light conditions were also the same as previously described.

*Gametophyte Characterization*

Each gametophyte from the C, N, and P limitation experiments was categorized as an ameristic male (lacking a lateral meristem and archegonia), a meristic female (the

presence of a meristem and developing archegonia), or a meristic hermaphrodite (archegonia and antheridia on a meristic plant) based on the Klekowski gender classification scheme (Klekowski, 1969). To evaluate meristic plant size and female reproductive output, all meristic gametophytes from each experimental replicate were preserved in 95% ethanol. Five meristic gametophytes from each replicate were subsequently stained with <0.1% Toluidine Blue and photographed with a Zeiss Primo Star light microscope equipped with an AxioCam ERc5s HD digital camera (Carl Zeiss, Göttingen, Germany). For each meristic gametophyte, three variables were scored: (1.) number of archegonia (eggs), (2.) gametophyte area (vegetative growth (V)), and (3.) the number of archegonia per unit area ($mm^2$). Each mature or developing archegonium was counted towards the total number of archegonia. Meristic gametophyte area was determined from digital photographs with Image J as described in Hill et al. (2011). The number of mature and developing archegonia per unit area per meristic gametophyte was used to quantify relative reproductive output (R).

Nutrient limitation will, by definition, reduce plant size (Sterner and Elser, 2002). In order to interpret results of nutrient limitation on *C. richardii* gametophytes, the allometric relationship between reproductive output and vegetative growth in unlimited nutrient conditions was first established. To estimate changes in R with respect to V in nutrient-unlimited environments, the number of archegonia/unit area (R) was plotted against total gametophyte area (V) for gametophytes from the elevated $CO_2$, N = 16, and P = 1 treatments. A logarithmic function was fit to the data. Deviations from that function in nutrient limited environments imply a change in the R-V relationship, unexplained by decreases in size.

*Statistical Analyses*

The effects of P (0, altered-1, and 1), N (0, 0.125, 0.5, 1, 5, and 16), and $CO_2$ (ambient or elevated) concentration on the percentage of ameristic gametophytes was tested using an analysis of covariance (ANCOVA), with total plant number serving as the concomitant variable. Assumptions of ANCOVA (e.g., equal treatment slopes, and general linear model constraints) were met for all analyses (see Supplementary Material Figs. S5, S6, S7). Given a significant effect of P concentration, pairwise tests were implemented to determine at which P concentrations percent ameristic males differed significantly, using Scheffe's procedure (Scheffe, 1953). Significant pairwise differences prompted the use of an upper-tailed, pooled-variance *t*-test to determine if the treatment with P = 0 resulted in a higher percentage of ameristic males than the new P = 1 treatment.

In order to determine if meristic gametophytes grown in nutrient limited environments (P = 0, N = 0, and ambient $CO_2$,) differed from their non-nutrient limited counterparts (P = 1, N = 16, and elevated $CO_2$, respectively) in area ($mm^2$) and number of archegonia per unit area, Welch, two-tailed *t*-tests were used. Sample sizes were larger than 30, allowing deferment of normality assumptions under the central limit theorem (Aho, 2013). One gametophyte in the ambient $CO_2$ treatment of the $CO_2$ limitation experiment was determined to be an outlier based on the IQR rule and was removed from the data set.

In order to determine if R and V exhibit a negative relationship when nutrients are not limiting, the data were sub-sampled by archegonia count (i.e., all gameotphytes with three, four, five six, seven, or eight archegonia) and log(R) was plotted against V for the observations with archegonia count three through eight (see Supplementary Material Fig.

S8). An analysis of covariance was used to determine if the number of archegonia, gametophyte area, or the interaction of those two variables influenced relative reproductive output (archegonia/unit area). The slope and y-intercept of the fitted line were estimated. All statistical analyses were performed using the R computational environment (R Core Team, 2014) with heavy reliance on the package asbio (Aho, 2014).

**Results**

*P-Limitation Experiment*

The percentage of ameristic gametophytes varied significantly between the treatments that contained P and the treatment that lacked P at $\alpha = 0.1$ (Tables 3.1, 3.2) and P-limitation resulted in ESD. Total gametophyte number did not influence the percent of ameristic males (Table 3.1). Based on the subsequent upper-tailed, pooled variance *t*-test, the percentage of ameristic males was significantly higher in the altered-BSM no-P treatment than in the altered-BSM P = 1 treatment ($t_{10} = 2.99$, *P*-value = 0.022). A Welch two-tailed, *t*-test indicated that meristic gametophytes in treatment P = 0 were significantly smaller in area than meristic gametophytes in treatment altered-BSM P = 1 at 14-DAS ($t_{44.12} = -6.02$, *P*-value <0.0001; Table 3.2). Additionally, the smallest meristic gametophyte in the P = 1 treatment was 0.263 $mm^2$ in area, and the smallest in the no-P treatments was 0.240 $mm^2$, resulting in a 0.023 $mm^2$ (9.6%) increase in area in the P = 1 treatment. The number of archegonia per unit area per meristic gametophyte did not differ between the P = 0 treatment and the altered-BSM P = 1 treatment ($t_{52.40} = 0.28$, *P*-value = 0.78; Table 3.2).

*N-Limitation Experiment*

At the N concentrations tested, the percentage of ameristic gametophytes was not influenced by N concentration, only total gametophyte number (Tables 3.1, 3.2); no ESD occurred. A Welch, two-tailed *t*-test indicated that meristic gametophytes in treatment N = 0 were significantly smaller in area at 14-DAS than meristic gametophytes in treatment N = 16 ($t_{40.50}$ = -5.23, *P*-value <0.0001; Table 3.2). The smallest meristic gametophyte in N = 16 treatment was 0.688 $mm^2$ in area, and the smallest in the N = 0 treatment was 0.575 $mm^2$. Thus, the smallest plant in N = 16 treatments was 0.113 $mm^2$ (19.6%) larger than the smallest gametophyte in the N = 0 treatment. Based on a Welch, two-tailed *t*-test, meristic gametophytes in treatment N = 0 had more archegonia per unit area than gametophytes grown in treatment N = 16 ($t_{60.50}$ = 3.22, *P*-value = 0.002; Table 3.2).

*C-Limitation Experiment*

When grown at the same C:N:P level in two different $CO_2$ levels, the percentage of ameristic *Ceratopteris richardii* gametophytes was not influenced by $CO_2$ concentration or total gametophyte population, i.e., ESD did not occur (Table 3.1). At ambient $CO_2$, after sex determination, both elevated $CO_2$ trials resulted in larger meristic gametophytes than the ambient $CO_2$ experiment at 14-DAS ($t_{70.35}$ = 22.02, *P*-value <0.0001; $t_{45.37}$ = 25.63, *P*-value <0.0001; Table 3.2). The smallest meristic gametophyte grown in the elevated $CO_2$ trial 1 was 0.457 $mm^2$ in area and in trial 2 was 0.408 $mm^2$, whereas the smallest plant grown at ambient $CO_2$ was 0.249 $mm^2$ in area. Therefore, the smallest gametophyte at elevated $CO_2$ was 0.16 $mm^2$ (64%) bigger than the smallest gametophyte at ambient $CO_2$. Additionally, both elevated $CO_2$ trials resulted in more archegonia per

unit area, based on Welch, two-tailed *t*-tests at $\alpha = 0.1$ ($t_{14.56} = 3.85$, *P*-value = 0.002; $t_{13.90} = 1.81$, *P*-value = 0.09; Table 3.2).

*R-V Allometric Patterns*

The R-V relationship for the unlimited environments examined followed a logarithmic function (Fig. 3.2). As plant size increased, reproductive output (number of archegonia/unit area (R)) decreased, but asymptotically. The characteristic steps observed in the plot were due to incremental differences in the number of archegonia because archegonia count was not given fractional values. The relationship between V and R was negative logarithmic, with a slope of approximately -2.20 (Fig. 3.2). Additionally, the interaction between meristic gametophyte area and the number of archegonia significantly influenced relative reproductive output (*P*-value <0.0001).

To illustrate how nutrient limitation influenced the R-V relationship of meristic gametophytes, data from the P-, N- and $CO_2$-limited treatments were plotted against the unlimited P, N, and $CO_2$ treatments, respectively (Fig. 3.3). The mean of R from the P-limited treatment did not significantly vary from that of the unlimited P treatment, though P-limited gametophytes were significantly smaller than non-limited gametophytes (Fig. 3.3A). The mean of R and V from both the N- and $CO_2$-limited treatments were significantly different than the means from the unlimited N and $CO_2$ treatments (Figs. 3.3B, C). N limitation resulted in an expected increase in R, correlated with a decrease in V (Figs. 3.3B), whereas $CO_2$-limitation caused both R and V to decrease (Fig. 3.3C).

**Discussion**

The allocation of resources that enable reproduction is key for progression of a plant's life cycle and is often used as a measure of overall individual plant fitness (Harper and

Ogden, 1970; Bazzaz and Ackerly, 1992; Reekie and Bazzaz, 1987b). In a heterogeneous environment, allocation patterns frequently vary as a result of environmental factors in order to strike a balance between investment in reproduction and continued vegetative growth (Mandák and Pyšek, 1999). However, the expression of environmental sex determination (ESD) also exhibits adaptive variation (Blackmore and Charnov, 1989; Goodnoe et al., 2016; Chapter 2) and variation in the sex ratio in response to local conditions can provide an advantage for species with ESD (Rhen and Lang, 1995; Freedberg and Taylor, 2007). The current study demonstrates that the extent to which nutrient availability influenced sex determination (i.e., whether ESD was expressed or not) also appeared to predict the subsequent degree of plasticity of resource allocation to female reproduction in resultant meristic *Ceratopteris richardii* gametophytes.

In the unlimited nutrient conditions specified, *C. richardii* gametophyte growth followed a characteristic negative, nonlinear allometric R-V function (Fig. 3.2) and gametophytes in the P-limited environment did not significantly diverge from that function (Fig. 3.3A). When N was limited or removed or $CO_2$ was limited, sizeable differences in area between the smallest gametophyte in the nutrient-unlimited environments and the smallest gametophyte in the nutrient-limited environments were evident. However, that same strong effect of a limiting nutrient reducing growth was not observed when P was removed from the environment. Though plant area was reduced in the P-limited treatment, the difference in area between the smallest gametophyte grown without P and the smallest grown with P was a fraction of that in the other two limitation experiments in absolute and relative terms. Therefore, failure to significantly shift the R-

V relationship as a result of P-limitation was specifically due to a relatively small change in V among meristic gametophytes (Fig. 3.3A).

Under P-limitation, ESD increased the proportion of undetermined gametophytes that develop as male, thus shifting them out of the future population of meristic phenotypes. That suggests that at least some individuals emerging from spore coats in those cultured populations were P-limited, and sex expression among individuals was partitioned so that gametophytes in the population that would have become the smallest females instead developed into males (Fig. 3.4A) in a manner consistent with ESD theory (Charnov and Bull, 1977). That shift appeared to constrain the decrease in V of remaining meristic gametophytes, reducing the need for plasticity of R and no significant change in the R-V relationship. Consequently, resulting populations of meristic gametophytes were better matched to the local nutrient environment and their relative reproductive output was not affected by P availability; the observed plasticity of reproductive allocation owing to variation in the nutrient environment decreased. It was impossible to explicitly prove the inference that spores that became males due to ESD would have otherwise became small females with modified reproductive allocation. However, the increase in the percentage of males—the smaller sex in this dimorphic species— due to ESD, when P was removed, and the increased frequency of small females in the N- and $CO_2$-limited treatments, where ESD did not occur, is consistent with our hypothesis.

