FACTORS AFFECTING THE NUTRIENT STRESS RESPONSE OF THE HYBRID SUNFLOWER *HELIANTHUS ANOMALUS*

by

LARRY C. BROUILLETTE

(Under the Direction of Lisa A. Donovan)

ABSTRACT

Hybridization is an important evolutionary process leading to the establishment of new species. Ecological speciation, or the formation of new species by colonization of novel niche, is a process by which newly-formed hybrids may persist alongside parental lineages. *Helianthus annuus* and *H. petiolaris* hybridized multiple times, giving rise to three hybrid species, which currently occupy different habitats. One of these hybrid species, *H. anomalus*, grows on sand dunes in the western United States, which have much lower fertility than the soils where the parental species are found. We employ a comparative approach within this known phylogenetic context to explore the role of nutrient limitation in the evolution of *H. anomalus*. We find that *H. anomalus* is more tolerant of nutrient stress than *H. annuus* and *H. petiolaris*. This tolerance appears to be conferred by increased tissue longevity that allows nutrients to be used more efficiently but comes at the cost of a reduced maximum relative growth rate. We present evidence that stress response genes, including one that is likely to affect leaf turnover rates, respond differently to nutrient stress in *H. anomalus* compared to its parental species. We find evidence of selection on several physiological traits important in plant water and nitrogen use strategies, suggesting that water and nutrients have been important forces in the evolution of *H.*

anomalus. Finally, we detect a pair of large-effect quantitative trait loci that affect foliar nitrogen concentrations, which may have been important in the rapid evolution of *H. anomalus*.

INDEX WORDS: ecological speciation, relative growth rate, ecophysiology, gene expression, QTL

FACTORS AFFECTING THE NUTRIENT STRESS RESPONSE OF THE HYBRID SUNFLOWER *HELIANTHUS ANOMALUS*

by

LARRY C. BROUILLETTE

B.S., Millsaps College, 2003

M.S., The University of Georgia, 2007

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2009

FACTORS AFFECTING THE NUTRIENT STRESS RESPONSE OF THE HYBRID SUNLFLOWER *HELIANTHUS ANOMALUS*

by

LARRY C. BROUILLETTE

Major Professor: Lisa A. Donovan

Committee: Kelly Dawe
Steven Knapp
Russell Malmberg
Rodney Mauricio

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2009

ACKNOWLEDGEMENTS

Several people have helped me in my academic career. My parents taught me the value of an education and encouraged me to seek the opportunities to learn new things. I have had excellent teachers at every stage of my education, but two were particularly outstanding. Dr. Eric Griffin helped me develop my skills as a writer. Dr. Mike Scanlon taught me techniques of molecular biology. Far more than this, both were outstanding mentors who gave me guidance during particularly difficult times. I fall short of their excellent examples, but they are two people I hope to be more like.

My doctoral advisor, Dr. Lisa Donovan, was instrumental in the design of studies and writing the dissertation. My advisory committee gave valuable feedback on the study designs and provided me with lab space, training, and equipment to complete much of the molecular work.

Several of my fellow graduate students, especially Cara Gormally and David Rosenthal, helped me think through the many logistical issues that came up through the course of my work. Michael Boyd and other members of the greenhouse staff worked tirelessly to keep my plants healthy. Undergraduate workers Charlie Darragh, Renee Pascal, Suzie Fatkin and Mechel DeSears helped with the very busy harvests. Roger Nilsen helped me overcome several problems with the microarray project. The UGA Plant Biology Department, the UGA Graduate School, and the Garden Club of America provided generous financial support.

This dissertation could not have been put together without the support I received, and I will always be grateful for all of the help I had along the way.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iv

LIST OF TABLES vii

LIST OF FIGURES viii

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW 1

2 TESTING HYPOTHESIZED SHIFTS TOWARD STRESS TOLERANCE IN HYBRID *HELIANTHUS* SPECIES 6

Abstract 7

Introduction 8

Materials and Methods 10

Results 15

Discussion 17

3 VARIATION IN RELATIVE GROWTH RATE AND ITS COMPONENTS IN WILD *HELIANTHUS* SPECIES 31

Abstract 32

Introduction 32

Materials and Methods 36

Results 40

Discussion 42

4 NUTRIENT STRESS RESPONSE OF A HYBRID SUNFLOWER: A GENE EXPRESSION STUDY 60

Abstract 61

Introduction 61

Materials and Methods 63

Results 67

Discussion 68

5 VARIATION IN FUNCTIONAL PLANT TRAITS RELATIVE TO NEUTRAL GENETIC VARIANCE IN *HELIANTHUS ANOMALUS* 79

Abstract 80

Introduction 80

Materials and Methods 82

Results 87

Discussion 87

6 GENETIC ARCHITECTURE OF LEAF ECOPHYSIOLOGICAL TRAITS IN *HELIANTHUS* 101

Abstract 102

Introduction 102

Materials and Methods 105

Results 107

Discussion 107

7 CONCLUSIONS 118

LIST OF TABLES

Page

Table 2.1: Analysis of plant growth variables and total biomass 27

Table 2.2: Plant functional trait means and data analysis 28

Table 2.3: Photosynthetic trait means and data analysis 29

Table 2.4: Analysis of selected plant functional traits 30

Table 3.1: Statistical analysis of RGR 57

Table 3.2: Statistical analysis of RGR components 58

Table 3.3: Statistical analysis of plant functional traits 59

Table 4.1: Candidate genes 77

Table 4.2: Contingency table of GO classifications 78

Table 5.1: Populations sampled 97

Table 5.2: Traits measured 99

Table 5.3: SSR markers 100

Table 6.1: Composite interval mapping results 116

Table 6.2: Epistasis results 117

LIST OF FIGURES

Page

Figure 2.1: Total biomass at reproductive maturity..23

Figure 2.2: Stem height and diameter growth..24

Figure 2.3: Plant functional traits...25

Figure 2.4: Maximum early seedling relative growth rate..26

Figure 3.1: Plant growth 52

Figure 3.2: Relative growth rate and its carbon components...53

Figure 3.3: Nitrogen use components of RGR..54

Figure 3.4: Plant functional traits...56

Figure 4.1: Loop hybridization design...75

Figure 4.2: Comparison of microarray and qPCR results...76

Figure 5.1: F_{ST} and Q_{ST} estimates..96

Figure 6.1: QTL map 115

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Hybridization is an important evolutionary process leading to the establishment of new plant and animal species (Arnold 1997). The genus *Helianthus* presents an ideal system for studying hybridization because the same pair of parental species hybridized to produce three ancient hybrid species, essentially a naturally replicated experiment in hybrid speciation (Rieseberg 1991). Ecological differentiation is thought to be an important driving force in the evolution of new species via selection under contrasting environmental pressures (Schluter 2001). Hybrid zones are often observed in disturbed areas, and these are often areas where hybrid lineages can be established (Seehausen 2004). *Helianthus* hybrids have overwhelmingly transgressive phenotypes when compared to the parental species (Rosenthal et al. 2002). The transgressive phenotypes exist in early generation hybrids, and those individuals are thought to be preadapted to novel, more stressful habitats (Rieseberg et al. 2003, Rosenthal et al. 2005b). A thorough understanding of the selective pressures and genetic changes that led to the establishment of the species would be a valuable tool in understanding speciation.

Helianthus is a good system for looking at adaptation to low nutrient habitats. *Helianthus annuus* and *H. petiolaris* hybridized multiple times to form the homoploid hybrid species *H. anomalus*, *H. deserticola*, and *H. paradoxus* (Rieseberg 1991). The two parental species occur on relatively benign substrates compared to the desert sand dune, desert floor, and salt marsh habitats of *H. anomalus*, *H. deserticola*, and *H. paradoxus*, respectively. Because *H. anomalus* and *H. deserticola* occur on soils that are much less fertile than their parental species

(L.A. Donovan, in prep.), phenotypes of hybrid species can be compared to those of the parental species for putative adaptations to low nutrient habitats (Rosenthal et al. 2002).

Previous work in the sunflower hybrids has pointed toward nutrient limitation as an important factor in the evolution of *H. anomalus*. Ludwig and colleagues (2004) demonstrated that selection in the *H. anomalus* habitat favors higher foliar nitrogen. Rosenthal and others (2005a) showed that, although it appears dry, the active sand dune habitat of *H. anomalus* contains deep water stores that are available year round. In a resource addition experiment, naturally-occurring *H. anomalus* did not have increased biomass when given nutrients or water alone (Ludwig et al. 2006). These results suggest that the ability to tolerate nutrient stress is one important determinant of success in the *H. anomalus* habitat.

Nutrient availability limits plant growth and productivity in many habitats (Verhoeven et al. 1996, Aerts & Chapin 2000). Nitrogen, in particular, is limiting due to high biochemical demands and relatively low supply, especially in newer soils. Plant adaptations to low nutrient habitats generally decrease demand of the limiting nutrient, increase uptake, or increase productivity (Grime 1979, Chapin 1980, Aerts & Chapin 2000). Traits decreasing demand for resources include the ability to re-absorb nutrients from senescent tissue, increased tissue longevity, and reduced losses due to leaching (Aerts & Chapin 2000). Specific leaf area, or the leaf area per unit of dry leaf mass, is known to be an important correlate for several traits affecting resource demand (Reich et al. 1997). Specific leaf area is known to come at a physiological cost of a reduced maximum growth rate (Lambers & Poorter 2004).

We ask the following questions about factors affecting the nutrient stress response of *H. anomalus*:

1. Do the hybrid species show a greater tolerance of nutrient stress?

2. How does nutrient availability affect the growth and allocation of *H. anomalus* and its parental species?
3. What effect does nutrient availability have on the gene expression in *H. anomalus*, *H. petiolaris*, and *H. annuus*?
4. Do traits important in the nutrient stress response of *H. anomalus* show evidence of selection?
5. What is the genetic architecture of traits related to nutrient use?

To address these questions, we present a series of experiments using techniques from across the subdisciplines of the biological sciences. These questions address the effects of nutrient stress on several spatial and temporal scales. Gene expression is regulated on very short temporal scales at the cellular level. Comparisons of plant phenotypes under contrasting growing conditions addresses plant acclimation over longer time scales at the whole-plant level. Making comparisons using these two methods within a known phylogenetic context allows us to make inference about selective pressures during the speciation of *H. anomalus*. In addition to the comparative approach, we look for evidence of selection with *H. anomalus* by comparing variation in plant phenotypes to neutral genetic variance. Four of these independent studies point toward leaf lifespan as an important trait, lending support to the idea that longer tissue longevity confers some selective advantage in the *H. anomalus* habitat.

References

Aerts R, Chapin III FS (2000) The mineral nutrition of wild plants revisited: A re-evaluation of processes and patterns. Advances in Ecological Research, 30, 1-67.

Arnold M L (1997) Natural Hybridization and Evolution. Oxford University Press, Oxford.

Chapin FS III (1980) The mineral-nutrition of wild plants. Annual Review of Ecology and Systematics, 11, 233-260.

Grime JP (1979) Plant Strategies and Vegetation Processes. John Wiley, New York.

Lambers H, Poorter H (2004) Inherent variation in growth rate between higher plants: A search for physiological causes and consequences. Advances in Ecological Research, 34, 283-362.

Ludwig F, Jewett RA, Donovan LA (2006) Nutrient and water addition effects on day and night-time conductance and transpiration in a C3 desert annual. Oecologia, 148, 219-225.

Ludwig F, Rosenthal DM, Johnston JA, et al. (2004) Selection on leaf ecophysiological traits in a desert hybrid *Helianthus* species and early-generation hybrids. Evolution, 58, 2682-2692.

Reich PB, Walters MB, Ellsworth DS (1997) From tropics to tundra: Global convergence in plant functioning. Proceedings of the National Academy of Sciences, USA, 94, 13730-13734.

Rieseberg LH (1991) Homoploid reticulate evolution in *Helianthus* (Asteraceae): Evidence from ribosomal genes. American Journal of Botany, 78, 1218-1237.

Rieseberg LH, Raymond O, Rosenthal DM, et al. (2003) Major ecological transitions in wild sunflowers facilitated by hybridization. Science, 301, 1211-1216.

Rosenthal DM, Ludwig F, Donovan LA (2005a) Plant responses to an edaphic gradient across an active sand dune/desert boundary in the great basin desert. International Journal of Plant Sciences, 166, 247-255.

Rosenthal DM, Rieseberg LH, Donovan LA (2005b) Recreating ancient hybrid species' complex multi-trait phenotypes from early generation synthetic hybrids: three examples using wild sunflowers. American Naturalist, 166, 26-41.

Rosenthal DM, Schwarzbach AE, Donovan LA, Raymond O, Rieseberg LH (2002) Phenotypic differentiation between three ancient hybrid taxa and their parental species. International Journal of Plant Sciences, 163, 387-398.

Seehausen O (2004) Hybridization and adaptive radiation. Trends in Ecology and Evolution, 19, 198-207.

Schluter D (2001) Ecology and the origin of species. Trends in Ecology and Evolution, 16, 372-380.

Verhoeven JTA, Koerselman W, Meuleman AFM (1996) Nitrogen- or phosphorus-limited growth in herbaceous, wet vegetation: relations with atmospheric inputs & management regimes. Trends in Ecology and Evolution, 11, 494-497.

CHAPTER 2

TESTING HYPOTHESIZED SHIFTS TOWARD STRESS TOLERANCE IN HYBRID *HELIANTHUS* SPECIES[1]

---

[1] Brouillette LC, Gebremedhin M, Rosenthal DM, Donovan LA. 2006. *Western North American Naturalist* 66: 409-419. Reprinted here with permission of publisher.

Abstract

We examined how plant traits related to growth and resource use have evolved during hybrid speciation and specialization into stressful habitats. Two desert sunflower species of homoploid hybrid origin are endemic to habitats with lower soil nutrient levels than those of their ancestral parent species. We hypothesized that the hybrid species would exhibit greater tolerance to low levels of soil nutrients than their parental species. The two hybrid species, *Helianthus anomalus* and *H. deserticola*, and their parental species, *H. annuus* and *H. petiolaris*, were compared for plant traits and growth through reproduction under three nutrient levels in a glasshouse study. An additional seedling study compared species for maximum seedling relative growth rate under optimum conditions. The hybrid species did have greater tolerance of nutrient limitation than the parental species, demonstrated by a resistance to change in stem height and diameter growth across treatments. A similar pattern was observed in total biomass at final harvest. This ability to maintain growth may be partially explained by maintained investment in photosynthetic enzymes regardless of nutrient treatment. Though the hybrid species were more tolerant of nutrient stress, differences in the hybrid response to nutrient stress compared to the parental species' response were much smaller than expected from habitat comparisons. *Helianthus anomalus* has evolved a classic stress tolerant phenotype, having long leaf lifespan, tough leaves, and slower early seedling relative growth rate. While both hybrid species have a conservative growth strategy conferring greater stress tolerance than parental species, functional

trait differences among the hybrids suggest that the two species have experienced vastly different selective pressures.

Introduction

Evolution of tolerance to stressful environments is a major area of ecological research with important applications to conservation biology and crop breeding (Wang et al. 2003). Many studies observe correlations between plant functional traits and environmental parameters, falling short of demonstrating that traits of interest were derived under a specific set of environmental conditions (Grime 1977). Organisms with known evolutionary relationships, such as hybrid species and their ancestral parents, can be used to make stronger inferences that certain plant functional traits are adaptive. In this study, we use a hybrid system to examine how environmental pressures directed the evolution of tolerance and functional trait responses to nutrient stress.

We focus on four species of sunflower that occur in the Great Basin. Due to variation in climatic and edaphic conditions, the Great Basin contains a patchwork of habitats spanning broad ranges of water and nutrient levels (Smith & Nowak 1990, Comstock & Ehleringer 1992). *Helianthus annuus* is a widely-distributed annual sunflower that occurs on mesic, clay-based soils in the Western United States. *Helianthus petiolaris* has a more restricted distribution, occurring on relatively more xeric, sandy soils (Heiser 1947). These two species hybridized multiple times to produce three ancient homoploid hybrid species (Rieseberg 1991). Hybridization produced individuals with functional trait values outside the range of the parental species, potentially making hybrids suited for colonization of extreme habitats (Rieseberg et al. 2003, Rosenthal et al. 2005b).

We compare the parental *Helianthus* species to the two hybrid species that occur on sandy, nutrient-poor soils in the Great Basin Desert. *Helianthus anomalus* is endemic to active sand dunes, which are very poor in fertility and organic content (N ~0.01%, P ~0.4 mg g^{-1}, organic content ~0.01%; Rosenthal et al. 2005a, F. Ludwig in preparation). *Helianthus deserticola* occurs on stabilized sand dunes with higher fertility than the *H. anomalus* habitat (N ~0.03%, P ~0.4 mg g^{-1}, organic content ~0.02), but lower fertility than the nearby habitats of both parental species (N ~0.6%, P ~0.5-.06 mg g^{-1}, organic content ~0.04-0.05%; Rosenthal et al. 2005a, L.A. Donovan, in prep.). Previous studies have pointed toward nutrient availability as an important selective pressure in the habitats of the hybrid species (Ludwig et al. 2004, Rosenthal et al. 2005a). However, this is the first study to examine differences in nutrient stress tolerance in the hybrid species relative to the parental species. We determine how functional trait values in the hybrid species have shifted as the species became established in and adapted to resource poor habitats in the Great Basin Desert.