Significant changes in R allocation appeared to be unavoidable in the absence of ESD (Figs. 3.3B, C). Specific patterns of resource re-allocation to different structures can be either (1.) a response to a proportional or ratio-driven process, because environmental resource pools are finite at any given time (Weiner, 2004), or (2.) a consequence of plant

size, where allometric patterns evolve in response to selection pressures and constraints (Müller et al., 2000). In this study system, meristic gametophytes were previously shown to be the more N-demanding sex in both absolute and relative terms (Goodnoe and Hill, 2016; Chapter 1). Yet even when N was completely removed from the nutrient environment, the percentage of male gametophytes did not increase, suggesting that meristem formation in *C. richardii* gametophytes required that element in very small absolute amounts.

Although elimination of N from the environment subsequently reduced growth and size of meristic gametophytes at 14-DAS, those gametophytes actually increased allocation to reproductive structures compared to meristic gametophytes in the highest N treatment (Fig. 3.3C). However, an increase in R is expected when considered in the context of allometric changes in allocation expressed during normal meristic gametophyte growth in non-limiting nutrient conditions (Fig. 3.2). In *C. richardii*, reproductive allocation to egg production was initially high and gradually decreased as meristic gametophyte size increased (Fig. 3.2). Therefore, though the R-V relationship of meristic gametophytes changed when gametophytes were N-limited, that change was largely attributable to a shift in position along the normal R-V growth function owing to small size (Figs. 3.2, 3.4B); it was an allometric response resulting in apparent plasticity (Bishop et al., 2010; Delerue et al., 2013). I would expect that if gametophytes in the N-limited treatment could escape nutrient limitation and continue growth beyond 14-DAS, they would decrease their relative reproductive output as a correlated response to increasing individual plant size, and thus behave like the gametophyte in the non-N-limited treatment in terms of allocation strategy.

Increasing the concentration of $CO_2$ also did not elicit ESD (Table 3.1), suggesting that sex determination in the population was established based on the concentration of antheridiogen sex pheromone alone. A consistent sex ratio between $CO_2$ treatments indicates that the concentration of $CO_2$ in the ambient environment was sufficient to initiate formation of an active meristem. However, C-limitation subsequently resulted in a disproportionate decrease in R relative to the decrease in V of meristic *C. richardii* gametophytes, which was accomplished by a significant phenotypic departure away from the normal R-V growth function (Figs. 3.3C). Smaller gametophytes in ambient $CO_2$ exhibited reduced reproductive allocation unexplained by a change in size, thus resulting in a truly plastic response. This response is similar to sex-allocation plasticity observed in angiosperm sporophytes, where hermaphroditic individuals greatly differ in their investment in female sexual function as a result of nutrient availability variation (Dorken and Mitchard, 2008; Bishop et al., 2010).

Elevated environmental $CO_2$ concentrations have the potential to critically alter plant communities via changes in strategies for allocation to growth and reproduction (Wang et al., 2015). In the present study, experimental manipulation of $CO_2$ resulted in the largest discrepancy in reproductive output between gametophytes grown in limited and unlimited conditions. Furthermore, vegetative growth was greatly influenced by variation in $CO_2$ concentration; meristic gametophytes grown at elevated $CO_2$ were on average three times larger than those grown at ambient $CO_2$, suggesting *C. richardii* gametophytes were C-limited when grown *in vitro* at ambient $CO_2$ concentrations, even when a low level of exogenous glucose was available. Growth at elevated $CO_2$ also allowed meristic gametophytes to allocate a greater proportion of total resources to

reproductive development (Fig. 3.3C). Furthermore, Ong et al. (1998) also observed that size and growth rate of *Pyrrosia piloselloides* gametophytes increased as a result of growth under elevated $CO_2$ conditions. It is possible that $CO_2$-limitation in gametophyte ecology has broad importance, functioning to significantly alter the R-V relationship, resulting in novel phenotypes that are far from the normal allometric function plants follow when nutrients are not limiting. Because C is the most abundant element in meristic *C. richardii* gametophytes, comprising 40-60% of gametophyte dry biomass, the probability of eliciting true plasticity of reproductive allocation based on natural environmental variation in resource availability might correlate with the absolute demand for that resource. The $CO_2$-limited gametophytes appeared to prioritize investment in a certain amount of meristematic growth in order to accomplish formation of at least one mature archegonium and then reduced any further investment in vegetative growth, whereas gametophytes grown in elevated $CO_2$ continued growth well after the minimum size for archegonial development had been reached. In order to successfully accomplish sporophyte formation, meristic gametophytes must reach a critical size (Sakamaki and Ino, 1999). The onset of sexual maturity at a small size may result in a shorter lifespan and accelerated, though unsuccessful, sporophyte formation (Greer and McCarthy, 1999).

*Conclusions*

Though a negative relationship between reproductive and vegetative allocation is theoretically mandatory, demonstrating that relationship has previously proven difficult because the collective noise of multiple allocation strategies operating concurrently to regulate the development of numerous labile structures often confounds the signal of interest (Van Noordwijk and de Jong, 1986; Metcalf, 2016). The current work

exemplifies the expected negative relationship between R and V (Fig. 3.2) by empirically testing the theory of allocation trade-offs in individuals within a population by means of a simple, land plant model system. I was able to discern the effects of labile sex determination on subsequent plasticity of nutrient allocation to reproductive output by creating environments where populations of genetically identical individuals did and did not exhibit ESD. In this case, ESD acted as an avoidance strategy by reducing the probability of low quality females developing in a population. The available evidence suggests those females would not have reached the minimum size threshold for reproduction through female function (Fig. 3.4A). Thus, in *C. richardii*, population-level ESD allowed individuals to avoid the need for large, potentially futile adjustments in future resource allocation as a result of decreased size caused by nutrient limitation by pre-emptively shifting the population sex ratio towards males. To our knowledge, there exists no other work that explicitly examines the effects of variation in ESD on resultant whole-organism female resource allocation in a plant species.

**References**


Aho, K. 2013. *Foundational and applied statistics for biologists using R*. CRC Press.

Aho, K. 2014. Asbio: A collection of statistical tools for biologists. R package version 1.1-1, http://cran.r-project.org/web/packages/asbio/index.html.

Banks, J. A. 1999. Gametophyte development in ferns. Annual Review of Plant Biology, **50**(1): 163-186. doi: 10.1146/annurev.arplant.50.1.163.

Bazzaz, F. A., and Ackerly, D. D. 1992. Reproductive allocation and reproductive effort in plants. Seeds: the ecology of regeneration in plant communities, 1: 26.

Bazzaz, F. A., and Grace, J. (Eds.). 1997. *Plant Resource Allocation*. Academic press.

Bennett, E., Roberts, J. A., and Wagstaff, C. 2011. Manipulating resource allocation in plants. Journal of Experimental Botany, **63**(9): 3391-3400. doi: 10.1093/jxb/err442.

Bishop, E. J., Spigler, R. B., and Ashman, T. L. 2010. Sex-allocation plasticity in hermaphrodites of sexually dimorphic *Fragaria virginiana* (Rosaceae). Botany, **88**(3): 231-240.

Blackmore, M. S., and Charnov, E. L. 1989. Adaptive variation in environmental sex determination in a nematode. American Naturalist, **134**(5): 817-823.

Bradshaw, A. D. 1965. Evolutionary significance of phenotypic plasticity in plants. Advances in Genetics, **13**: 115-155.

Bull, J. J. 1983. Evolution of sex determining mechanisms. The Benjamin/Cummings Publishing Company, Inc..

C-Fern Web Manual. 2009. http://www.magrinscience.com/images/biology/ferns/C-Fern_Manual.pdf.

Charlesworth, D. and Charlesworth, B. 1981. Allocation of resources to male and female functions in hermaphrodites. Biological Journal of the Linnean Society, **15**(1): 57-74. doi: 10.1111/j.1095-8312.1981.tb00748.x.

Charnov, E. L., and Bull, J. 1977. When is sex environmentally determined?. Nature. **266**: 828-830. doi:10.1038/266828a0.

Cleveland, C. C., and Liptzin, D. 2007. C: N: P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass?. Biogeochemistry. **85**(3): 235-252.

DeBiasse, M. B., and Kelly, M. W. 2016. Plastic and Evolved Responses to Global Change: What Can We Learn from Comparative Transcriptomics?. Journal of Heredity, **107**(1): 71-81. doi:10.1093/jhered/esv073.

Delerue, F., Gonzalez, M., Atlan, A., Pellerin, S., and Augusto, L. 2013. Plasticity of reproductive allocation of a woody species (Ulex europaeus) in response to variation in resource availability. Annals of Forest Science, **70**(3): 219-228. doi: 10.1007/s13595-012-0260-x.

Delph, L. F. 2003. Sexual dimorphism in gender plasticity and its consequences for breeding system evolution. Evolution & development, **5**(1): 34-39.

Dorken, M. E. and Mitchard, E. T. A. 2008. Phenotypic plasticity of hermaphrodite sex allocation promotes the evolution of separate sexes: an experimental test of sex-differential plasticity hypothesis using *Sagittaria latifolia* (Alismataceae). Evolution, **62**(4): 971–978. doi: 10.1111/j.1558-5646.2008.00336.x

Dorken, M. E., and Barrett, S. C. 2004. Phenotypic plasticity of vegetative and reproductive traits in monoecious and dioecious populations of *Sagittaria latifolia* (Alismataceae): a clonal aquatic plant. Journal of Ecology, **92**(1): 32-44. doi: 10.1111/j.1365-2745.2004.00857.x.

Freedberg, S., and Taylor, D. R. 2007. Sex ratio variance and the maintenance of environmental sex determination. Journal of Evolutionary Biology, **20**(1): 213-220. doi:10.1111/j.1420-9101.2006.01209.x.

Goodnoe, T. T., and Hill, J. P., 2016. Absolute and Relative Content of Carbon and Nitrogen Differ by Sex in *Ceratopteris richardii* Gametophytes. Botany. doi: 10.1139/cjb-2015-0254.

Goodnoe, T. T., Hill, J. P., and Aho, K. 2016. Effects of Variation in Carbon, Nitrogen, and Phosphorus Molarity and Stoichiometry on Sex Determination in the Fern *Ceratopteris richardii.* Botany. doi: 10.1139/cjb-2015-0187.

Greer, G. K., and McCarthy, B. C. 1999. Gametophytic plasticity among four species of ferns with contrasting ecological distributions. International Journal of Plant Sciences, **160**(5): 879-886. doi: 10.1086/314188.

Harper, J. L., and Ogden, J. 1970. The reproductive strategy of higher plants: I. The concept of strategy with special reference to *Senecio vulgaris L*. The Journal of Ecology, **58**(3): 681-698.

Hendry, A. P. 2016. Key Questions on the Role of Phenotypic Plasticity in Eco-Evolutionary Dynamics. Journal of Heredity, **107**(1): 25-41. doi:10.1093/jhered/esv060

Hickok, L. G., Warne, T. R., and Slocum, M. K. 1987. *Ceratopteris richardii*: applications for experimental plant biology. American Journal of Botany, **74**(8):1304-1316.

Hickok, L. G., Warne, T. R., and Fribourg, R. S. 1995. The biology of the fern *Ceratopteris* and its use as a model system. International Journal of Plant Sciences, **156**(3): 332-345.

Hiddink, J. G., and Kaiser, M. J. 2005. Implications of Liebig's law of the minimum for the use of ecological indicators based on abundance. Ecography, **28**(2): 264-271. doi: 10.1111/j.0906-7590.2005.04063.x

Hill, J. P., Germino, M. J., and Alongi, D. A. 2011. Carbon-use efficiency in green sinks is increased when a blend of apoplastic fructose and glucose is available for uptake. Journal of Experimental Botany. **62**: 2013-2022. doi: 10.1093/jxb/erq407.

Janzen, F. J., and Phillips, P. C. 2006. Exploring the evolution of environmental sex determination, especially in reptiles. Journal of Evolutionary Biology, **19**(6): 1775-1784. doi: 10.1111/j.1420-9101.2006.01138.x.

Kaplan, F., Zhao, W., Richards, J. T., Wheeler, R. M., Guy, C. L., and Levine, L. H. 2012. Transcriptional and metabolic insights into the differential physiological responses of Arabidopsis to optimal and supraoptimal atmospheric $CO_2$. PloS One. **7**(8): e43583. doi: 10.1371/journal.pone.0043583.

Klekowski, E. J. 1969. Reproductive biology of the Pteridophyta. III. A study of the Blechnaceae. Botanical Journal of the Linnean Society, **62**(3): 361-377.

Klinkhamer, P. G. L., Meelis, E., De Jong, T. J., and Weiner, J. 1992. On the analysis of size-dependent reproductive output in plants. Functional Ecology, **6**(3): 308-316. doi: 10.2307/2389522.

Korpelainen, H. 1998. Labile sex expression in plants. Biological Reviews. **73**(2): 157-180. doi: 10.1111/j.1469-185X.1997.tb00028.x.

Liebig, J. 1842. Animal Chemistry, or Organic Chemistry in its Application to Physiology and Pathology. Johnson Reprint Corporation, New York, USA.

Mandák, B., and Pyšek, P. 1999. Effects of plant density and nutrient levels on fruit polymorphism in *Atriplex sagittata*. Oecologia, **119**(1): 63-72.

Mathews, L., and Chandramohanakumar, N. 2003. The ratios of carbon, nitrogen, and phosphorus in a wetland coastal ecosystem of Southern India. International Review of Hydrobiology. **88**(2): 179-186. doi: 10.1002/iroh.200390013.

McGroddy, M. E., Daufresne, T., and Hedin, L. O. 2004. Scaling of C: N: P stoichiometry in forests worldwide: implications of terrestrial Redfield-type ratios. Ecology. **85**(9): 2390-2401.

Metcalf, C. J. E. 2016. Invisible Trade-offs: Van Noordwijk and de Jong and Life-History Evolution. American Naturalist. **157**. doi: 10.1086/685487.

Müller, I., Schmid, B., and Weiner, J. 2000. The effect of nutrient availability on biomass allocation patterns in 27 species of herbaceous plants. Perspectives in Plant Ecology, Evolution and Systematics, **3**(2): 115-127. doi: 10.1078/1433-8319-00007.

Nunney, L. 2016. Adapting to a Changing Environment: Modeling the Interaction of Directional Selection and Plasticity. Journal of Heredity, **107**(1): 15-24. doi:10.1093/jhered/esv084.

Obeso, J. R. 2002. The costs of reproduction in plants. New Phytologist, **155**(3): 321-348. doi: 10.1046/j.1469-8137.2002.00477.x.

Ong, B. L., Koh, C. K., and Wee, Y. C. 1998. Effects of $CO_2$ on growth and photosynthesis of *Pyrrosia piloselloides* (L.) Price gametophytes. Photosynthetica, **35**(1): 21-27.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. [Computer program]. Available from http://www.R-project.org/.

Reekie, E. G., and Avila-Sakar, G. 2005. The shape of the trade-off function between reproduction and growth. *Reproductive Allocation in Plants*, pp. 189-214.

Reekie E. G., and Bazzaz F. A. 1987a. Reproductive effort in plants. 2. Does carbon reflect the allocation of other resources?. American Naturalist. **129**(6): 897–906.

Reekie, E. G., and Bazzaz, F. A. 1987b. Reproductive effort in plants. 1. Carbon allocation to reproduction. American Naturalist, **129**(6): 876-896.

Rhen, T., and Lang, J. W. 1995. Phenotypic plasticity for growth in the common snapping turtle: effects of incubation temperature, clutch, and their interaction. American Naturalist, **146**(5): 726-747.

Reznick, D. 1985. Costs of reproduction: an evaluation of the empirical evidence. Oikos, **44**(2): 257-267. doi: 10.2307/3544698.

Roff, D. A. 1992. *Evolution of life histories: theory and analysis*. Springer Science and Business Media.

Sakamaki, Y., and Ino, Y. 1999. Contribution of fern gametophytes to the growth of produced sporophytes on the basis of carbon gain. Ecological Research, **14**(1): 59-69.

Scheffe, H. 1953. A method for judging all contrasts in the analysis of variance*. Biometrika, 40(1-2): 87-110. doi: 10.1093/biomet/40.1-2.87.

Scott, R. J., and Hickok, L. G. 1987. Genetic analysis of antheridiogen sensitivity in *Ceratopteris richardii*. American Journal of Botany. **74**(12): 1872-1877.

Sterner, R. W., and Elser, J. J. 2002. Ecological stoichiometry: the biology of elements from molecules to the biosphere. Princeton University Press.

Tanurdzic, M., and Banks, J. A. 2004. Sex-determining mechanisms in land plants. The Plant Cell Online. **16**(sup 1): S61-S71. doi: http://dx.doi.org/10.1105/tpc.016667.

Van Noordwijk, A. J., and de Jong, G. 1986. Acquisition and allocation of resources: their influence on variation in life history tactics. The American Naturalist, **128**(1): 137-142.

Via, S., and Lande, R. 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution, **39**(3): 505-522. doi: 10.2307/2408649.

Wang, X., Taub, D. R., and Jablonski, L. M. 2015. Reproductive allocation in plants as affected by elevated carbon dioxide and other environmental changes: a synthesis using meta-analysis and graphical vector analysis. Oecologia, **177**(4): 1075-1087. doi: 10.1007/s00442-014-3191-4.

Weiner, J. 2004. Allocation, plasticity and allometry in plants. Perspectives in Plant Ecology, Evolution and Systematics, **6**(4): 207-215. doi:10.1078/1433-8319-00083.

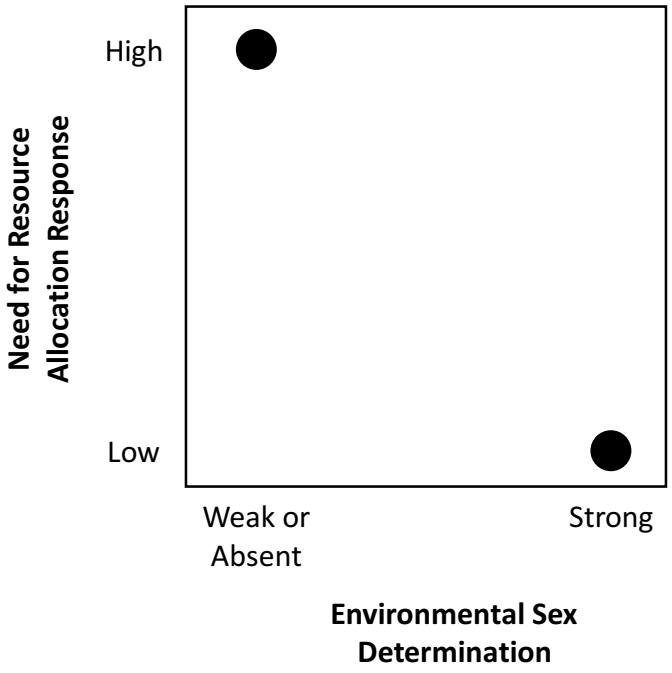



Figure 3.1- Hypothesized effect of environmental sex determination (ESD) on expected level of plasticity of resource allocation. When ESD is weak or absent, the need for plasticity in R:V resource allocation is predicted to be strong because the individual has not been preferentially matched to the given environmental patch. However, when ESD is present, there is less need for an individual to alter their resource allocation response because their gender has been previously matched to the availability of resources in the environment.

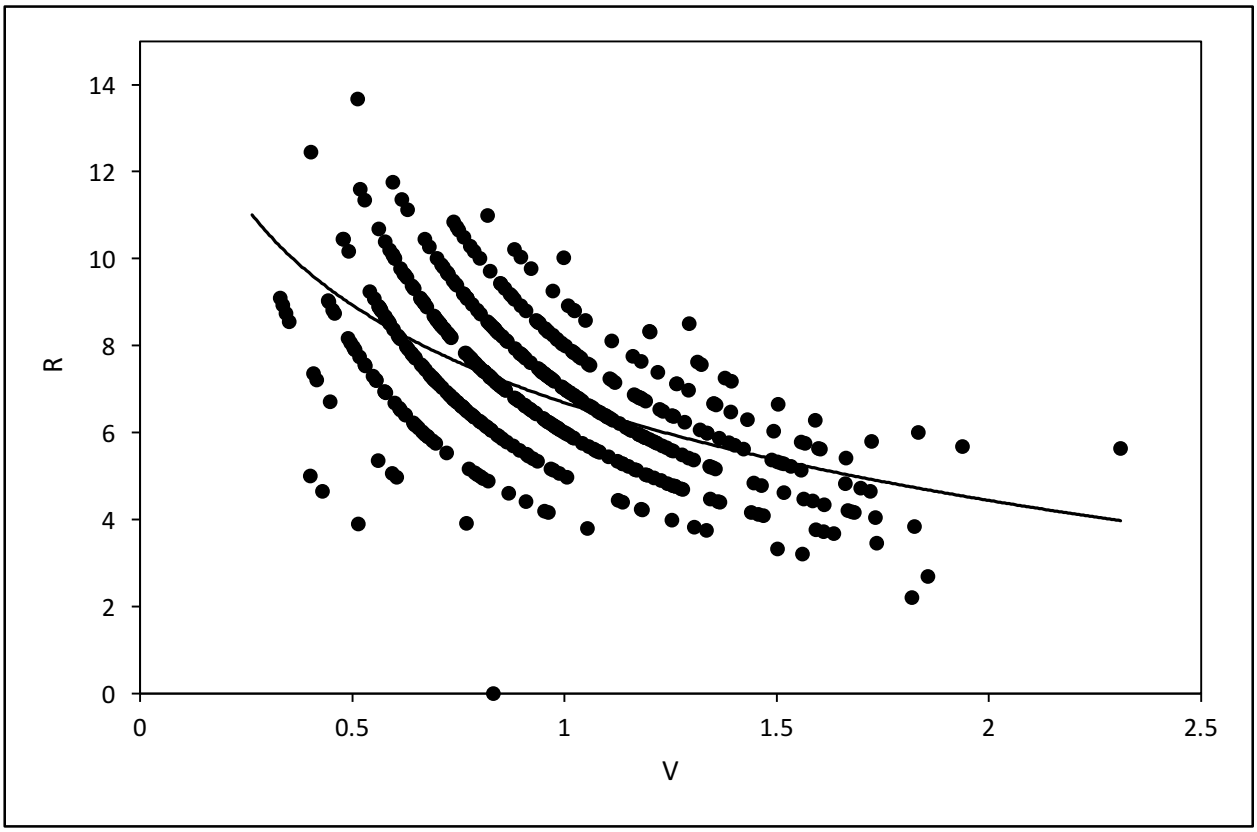



Figure 3.2- Plot of relative reproductive output (R)—measured as the number of archegonia per unit area—as a function of total gametophyte area (V), for all meristic gametophytes in the unlimited $CO_2$, N, and P treatments. Normal gametophyte growth and development in non-nutrient-limited conditions follow a logarithmic function, depicted by the black line. As gametophyte area increases, reproductive output decreases asymptotically. Additionally, the observations separate incrementally based on whole number differences in the number of archegonia.