Because the hybrid species occur on nutrient poor soil, we hypothesized that they would exhibit greater tolerance to soil nutrient limitations than the parental species. We define nutrient stress tolerance as the ability to resist change in plant growth or biomass accumulation when nutrients vary from optimum to limiting levels. We also expected pronounced plant traits associated with low-resource habitats in the hybrid species, compared to the parental species.

Suites of plant functional traits such as increased root mass ratios, lowered tissue turnover rates, and decreased transpiration rates have been proposed as adaptations that allow plants to inhabit resource-poor habitats. These traits may come at a physiological cost, exhibited as lowered photosynthetic rates, decreased capacity for nutrient uptake, and/or reduction in maximum growth rate (Chapin et al. 1993). Variation in specific leaf area (SLA, leaf area per unit dry mass) may also be an important trait in stressful environments. Traits such as leaf lifespan, leaf longevity, and maximum photosynthetic rate are strongly correlated with SLA across plant functional groups (Reich et al. 1997).

In this study, we experimentally compare the growth and performance responses of two hybrid *Helianthus* species to their ancestral parent species at different nutrient levels. We expect the hybrid species to exhibit greater tolerance to nutrient stress than their parental species. We also examine a suite of traits important in nutrient-poor habitats to identify putative mechanisms of nutrient stress tolerance.

Materials and Methods

Seeds were collected from a single natural population per species in 2002 and stored at 6°C until use. Seeds from *H. annuus*, *H. anomalus*, and *H. deserticola* were collected at the Little Sahara Recreational area, Juab County, UT. Those populations were chosen because it is the only known place where the species presently co-occur. Seeds from *H. petiolaris* were collected from population used by Rieseberg et al. (2003) located 10 miles south of Page along Hwy 89, Coconino County, AZ. For all experiments, seeds of all four species were pre-treated for germination by cold stratification on moist filter paper for 4 weeks. Seeds were removed from cold treatment, stored in the dark at room temperature overnight, and transferred to fresh filter paper in Petri dishes. Petri dishes were placed under florescent lights (~115 μmol m^{-2} s^{-1}) with a 12-hour photoperiod.

Nutrient Limitation Experiment

The nutrient limitation experiment was a complete randomized block design, with 4 species, 3 nutrient treatment, 3 blocks, and 3 replicates per block, totaling 108 plants. Seedlings with 0-2 true leaves were transplanted into 25 cm pots in the University of Georgia greenhouses on 17-19 March 2004. Pots contained 3:1 sand/baked clay mixture (Turface, Profile Products, Buffalo Grove, IL). The high, medium, and low nutrient treatments were 40g, 4g, and 2g, respectively, of slow-release fertilizer with macro and micronutrients applied on 23 March to the upper 2cm of the soil (Osmocote Plus, Scotts-Sierra Horticultural Products, Marysville, OH). Nutrient levels were chosen based on a preliminary experiment where the 40g and 4g treatment was applied to all four species and a 2g treatment was applied to *H. anomalus*, which showed a significant decrease in biomass over the small change in nutrient levels. The plants were watered daily to maintain field capacity. Seven plants died from transplant shock and were excluded from all analyses.

We measured stem height 5 times during the experiment, starting on 23 March and continuing at approximate three-week intervals. Measurements for the last three dates included the diameter of the stem at soil level. We determined leaf lifetime by marking the most recently fully expanded mature leaf on 5 May and recording the date when 50% of the leaf turned brown. On 27 May, each plant was sampled for mature leaf traits by harvesting the most recently expanded fully mature leaf in early morning, when leaves were fully hydrated. Leaf area (LI-3100, LiCor Biosciences, Lincoln, NE), wet weight, and dry weight were measured to estimate SLA and leaf succulence (Jennings 1976). On 9 June, a fruit ripeness penetrometer was used to estimate leaf toughness on a mature leaf (McCormick Fruit Tech., Yakima, WA). Variability of individual measurements was large, so the mean of 5-7 measurements was the estimate of leaf toughness for the plant.

Gas exchange traits were measured on 5-6 July for a subset of the plants (4 replicates, 4 species, 2 treatments: high and low) with a portable gas exchange system (LI-6400, LiCor Biosciences). Chamber conditions were photosynthetically active radiation = 2000 µmol m^{-2} s^{-1} and air temperature ≈ 30°C. Measurements were taken at multiple external CO_2 concentrations (c_a) to determine rates of photosynthesis (A) over a range of internal leaf CO_2 concentrations (c_i). In order, measurements were taken at c_a equal to 400, 300, 200, 100, 50 and 400 ppm. Because photosynthesis at low c_i is limited by rubisco and is linearly related to the values of c_i, we estimated investment in the enzyme as initial linear the slope of regression of A with c_i. We averaged the two measurements at 400 ppm to estimate maximum photosynthesis (A_{max}). Date had no significant effect on measurements ($p > 0.90$) and was excluded from the final model. All measurements were corrected for leaf area (LI- 3100).

Gas exchange leaves were individually dried at 60°C, ground, and analyzed for N concentration (mg N/ g biomass) (NA1500, Carbo Erba Strumentazione, Milan, Italy) and leaf carbon isotopic composition (leaf $\delta^{13}C$, continuous flow mass spectrometer, Finnegan, Bremen, Germany). Leaf $\delta^{13}C$ provides an integrated measure of c_i over the lifetime of the leaf. Integrated c_i is, in turn, a relative measure of integrated water use efficiency (WUE), provided leaf temperatures are similar (Farquhar et al. 1989, Ehleringer et al. 1992). A higher (less negative) leaf $\delta^{13}C$ reflects greater WUE. Maximum photosynthetic rate, leaf N, and specific leaf area were used to calculate photosynthetic nitrogen-use efficiency (PNUE) following Field and Mooney (1986).

All plants were harvested on 15-16 June. Biomass was partitioned into belowground, vegetative, and reproductive components. Counts were taken of number of buds, flowers, and seed-heads to estimate life history stage at harvest. Biomass components were dried at 60°C and weighed.

We modeled each variable as a function of categorical variables treatment, species, and their interaction, with block as a random effect in a mixed model ANOVA (PROC MIXED, SAS 8, Cary, NC). All biomass components were log-transformed to fit ANOVA assumptions. Visual inspection of residual plots revealed extreme outliers for leaf succulence ($n = 2$), specific leaf area ($n = 2$), total biomass ($n = 1$), and stem growth ($n = 1$). These observations were excluded from our analyses. Unadjusted values from the final three measurements of stem height and stem diameter were analyzed in a repeated-measurement mixed model in PROC

MIXED with an AR(1) covariance matrix. We used only the final three measurements because they were available for both growth measurements. Inclusion of the first two dates of plant height did not change conclusions of statistical tests. For traits measured in all treatments, we broke the interaction term into six components: linear and quadratic trends between and within the hybrid and parental species using a contrast statement. Partitioning the interaction term between hybrid and parental species tested the hypothesis that parental species respond differently to nutrient stress than the hybrid species. Further breaking the interaction into within hybrids and within parentals tested for species differences in response. For traits assessed only in high and low treatments, only linear components were examined. Evaluating these *a priori* comparisons retained precision that is lost in post-hoc tests.

Seedling Relative Growth Rate Experiment

The relative growth rate study was a completely randomized design with 4 species, 2 harvests, and 12 replicates, giving a sample size of 96. On 17-19 March, seedlings were transplanted into 6.5 cm x 25 cm pots (Deepots, Stuewe & Sons, Corvallis, OR) containing the soil mixture described for the nutrient limitation experiment. Seedlings were watered to field capacity daily and received non-limiting nutrient solution weekly (300 ppm Pete Lite, J.R. Peters Lab., Allentown, PA). The plants were used to estimate maximum early seedling relative growth rate (RGR_{max}) under optimum conditions for each of the four species. Plants were harvested on 29 March and 12 April, dried at 60°C, and measured for total biomass. Log-transformed total biomass was regressed against harvest date, species, and their interaction (PROC GLM, SAS 8, Poorter and Lewis 1986). We used Fisher's protected least significant difference tests to determine significant differences in the interaction term, indicating species with statistical differences in RGR_{max}.

Results

Nutrient Limitation Experiment

Plant performance was assessed as stem height and diameter growth and total biomass at harvest. All four species showed substantial reduction in growth and biomass production in response to lowered nutrient levels (Figures 2.1 & 2.2; Table 2.1). Hybrid species had a less drastic reduction in stem height and diameter growth than the parental species when comparing the low nutrient treatment to the high nutrient treatment, seen as a significant difference in linear response of hybrid and parental species (Table 2.1). This was paralleled by a similar, but not significant, pattern in total biomass (Figure 2.1; Table 2.1, F=0.14). Individuals of the hybrid species under low nutrients produced approximately 85% less biomass than those under high nutrients. By comparison, parental species showed a 90% reduction. We used percentage of inflorescences at the seed head stage to assess life history status. These data suggest differences in life history stage between species and treatments at harvest, with *H. anomalus* in the high treatment having the lowest percentage of inflorescences at the seed head stage (Table 2.2).

There was a highly significant effect of treatment on leaf percent nitrogen, indicating that foliar nitrogen concentration paralleled soil nutrient levels. Hybrid species tended to have less of a difference between treatments than the parental species (Table 2.3). The opposite was true of leaf $\delta^{13}C$, with parental species overall having less of a change in WUE as a response to treatment than the hybrid species (Table 2.3, F=2.93+). There was also a difference between hybrid and parental species responses to nutrient stress for leaf succulence; hybrid species tended to maintain higher leaf succulence under low nutrients (Table 2.2, F=3.93+). Hybrid species maintained higher investment in rubisco than parental species under low nutrient conditions, as seen in the significant difference in linear response of the initial slope of the A/c_i curve (Figure 2.3; Table 2.4, F=4.36*). Though we examined A_{max} and leaf-level PNUE, only marginally significant differences in the linear response of hybrid and parental species were detected for A_{max} (Table 2.3, F=3.31+).

In addition to the anticipated differences between the hybrid and parental species, there were also interesting differences in the responses of the two hybrid species to nutrient treatment. *Helianthus anomalus* produced leaves that were tougher than those of the other species (Figure 2.3; Table 2.4, F=94.82***). Additionally, *H. anomalus* produced longer-lived leaves in response to the lowered nutrients assessed as a difference in linear nutrient stress response between *H. anomalus* and *H. deserticola* (Figure 2.3; Table 2.4, F=9.31**). We also observed a marginally significant quadratic response of *H. anomalus* root mass ratio to the treatment levels, suggesting a lower threshold for stress-induced root allocation (Table 2.2, F=3.43+).

Under low resources, *H. deserticola* had a mean maximum photosynthetic rate that comparable to high nutrient plants, but the response of the two hybrid species was not significantly different (Table 2.3, F=1). Additionally, the linear response of specific leaf area to the nutrient levels in *H. deserticola* indicates it produces thicker leaves in response to nutrient stress, unlike the other study species (Figure 2.3; Table 2.4, F=5.90*).

Seedling Relative Growth Rate Experiment

Helianthus anomalus had a significantly slower early seedling RGR_{max} than *H. deserticola* and *H. petiolaris*. We estimate seedling RGR_{max} for *H. anomalus* to be lower than that of *H. annuus*, but the difference was not significant (Figure 2.4).

Discussion

We conclude that the hybrid species are more tolerant of nutrient stress than their parental species. We observed significant differences in stem height and diameter growth that suggested higher stress tolerance in the hybrid species. A similar trend was seen in total biomass, but differences were not significant. We found evidence that hybrid species have higher tolerance of nutrient limitation, but differences were much subtler than expected. The final harvest date arbitrarily truncated the life cycles of the species, and the failure to detect expected differences in biomass could be due to early cutoff of *H. anomalus*, which tends to persist in the field until first frost in many populations. A better test of the effects of nutrient limitation on fitness would have been a direct measure of seedset. Pollinators are excluded from the glasshouse, so seed weight and number are impossible to obtain without frequent crossing throughout the experiment.

Foliar nitrogen concentration was less responsive to treatment in hybrid species compared to parental species. This seems to be important in the field because selection analyses show a strong association between foliar nitrogen content and fitness in the *H. anomalus* habitat

(Ludwig et al. 2004). High levels of foliar nitrogen may account for the greater investment in rubisco and marginally higher ability to maintain A_{max} found in hybrid species under low nutrient conditions. The resistance to changing investment in photosynthesis despite lower nitrogen availability is one possible explanation for increased nutrient stress tolerance of the hybrid species. Under low nutrients, hybrid species also tended to have higher water-use efficiency and leaf succulence, which are putative drought resistance traits. Water does not appear to be a major limiting factor for *H. anomalus* in its home habitat, so drought-resistance traits in *H. anomalus* may result from genetic correlations among stress resistance traits (Chapin et al. 1993).

Helianthus anomalus produced tougher, longer-lived leaves than the other species and showed a reduced early seedling RGR_{max}. While RGR_{max} was not significantly lower than both parentals in this study, a subsequent experiment showed *H. anomalus* had a significantly lower RGR_{max} than *H. annuus* (data not shown). *Helianthus anomalus* also tended to increase investment in roots in the intermediate nutrient treatment. These observations suggest *H. anomalus* has a classic stress-tolerant phenotype. Under this paradigm, long leaf lifespan decreases demand and higher root allocation increases uptake of the limiting resource. These traits probably come at a cost of reduced seedling RGR_{max} (Chapin et al. 1993).

Our data suggest that *H. deserticola* has a different strategy than *H. anomalus*. Unlike in the *H. anomalus* habitat, water becomes scarce in the *H. deserticola* habitat early in the growing season (Rosenthal et al. 2005a). Water and nutrients likely co-limit growth in the *H. deserticola* habitat, imposing selective pressures different from those in the *H. anomalus* habitat (Ludwig et al. 2004). Under low nutrients, *H. deserticola* produced leaves with low SLA, a trait correlated to putative adaptations to multiple abiotic stresses (Riech et al. 1997). It is also interesting that some photosynthetic traits were unresponsive to treatment in hybrids because we have observed unexpectedly high photosynthetic rates in *H. deserticola* growing in its nutrient-poor home habitat (D. M. Rosenthal, unpublished).

Though we initially expected more obvious differences, we demonstrate that *H. anomalus* and *H. deserticola* are more tolerant of nutrient stress than their parental species. Additionally, we report shifts in *H. anomalus* trait means predicted by correlational studies of plant adaptations to abiotic stress. *Helianthus anomalus* also has a reduced seedling RGR_{max}, which is likely a physiological cost of stress resistance. *Helianthus deserticola* appears to acclimate to stress by shifting specific leaf area. Hybrid species maintain greater investment in photosynthetic enzymes despite severe nutrient limitation. While the two species exhibit similar growth responses to nutrient stress, *H. anomalus* and *H. deserticola* appear to have evolved different suites of traits in order to adapt to distinct low resource habitats.

Acknowledgements

The authors thank A Howard, C Gormally, and two anonymous reviewers for comments on earlier drafts. Research was supported by NSF grant IBN-0131078 to LAD and a UGA Plant Biology Department small grant to LCB.

References

Chapin FS, Autumn K, Pugmaire F (1993) Evolution of suites of traits in response to stressful environments. American Naturalist, 142, S78-S92.

Comstock JP, Ehleringer JR (1992) Plant adaptation in the Great Basin and Colorado Plateau. Great Basin Naturalist, 52, 195-215.

Ehleringer JR, Phillips SL, Comstock JP (1992) Seasonal variation in the carbon isotopic composition of desert plants. Functional Ecology, 6, 396-404.

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology, 40, 503-537.

Field F, Mooney HA (1986) The photosynthesis-nitrogen relationship in wild plants. In: On the Economy of Plant Form and Function (ed. Givnish TJ), pp.25-55. Cambridge University Press, Cambridge.

Grime JP (1977) Evidence for existence of 3 primary strategies in plants and its relevance to ecological and evolutionary theory. American Naturalist, 111, 1169-1194.

Heiser CB (1947) Hybridization between the sunflower species *Helianthus annuus* and *Helianthus petiolaris*. Evolution, 1, 249-262.

Jennings DH (1976) The effects of sodium chloride on higher plants. Biology Review, 51, 453-486.

Ludwig, F, Rosenthal DM, Johnston JA, et al. (2004) Selection on leaf ecophysiological traits in a desert hybrid *Helianthus* species and early-generation hybrids. Evolution, 58, 2682-2692.