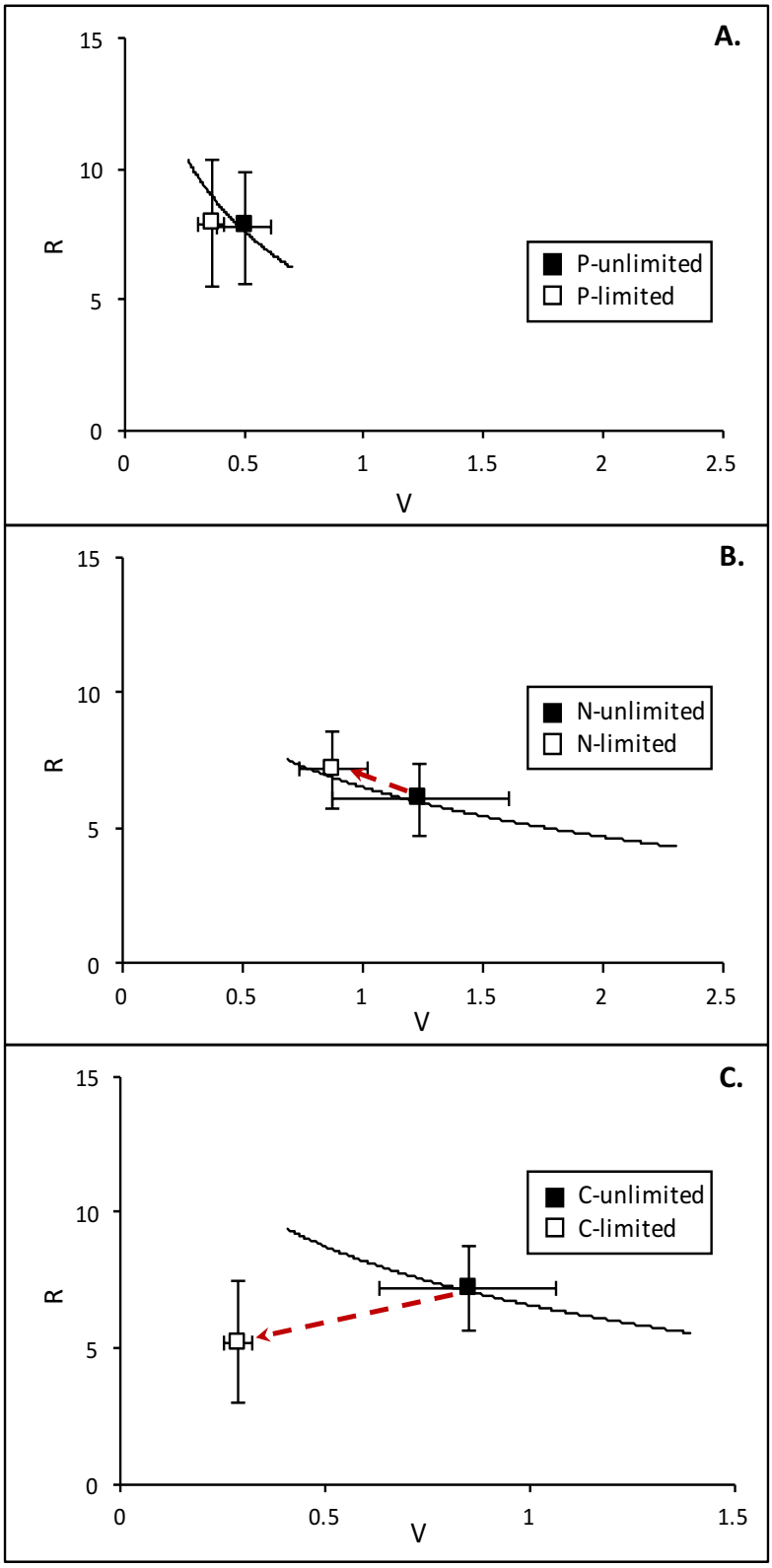

A.
15
10
5
0
R
0
0.5
1
1.5
2
2.5
V
P-unlimited
P-limited
B.
15
10
5
0
R
0
0.5
1
1.5
2
2.5
V
N-unlimited
N-limited
C.
15
10
5
0
R
0
0.5
1
1.5
V
C-unlimited
C-limited

Figure 3.3- Changes in relative reproductive output (R) with respect to total gametophyte area (V), as a result of Liebig limitation. In each panel, the solid black line represents a function fit to the unlimited R-V data, with the black square denoting the mean R and V value, with error bars representing ± 1 standard deviation. Open circles denote the mean R and V value, ± 1 standard deviation, for the nutrient-limited data. This study was explicitly designed to examine the effect of nutrient limitation. Nutrient limitation is defined as any nutrient level that reduces growth (i.e., V is experimentally reduced). A reduction of growth over a set time interval translates to smaller individual size. Thus, by definition, the current study does not consider potential responses where V remains constant or increases. (A.) With prior ESD, when the environment varies from unlimited to limited P, R does not significantly change, though V increases. (B.) Under N limitation, greater relative allocation to reproductive structures is due to an allometric response, in particular smaller meristic gametophytes develop more archegonia per unit area than larger gametophytes, even when N is not limiting (see Fig. 3). Without prior ESD, N-limitation causes a significant change in the R-V relationship. (C.) When the environment varies from non-$CO_2$-limited (n = 100) to $CO_2$-limited (n = 13), ESD is not present and meristic gametophytes significantly alter their reproductive allocation in response to inadequate nutrient availability. Meristic gametophytes in ambient $CO_2$ are much smaller, and have reduced reproductive effort when compared to gametophytes grown at elevated $CO_2$.

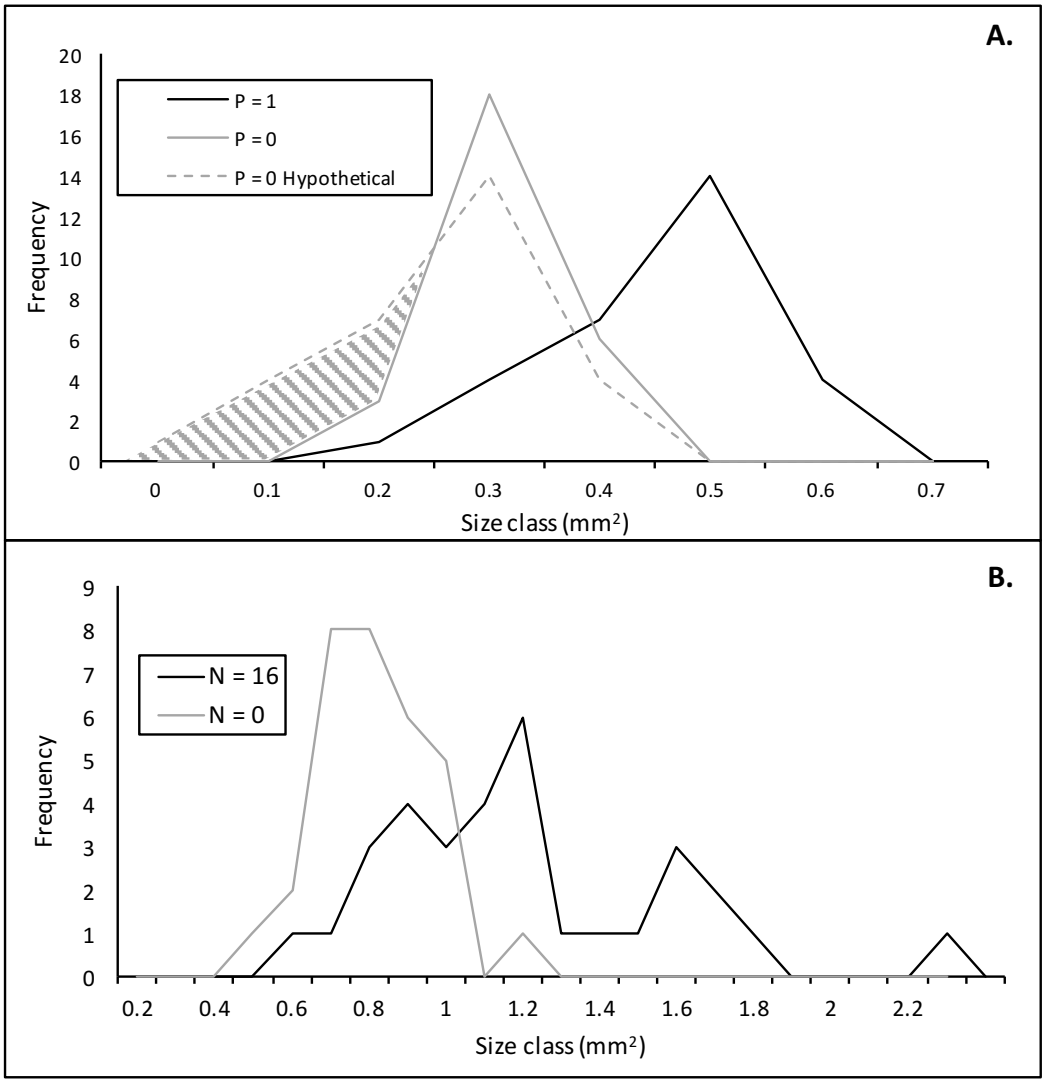



Figure 3.4- Population-level effects of (A.) phosphorus and (B.) nitrogen limitation on gametophyte size (area). (A.) Though both the P-unlimited (black line) and the P-limited (grey line) data resulted in left-skewed distributions, the size difference between the smallest gametophyte in the P-limited environment and the smallest gametophyte in the unlimited P environment was insufficient (9.6%) to prompt a substantial change in the R-V relationship. If gametophyte size had decreased proportionately as a result of P-limitation (grey dotted line), a size difference between the limited and unlimited

environments comparable to that observed when N was limited (grey hashed area) would be expected. However, as a result of environmental sex determination (ESD), gametophytes that would have developed into the smallest of females appear to have developed into males instead. In the N limitation experiment (B.), data from both the N-unlimited and the N-limited environments resulted in distributions that were skewed right; the mean gametophyte size was larger than the most prevalent gametophyte size. The smallest meristic gametophyte in the limited environment (grey line) was considerably smaller (19%) than the smallest gametophytes in the unlimited environment (black line). Therefore, the lack of ESD resulted in a large size effect was not observed when P was removed.

Table 3.1- Summary of ANCOVA results for the effects of nutrients ($CO_2$, N, or P concentration) on the percentage of ameristic male *Ceratopteris richardii* gametophytes. Total plant number served as the concomitant variable in each analysis.

| Experiment | Effect in model | Type II Sums of Squares | d.f. | Mean Square | *F* | *P*-value |
|---|---|---|---|---|---|---|
| C Limitation | $CO_2$ concentration | 240.6 | 2 | 120.30 | 1.35 | 0.27 |
| | Total plant number | 138.6 | 1 | 138.60 | 1.55 | 0.22 |
| | Residuals | 3659.6 | 41 | 89.26 | | |
| N Limitation | N concentration | 2.8 | 1 | 2.80 | 0.02 | 0.89 |
| | Total plant number | 2582.4 | 1 | 2582.40 | 16.78 | 0.0003** |
| | Residuals | 5079.9 | 33 | 153.94 | | |
| P Limitation | P concentration | 1198.7 | 2 | 599.35 | 3.60 | 0.05* |
| | Total plant number | 249.1 | 1 | 249.10 | 1.50 | 0.24 |
| | Residuals | 2331.8 | 14 | 166.56 | | |

Note: *P-*value* < *0.1,* **P-*value* < *0.05.*

Table 3.2- Summary of factor level means and standard error of the mean (SEM) for percent ameristic males in Ceratopteris richardii gametophyte populations, as well as meristic gametophyte area ($mm^2$) and the number of eggs/unit area per meristic gametophyte in all nutrient limitation experiments (i.e., $CO_2$, N, and P).

| Experiment | Treatment | Percent Males | | Area | | Eggs/Unit Area | |
|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM |
| $CO_2$ Limitation | Ambient | 48.9 | 5.7 | 0.3 | 0.009 | 5.2 | 0.5 |
| | Elev., #1 | 47.8 | 1.5 | 0.7 | 0.02 | 7.7 | 0.2 |
| | Elev., #2 | 57.3 | 10.6 | 1.04 | 0.03 | 6.4 | 0.2 |
| N Limitation | 0 | 62.6 | 6.3 | 0.9 | 0.03 | 7.2 | 0.3 |
| | 0.125 | 66 | 7 | — | — | — | — |
| | 0.5 | 70 | 4.7 | — | — | — | — |
| | 1 | 50.9 | 5.8 | — | — | — | — |
| | 5 | 56.9 | 7.2 | — | — | — | — |
| | 16 | 65.2 | 3.7 | 1.2 | 0.1 | 6.1 | 0.2 |
| P Limitation | Altered 0 | 71.2 | 3.5 | 0.4 | 0.01 | 7.9 | 0.5 |
| | Altered 1 | 48.2 | 7.2 | 0.5 | 0.02 | 7.7 | 0.4 |
| | 1-1/500 BSM | 57.4 | 4.7 | — | — | — | — |

# EPILOGUE

This project evaluated the growth and development of *Ceratopteris richardii* fern gametophytes under defined nutrient levels and combinations most likely encountered in natural environments, in order to prompt ecologically relevant behavior. Specifically, the principles of ecological stoichiometry were applied to better understand the phenomenon of environment sex determination (ESD) and resource allocation in gametophyte populations of this species. Sexual lability provided the opportunity to turn ESD on or off *in vitro* by means of changes in nutrient availability, which resulted in associated changes in the proportions of males and hermaphrodites. Similarly, Rhen and Lang (1995) succeeded in supressing ESD in common snapping turtle hatchlings via hormonal manipulation of eggs, which allowed same-sex comparisons of hatchling growth across a range of incubation temperatures. The ability to create situations with and without ESD enables examination of subsequent differences between individuals that are and are not preferentially matched to their local environment. However, instances where the expression of ESD is a phenotypically plastic trait in natural environments may be rare or absent for most organisms, adding to the significance of the current work.