Poorter H, Lewis C (1986) Testing differences in relative growth-rate – A method avoiding curve fitting and pairing. Pyhsiologia Plantarum, 67, 223-226.

Reich PB, Walters MB, Ellsworth DS (1997) From tropics to tundra: Global convergence in plant functioning. Proceedings of the National Academy of Sciences, USA, 94, 13730-13734.

Rieseberg LH (1991) Homoploid reticulate evolution in *Helianthus* (Asteraceae): Evidence from ribosomal genes. American Journal of Botany, 78, 1218-1237.

Rieseberg LH, Raymond O, Rosenthal DM, et al. (2003) Major ecological transitions in wild sunflowers facilitated by hybridization. Science, 301, 1211-1216.

Rosenthal DM, Ludwig F, Donovan LA (2005a) Plant responses to an edaphic gradient across an active sand dune/desert boundary in the great basin desert. International Journal of Plant Sciences, 166, 247-255.

Rosenthal DM, Rieseberg LH, Donovan LA (2005b) Recreating ancient hybrid species' complex multi-trait phenotypes from early generation synthetic hybrids: three examples using wild sunflowers. American Naturalist, 166, 26-41.

Smith SD, Nowak RS (1990) Ecophysiology of plants in the intermountain lowlands. In: Plant Biology of the Basin and Range (eds. Osmond CB, Pitelka LF, Hidy GM), pp 179-241. Springer-Verlag, Berlin.

Wang W, Vincour B, Altman A (2003) Plant responses to drought, salinity, and extreme temperatures: Toward genetic engineering for stress tolerance. Planta, 218, 1-14.

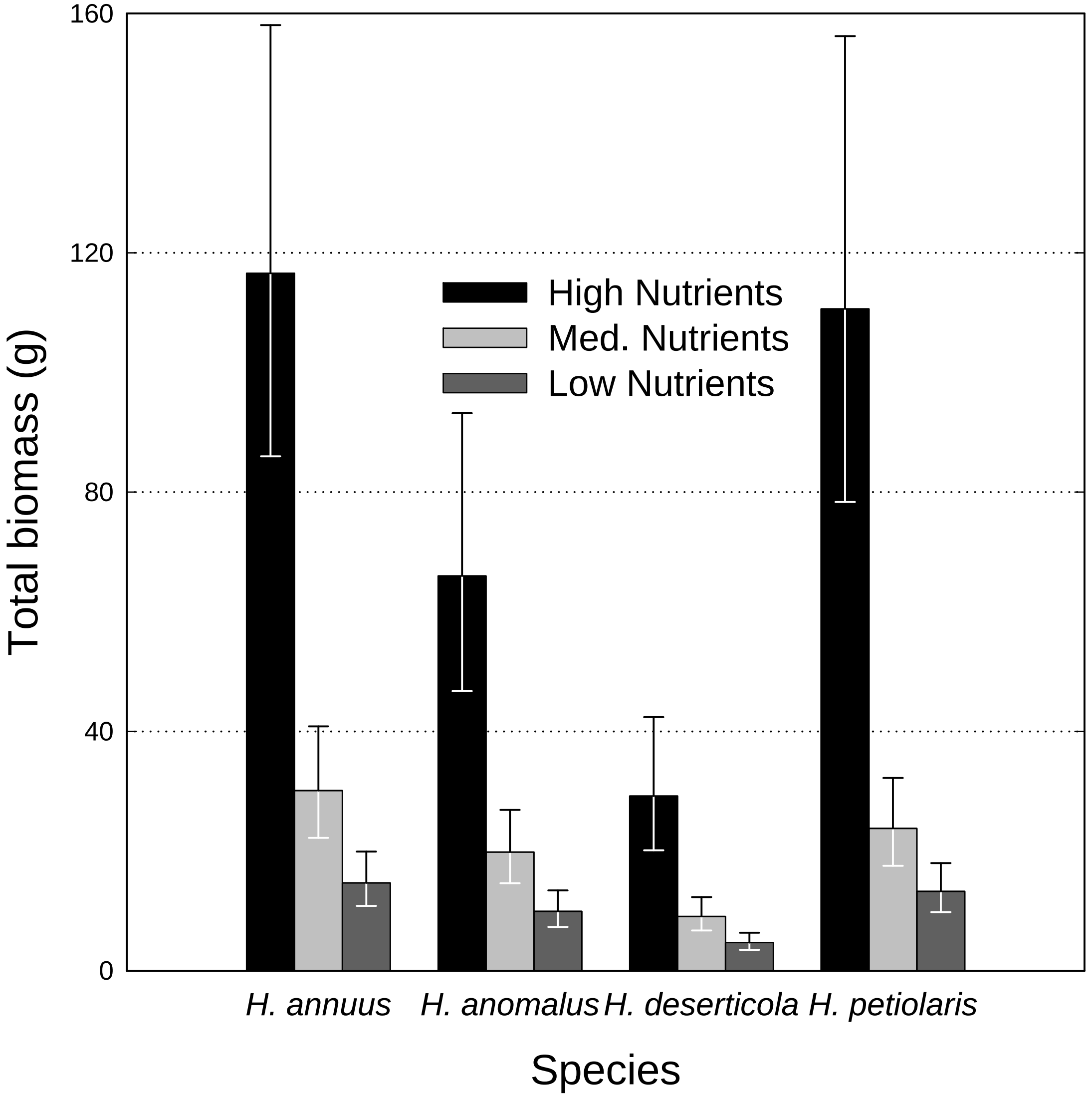

Figure 2.1: Total biomass at reproductive maturity. Mean total biomass measurements and 95% confidence intervals for four *Helianthus* species grown in a glasshouse at three nutrient levels (n = 101).

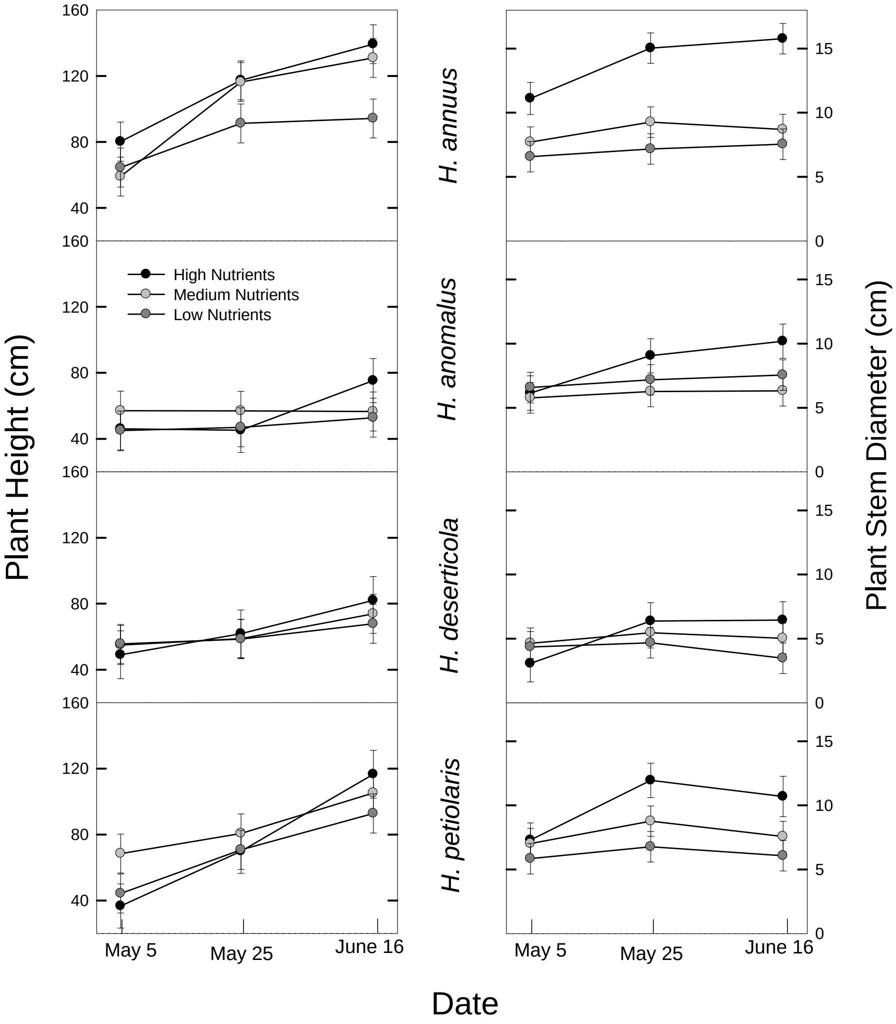

Figure 2.2: Stem height and diameter growth. Stem height and diameter for four *Helianthus* species grown under three nutrient levels in a glasshouse (n = 101). Plotted values are arithmetic means at each date for each species/treatment combination. Least squared estimates of the means are given for each of three dates used in the repeated measures analysis. Each time, measurements were repeated over all individual plants in the experiment. Error bars represent 95% confidence intervals.

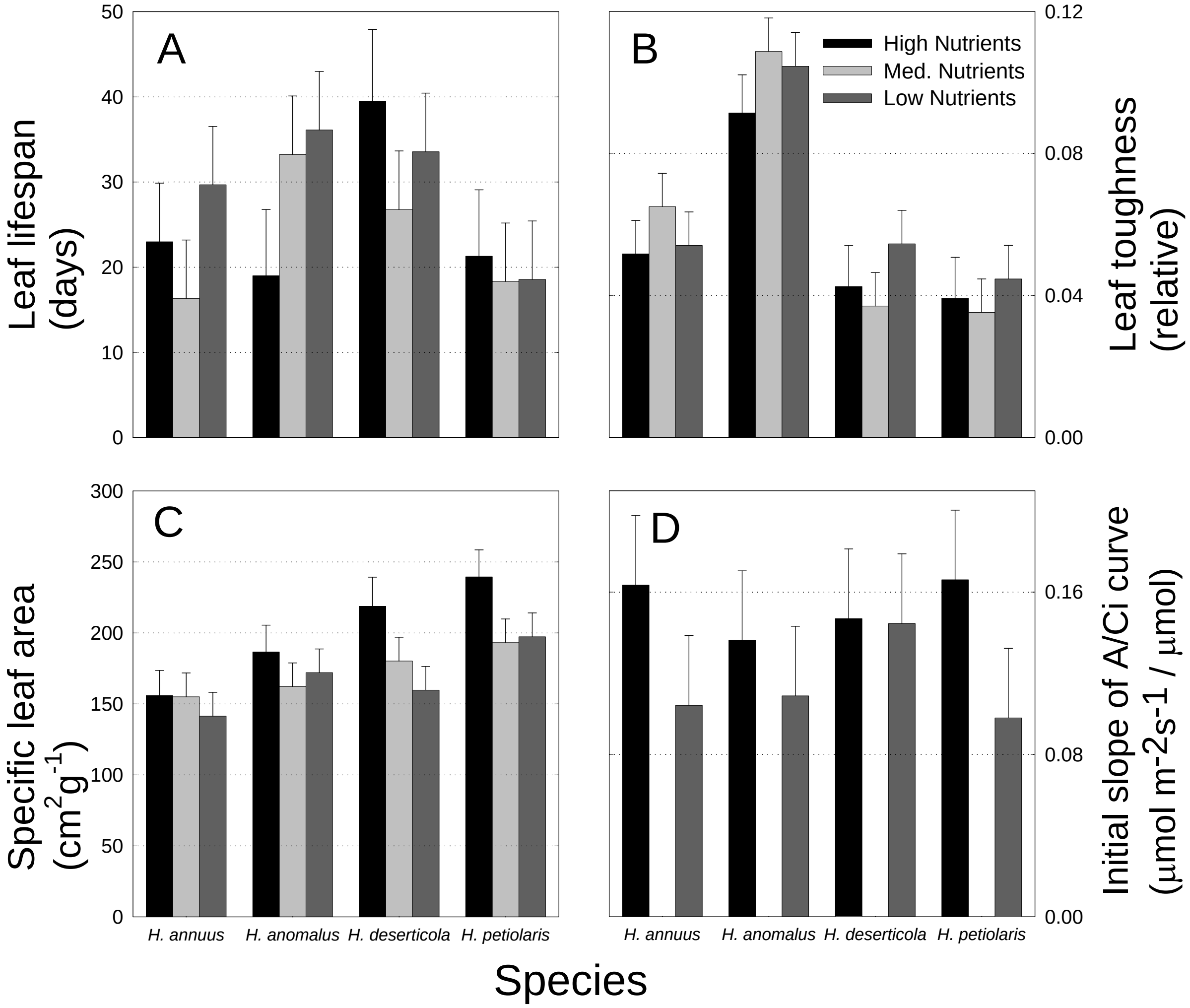

Figure 2.3: Plant functional traits. Model least squared estimates of the means of plant functional traits leaf lifespan (A), leaf toughness (B), specific leaf area (C), and initial slope of A/c_i curve (D). Traits were measured on four *Helianthus* species grown in a glasshouse under three nutrient levels. The total number of individuals assayed was 101, except for A/c_i where 32 plants were subsampled. For gas exchange measurements, such as initial slope of A/c_i curve, we subsampled individuals from high and low nutrient levels. Error bars represent 95% confidence intervals about the mean.

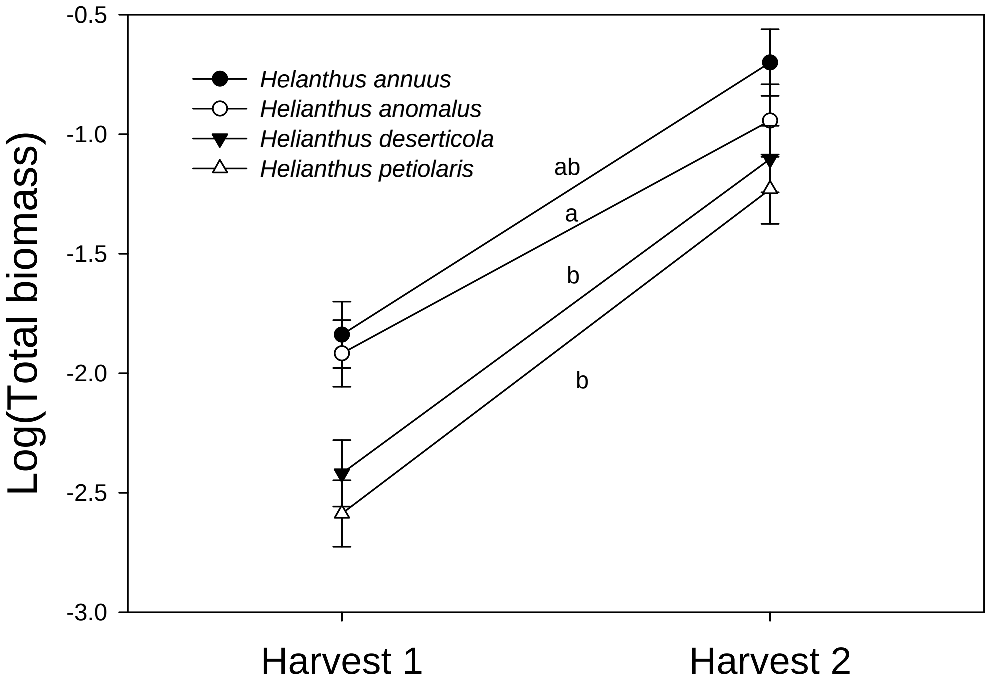

Figure 2.4: Maximum early seedling relative growth rate. LS means of log-transformed total seedling biomass is plotted for 2 harvests and 4 species (n = 96). Slopes with the same letter are not significantly different from each other, indicating that corresponding species have indistinguishable early seeding RGR at the p=0.05 level. Error bars represent 95% confidence intervals.

Table 2.1: Analysis of plant growth variables and total biomass. Degrees of freedom and F-values for the appropriate mixed model analysis of our three measures of tolerance: height growth, stem diameter growth, and total biomass at harvest ($n = 101$). Significant F-values are denoted + ($p \leq 0.10$), * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.0001$).

	Height		Stem diameter		Total biomass	
Source	df	F	df	F	df	F
Species	3,264	94.67***	3,260	122.66***	3,85	41.79***
Treatment	2,264	13.38***	2,260	118.00***	2,85	284.81***
Species*Treatment	6,264	3.58**	6,260	14.63***	6,85	0.68
Time	2,264	95.83***	2,260	29.38***		
Species*Time	6,264	11.47***	6,260	0.9		
Treatment*Time	4,264	4.63**	4,260	11.25***		
Species*Treatment*Time	12,264	1.60+	12,260	0.22		
Linear parental*hybrid	1,264	4.91*	1,260	30.32***	1,85	0.14
Linear annuus*petiolaris	1,264	10.84**	1,260	25.15***	1,85	0.04
Linear anomalus*deserticola	1,264	0.23	1,260	13.56***	1,85	1.12
Quadratic parental*hybrid	1,264	1.99	1,260	5.73*	1,85	1.78
Quadratic annuus*petiolaris	1,264	2.03	1,260	10.34**	1,85	0.65
Quadratic anomalus*deserticola	1,264	0.61	1,260	1.96	1,85	0.23

Table 2.2: Plant functional trait means and data analysis. Least squared estimates of mean trait values with 95% confidence interval bounds (in parenthesis) for functional traits measured at all treatment levels (n = 101). F-values and degrees of freedom from the mixed model analysis are reported below the trait values. Significant F-values are denoted + ($p \leq 0.10$), * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.0001$).