Though *C. richardii* is a homosporous fern species—meaning sporophytes produce spores that are not differentiated by sex as they are in heterosporous, non-seed plants—significant variation in spore size does exist (Banks, 1999; Ganger and Sturey, 2012). A pool of nutrients provided to the gametophyte in the spore cytoplasm is available during germination (Banks, 1999) and dry *C. richardii* spores average 65% C, 0.7% N, and 0.28% P (K. Boydstun and J. Hill , unpublished data). If small spores contain fewer resources, ESD may be driving gametophytes that germinate from those

small spores to develop as males due to the constraints of nutrient limitation, whereas gametophytes that germinate from large spores may escape that constraint and determine gender based mainly on *Ceratopteris* antheridiogen signals. On full strength C-fern media (i.e., roughly 500 times more concentrated than the media used in this work), tests of variation in spore size as a predictor of gender have reported conflicting results (Sayers and Hamilton, 1995; Ganger and Sturey, 2012). By sorting spores based on size and by ensuring important nutrients like C are not simultaneously limiting, preliminary tests have shown that elimination of P from the *in vitro* environment results in an increased proportion of male gametophytes when the population is derived from only small *C. richardii* spores. However, P-limitation did not have the same effect when only large spores were examined (K. Boydstun, T. Goodnoe, and J. Hill, unpublished data). Therefore, it appears that the amount of P available in small spores when they are shed from the parent sporophyte is inadequate for *C. richardii* gametophytes to support meristem formation, making them sensitive to extra-cellular P levels, thereby resulting in ESD.

Though elimination of P from the growth media elicited a sex determination response (Goodnoe et al., 2016; Chapter 2), it is not clear if such an environment is encountered by gametophytes in natural settings; a "zero-P" nutrient environment is feasible experimentally, but possibly not ecologically. Furthermore, natural environments are cohabited by complex microbial communities (Van Der Heijden et al., 2008). Plant surfaces interact extensively with microorganisms present in soils or water (Richardson et al., 2009), and those microbes impact plant growth in various ways (Kutschera, 2007; Berg, 2009; Richardson et al., 2009; Corrêa et al., 2012; Smith et al., 2015). Though I

have endeavored to emulate natural gametophyte environments, in this study, I did not address the ancient and complex relationship between microbial communities and gametophytes (Knack et al., 2015). Preliminary studies suggest that when fungi and *C. richardii* gametophytes are grown together *in vitro* on media that contains no exogenous P source, meristic *C. richardii* gametophyte growth is greatly increased compared to when fungi are absent (K. Boydstun and J. Hill, unpublished data), suggesting that fungi provide gametophytes better access to the trace amounts of P found in agar (Jain et al., 2009; Bornman and Barnard, 1993), which could potentially alter the occurrence of ESD in that same environment.

Anthropogenic activity has already irreversibly transformed many ecosystems and dramatically influenced macronutrient concentrations worldwide (Mackenzie et al., 2002; Ellis, 2011; Bracken et al., 2015). Combustion of fossil fuels has significantly raised atmospheric $CO_2$ concentrations (Peñuelas et al., 2012; Intergovernmental Panel on Climate Change, 2013), while burning fossil fuels in combination with fertilizer production and human-induced $N_2$ fixation via the cultivation of crops have considerably increased the release of biologically active sources of N into the environment. Theoretically, those two practices should have stimulating effects on plant primary productivity (Reich et al., 2006; Peñuelas et al., 2012). However, the dramatic increases in C and N have created a strong imbalance with P—which is also increasing globally, though at a much slower rate than the increases in the availabilities of C and N—resulting in higher biospheric C:P and N:P ratios (Peñuelas et al., 2012). This has contributed to growing concern over how organisms, especially vegetation, will respond or adapt to such changes (Lammertsma et al., 2011). The study of organismal responses to nutrient

variation has long been a topic of interest, but has primarily focused on sporophytic angiosperms. My work demonstrates that non-seed plant gametophyte growth dramatically increases under elevated $CO_2$ conditions (Chapter 3; T. Goodnoe and J. Hill, unpublished data). Those responses may be similar to the initial response of angiosperms when exposed to elevated $CO_2$, which results in increased productivity. However, angiosperms eventually respond with acclimation and the gains in productivity decline (Sage et al., 1989; Caporn et al., 1999; Newingham et al., 2013). It is possible that because *C. richardii* gametophytes are short-lived (or the current experiments were too short term), detecting acclimation responses was not possible. Alternatively, the small nonvascular phase of non-seed plants may possess specific adaptations to high $CO_2$ conditions because their natural environment normally consists of elevated $CO_2$ levels above what is present in ambient air (Kimmerer, 2003). Because these sessile gametophytes respond to variation in $CO_2$ availability in markedly dissimilar ways (Saxe et al., 1998; Long et al., 2004; Coe et al., 2012), they may provide unique insights into how natural systems solve issues of macronutrient limitation and distribution in a changing world.

REFERENCES

Banks, J. A. 1999. Gametophyte development in ferns. Annual Review of Plant Biology, **50**(1): 163-186. doi: 10.1146/annurev.arplant.50.1.163.

Berg, G. 2009. Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied microbiology and biotechnology, **84**(1): 11-18.

Bornman, J. J., and Barnard, R. O. 1993. The possible use of agar gel in plant nutritional studies. South African Journal of Plant and Soil, **10**(3): 146-149. doi: 10.1080/02571862.1993.10634661.

Bracken, M. E. S., Hillebrand, H., Borer, E. T., Seabloom, E. W., Cebrian, J., Cleland, E. E., Elser, J. J., Gruner, D. S., Harpole, W. S., Ngai, J. T. and Smith, J. E. 2015. Signatures of nutrient limitation and co-limitation: responses of autotroph internal nutrient concentrations to nitrogen and phosphorus additions. Oikos. **124**(2): 113–121. doi: 10.1111/oik.01215

Caporn, S. J., Brooks, A. L., Press, M. C., and Lee, J. A. 1999. Effects of long-term exposure to elevated $CO_2$ and increased nutrient supply on bracken (*Pteridium aquilinum*). Functional ecology, **13**(s1): 107-115. doi: 10.1046/j.1365-2435.1999.00013.x.

Coe, K. K., Belnap, J., Grote, E. E., and Sparks, J. P. 2012. Physiological ecology of desert biocrust moss following 10 years exposure to elevated $CO_2$: evidence for enhanced photosynthetic thermotolerance. Physiologia Plantarum, **144**(4): 346-356. doi: 10.1111/j.1399-3054.2012.01566.x.

Corrêa, A., Gurevitch, J., Martins-Loução, M. A., and Cruz, C. 2012. C allocation to the fungus is not a cost to the plant in ectomycorrhizae. Oikos, **121**(3): 449-463. doi: 10.1111/j.1600-0706.2011.19406.x.

Ellis, E. C. 2011. Anthropogenic transformation of the terrestrial biosphere. Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences, **369**(1938): 1010-1035. doi: 10.1098/rsta.2010.0331.

Ganger, M., and Sturey, T. 2012. Antheridiogen concentration and spore size predict gametophyte size in *Ceratopteris richardii*. Botany. **90**(3): 175-179.

Goodnoe, T. T., Hill, J. P., and Aho, K. 2016. Effects of Variation in Carbon, Nitrogen, and Phosphorus Molarity and Stoichiometry on Sex Determination in the Fern *Ceratopteris richardii.* Botany. doi: 10.1139/cjb-2015-0187.

Intergovernmental Panel on Climate Change. 2013. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge U. Press, Cambridge.

Jain, A., Poling, M. D., Smith, A. P., Nagarajan, V. K., Lahner, B., Meagher, R. B., and Raghothama, K. G. 2009. Variations in the composition of gelling agents affect morphophysiological and molecular responses to deficiencies of phosphate and other nutrients. Plant Physiology, **150**(2): 1033-1049. doi: 10.1104/pp.109.136184.

Kimmerer, R. W. 2003. Gathering moss. A Natural and Cultural History of Mosses. Oregon State University Press. Corvallis.

Knack, J. J., Wilcox, L. W., Delaux, P. M., Ané, J. M., Piotrowski, M. J., Cook, M. E., Graham, J.M. and Graham, L. E. 2015. Microbiomes of streptophyte algae and bryophytes suggest that a functional suite of microbiota fostered plant colonization of land. International Journal of Plant Sciences, **176**(5): 405-420. doi: 10.1086/681161.

Kutschera, U. 2007. Plant-associated methylobacteria as co-evolved phytosymbionts: a hypothesis. Plant signaling & behavior, **2**(2): 74-78. doi: 10.4161/psb.2.2.4073.

Lammertsma, E. I., de Boer, H. J., Dekker, S. C., Dilcher, D. L., Lotter, A. F., and Wagner-Cremer, F. 2011. Global $CO_2$ rise leads to reduced maximum stomatal conductance in Florida vegetation. Proceedings of the National Academy of Sciences, **108**(10): 4035-4040. doi: 10.1073/pnas.1100371108.

Long, S. P., Ainsworth, E. A., Rogers, A., and Ort, D. R. 2004. Rising atmospheric carbon dioxide: plants FACE the Future. Annual Review of Plant Biology, **55**: 591-628. doi: 10.1146/annurev.arplant.55.031903.141610.

Mackenzie, F. T., Ver, L. M., and Lerman, A. 2002. Century-scale nitrogen and phosphorus controls of the carbon cycle. Chemical Geology, **190**(1): 13-32. doi: 10.1016/S0009-2541(02)00108-0.

Newingham, B. A., Vanier, C. H., Charlet, T. N., Ogle, K., Smith, S. D., and Nowak, R. S. 2013. No cumulative effect of 10 years of elevated [$CO_2$] on perennial plant biomass components in the Mojave Desert. Global change biology, **19**(7): 2168-2181. doi: 10.1111/gcb.12177.

Peñuelas, J., Sardans, J., Rivas-ubach, A., and Janssens, I. A. 2012. The human-induced imbalance between C, N and P in Earth's life system. Global Change Biology, **18**(1): 3-6. doi: 10.1111/j.1365-2486.2011.02568.x.

Reich, P. B., Hungate, B. A., and Luo, Y. 2006. Carbon-Nitrogen Interactions in Terrestrial Ecosystems in Response to Rising Atmospheric Carbon Dioxide. Annual Review of Ecology, Evolution, and Systematics, **37**: 611–636.