Treatment	Species	Percent seedheads at harvest (%)		Date of first flower (Julian day)		Root mass ratio (g/g)		Leaf succulence (mg/cm^2)	
High	*H. annuus*	40.1	(26.4, 53.7)	126.7	(123.1, 130.3)	0.113	(0.093, 0.138)	24.5	(21.3, 27.8)
	H. anomalus	16	(1.4, 30.6)	120.9	(116.9, 124.9)	0.09	(0.072, 0.112)	37	(33.4, 40.6)
	H. deserticola	37.5	(22.3, 52.8)	125.7	(121.3, 130.0)	0.073	(0.058, 0.089)	25.4	(21.6, 29.3)
	H. petiolaris	39.1	(23.9, 54.4)	127	(123.0, 131.1)	0.071	(0.057, 0.089)	24.2	(20.6, 27.8)
Medium	*H. annuus*	34	(20.4, 47.7)	131.4	(127.8, 135.1)	0.146	(0.120, 0.178)	22.5	(19.3, 25.8)
	H. anomalus	36.7	(23.0, 50.3)	118	(114.4, 121.6)	0.156	(0.128, 0.191)	41.2	(37.8, 44.6)
	H. deserticola	54.6	(40.9, 68.2)	121.1	(117.5, 124.7)	0.093	(0.077, 0.114)	30.8	(27.6, 34.0)
	H. petiolaris	56	(42.3, 69.6)	122.7	(119.1, 126.3)	0.095	(0.078, 0.115)	25.7	(22.5, 29.0)
Low	*H. annuus*	44.6	(30.9, 58.2)	128.2	(124.6, 131.8)	0.198	(0.163, 0.242)	21.9	(18.7, 25.2)
	H. anomalus	35.7	(22.1, 49.4)	117	(113.4, 120.6)	0.148	(0.122, 0.181)	39.5	(36.1, 42.9)
	H. deserticola	45.4	(31.7, 59.0)	121.9	(118.3, 125.5)	0.123	(0.101, 0.150)	29.5	(26.2, 32.7)
	H. petiolaris	54.2	(40.5, 67.8)	125.7	(122.1, 129.3)	0.111	(0.091, 0.135)	24.3	(21.0, 27.5)
	Source	df	F	df	F	df	F	df	F
	Species	3,86	8.93***	3,87	17.41***	3,87	17.06***	3,85	62.14***
	Treatment	2,86	6.72**	2,87	1.27	2,87	24.42***	2,85	2.04
	Species*treatment	6,86	1.8	6,87	1.79	6,87	0.87	6,85	1.26
	Linear parental*hybrid	1,86	0.29	1,87	2.32	1,87	0	1,85	3.93+
	Linear annuus*petiolaris	1,86	1.06	1,87	0.67	1,87	0.31	1,85	0.74
	Linear anomalus*deserticola	1,86	1.25	1,87	0	1,87	0	1,85	0.22
	Quadratic parental*hybrid	1,86	3.55+	1,87	0.84	1,87	1.05	1,85	2.05
	Quadratic annuus*petiolaris	1,86	4.26*	1,87	6.51*	1,87	0.29	1,85	0.72
	Quadratic anomalus *deserticola	1,86	0.07	1,87	0.31	1,87	3.43+	1,85	0.03

Table 2.3: Photosynthetic trait means and data analysis. Least squared estimates of mean trait values with 95% confidence interval bounds (in parenthesis) for plant functional traits measured only at high and low nutrient levels (n = 32). F-values and degrees of freedom from the mixed model analysis are reported below the trait values. Significant F-values are denoted + ($p \leq 0.10$), * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.0001$).

Treatment	Species	A_{max} (μmol CO$_2$ m^{-2}s^{-1})		PNUE (μmol CO$_2$(mol N)$^{-1}$s^{-1})		Leaf % nitrogen (%)		Leaf δ^{13}C (ppt)	
High	*H. annuus*	36.4	(28.6, 44.3)	252.8	(184.2, 321.5)	3.9	(3.43, 4.37)	-30.4	(-30.86, -29.93)
	H. anomalus	33.8	(26.0, 41.7)	272.1	(208.8, 335.5)	3.32	(2.82, 3.82)	-31.89	(-32.38, -31.39)
	H. deserticola	33.4	(25.6, 41.2)	253	(192.3, 313.6)	3.85	(3.31, 4.39)	-31.75	(-32.29, -31.22)
	H. petiolaris	39.5	(31.6, 47.3)	340.5	(271.2, 409.8)	4.08	(3.50, 4.67)	-31.19	(-31.78, -30.61)
Low	*H. annuus*	25.1	(17.3, 32.9)	305.5	(242.0, 368.9)	1.58	(1.08, 2.08)	-29.66	(-30.16, -29.17)
	H. anomalus	26.9	(19.1. 35.0)	349.4	(286.1, 412.8)	1.82	(1.35, 2.29)	-31.04	(-31.51, -30.58)
	H. deserticola	34	(26.1, 41.8)	313.7	(253.0, 374.5)	2.39	(1.85, 2.92)	-30.68	(-31.21, -30.14)
	H. petiolaris	25.1	(17.2, 32.9)	319	(257.0, 381.1)	2.09	(1.59, 2.59)	-31.23	(-31.73, -30.73)
	Source	df	F	df	F	df	F	df	F
	Species	3,22	0.32	3,22	1.62	3,22	2.48+	3,22	14.43***
	Treatment	1,22	9.10**	1,22	5.28*	1,22	112.36***	1,22	13.41***
	Species*treatment	3,22	1.49	1,22	1.28	3,22	1.54	3,22	1.64
	Linear parental*hybrid	1,22	3.31+	1,22	2.17	1,22	3.88+	1,22	2.93+
	Linear annuus*petiolaris	1,22	0.16	1,22	1.67	1,22	0.45	1,22	2.29
	Linear anomalus*deserticola	1,22	1	1,22	0.11	1,22	0	1,22	0.2

Table 2.4. Analysis of selected plant functional traits. Results of mixed model analysis of variance analyses of traits with means and standard errors plotted in figure 2.2. Significant F-values are denoted + ($p \leq 0.10$), * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.0001$). The total number of individuals assayed was 101, except for A/c_i where 32 plants were subsampled.

	Leaf Lifetime		Leaf Toughness		Specific Leaf Area		Initial slope of A/c_i curve	
Source	df	F	df	F	df	F	df	F
Species	3,87	8.81***	3,86	94.82***	3,86	24.38***	3,22	0.82
Treatment	2,87	2.90+	2,86	2.64+	2,86	14.64***	1,22	11.27**
Species*treatment	6,87	3.23**	6,86	2.31*	6,86	2.30*	3,22	1.67
Linear parental*hybrid	1,87	0.48	1,86	1.42	1,86	0.44	1,22	4.36*
Linear annuus*petiolaris	1,87	1.73	1,86	0.09	1,86	2.47	1,22	0.07
Linear anomalus*deserticola	1,87	9.31**	1,86	0.01	1,86	5.90*	1,22	0.57
Quadratic parental*hybrid	1,87	0.75	1,86	0.28	1,86	0.12		
Quadratic annuus*petiolaris	1,87	1.93	1,86	5.02*	1,86	4.63*		
Quadratic anomalus*deserticola	1,87	6.24*	1,86	6.85*	1,86	0.29		

CHAPTER 3

VARIATION IN RELATIVE GROWTH RATE AND ITS COMPONENTS IN WILD *HELIANTHUS* SPECIES[1]

---

[1] Brouillette LC, Donovan LA. To be submitted to *Oecologia*.

Abstract

We examined relative growth rate (RGR) and its nitrogen and carbon use components in the *Helianthus* hybrid system to explore the nutrient stress response of the homoploid hybrid sunflower *H. anomalus*. The active sand dune habitat of *H. anomalus* contains lower levels of soil nutrients than the habitats of the parental species *H. annuus* and *H. petiolaris*. Therefore, we expected the hybrid species to have a lower seedling RGR measured under optimum conditions as a consequence of a growth strategy that conserves nitrogen. Partitioning RGR into its components, we expected a longer mean residence time of nitrogen (MRT) and lower specific leaf area (SLA) in *H. anomalus* because longer MRT and SLA are key traits under selection in nutrient-limited habitats. All three species were grown in a glasshouse experiment with two treatments: non-limiting and limiting nitrogen. Plants were destructively harvested at four time points to estimate relative growth rate and its components during the seedling, juvenile, and adult stages. As expected, *H. anomalus* had a lower seedling maximum RGR than the parental species. Additionally, we found the seedling growth rate of *H. anomalus* was less affected by nitrogen limitation than its parental species. MRT was longer for *H. anomalus* suggesting that tissue longevity is an important component of the nitrogen use strategy of the species. This is supported by data showing *H. anomalus* increased leaf lifespan in response to nutrient stress. Also, *H. anomalus* appears to have a longer growing season, with fewer of its reproductive heads mature and a large proportion of biomass still invested in green leaves at the end of the study, the length of which approximates the average lifespan of these three annual plant species.

Introduction

Nutrient availability is a limiting factor in many natural systems (Verhoeven et al. 1996, Aerts & Chapin 2000). The ability to maintain growth under nutrient-limited conditions is an important part of establishment, growth, and persistence of plant populations in these widely-distributed habitats. Maintenance of growth rates under nutrient-limited conditions is likely to arise through a suite of traits that tolerate or avoid nutrient stress by using limiting resources more efficiently, preventing their loss, or increasing their uptake (Grime 1979, Chapin 1980, Aerts & Chapin 2000). Plant functional traits that confer tolerance of nutrient stress are thought to come at a cost in the form of reduced relative growth rate (RGR, growth rate scaled for initial size, $g*g^{-1}*day^{-1}$, Lambers & Poorter 2004).

Relative growth rates are highest when plants are young and grown under non-limiting resources, so the seedling RGR under optimum conditions (RGR_{max}) has been used as a standard for comparison of the stress tolerance of a broad range of plant accessions (Grime & Hunt 1975, Poorter & Remkes 1990, Chapin et al. 1993, Lambers et al. 2008). Less commonly, estimates of RGR have been used to determine the effect of various stresses on plant growth by examining the acclimation of accessions to an environmental stress imposed during an experiment (Boot et al. 1992, Chiba & Hirose 1993, van der Werf et al. 1993, Deng & Woodward 1998, Yuan et al. 2005, James 2008).

Relative growth rate can be mathematically partitioned into components that describe the growth strategy of the plant in terms of carbon or nitrogen use. Measured under contrasting growing conditions on different accessions, these components provide insight into putatively adaptive plant strategies in divergent habitats (Verhoeven et al. 2004) and the ability to acclimate to different environments. It is also useful to break RGR into its components when considering the effect of selection on growth rates because it is unlikely that selection would directly favor a lower RGR due to downstream effects on reproductive output. The components of RGR are likely to be under selection under adverse growing conditions, and those components can cause decreases in RGR (Lambers & Poorter 2004).

In this study, we examine employ a comparative approach within a known phylogenetic context to examine the evolutionary response of RGR and its components to nitrogen limitation. *Helianthus annuus*, the common sunflower, is widely-distributed in North America, and commonly occurs in disturbed habitats on mesic, clay-based soils. *Helianthus petiolaris*, the prairie sunflower, is common in the western United States, where it tends to occur on soils that are drier and sandier but with similar fertility compared to *H. annuus*. *Helianthus anomalus* is a hybrid of *H. annuus* and *H. petiolaris* that is restricted to sand dune habitats in Utah and Arizona (Rieseberg 1991). The substrates where *H. anomalus* occurs are very low in organic content than soils from *H. annuus* and *H. petiolaris* populations (L.A. Donovan, in prep.). Previous work with these plants has shown that growth in the habitat of *H. anomalus* is nutrient-limited or co-limited by water and nutrients (Ludwig et al. 2006) and that some functional traits in *H. anomalus* appear to have shifted toward a nutrient-stress tolerant syndrome compared to its parental species (Brouillette et al. 2006).

Here, we investigate species differences in resource use by breaking RGR into its nitrogen and carbon use components. The nitrogen-use components are nitrogen productivity (NP, $g\ g_N^{-1}\ day^{-1}$), plant nitrogen content (PNC, $g_N\ g^{-1}$), mean residence time (MRT, days), and nitrogen use efficiency (NUE, $g\ g_N^{-1}$). These traits are mathematically related to RGR by the equations (Berendse & Aerts 1987):

$$RGR = NP * PNC$$

$$NUE = NP*MRT$$

Higher RGR may be a result of an increase in NP, PNC, or both (Poorter et al. 1990). NUE is not a component of RGR, but NP is a component of both NUE and RGR. In order to increase NUE, plants must either increase NP or MRT. MRT is a measure of the amount of time a unit of nitrogen remains in the plant, which is determined by factors such as tissue longevity and resorption efficiency (Aerts 1990). Plants that have longer MRT tend to have lower NP (Berendse & Aerts 1987, Eckstein & Karlsson 1997), so NUE is constrained by a tradeoff between its components. Additionally, an increase in MRT will tend to decrease RGR through the antagonistic association between MRT and NP.

Carbon use components of RGR are net assimilation rate (NAR, g cm^{-2} day^{-1}), leaf area ratio (LAR, cm^2 g^{-1}), leaf mass ratio (LMR, g g^{-1}), and specific leaf area (SLA, cm^2 g^{-1}). Relative growth rate has this relationship to its carbon components (Lambers et al. 2008):

$$RGR = NAR*LAR$$

$$LAR = LMR*SLA$$

Similar to case with nitrogen use components, RGR is constrained by tradeoffs among the carbon components. NAR is a measure of productivity per unit leaf area, and LAR is ratio of leaf area to total biomass. SLA, one of the two components of LAR, is a trait that appears to be a key physiological trait in plant adaptations to abiotic stress, potentially due to tight correlations with other traits such as leaf lifespan (Reich et al. 1997). Plants from more stressful environments tend to have lower SLA, or thicker leaves, than plants from less stressful habitats. Empirical data show a strong inverse correlation between SLA and RGR (Lambers & Poorter 2004).

When grown under optimum conditions, we expect to see a lower seedling RGR in *H. anomalus* relative to its parents based on differences between the habits and preliminary data for these species (Brouillette et al. 2006). We also expect *H. anomalus* will have higher NUE as a consequence of longer MRT and greater ability to reabsorb nitrogen from senescing leaves because nitrogen appears to be a limiting resource in the *H. anomalus* habitat (Ludwig et al. 2006). Based on previous data (Rosenthal et al. 2002, Brouillette et al. 2006), we also expect to see a lower SLA in *H. anomalus* relative to its parents. Longer MRT and lower SLA are likely the root cause of a lower RGR in *H. anomalus* because of their proximate benefits under low nutrient conditions and the constraints they impose on growth.

Materials and Methods

Bulked seeds were collected in 2002 from field sites at Little Sahara Recreation Area, Juab Co., Utah (*H. annuus & H. anomalus*, 39°44'N 112°18'W), and 10 miles south of Page, AZ along US Hwy. 89 (*H. petiolaris*, 36°48'N 111°31'W*)*. Seeds were treated for germination by cold stratification on moist filter paper at 6°C for four weeks. Upon removal from cold stratification, seeds were placed in Petri dishes containing moist filter paper and placed under fluorescent light (80-90 μmol m^{-2} s^{-1}) with an 11-hour photoperiod. Additionally, seed coats were removed from *H. anomalus* immediately following stratification to remove physical inhibition to germination. After seven days (7 May 2007), seedlings of all species were transplanted into 5-gallon pots filled with a 3:1 mixture of sand-to-Turface (Profile Products, Buffalo Grove, IL) at the UGA Plant Biology Greenhouses (Athens, GA). Pots were watered to field capacity daily by an automated drip irrigation system. Seven days after transplant, nutrient treatment was initiated. Nutrient treatments consisted of watering pots to field capacity three times each week with nutrient solution, with applications approximately two hours after irrigation. The high nitrogen treatment was Hoagland's solution at half-strength (Epstein & Bloom 2005), and the low nitrogen treatment was the same as the high, excepting concentrations of nitrate and ammonium were reduced by a factor of 20, and compensating changes to calcium, phosphorus, and sulfur concentrations were made to restore balance of anions to cations (0.175 mmol L^{-1}, 1.05 mmol L^{-1}, and 2.5 mmol L^{-1}, respectively).

At 7, 21, 42, and 140 days after treatment initiation, 2 randomly-selected plants of each species/treatment combination were harvested from each of the six blocks (N = 288). At each harvest, biomass was partitioned into roots, stems, leaves, and, if present, cotyledons and reproductive biomass. At harvest 3, roots were further partitioned into coarse and fine samples according to size (> or < 1mm in diameter) and a subsample of fine roots was stored in water for estimation of specific root length (SRL, total length / mass of root sample, WinRhizo, Regent Instruments, Quebec, QC, Canada). When present, inflorescences were classified as buds (disk not visible), flowers (disk visible), and seedheads (inflorescence and stem leading to it desiccated) and counted. All biomass components were dried at 60°C until constant mass before weighing.

From 11 July through 19 July 2007, estimates of maximum photosynthetic rate (A_{max}) were obtained (LI-6400, LiCor Biosciences, Lincoln, NE). LiCor chamber conditions were 2000 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 380 ppm CO_2, with relative humidity and block temperature adjusted to reflect ambient conditions on the date measurements were taken (29-31°C, 55-57%). After gas exchange measurement, the leaf used was excised, measured for total area and area enclosed within the chamber, dried, weighed, and analyzed for foliar nitrogen concentration (continuous flow mass spectrometer, Finnegan, Bremen, Germany). Photosynthetic nitrogen-use efficiency (PNUE) was calculated after Field and Mooney (1986). Date of measurement was confounded with block to facilitate statistical analysis.

At each harvest, total leaf area (LI-3100, LiCor Biosciences, Lincoln, NE) and mass of fresh leaf tissue were estimated. Additionally, at harvest 3, the toughness of a randomly selected recently fully expanded leaf was estimated using a digital force meter (E-DFE-002, Chatillon/Ametek, Largo, FL). Variability of measurements within a leaf was large, so seven replicate measurements made within one leaf were averaged to calculate leaf toughness.

On the date of the second harvest, the most recently fully-expanded leaf was marked to track relative leaf lifespan. Leaves did not begin senescing until after the second harvest. Leaves were examined every two days, and the date when the marked leaf turned 25% yellow was recorded. As leaves senesced, they were collected from each plant. The dry mass of the senescent leaves was measured at the third and fourth harvests. After the final harvest, all senescent leaves were bulked by plant, ground, and subsampled to obtain estimates of percentage of nitrogen present in senescent leaves, which estimates foliar nitrogen resorption proficiency. Calculation of resorption efficiency requires an estimate of foliar nitrogen in green leaves ($\{[N_{leaf} - N_{scen}] / N_{leaf}\}*100\%$, %, Killingbeck 1996). To calculate resorption efficiency, the nitrogen content of bulked leaves was used for harvest 3 plants, but the nitrogen content of the gas exchange leaf was used for harvest 4 plants because leaves were mostly senescent at the end of the experiment. Beginning 6 June 2007 and continuing every two days, plants were also examined to determine date of the emergence of the terminal bud and the date of the first flower.

Beginning 6 weeks after treatment initiation and continuing for 6 weeks, pollen was bulked by species and applied to all receptive flowers three times per week. After ligules were shed, pollinated inflorescences were bagged with fine mesh nylon to prevent loss of seeds. After maturing, seeds were removed, counted, dried at 60°C, and weighed. Desiccated seedheads from all plants were counted and removed periodically to prevent their loss. All reproductive tissue was pooled for total biomass calculation and nitrogen content, which included seeds for harvest four plants. For plants in the first and second harvest, all biomass was pooled, ground, and analyzed for % carbon and % nitrogen (NA1500, Carlo Erba Strumentazione, Milan). For plants in harvests 3 and 4, root, stem, leaf, and reproductive biomass components were separately ground and analyzed for % carbon and % nitrogen. These percentages were weighted by the biomass of the component to obtain an estimate for total plant carbon and nitrogen content.

Statistical analysis

Plant traits leaf lifespan, percentage of inflorescences at the seedhead stage, A_{max}, PNUE, leaf toughness, proportion of nitrogen invested in leaves, the ratio of fine root length to leaf area, and senescent leaf resorption efficiency and proficiency were modeled as a function of species, treatment, and their interaction, with block included as a random factor in a mixed model (PROC MIXED, SAS 9, Cary, NC). The percentage of seedheads present at harvest 4 was arcsine-square root transformed to meet the assumptions of the model (Sokal & Rohlf 1981). To estimate relative growth rate, a piecewise linear regression model was fit to the data, with log-transformed biomass modeled as a function of species, treatment, time, and the interactions between time and species, treatment, and species x treatment (Fitzmaurice et al. 2004), with block treated as a random variable. The knots of the piecewise linear mixed model regression were set at harvests two (3 weeks) and three (6 weeks), yielding calculations of seedling, juvenile, and adult relative growth rates. To calculate NP, NUE, and MRT over the same intervals, individuals of the same species and treatment within a block were paired across consecutive harvests by relative biomass after Hunt (1978). Where only one seedling in a block survived to harvest, one of the two possible matches was chosen randomly. Nitrogen-use components were calculated using the r_{out} method (Hirose 1971), which assumes no leaching of nitrogen from the plant but does not assume nitrogen to be in a steady state of uptake and loss (Frissel 1981). Senescent leaves were collected from each plant to estimate the amount of nitrogen lost from the plant. Carbon components LAR, LMR, SLA, and NAR were calculated after Hunt (1990), using the same pairings as the nitrogen-use components. All components of RGR were modeled as a function of fixed effects species, treatment, harvest, and all possible interactions, with block treated as a random factor in the mixed model. Nitrogen productivity, NUE, MRT, PNC, and NAR were log-transformed to fit the assumptions of the model (Sokal & Rohlf 1981).

Harvest three stem samples from 3 of the 6 blocks were lost in a drying oven fire after their dry mass was estimated but before their nitrogen content could be determined. This reduced the sample size for nitrogen components of RGR by one half but did not affect calculation of carbon components.

Seed number was modeled as a function of bagged reproductive biomass, excluding the mass of the seeds. Separate simple linear regression models were fit for each species to determine the predictive power of reproductive biomass for seed number.

Results

RGR

Biomass increased and RGR decreased for all species and treatments through the seedling, juvenile, and adult stages (Figures 3.1 & 3.2; Table 3.1). As expected, seedling RGR_{max} was lower for *H. anomalus* than for its ancestral parents, *H. anomalus* and *H. petiolaris* (Figure 3.2).

The *H. anomalus* seedling RGR was significantly less affected by nutrient treatment than the growth rates of the two parental species (Table 3.1, $F_{1,253}$=24.46***). At the juvenile stage, the relative growth rate of *H. anomalus* was more responsive to nutrient stress than the parental species ($F_{1,253}$=12.72***), but at the adult stage the differential species response of RGR to nutrient stress was similar to the seedling stage ($F_{1,253}$=3.23+).

RGR components

Breaking RGR into its nutrient-use components, we found that *H. anomalus* had higher NUE (nitrogen use efficiency) and MRT (mean residence time) than its parental species (Figure 3.3; Table 3.2, $F_{1,52}$=10.7**, $F_{1,48}$=19.06***, respectively). Note that calculation of MRT and NUE requires senescent leaves, so no estimates were available for analysis during the seedling stage of the experiment. Nitrogen productivity did not differ markedly by species, though *H. anomalus* appears to respond differently over time than its parental species ($F_{1,106}$=16.56***, $F_{1,106}$=10.35***). Not surprisingly, nitrogen treatment appears to drive major differences in PNC ($F_{1,106}$=265.58***).

Carbon-use RGR components LAR, LMR, and SLA generally decreased over time (Figure 3.2). NAR was slower for the low nitrogen plants in the seedling and juvenile intervals, but were similar to high-nitrogen plants in the adult stage (Table 3.2, $F_{2,180}$=24.54***). SLA was generally lower for *H. anomalus* ($F_{1,181}$=145.77***). Differences in SLA were greatest at the seedling interval, with parental species showing a more of decline in SLA through time ($F_{1,181}$=26.86***).

Functional traits

Functional traits differed as to whether species, treatment, or an interaction was more important. *Helianthus anomalus* had significantly higher leaf toughness (Figure 3.4; Table 3.3, $F_{1,60}$=113.92***) and photosynthetic rates ($F_{1,57}$=38.25***) and tended to have significantly higher PNUE than its parental species ($F_{1,54}$=2.9+), though all species responded similarly to nitrogen stress. Leaf lifespan was longer in the low-nutrient *H. anomalus* plants than in the high nutrient *H. anomalus* plants. The parental species showed the opposite response ($F_{1,130}$=15.07***), having longer leaf lifespan under high nutrient conditions compared to low nutrient conditions. The amount of nitrogen left in senescent leaves, resorption proficiency, differed significantly by treatment (resorption proficiency, $F_{1,124}$=49.83***). In contrast, resorption efficiency did not differ by species, treatment, or their interaction (Table 3.3), indicating that, taken as a proportion of nitrogen present in green leaves, all plants in the study recovered similar amounts of nitrogen through resorption.

At the end of the experiment, *H. anomalus* had a higher proportion of its reproductive heads at the seedhead stage compared to the other species ($F_{1,58}$=44.43***). For seed heads that were hand pollinated and bagged to capture seed, the number of seeds was explained well by bagged reproductive biomass excluding seeds for *H. annuus* ($p<0.0001$, $r^2=0.728$) and *H. petiolaris* ($p<0.0001$, $r^2=0.886$), with a weaker, though still significant, relationship in *H. anomalus* ($p=0.0002$, $r^2=0.500$).

Discussion

Helianthus anomalus has a suite of traits that are indicative of its being more stress tolerant than its parental species. Seedling RGR_{max} is lower for *H. anomalus* than for *H. annuus* and *H. petiolaris*. The nitrogen use strategy of *H. anomalus* is more conservative of nitrogen than that of its parents, largely due to increased MRT. Similarly, SLA is lower for *H. anomalus* at the seedling stage. Recall that RGR_{max} is directly correlated with SLA and inversely correlated with MRT, so observed differences in seedling growth rate are likely the result of changes in the two components RGR and MRT. SLA and MRT are themselves inversely correlated, with species having low SLA also having a longer leaf lifespan (Reich et al. 1997).

These major results are part of a suite of traits associated with plants tolerant of nutrient stress (Aerts & Chapin 2000).

Results from the growth rate analysis suggest that both magnitude of RGR and the response of RGR to nutrient stress are highly dependent on life history stage. Early on, when growth rates were highest, *H. anomalus* had less of a response to nutrient stress than did its parental species. The seedling stage of growth is arguably the most influential because growth rates are highest and, because of the compounding effect of relative growth rate, the seedling RGR has a strong influence on the final size of the plant. It has been posited that the resistance to change in relative growth rate to abiotic stress is an adaptation to resource-limited habitats (Lambers et al. 2008), though evidence for this is weak (Fichtner & Schulze 1992). The less dramatic response to nutrient stress may be partially a function the fact that lower growth rates, by virtue of being closer to zero, cannot decrease as much as high growth rates (Lambers et al. 2008).

At the juvenile stage, we observed the unexpected result of a greater response to nutrient limitation in *H. anomalus* than in the parental species. This was accompanied by large differences in the nitrogen-use components mean residence time and nitrogen use efficiency. MRT is affected by the resorption of nitrogen from senescent leaves and the rate of leaf senescence. We did not find evidence of a difference in resorption efficiency or proficiency by species, with a similar response to nutrient stress by all three species in proficiency. A survey of 60 plant species showed similar results, with most species having no change in resorption proficiency in response to nutrient stress (Aerts 1996). In the absence of different amounts of nitrogen resorption, the patterns in MRT among the species appear to be driven by differences in leaf lifespan, which ultimately led to different amounts of senescent leaf mass. The response of leaf lifespan to nutrient stress in *H. anomalus* was more conservative of nitrogen compared the parental species. Under low nitrogen, *H. anomalus* increased leaf lifespan while the parental species decreased lifespan. While it is thought that all plants should increase leaf lifespan under nutrient limited conditions (Lambers et al. 2008), previous studies have found increased, decreased, or unchanged leaf lifespan in response to nutrient stress (Aerts & Chapin 2000 and references therein).

At the adult stage, the tendency for *H. anomalus* to be less responsive to nutrient stress was observed again, accompanied by a shift in the nitrogen productivity of the nutrient-stressed *H. anomalus* plants. Nitrogen productivity is affected by many plant traits that affect the efficiency of the use of nitrogen for biomass accumulation, including differences in allocation to leaves and photosynthetic rates. *Helianthus anomalus* had photosynthetic rates and marginally higher PNUE than its parentals. The analysis of the carbon-use components of RGR suggests that relative biomass allocation to leaves in *H. anomalus* is unresponsive to nitrogen limitation. SLA, LMR, and LAR remain more or less unchanged for *H. anomalus* as nitrogen levels shift. Allocational traits of leaf area ratio and fine root length-to-leaf area ratio were less responsive to treatment in *H. anomalus* than in parentals. Allocation in *H. anomalus* appears to be less affected by nitrogen availability, which could partially explain the pattern in growth rates observed in the seedling and adult stages.

It is thought to be a general rule that fast-growing species achieve their growth rate through higher nitrogen productivity and that slow-growing species achieve success in their home habitat through increased tissue longevity (Golluscio 2007). While not a direct tradeoff, longer MRT constrains RGR through its effects on NP. We did not find significant differences in nitrogen productivity by species in this experiment. The nitrogen-use components of RGR are difficult to estimate, and the lack of significant differences may be attributable to small sample size. However, it may also be that differences in nitrogen-use components of RGR are small in these closely-related study species and that broader taxonomic groups must be compared in order to be able to detect differences in NP (Garnier et al. 1995). While several studies note that fast RGR is attributable to high NP (Boot et al. 1992, van der Werf et al. 1993, James 2008), the paradigm may not be broadly applicable (Chiba & Hirose 1993).

Though we cannot attribute high RGR to high NP in this study, our results do support the idea that low nutrient adapted plants have increased tissue longevity (Garnier et al. 1995, Reich et al. 1997). Higher NUE in *H. anomalus* is conferred by increased tissue longevity rather than higher NP or an improved ability to reabsorb nitrogen from senescent leaves. Theoretical results show an advantage for increased tissue longevity when nutrients limit growth (Aerts & van der Peijl 1993, Golluscio 2007).

It appears that maintaining vegetative function later into the growing season is also a part of the *H. anomalus* strategy. At the end of the experiment, *H. anomalus* had fewer of its reproductive heads at the seedhead stage than its parental species (Figure 3.4; Table 3.3). Additionally, low-nitrogen *H. anomalus* plants had a higher proportion of plant nitrogen invested in green leaves than other treatment groups (data not shown). These data suggest that *H. anomalus* may compensate for slower growth rates with a longer growing season. Stanton and colleagues (2000) found that selection on wild mustard under a range of abiotic stresses altered flowering time, suggesting that life history traits may be major targets of selection under resource-limited conditions. While selection under low nutrients in that study favored earlier flowering time, selection may act more broadly on phenology. Depending on a longer growing season may be a successful strategy in years when first frost comes late, but may be detrimental in others.

Helianthus anomalus has a slower maximum inherent relative growth rate than its parental species, and this early relative growth rate is less responsive to nitrogen limitation. For all three species, relative growth rate decreased as plants aged or were grown under limiting nitrogen. Mean residence was higher for *H. anomalus* plants, yielding a more conservative nitrogen-use strategy in the species. Toward the end of the experiment, *H. anomalus* maintained more nitrogen in green leaves, suggesting that an extended growing season is also a part of the *H. anomalus* strategy.

Acknowledgements

We wish to thank S Gevaert and A Howard for help with gas exchange measurements, J Maddox for help with pollinations, C Gormally, C Darragh, S Fatkin, R Pascal, and M DeSears for help with harvests and sample processing, and the Donovan lab group for suggestions for the manuscript. This research was funded by NSF grant #0614739 to LAD. LCB was supported by a Dissertation Completion Award from the University of Georgia Graduate School. The experiments presented in this manuscript comply with current laws of the United States, where the work was carried out.

References

Aerts R (1990) Nutrient use efficiency in evergreen and deciduous species from heathlands. Oecologia, 84, 391-397.

Aerts R (1996) Nutrient resorption from scenesing leaves of perennials: are there general patterns? Journal of Ecology, 84, 597-608.

Aerts R, Chapin III FS (2000) The mineral nutrition of wild plants revisited: A re-evaluation of processes and patterns. Advances in Ecological Research, 30, 1-67.

Aerts R, van der Peijl MJ (1993) A simple model to explain the dominance of lo-productive perennials in nutrient-poor habitats. Oikos, 57, 310-318.

Berendse F, Aerts R (1987) Nitrogen use efficiency: A biologically meaningful definition? Functional Ecology, 1, 293-296.

Boot RGA, Schildwacht PM, Lambers H (1992) Partitioning of nitrogen and biomass at a range of N-addition rates and their consequences for growth and gas exchange in two perennial grasses from inland dunes. Physiologia Plantarum, 86, 152-160.