Rhen, T., and Lang, J. W. 1995. Phenotypic plasticity for growth in the common snapping turtle: effects of incubation temperature, clutch, and their interaction. American Naturalist, **146**(5): 726-747.

Richardson, A. E., Barea, J. M., McNeill, A. M., and Prigent-Combaret, C. 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant and Soil, **321**(1-2): 305-339.

Sage, R. F., Sharkey, T. D., and Seemann, J. R. 1989. Acclimation of photosynthesis to elevated $CO_2$ in five C3 species. Plant Physiology, **89**(2), 590-596. doi: http://dx.doi.org/10.1104/pp.89.2.590.

Saxe, H., Ellsworth, D. S., and Heath, J. 1998. Tree and forest functioning in an enriched $CO_2$ atmosphere. New Phytologist, **139**(3): 395-436. doi: 10.1046/j.1469-8137.1998.00221.x.

Sayers, A., and Hamilton, R. G. 1995. The Effect of Neighbors on Gametophyte Development in *Ceratopteris richardii*. American Fern Journal, **85**(2): 47–53. doi: 10.2307/1547465.

Smith, D. L., Subramanian, S., Lamont, J. R., and Bywater-Ekegärd, M. 2015. Signaling in the phytomicrobiome: breadth and potential. Frontiers in plant science, **6**: 709. doi: 10.3389/fpls.2015.00709.

Van Der Heijden, M. G., Bardgett, R. D., and Van Straalen, N. M. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology letters, **11**(3): 296-310. doi: 10.1111/j.1461-0248.2007.01139.x.

SUPPLEMENTARY MATERIAL

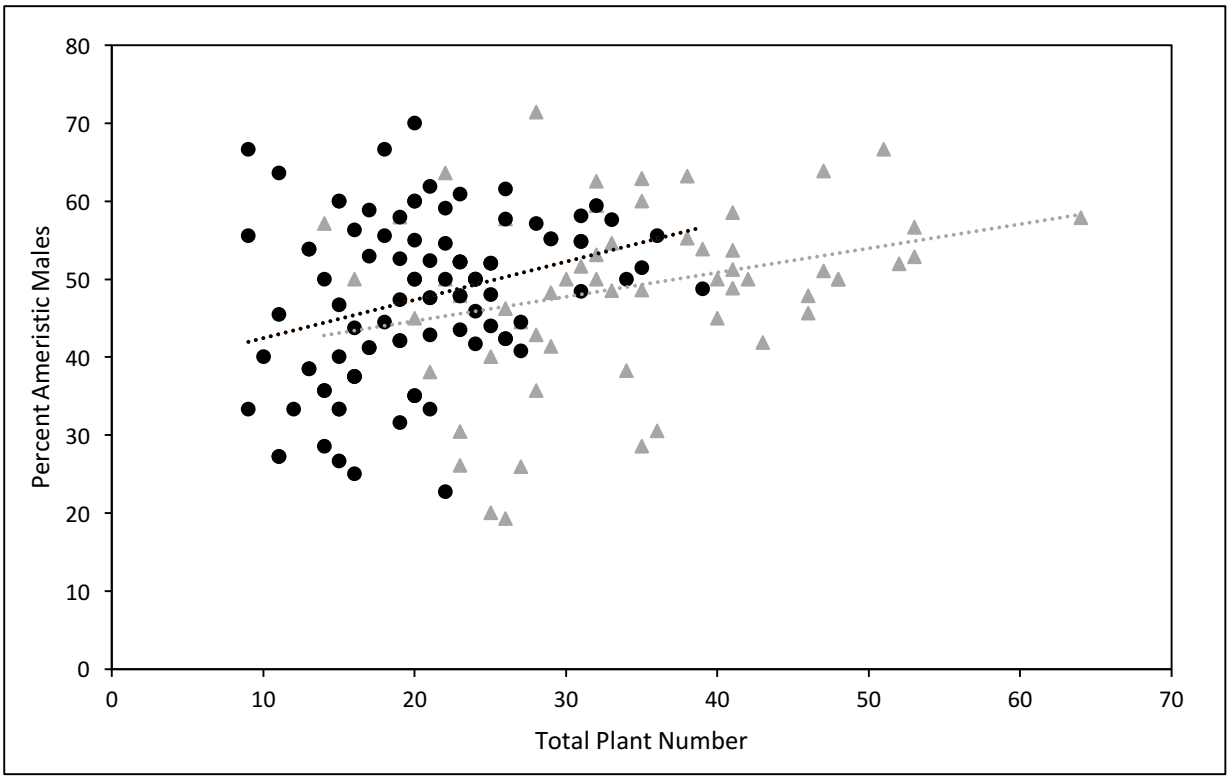



Figure S1. Plot of percent ameristic males as a function of total plant number, for both ambient $CO_2$ (black circles) and elevated $CO_2$ (grey triangles). At ambient $CO_2$, the slope of the trend line is equal to 0.49 and the $R^2$ value for the regression is equal to 0.097. At elevated $CO_2$, the slope of the trend line is equal to 0.31 and the $R^2$ value for the regression is 0.081.

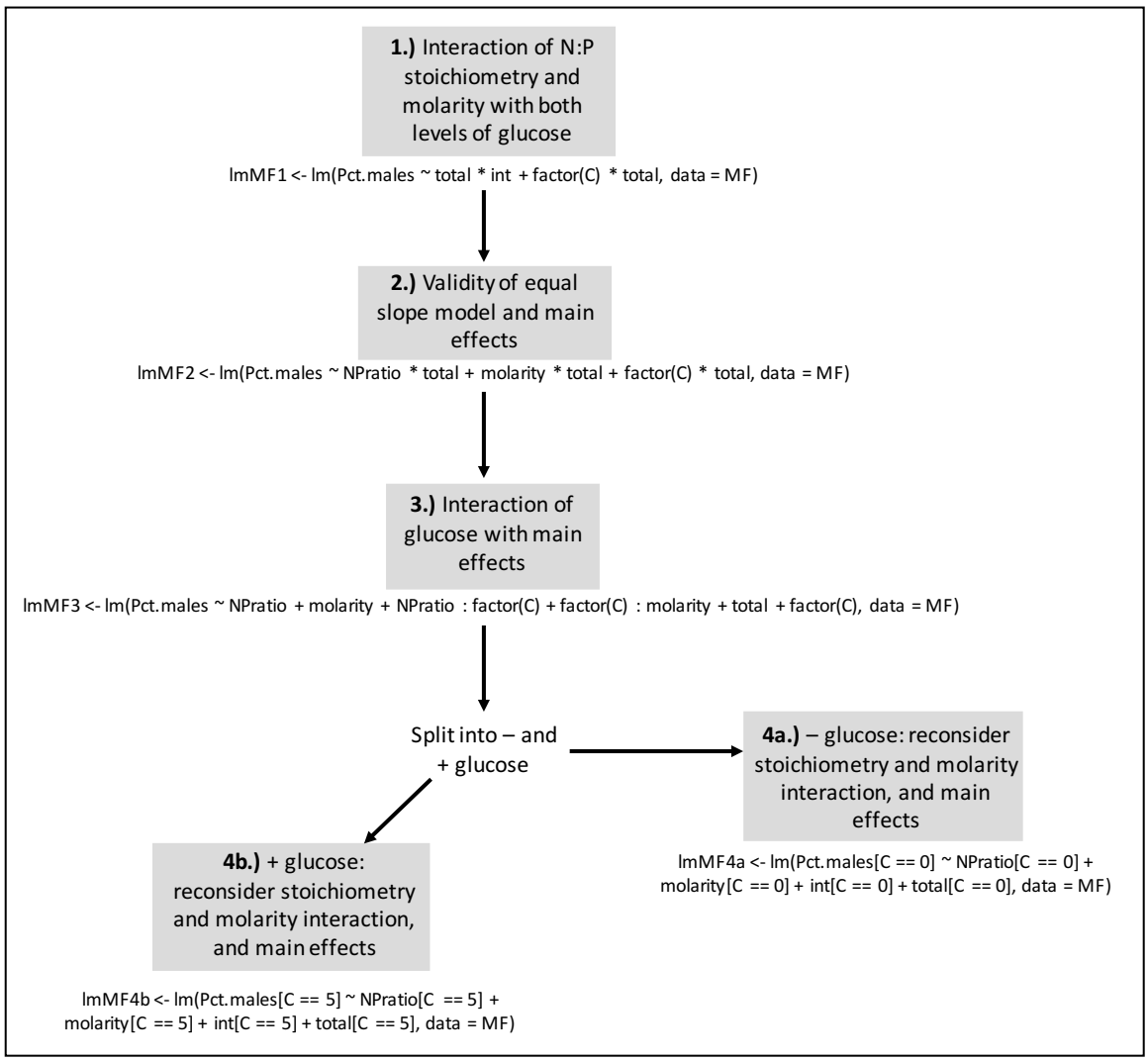
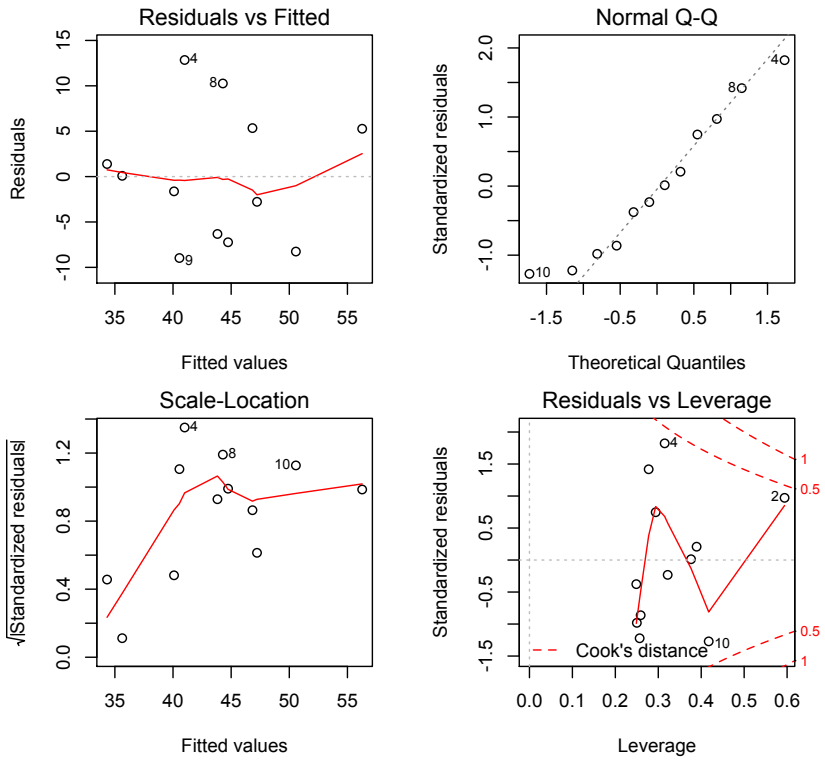



Figure S2. Model selection flow chart, including R model code for each step. Insignificance of the interaction in step (1) prompted analysis of molarity and stoichiometry main effects. Insignificance of main effects in step (2) prompted analysis of interaction of main effects and glucose level. Significant interaction between N:P ratio and glucose level led to the division of the data set into without (-) and with (+) glucose, and analysis of – and + glucose separately. The model selection process was completed for both the ambient and elevated $CO_2$ experiments.