Brouillette LC, Gebremedhin M, Rosenthal DM, Donovan LA (2006) Testing hypothesized evolutionary shifts toward stress tolerance in hybrid *Helianthus* species. Western North American Naturalist, 66, 409-419.

Chapin FS III (1980) The mineral-nutrition of wild plants. Annuual Review of Ecology and Systematics, 11, 233-260.

Chapin FS III, Autumn K, Pugnaire F (1993) Evolution of suites of traits in response to environmental stress. American Naturalist, 142, S78-S92.

Chiba N, Hirose T (1993) Nitrogen acquisition and use in three perennials in the early stage of primary succession. Functional Ecology, 7, 287-292.

Deng X, Woodward FI (1998) The growth and yield responses of *Fragaria ananassa* to elevated CO_2 and N supply. Annals of Botany, 81, 67-71.

Epstein E, Bloom AJ (2005) Mineral Nutrition of Plants: Principles and Perspectives, 2nd edn. Sinauer Associates, Sunderland, MA.

Eckstein RL, Karlsson PS (1997) Above-ground growth and nutrient use by plants in a subarctic environment: effects of habitat, life-form and species. Oikos, 79, 311-324.

Fichtner K, Schulze ED (1992) The effect of nitrogen nutrition on growth and biomass partitioning of annual plants originating from habitats of different nitrogen availability. Oecologia, 92, 236-241.

Field F, Mooney HA (1986) The photosynthesis-nitrogen relationship in wild plants. In: On the Economy of Plant Form and Function (ed. Givnish TJ), pp 25-55. Cambridge University Press, Cambridge, pp 25-55.

Fitzmaurice GM, Laird NM, Ware JH (2004) Applied longitudinal analysis. Wiley-Interscience, Hoboken, NJ.

Frissel MJ (1981) The definition of residence times in ecological models. Ecological Bulletins, 33, 117-122.

Garnier E, Gobin O, Poorter H (1995) Nitrogen productivity depends on photosynthetic nitrogen use efficiency and on nitrogen allocation with the plant. Annals of Botany, 76, 667-672.

Golluscio RA (2007) On the link between nitrogen productivity and mean residence time: Two opposite nitrogen use strategies? Journal of Arid Environments, 68, 165-169.

Grime JP (1979) Plant strategies and vegetation processes. John Wiley, New York.

Grime JP, Hunt R (1975) Relative growth rate: Its range an adaptive significance in local flora. Journal of Ecology, 63, 393-422.

Hirose T (1971) Nitrogen turnover and dry matter production of a *Solidago altissima* population. Japanese Journal of Ecology, 21, 18-32.

Hunt R (1978) Plant Growth Analysis. Edward Arnold Publishers, London.

Hunt R (1990) Basic Growth Analysis: Plant Growth Analysis for Beginners. Unwin Hyman, London.

James JJ (2008) Leaf nitrogen productivity as a mechanism driving the success of invasive annual grasses under low and high nitrogen supply. Journal of Arid Environments, 72, 1775-1784.

Killingbeck KT (1996) Nutrients in scenesced leaves: Keys to the search for potential resorption and resorption proficiency. Ecology, 77, 1716-1727.

Lambers H, Chapin III FS, Pons TL (2008) Plant Physiological Ecology, 2nd edn. Springer, Berlin.

Lambers H, Poorter H (2004) Inherent variation in growth rate between higher plants: A search for physiological causes and consequences. Advances in Ecological Research, 34, 283-362.

Ludwig F, Jewett RA, Donovan LA (2006) Nutrient and water addition effects on day and night-time conductance and transpiration in a C3 desert annual. Oecologia, 148, 219-225.

Poorter H, Remkes C (1990) Leaf area ration and net assimilation rate of 24 wild species differing in relative growth rate. Oecologia, 83, 553-559.

Poorter H, Remkes C, Lambers H (1990) Carbon and nitrogen economy of 24 wild species differing in relative growth rate. Plant Physiology, 94, 621-627.

Reich PB, Walters MB, Ellsworth DS (1997) From tropics to tundra: Global convergence in plant functioning. Proceedings of the National Academy of Sciences, USA, 94, 13730-13734.

Rieseberg LH (1991) Homoploid reticulate evolution in *Helianthus* (Asteraceae): Evidence from ribosomal genes. American Journal of Botany, 78, 1218-1237.

Rosenthal DM, Schwarzbach AE, Donovan LA, Raymond O, Rieseberg LH (2002) Phenotypic differentiation between three ancient hybrid taxa and their parental species. International Journal of Plant Sciences, 163, 387-398.

Sokal RR, Rohlf FJ (1981) Biometry: The Principles and Practice of Statistics in Biological Research, 2nd edn. W.H. Freeman, San Francisco.

Stanton ML, Roy BA, Thiede DA (2000) Evolution in stressful environments. I. Phenotypic variability, phenotypic selection, and response to selection in five distinct environmental stresses. Evolution, 54, 93-111.

van der Werf A, van Nuenen M, Visser AJ, Lambers H (1993) Contribution of physiological and morphological plant traits to a species' competitive ability at high and low nitrogen supply. Oecologia, 94, 434-440.

Verhoeven JTA, Koerselman W, Meuleman AFM (1996) Nitrogen- or phosphorus-limited growth in herbaceous, wet vegetation: relations with atmospheric inputs & management regimes. Trends in Ecology and Evolution, 11, 494-497.

Verhoeven KJF, Biere A, Novo E, van Damme JMM (2004) Differential selection of growth rate-related traits in wild barley, *Hordeum spontaneum*, in contrasting greenhouse nutrient environments. Journal of Evolutionary Biology, 17, 184-196.

Yuan ZY, Li LH, Huang JH, Han XG, Wan SQ (2005) Effect of nitrogen supply on the nitrogen use efficiency of an annual herb, *Helianthus annuus* L. Journal of Integrative Plant Biology, 47, 539-548.

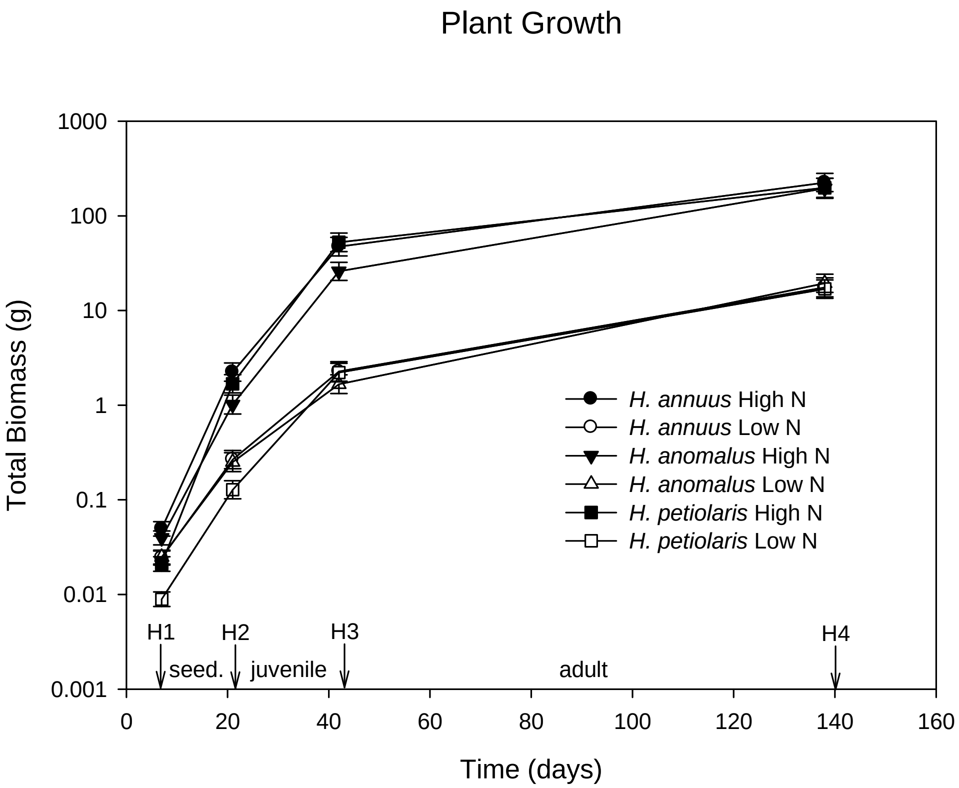

Figure 3.1. Plant growth. Means and 95% confidence intervals for total plant biomass are plotted for each of the four harvests. The first through fourth harvests are marked as H1-H4, respectively. Also shown are the seedling, juvenile, and adult intervals over which RGR and its components are calculated. Note that the y-axis is in a $\log_{10}$ scale.

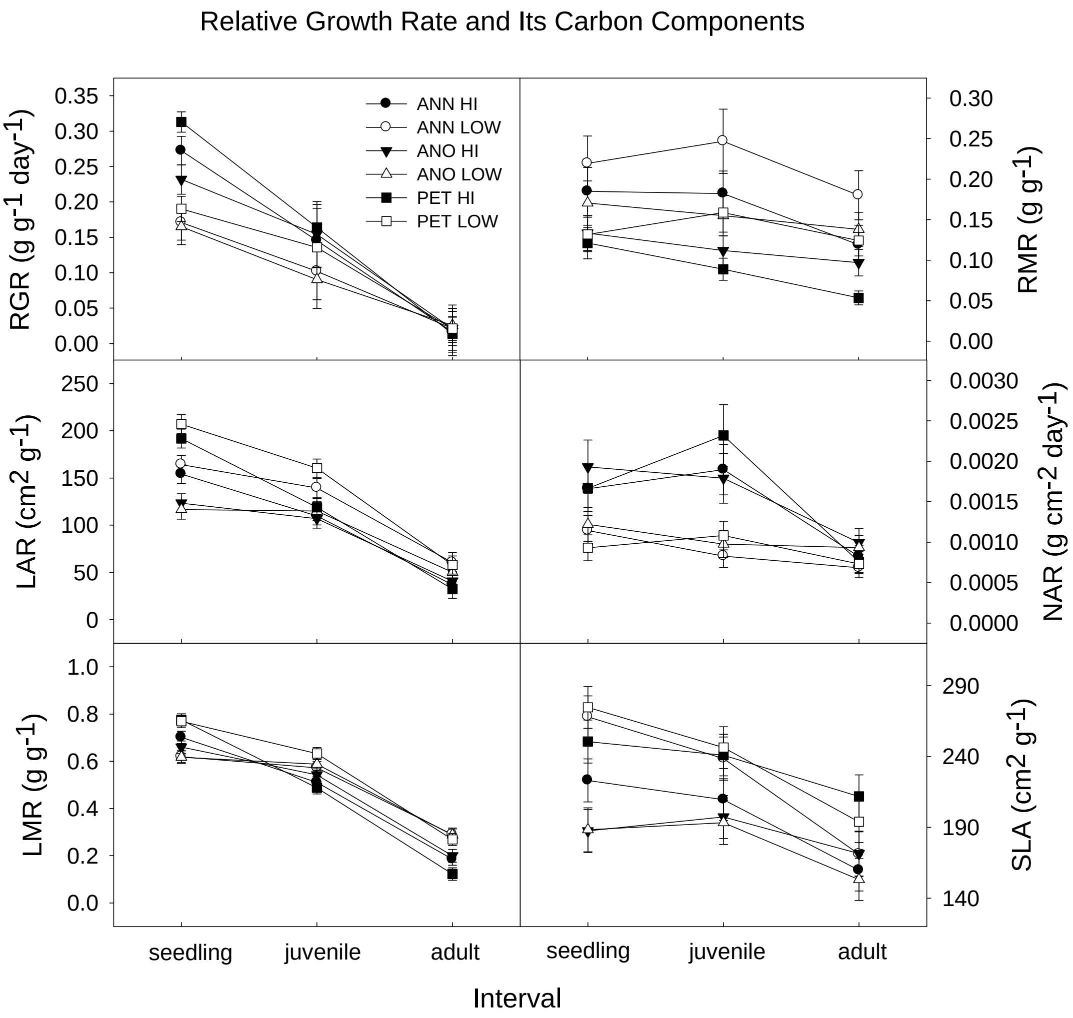

Figure 3.2. Relative growth rate and its carbon components. Means and 95% confidence intervals for relative growth rate (RGR), root mass ratio (RMR), leaf area ratio (LAR), net assimilation rate (NAR), leaf mass ratio (LMR), and specific leaf area (SLA).

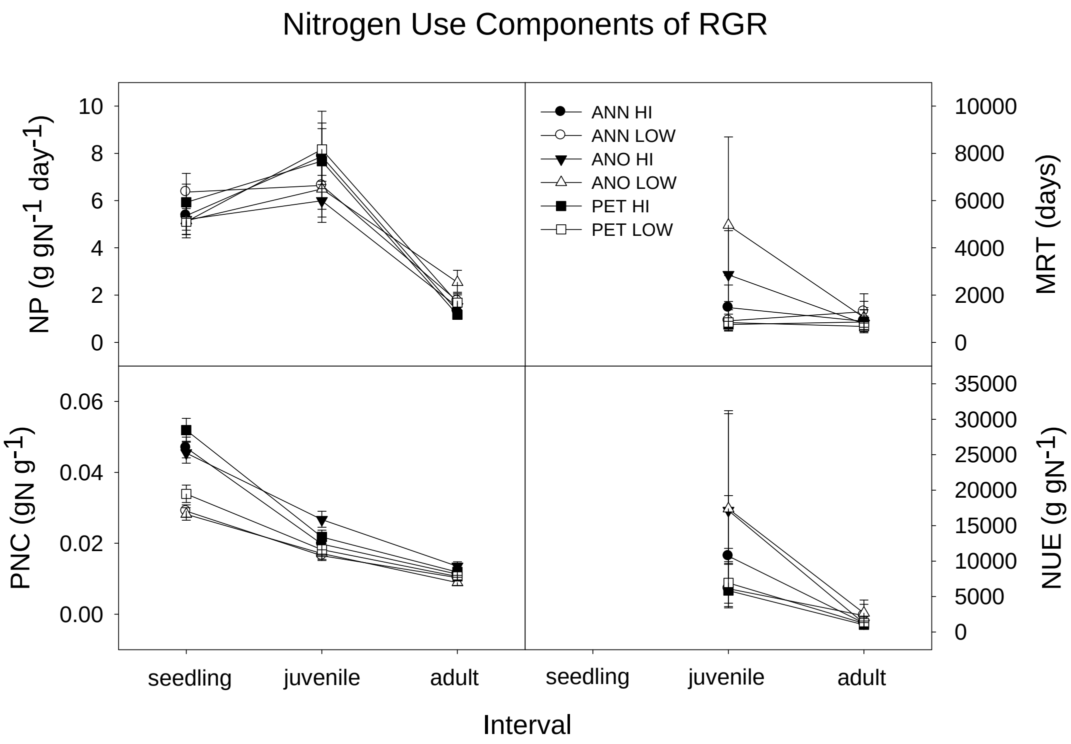

Figure 3.3. Nitrogen use components of RGR. Means and 95% confidence intervals for nitrogen productivity (NP), mean residence time (MRT), plant nitrogen concentration (PNC), and nitrogen use efficiency (NUE). It was not possible to estimate MRT or NUE using the r_{out} method for interval 1 because no plants had shed leaves by harvest 2.

Figure 3.4. Plant functional traits. Means and 95% confidence intervals are plotted for plant functional traits measured once during the experiment. Leaf lifespan is the number of days from marking the most recently fully expanded leaf until 25% of the leaf turned yellow. Flowering is the number of days from transplant until flowering. Resorption proficiency (proportion of healthy leaf nitrogen remaining at scenescence) was calculated using leaves that had turned completely brown. All three of these characters were estimated over plants allocated to the third and fourth harvests, though flowering and leaf lifespan data from a few of the harvest 3 plants were truncated. Fine root length to leaf area ratio and leaf toughness data were collected during the third harvest. Maximum photosynthetic rate (A_{max}) and photosynthetic nitrogen use efficiency (PNUE) were estimated shortly after the third harvest on all remaining plants. Percent seedheads was calculated for harvest 4 plants at the end of the experiment.

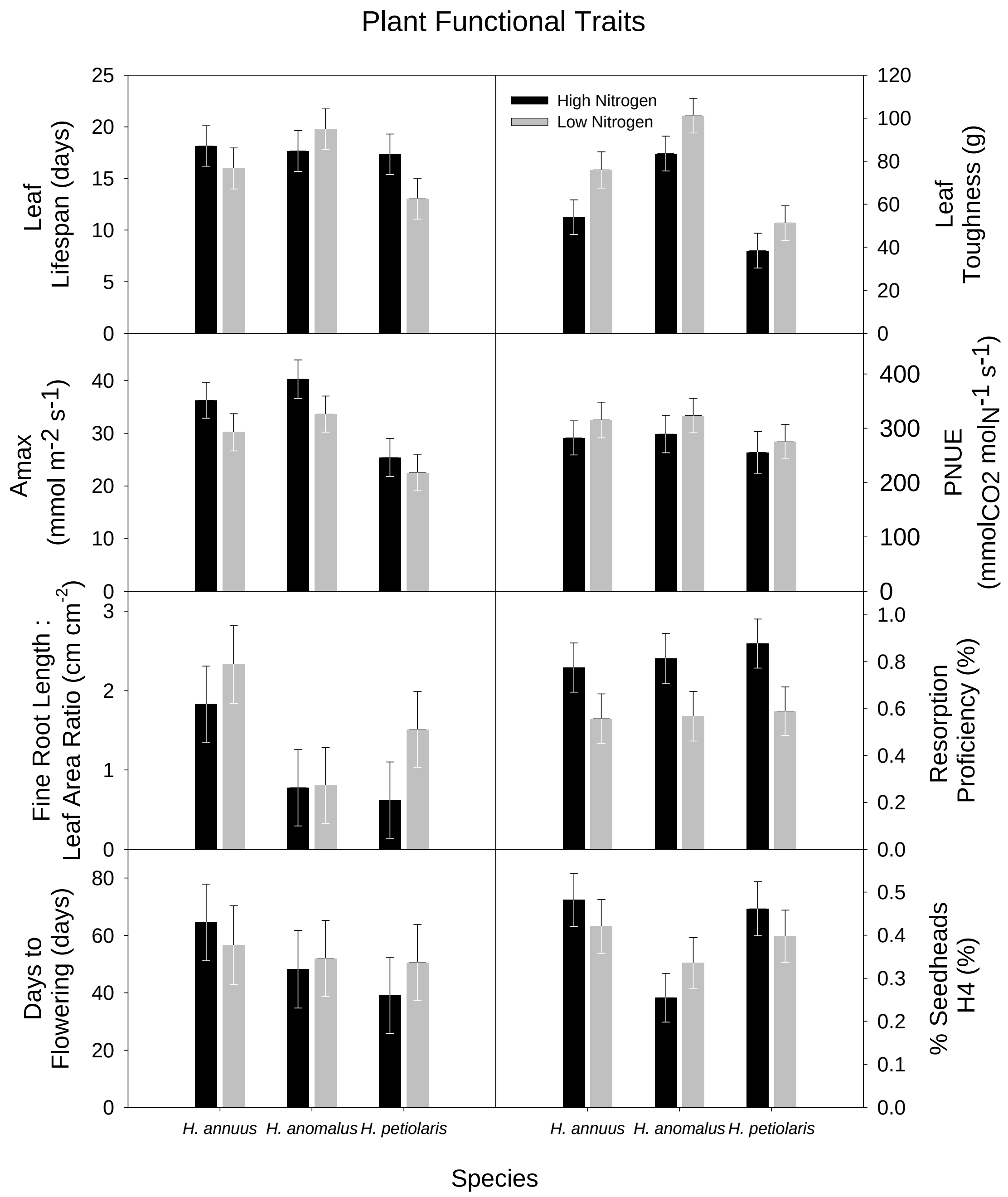
Plant Functional Traits
High Nitrogen
Low Nitrogen
Leaf Lifespan (days)
25
20
15
10
5
0
Leaf Toughness (g)
120
100
80
60
40
20
0
Amax (mmol m-2 s-1)
40
30
20
10
0
PNUE (mmolCO2 molN-1 s-1)
400
300
200
100
0
Fine Root Length : Leaf Area Ratio (cm cm-2)
3
2
1
0
Resorption Proficiency (%)
1.0
0.8
0.6
0.4
0.2
0.0
Days to Flowering (days)
80
60
40
20
0
% Seedheads H4 (%)
0.5
0.4
0.3
0.2
0.1
0.0
H. annuus
H. anomalus
H. petiolaris
H. annuus
H. anomalus
H. petiolaris
Species

Table 3.1. Statistical analysis of RGR. Log-transformed biomass was fit to a piecewise regression model, yielding estimates of relative growth rate at the seedling, juvenile, and adult stage for each of the six species-by-treatment combinations. Terms with multiple numerator degrees of freedom (d.f.) are broken into their components dealing with the comparison of *H. anomalus* to its parental species and the comparison of the two parental species. Significant F-values are denoted by +(p<0.1), *(p<0.05), **(p<0.01), and ***(p<0.001).

Source	**d.f.**	**F**
Species	2,253	26.52***
ANO vs. Parentals	1,253	16.04***
ANN vs. PET	1,253	37.57***
time (seedling)	1,253	2189.79***
time (juvenile)	1,253	175.66***
time (adult)	1,253	994.47***
time*treatment (seedling)	1,253	516.28***
time*treatment (juvenile)	1,253	28.82***
time*treatment (adult)	1,253	53.27***
time*species (seedling)	2,253	10.28***
ANO vs. Parentals	1,253	14.19***
ANN vs. PET	1,253	6.50*
time*species (juvenile)	2,253	1.32
ANO vs. Parentals	1,253	2.59
ANN vs. PET	1,253	0.05
time*species (adult)	2,253	8.43***
ANO vs. Parentals	1,253	6.7*
ANN vs. PET	1,253	10.04**
time*treatment*species (seedling)	2,253	14.38***
ANO vs. Parentals	1,253	24.46***
ANN vs. PET	1,253	4.33*
time*treatment*species (juvenile)	2,253	7.59***
ANO vs. Parentals	1,253	12.72***
ANN vs. PET	1,253	2.44
time*treatment*species (adult)	2,253	1.91
ANO vs. Parentals	1,253	3.23+
ANN vs. PET	1,253	0.56

Table 3.2. Statistical analysis of RGR components. Terms with multiple numerator degrees of freedom (d.f.) are broken into their components dealing with the comparison of *H. anomalus* to its parental species and the comparison of the two parental species. Significant F-values are denoted by +(p<0.1), *(p<0.05), **(p<0.01), and ***(p<0.001).

	NAR		SLA		LAR		LMR		RMR		PNC		NP		MRT		NUE	
Source	**df**	**F**	**df**	**F**	**df**	**F**	**df**	**F**	**df**	**F**	**df**	**F**	**Df**	**F**	**df**	**F**	**df**	**F**
Species	2,180	4.63*	2,181	92.97***	2,180	92.86***	2,180	11.12***	2,180	81.17***	2,106	7.56***	2,106	0.09	2,48	11.78***	2,52	6.97**
ANO vs. Parentals	1,180	8.46**	1,181	145.77***	1,180	141.82***	1,180	4.00*	1,180	2.69	1,106	0.09	1,106	0	1,48	19.06***	1,52	10.7**
ANN vs. PET	1,180	0.83	1,181	38.66***	1,180	42.97***	1,180	18.15***	1,180	161.85***	1,106	14.93***	1,106	0.18	1,48	4.51*	1,52	2.94+
Treatment	1,180	133.52***	1,181	6.54*	1,180	64.78***	1,180	81.36***	1,180	110.27***	1,106	265.58***	1,106	11.43**	1,48	0.47	1,52	1.45
Interval	2,180	88.42***	2,181	105.37***	2,180	955.29***	2,180	2333.12***	2,180	36.43***	2,106	1602.29***	2,106	524.14***	1,48	12.77***	1,52	130.97***
Linear	1,180	132.47***	1,181	188.53***	1,180	1818.3***	1,180	4406.74***	1,180	62.12***	1,106	3091.76***	1,106	779.53***				
Quadratic	1,180	44.63***	1,181	23.05***	1,180	94.16***	1,180	264.37***	1,180	10.83**	1,106	3.10+	1,106	427.13***				
species*treatment	2,180	0.56	2,181	10.24***	2,180	10.93***	2,180	15.06***	2,180	3.67*	2,106	11.74***	2,106	0.83	2,48	1.17	2,52	0.42
ANO vs. Parentals	1,180	1.12	1,181	11.12**	1,180	20.31***	1,180	6.56*	1,180	1.5	1,106	23.47***	1,106	1.66	1,48	2.33	1,52	0.64
ANN vs. PET	1,180	0	1,181	9.55**	1,180	1.5	1,180	23.43***	1,180	5.81*	1,106	0.05	1,106	0	1,48	0.01	1,52	0.18
species*interval	4,180	5.26***	4,181	8.11***	4,180	38.72***	4,180	35.36***	4,180	2.17+	4,106	5.50***	4,106	6.39***	2,48	9.36***	2,52	1.57
ANO vs. Parentals linear	1,180	0.93	1,181	26.86***	1,180	105.01***	1,180	49.55***	1,180	1.54	1,106	3.47+	1,106	16.56***	1,48	18.7***	1,52	2.96+
ANN vs. PET linear	1,180	0.67	1,181	4.37*	1,180	45.97***	1,180	81.4***	1,180	1.29	1,106	2.6	1,106	0.07	1,48	0.01	1,52	0.21
ANO vs. Parentals quadratic	1,180	10.13**	1,181	0.55	1,180	3.55+	1,180	9.43**	1,180	5.52*	1,106	13.31***	1,106	10.35**				
ANN vs. PET quadratic	1,180	9.14**	1,181	0.84	1,180	0.37	1,180	1.29	1,180	0.35	1,106	0.09	1,106	2.67				
treatment*interval	2,180	24.54***	2,181	7.80***	2,180	7.67***	2,180	71.2***	2,180	9.32***	2,106	19.41***	2,106	11.07***	1,48	0.08	1,52	3.91+
Linear	1,180	16.8***	1,181	15.47***	1,180	6.32*	1,180	125.48***	1,180	18.17***	1,106	32.55***	1,106	19.04***				
Quadratic	1,180	32.35***	1,181	0.15	1,180	9.01**	1,180	16.79***	1,180	0.47	1,106	2.4	1,106	5.95*				
species*treatment*interval	4,180	0.93	4,181	0.36	4,180	0.47	4,180	1.46	4,180	2.73*	4,106	3.08*	4,106	2.43+	2,48	1.9	2,52	1.13
ANO vs. Parentals linear	1,180	0.02	1,181	1.19	1,180	0.15	1,180	1.67	1,180	4.57*	1,106	9.09**	1,106	1.4	1,48	0.67	1,52	0.05
ANN vs. PET linear	1,180	2.56	1,181	0.18	1,180	0.07	1,180	1.02	1,180	5.94*	1,106	0.53	1,106	3.57+	1,48	3.14+	1,52	2.18
ANO vs. Parentals quadratic	1,180	0.88	1,181	0.05	1,180	1.11	1,180	2.36	1,180	0.06	1,106	1.03	1,106	0.05				
ANN vs. PET quadratic	1,180	0.26	1,181	0.01	1,180	0.55	1,180	0.75	1,180	0.37	1,106	0.01	1,106	3.08+				

Table 3.3. Statistical analysis of plant functional traits. Terms with multiple numerator degrees of freedom (d.f.) are broken into their components dealing with the comparison of *H. anomalus* to its parental species and the comparison of the two parental species. Significant F-values are denoted by +(p<0.1), *(p<0.05), **(p<0.01), and ***(p<0.001).

	Leaf Lifespan		**Amax**		**Leaf Toughness**		**Fine Root Length: Leaf Area Ratio**		**Flowering**		**PNUE**		**Resorption Proficiency**		**% Seedheads**		**Resorption Efficiency**	
Source	**df**	**F**	**df**	**F**	**df**	**F**	**df**	**F**	**df**	**F**	**df**	**F**	**df**	**F**	**df**	**F**	**df**	**F**
Species	2,130	9.74***	2,57	37.31***	2,60	69.81***	2,60	23.02***	2,130	3.1*	2,54	3.27*	2,124	1.25	2,58	22.56***	2,120	1.71
ANO vs. Parentals	1,130	14.07***	1,57	38.25***	1,60	113.92***	1,60	20.81***	1,130	0.22	1,54	2.9+	1,124	0.06	1,58	44.43***	1,120	1.36
ANN vs. PET	1,130	5.42*	1,57	36.43***	1,60	24.32***	1,60	25.86***	1,130	6*	1,54	3.97+	1,124	2.43	1,58	0.68	1,120	1.99
treatment	2,130	5.04*	1,57	16.96***	1,60	27.64***	1,60	8.48**	1,130	0.2	1,54	4.51*	1,124	49.83***	1,58	0.34	1,120	0.42
species*treatment	2,130	8.43***	2,57	0.83	2,60	0.63	2,60	2.40+	2,130	1.15	2,54	0.11	2,124	0.34	2,58	5.5**	2,120	0.43
ANO vs. Parentals	1,130	15.07***	1,57	0.62	1,60	0	1,60	3.80+	1,130	0.03	1,54	0.08	1,124	0.01	1,58	11.11**	1,120	0.67
ANN vs. PET	1,130	1.78	1,57	1.05	1,60	1.27	1,60	0.95	1,130	2.28	1,54	0.16	1,124	0.68	1,58	0	1,120	0.21

CHAPTER 4

NUTRIENT STRESS RESPONSE OF A HYBRID SUNFLOWER: A GENE EXPRESSION STUDY[1]

---

[1] Brouillette LC, Donovan LA. To be submitted to *Oecologia*.

Abstract

Low soil fertility limits growth and productivity in many natural and unammended agricultural systems. The ability to sense and respond to nutrient limitation is an important determinant of success those environments. Genes involved in stress responses that improve growth and productivity under nutrient stress could be used to engineer crops to grow under lower soil fertility found in more marginal agricultural settings. *Helianthus anomalus* is an annual sunflower of hybrid origin that grows on substrates that have lower fertility than the habitats of its parental species, *H. annuus* and *H. petiolaris*. Previous studies have shown that the growth of *H. anomalus* is less affected by lower levels of soil nitrogen than the growth rates of its parental species. Here, we compare the nitrogen stress response of *H. anomalus* at the transcript level to its parental species using a microarray. Relative to the set of genes on the array, the set of genes that show differential expression patterns in the hybrid species relative to its parents are enriched in stress response genes, developmental genes, and genes involved in responses to biotic and abiotic stimuli. After a correction for multiple comparisons, five unique genes show a significantly different response to nitrogen limitation in *H. anomalus* compared to *H. petiolaris* and *H. annuus*. These genes will be examined further as candidate genes for the adaptive stress response in *H. anomalus*, with the potential for improvement of cultivated sunflower, *H. annuus*.

Introduction

Plant growth and productivity is often limited by soil nutrient levels (Aerts & Chapin 2000). Nitrogen, in particular, is frequently an important limiting factor because of high biochemical demands (Marschner 1995) and relatively low supply in soils, particularly newer soils (Aerts & Chapin 2000). Plants have evolved several strategies for dealing with nitrogen limitation. Changing allocation to roots or altering uptake kinetics can increase the amount of nitrogen that is taken up by the plant (Lambers et al. 2008). Boosting allocation to leaves or photosynthetic enzymes can increase productivity per unit nitrogen, though most low nutrient adapted plants increase the efficiency of nitrogen use by increasing retention time by longer tissue longevity or resorption from senescent tissues (Berendse & Aerts 1987, Aerts & Chapin 2000).

The *Helianthus* hybrid system is an excellent resource for looking at adaptation to nutrient stress, where the comparative approach within a known phylogenetic context can provide insight into the adaptation of plants to stressful habitats. *Helianthus anomalus* is an annual sunflower endemic to desert sand dunes in the southwestern US and is a homoploid hybrid of *H. annuus* and *H. petiolaris* (Rieseberg 1991). The soils where *H. anomalus* is found are much lower in fertility than the soils in parental species' habitats (L. A. Donovan, in prep.). *Helianthus anomalus* shows variation in plant functional traits outside the range of the parental species for a number of traits (Rosenthal et al. 2002), with key nitrogen use traits leaf lifespan and specific leaf area showing a shift toward a more stress-tolerant phenotype (L.C. Brouillette & L.A. Donovan, in prep.).

Rieseberg and colleagues (2003) showed that much of the transgressive variation in the hybrid sunflower species could be accounted for by reshuffling of parental genomes. Genotypes resembling hybrid species could be found among early generation hybrids between *H. annuus* and *H. petiolaris* (Rosenthal et al. 2005). It is also likely, however, that changes in gene expression also contribute to the novel phenotypes of the hybrid species (Lai et al. 2006). Activation of transposable elements is one common way that alterations in gene expression levels can be achieved quickly.

Hybridization and stress can cause changes in gene expression by the activation of transposable elements (McClintock 1984). Baack and colleagues (2005) showed that the genome size of *Helianthus anomalus* is greater than either of its two parents. Much of the increase can be attributed to proliferation of *gypsy*-like transposable elements (Ungerer et al. 2006), which may have been induced by the stressful conditions of the *H. anomalus* habitat (Wessler 1996, Baack et al. 2005).