**A.**


Residuals vs Fitted
Residuals
Fitted values
Normal Q-Q
Standardized residuals
Theoretical Quantiles
Scale-Location
√|Standardized residuals|
Fitted values
Residuals vs Leverage
Standardized residuals
Cook's distance
Leverage

**B.**

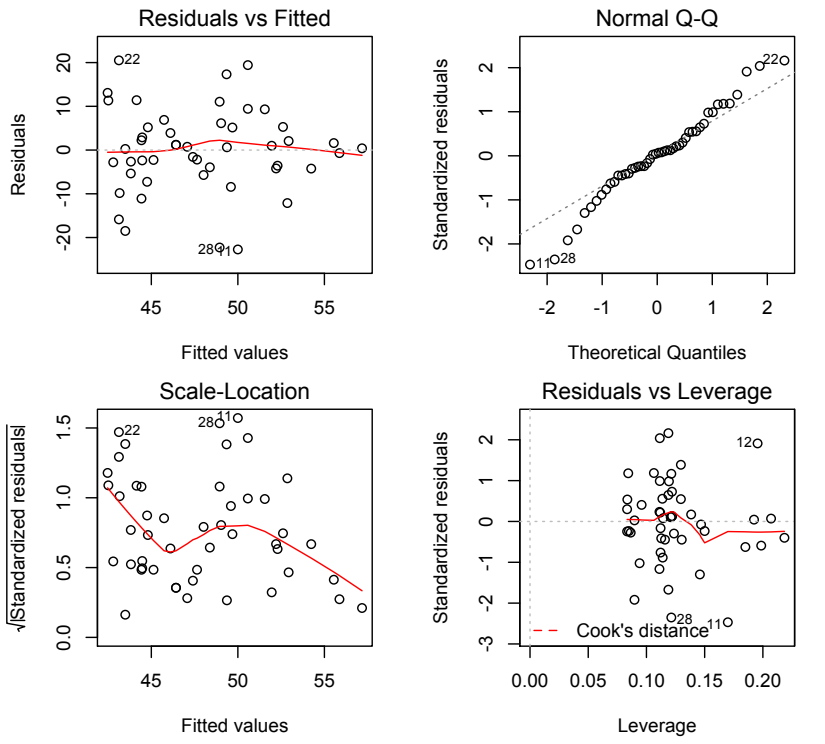
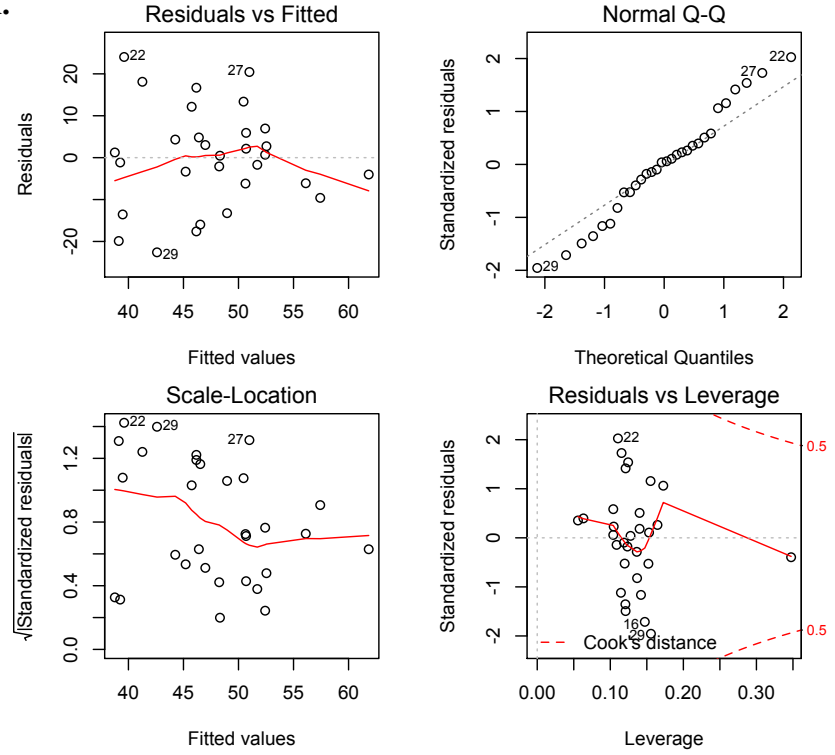

Residuals vs Fitted
Residuals
Fitted values
Normal Q-Q
Standardized residuals
Theoretical Quantiles
Scale-Location
√|Standardized residuals|
Fitted values
Residuals vs Leverage
Standardized residuals
Cook's distance
Leverage

Figure S3. Default diagnostic plots for the ambient $CO_2$ experiment described in Chapter 2, both (A.) without and (B.) with glucose. In the presence of glucose, factor-level populations are heteroscedastic, based on the Breusch-Pagan test (*P*-value 0.148). There is some evidence of non-constant variance when glucose is absent (*P*-value = 0.035), however, increasing variance in residual values is not a monotonic function of fitted values. Both without and with glucose, factor-level populations are normally distributed based on normal quantile plots and the Shapiro-Wilk normality test (*P*-value = 0.715 and 0.578, respectively).

**A.**


Residuals vs Fitted
Residuals
Fitted values
Normal Q-Q
Standardized residuals
Theoretical Quantiles
Scale-Location
√|Standardized residuals|
Fitted values
Residuals vs Leverage
Standardized residuals
Cook's distance
Leverage

**B.**

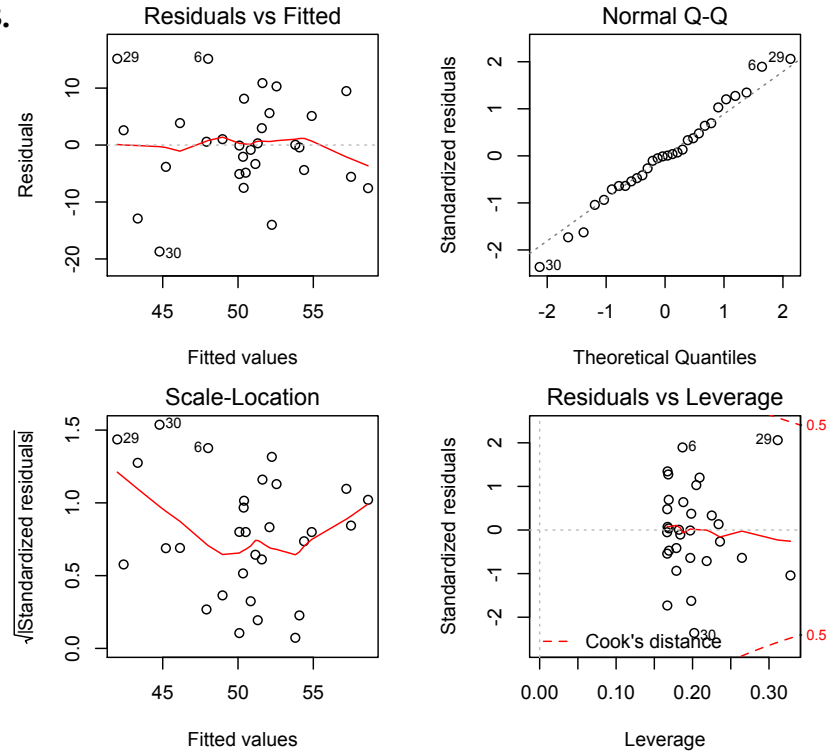

Residuals vs Fitted
Residuals
Fitted values
Normal Q-Q
Standardized residuals
Theoretical Quantiles
Scale-Location
√|Standardized residuals|
Fitted values
Residuals vs Leverage
Standardized residuals
Cook's distance
Leverage

Figure S4. Default diagnostic plots for the elevated $CO_2$ experiment described in Chapter 2, both (A.) without and (B.) with glucose. Without glucose, there is some evidence of non-constant variance when glucose is absent based on the Breusch-Pagan test (*P*-value = 0.035), however, increasing variance in residual values is not a monotonic function of fitted values. In the presence of glucose, factor-level populations are heteroscedastic (*P*-value = 0.336). Both without and with glucose, factor-level populations are normally distributed based on normal quantile plots and the Shapiro-Wilk normality test (*P*-value = 0.803 and 0.850, respectively).

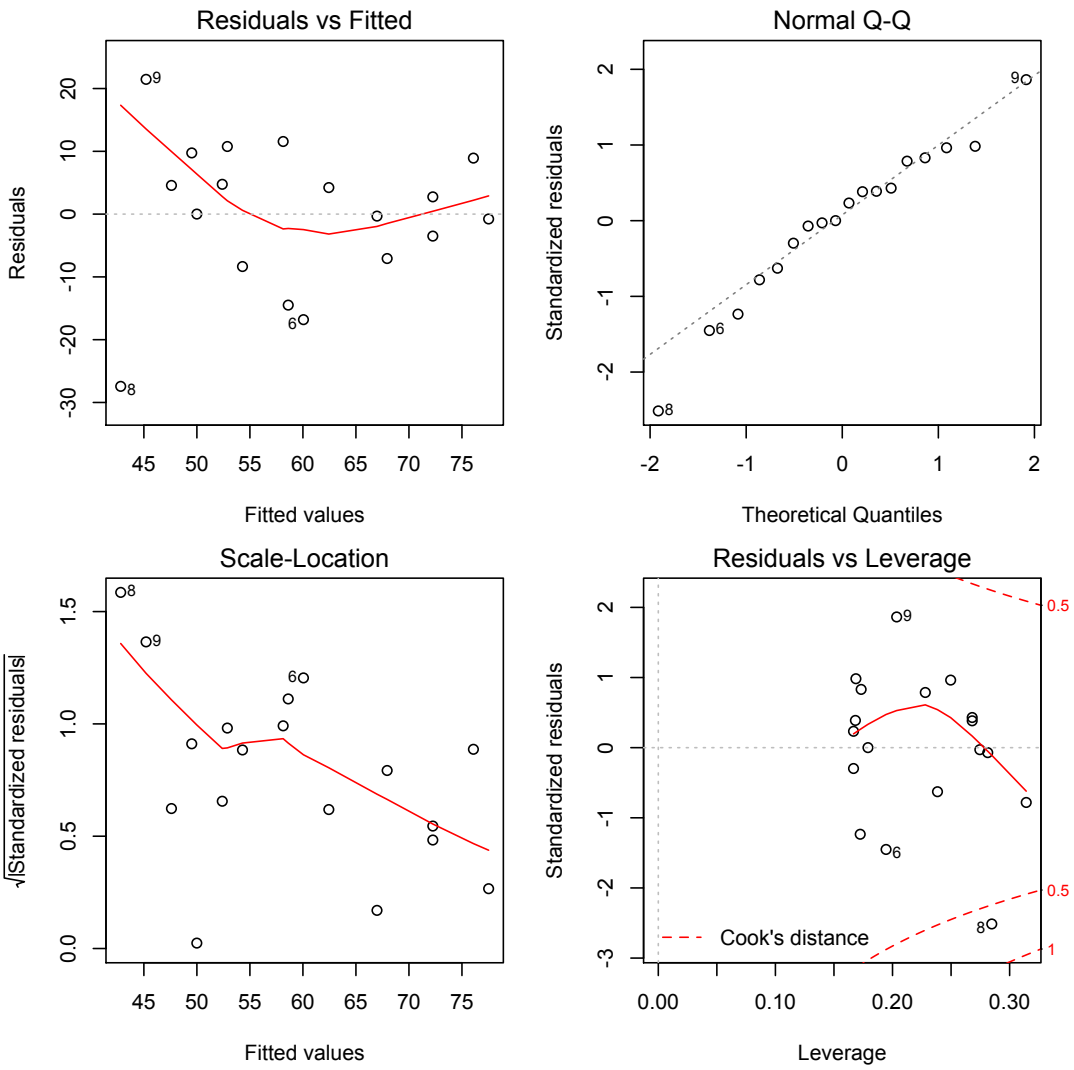



Figure S5. Default diagnostic plots produced for the P-limitation experiment. Individual observations are assumed independent because there is no obvious trend in the residuals vs. fitted plot. The linear relationship in the normal Q-Q plot and the Shapiro-Wilk normality test (*P*-value = 0.73) suggest that the data is normally distributed. Because there is not a shot-gun pattern in either the residuals vs. fitted plot or the scale-location plot homoscedasticity can be assumed. Based on the residuals vs. leverage plot, observation number 8 may be an outlier.

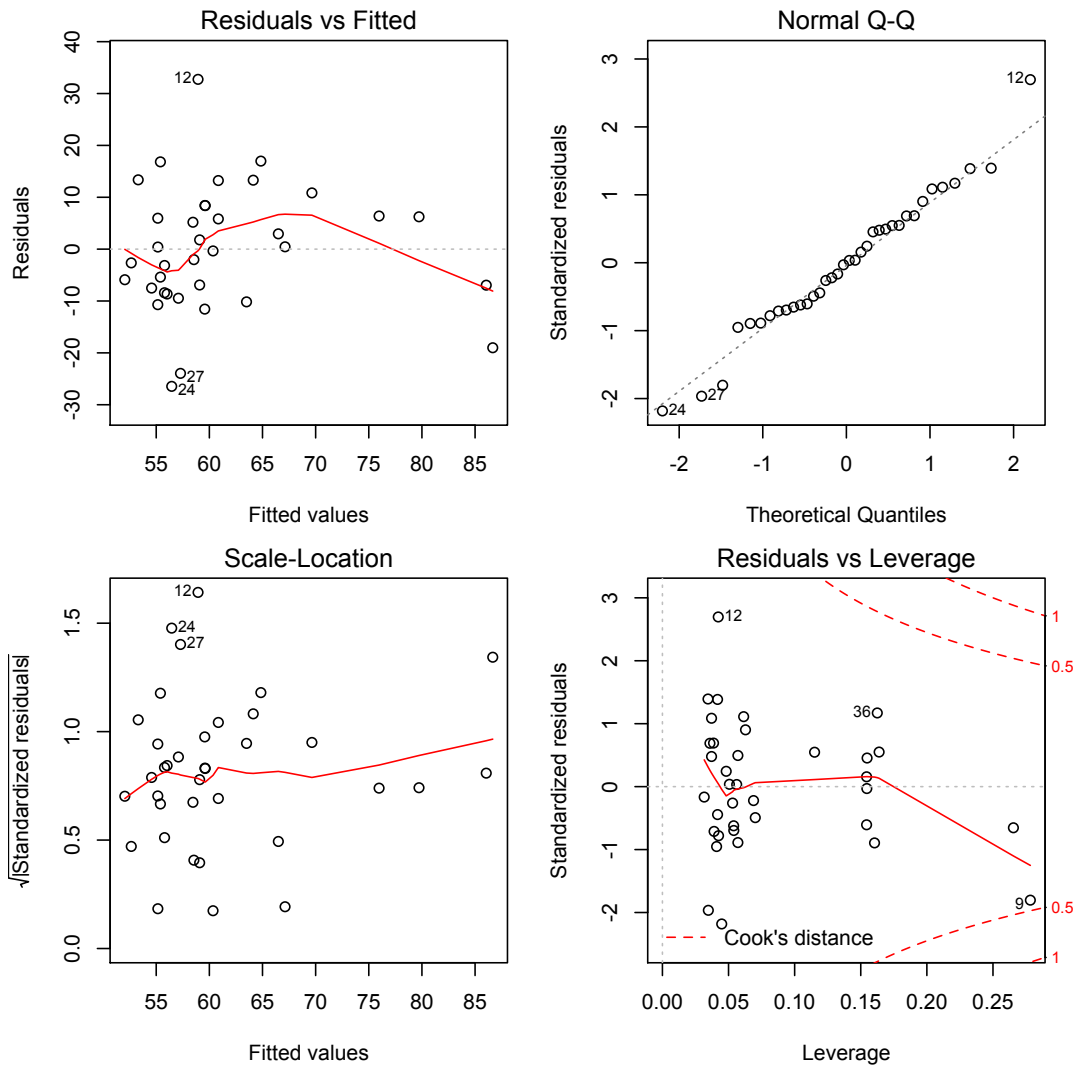



Figure S6. Default diagnostic plots produced for the N-limitation experiment. Individual observations are most likely independent because there is no obvious trend in the residuals vs. fitted plot. The linear relationship in the normal Q-Q plot and the Shapiro-Wilk normality test (*P*-value = 0.75) suggest that the data is normally distributed. Since there is not a shot-gun pattern in either the residuals vs. fitted plot or the scale-location plot homoscedasticity can be assumed.

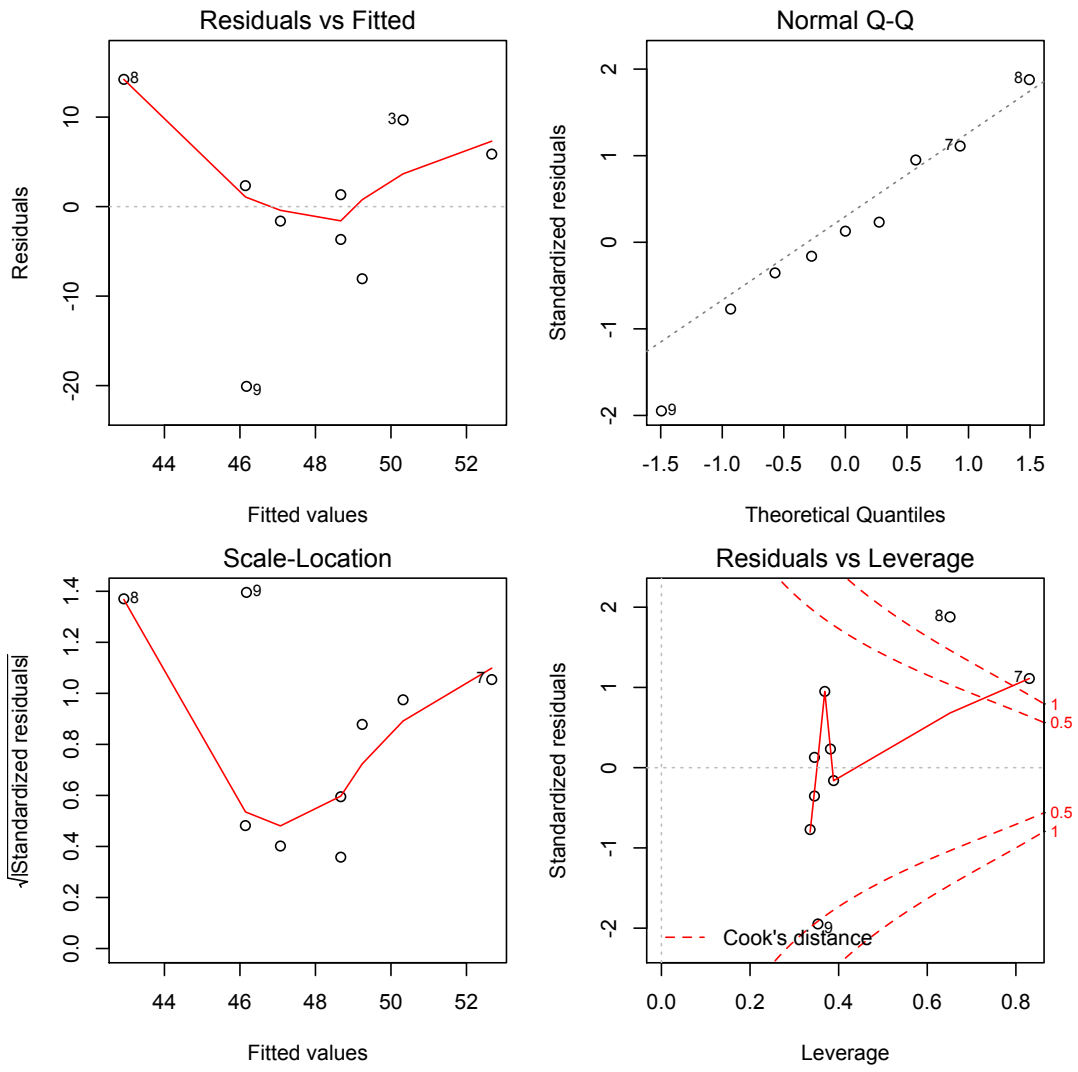



Figure S7. Default diagnostic plots produced for the C-limitation experiment. Individual observations are assumed independent because there is no trend in the residuals vs. fitted plot. The linear relationship in the normal Q-Q plot and the Shapiro-Wilk normality test (P-*value = 0.88*) suggest that the data is normally distributed. Since there is not a shot-gun pattern in either the residuals vs. fitted plot or the scale-location plot, homoscedasticity can be assumed. Based on the residuals vs. leverage plot, observations number 7, 8, and 9 may be outliers.

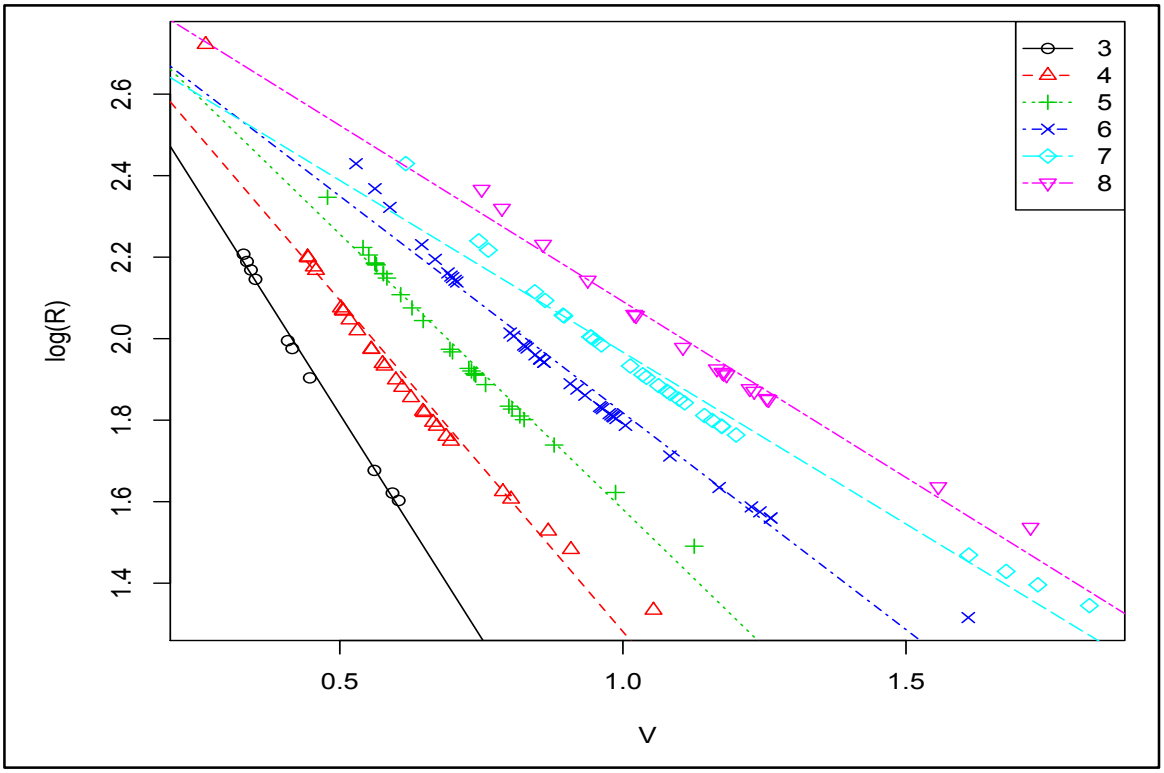



Figure S8. The log of R (relative reproductive output, measured as the number of archegonia per unit area per meristic gametophytes) plotted as a function of V (vegetative output, measured as gametophyte area) for meristic gametophytes with archegonia counts three through eight. Based on the ANCOVA, the interaction term (V:number of archegonia) significantly influenced R (*P*-value <0.0001) and the R-V relationship is negative with a slope of approximately -2.20.