In the present study, we compare the expression profile of *H. anomalus* grown under contrasting nutrient regimes to the profiles of *H. annuus* and *H. petiolaris*. A previous study compared gene expression in the hybrid system, with all plants grown under the same nutrient regime (Lai et al. 2006). The present study aims to ask which genes show differential regulation in response to nutrient stress in *H. anomalus* relative to its parental species. This will elucidate genes showing novel regulation in *H. anomalus* under nutrient stress, which can be considered candidate genes for the putatively adaptive nutrient stress response of *H. anomalus*.

Materials and Methods

Seeds were collected from natural populations of *H. anomalus* (White Sands, Little Sahara Recreational Area, Juab County, UT), *H. annuus* (Little Sahara Recreational Area), and *H. petiolaris* (I-15, exit 95, Iron County, UT) during the 2002 growing season. To reduce surface fungal contamination, seeds of all three species were soaked briefly in a 3% hypochlorate solution and rinsed repeatedly with deionized water. Immediately following surface sterilization, seeds were put into cold wet stratification at 4°C for 5 weeks. Upon removal from cold stratification, seeds were placed on moist filter paper in Petri dishes, and seedcoats were removed from *H. anomalus*. The Petri dishes were placed under fluorescent lights with an 11-hour photoperiod. Due to poor germination rates, seedcoats were removed from *H. petiolaris* 5-

7 days after cold stratification. Seedlings were transplanted after developing green cotyledons and healthy roots (4-10 days after cold stratification).

Plants were grown in a fully randomized design in 983 mL pots (D60 Deepots, Stewe & Sons, Corvallis, OR) containing a 3:1 sand-to-fritted clay mixture (Turface, Profile Products, Buffalo Grove, IL) in a growth chamber. Growing conditions in the growth chamber (PGW36, Conviron, Winnipeg, Manitoba) were set to 70% relative humidity, 27°C daytime and 20°C nighttime temperatures, with a 14-hour photoperiod. Beginning 14 days after cold stratification, nutrient treatments were initiated, with plants being watered to field capacity three times each week with nutrient solution. The high nutrient solution was a half-strength Hoagland's solution (Epstein & Bloom 2005), and the low nutrient solution was the same as the high except nitrate and ammonium were lowered by 95% and adjustments were made to calcium, phosphorus, and sulfur concentrations to restore the anion-cation balance (0.175 mmol L^{-1}, 1.05 mmol L^{-1}, and 2.5 mmol L^{-1}, respectively).

Nineteen days after treatment initiation, the most recently fully-expanded leaf was plucked from each plant, flash frozen in liquid N_2, and stored at -80°C. For each RNA sample, leaf tissue from three plants was pooled to reduce variation due to genotypes, thus the 48 samples come from 144 plants. Pooled samples were ground with a mortar and pestle in liquid N_2, and suspended in 0.1mL of Trizol (Invitrogen, Carlsbad, CA), precipitated with 250 μL isopropanol and 250 μL 1.2 M NaCl, 0.8 M sodium citrate. RNA was dried and resuspended in 100 μL RNAse-free water and 350 μL Qiagen RLT lysis buffer containing 1% polyvinylpyrrolidone, and the standard RNEasy protocol was followed to purify the sample (Qiagen, RNEasy plant mini kit, Valencia, CA). Samples were dried to 25 μL volume.

Yields of RNA were low, so a Genisphere labeling reaction was used instead of direct labeling of cDNA (Array900, Genisphere, Hatfield, PA). Approximately 4 μg of total RNA was used as starting material for cyanine 3 and cyanine 5 labeling reactions, following the Cherbas (2006) protocol. Hybridizations were done in a loop design with four replicates, 2 in each possible dye configuration (Figure 4.1). Hybridized slides were scanned at 10 μm resolution (ProScanArray, PerkinElmer, Waltham, MA) and segmented in ScanArray Express (PerkinElmer) using manual subarray alignment, automatic spot alignment, and adaptive circle quantitation. Raw quantitative data were imported into limmaGUI (Wettenhall & Smyth 2004, Smyth 2005). A linear model was fit to the data to estimate the effect of species, treatment, and their interaction (Smyth 2004, Smyth et al. 2005). A linear contrast was used to compare the *H. anomalus* response to nutrient treatment to the response of its parental species.

Confirmation of the five genes with significant contrast terms after using the Benjamini-Hochberg correction for multiple comparisons were evaluated using real-time RT-PCR (Benjamini & Hochberg 1995). Four additional genes were included in the analysis: one for use as a normalization gene and three others for additional confirmation of microarray results. Primers were designed using the oligoperfect algorithm at www.invitrogen.com. To produce cDNA for the two-step reaction, approximately 4 μg of total RNA was brought to 20 μL with DEPC-treated water. Four microliters of mastermix and 2 μL of Superscript enzyme mix (Invitrogen) were added to the RNA, and the plate was incubated at 25°C for 10 minutes, 42°C for 120 minutes, and 85°C for 5 minutes. Two-hundred thirty μL of DEPC-treated water was added to the cDNA, yielding the starting material for quantitative PCR. Ten μL of Express SYBR Greener SuperMix (Invitrogen), 0.04 μL of forward and reverse primers at 100 μM concentration, and 6 μL of DEPC-treated water were added to 4 μL of the diluted cDNA. The reaction mixture was arrayed in triplicate for each gene on 96-well plates. The plates were loaded onto a thermocycler with a fluorescence detector (PTC-200, Chromo4, MJ Research, Waltham, MA). Plates were heated to 50°C for 2 minutes, 95°C for 2 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. At the end of each 60°C step, the fluorescence level of each well was recorded. Following the amplification cycles, a melting curve was constructed by heating the reaction mixture to 72°C for 8 minutes, cooling the plate to 30°C for 10 minutes, and reading the fluorescence level of each well as the temperature was increased 1°C each minute until reaching 90°C.

Analysis of quantitative PCR data was carried out using Opticon Monitor 3 (Biorad, Hercules, CA). All default settings were used for quantification, except the fluorescence threshold was manually set to 1.0, which appeared reasonable for all reactions. If one of the three replicate reactions differed from the mean of the other two by more than 1 cycle, it was considered an outlier and deleted from the dataset. Replicate observations were then averaged and imported into SAS for statistical analysis. Quantitative PCR measures were normalized using the delta c(t) method, with QHF3H09 as the reference gene to correct for differences in initial cDNA concentrations (Litvak & Schmittgen 2001). Delta c(t) values were modeled as a function of species, treatment, and their interaction (PROC GLM, SAS 9, Cary, NC). The linear contrast between the parental and *H. anomalus* responses to nutrient treatment was extracted from the interaction term as in the microarray analysis.

Sequences of genes spotted on the microarray were compared to genes in *Arabidopsis thaliana* using BLAST searches with a cutoff e-value of 1e-10 as described by Lai and colleagues (2006). The 1,696 unigenes with homologues in *Arabidopsis* were partitioned into two categories based on whether the contrast term was significant for the gene at the 0.05 level.

Note that no correction for multiple comparisons was made for this analysis. This comparison allowed us to determine whether genes that appear to have a different response to nutrient stress in *H. anomalus* relative to its parents share some cellular localization, molecular function, or biosynthetic pathway according to gene ontology (GO) classifications (TAIR, www.arabidopsis.org). Categories with fewer than five expected observations were binned together according to functional similarity to satisfy assumptions of the statistical test (Sokal & Rohlf 1981). The distribution of genes with significant contrast terms was compared to the population of genes on the array using a chi-squared test.

Results

Five unique genes showed a novel response to nutrient stress in *H. anomalus* relative to its parental species *H. annuus* and *H. petiolaris* (Table 4.1). Based on sequence similarity, these five genes had apparent homology to genes with known functions in *A. thaliana*.

When messenger RNA levels were compared using qPCR, the significant interaction term was recovered for 3 of the 5 genes, though only at the 0.1 level of significance for QHL11N17. Expression levels from the two methods agree reasonably well, with some exceptions (Figure 4.2). The agreement of the two methods was comparable to that found in other studies (Lai et al. 2006). For gene QHG17D10, it appears that primers only amplified the *H. annuus* samples efficiently.

The population of genes showing a different expression response to nutrient stress in *H. anomalus* relative to its parents did not differ significantly from the population of genes spotted on the array with respect to cellular localization or molecular function. However, genes showing a novel expression pattern in *H. anomalus* did differ in biological function, being enriched in genes involved in stress response genes, genes responding to biotic or abiotic stimuli, and genes involved in development relative to the genes on the array (Table 4.2).

Discussion

One of the five candidate genes from the microarray analysis, QHL11N17, is known to play a role in leaf senescence of *A. thaliana* (Zimmerman et al. 2006). Additionally, genes involved in development appear to have a novel expression pattern in *H. anomalus* relative to its parents. These results suggest that leaf senescence is one of the major processes that have changed during the evolution of *H. anomalus*. The timing of leaf senescence is known to be an important trait in the adaptation of plants to low nutrient habitats (Aerts & Chapin 2000). In fact, increased leaf lifespan in *H. anomalus* appears to be the most important difference in nitrogen use and allocation compared to *H. annuus* and *H. petiolaris* (L.C. Brouillette & L.A. Donovan, in prep.). Because leaves of comparable age were collected from all plants in the study, it is unlikely that these differences were attributable to leaf age.

Genes involved in stress response were among the classes of genes that showed a significantly different pattern of expression in response to nutrient limitation in *H. anomalus* compared to its parental species. It is not surprising that stress response genes show different response to nutrient stress in *H. anomalus* because the species appears to tolerate nutrient stress better than *H. annuus* and *H. petiolaris* (Brouillette et al. 2006). Of the five candidate genes, all but the homologue of QHE22M10 appear to be involved in a generalized stress response of *Arabidopsis thaliana* (Gómez-Lim et al. 1993, Kreps et al. 2002, Laloi et al. 2007, Verslues et al. 2007). In addition to abiotic stress response in *Arabidopsis*, the homologue of QHL11K09 in poplar appears to show strong regulation in response to rust and caterpillar damage (Rinaldi et al.

2007, Ralph et al. 2008). Differential expression of stress response genes is likely to be among the adaptations to the *H. anomalus* habitat.

As a next step in determining the selective advantage of altered gene expression in *H. anomalus,* natural variation in candidate genes can be used to test an association between gene expression and plant fitness (Lai et al. 2006). Genes with an altered gene expression having a demonstrated fitness effect in the *H. anomalus* habitat may be regarded as putative speciation genes because they contribute to the ecological divergence between the hybrid species and its parents (Schluter 2009). Genes conferring stress tolerance have only recently received attention in plant species as potential speciation genes (Lexer & Fay 2005). In addition to examining naturally occurring gene expression, knockout or overexpression mutants or recombinant inbred lines can be constructed for each of the candidate genes. This approach can be used to find the specific phenotypic effects of each of the candidate genes in *H. annuus* to determine the feasibility of their use to improve cultivated sunflower.

As a general caveat to this study, it is worth noting that only genes present on the array were able to be assayed using this analysis. Because the array was constructed with cDNAs from *H. annuus*, *H. argophyllus*, and *H. paradoxus*, we may have been prevented from detecting genes that are more important in the *H. anomalus* stress response. The construction of cDNA libraries from *H. anomalus* will allow the further study of the stress response of the species. As sunflower genomic resources continue to advance, the use of next-generation sequencing technologies will allow for unbiased study of the *H. anomalus* transcriptome. It should also be noted that sequence divergence between the sunflower species in this study may have affected the results. However, the use of a cDNA microarray rather than an oligonucleotide array makes this less of an issue (Buckley 2007), and sequence divergence does not appear to be a major source of error using this particular array with these study species (Lai et al. 2006). Additionally, our choice of treatment and sampled tissue affected our results. The use of transient nutrient treatments is common (e.g. Palenchar et al. 2004), but the use of long-term treatments may be more appropriate for ecological questions (Bray 2008). We chose leaf tissue because of the abundance of leaf-level physiological data in the *Helianthus* hybrid system and to exclude possible microbial RNA contamination from roots.

Acknowledgements

The authors wish to thank C Darragh and M Boyd for assistance growing plant material, R Nilsen for assistance with protocols and RNA isolation, and the Dawe lab group, especially C Topp, for assistance with quantitative PCR, Z Lai for providing support for bioinformatics, and L Rieseberg for use of microarray slides. This research was funded by NSF grant #0614739 to LAD. LCB was supported by a Dissertation Completion Award from the University of Georgia Graduate School.

References

Aerts R, Chapin III FS (2000) The mineral nutrition of wild plants revisited: A re-evaluation of processes and patterns. Advances in Ecological Research, 30, 1-67.

Baack EJ, Whitney KD, Rieseberg LH (2005) Hybridization and genome size evolution: timing and magnitude of nuclear DNA content increases in *Helianthus* homoploid hybrid species. New Phytologist, 167, 623-630.

Benhamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society, Series B, 57, 289-300.

Berendse F, Aerts R (1987) Nitrogen use efficiency: A biologically meaningful definition? Functional Ecology, 1, 293-296.

Bray EA (2008) Molecular and physiological responses to water-deficit stress. In: Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops (eds. Jenks MA, Hasegawa PM, Jain SM), pp. 121-140. Springer, The Netherlands.

Brouillette LC, Gebremedhin M, Rosenthal DM, Donovan LA (2006) Testing hypothesized evolutionary shifts toward stress tolerance in hybrid *Helianthus* species. Western North American Naturalist, 66, 409-419.

Buckley BA (2007) Comparative environmental genomics in non-model species: using heterologous hybridization to DNA-based microarrays. Journal of Experimental Biology, 209, 1602-1606.

Cherbas L (2006) Dendrimer use and hybridization protocol. CGB Technical Report 2006-08, The Center for Genomics and Bioinformatics, Indiana University, Bloomington, Indiana.

Epstein E, Bloom AJ (2005) Mineral Nutrition of Plants: Principles and Perspectives, 2nd edn. Sinauer Associates, Sunderland, MA.

Gómez-Lim MA, Valdés-López V, Cruz-Hernandez A, Saucedo-Arias LJ (1993) Isolation and characterization of a gene involved in ethylene biosynthesis from *Arabidopsis thaliana*. Gene, 134, 217-221.

Kreps JA, Wu Y, Chang HS, et al. (2002) Transcriptome changes for Arabidopsis in response to Salt, Osmotic, and Cold Stress. Plant Physiology, 130, 2129-2141.

Lai Z, Gross BL, Zou Y, Andrews J, Rieseberg LH (2006) Microarray analysis reveals differential expression in hybrid sunflower species. Molecular Ecology, 15, 1213-1227.

Laloi C, Stachowiak M, Pers-Kamczyc E, et al. (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences, USA, 104, 672-677.

Lambers H, Chapin III FS, Pons TL (2008) Plant Physiological Ecology, 2nd edn. Springer, Berlin.

Lexer C, Fay MF (2005) Adaptation to environmental stress: a rare or frequent driver of speciation? Journal of Evolutionary Biology, 18, 893-900.

Litvak SK, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25, 402-408.

Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic Press, Amsterdam.

McClintock B (1984) The significance of responses of the genome to challenge. Science, 226, 792-801.

Palenchar PM, Kouranov A, Lejay LV, Coruzzi GM (2004) Genome-wide patterns of carbon and nitrogen regulation of gene expression validate the combined carbon and nitrogen (CN)-signaling hypothesis in plants. Genome Biology, 5, R91.

Ralph SG, Chun HJE, Cooper D, et al. (2008) Analysis of 4,664 high-quality sequence-finished popular full0length cDNA clones and their utility for the discovery of genes responding to insect feeding. BMC Genomics, 9, 57.

Rieseberg LH (1991) Homoploid reticulate evolution in *Helianthus* (Asteraceae): Evidence from ribosomal genes. American Journal of Botany, 78, 1218-1237.

Rieseberg LH, Raymond O, Rosenthal DM, et al. (2003) Major ecological transitions in wild sunflowers facilitated by hybridization. Science, 301, 1211-1216.

Rinaldi C, Kohler A, Frey P, et al. (2007) Transcript profiling of poplar leaves upon infection with compatible and incompatible strains of the foliar rust *Melampsora larici-populina*. Plant Physiology, 144, 347-366.

Rosenthal DM, Schwarzbach AE, Donovan LA, Raymond O, Rieseberg LH (2002) Phenotypic differentiation between three ancient hybrid taxa and their parental species. International Journal of Plant Sciences, 163, 387-398.

Rosenthal DM, Rieseberg LH, Donovan LA (2005) Recreating ancient hybrid species' complex multi-trait phenotypes from early generation synthetic hybrids: three examples using wild sunflowers. American Naturalist, 166, 26-41.

Schluter D (2009) Evidence for ecological speciation and its alternative. Science, 323, 737-741.

Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Statistical Applications in Genetics and Molecular Biology, 3, Article 3.

Smyth GK (2005) Limma: linear models for microarray data. In: Bioinformatics and Computational Biology Solutions Using R and Bioconductor (eds. Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W), pp 397-420. Springer, New York.

Smyth GK, Michaud J, Scott H (2005) The use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics, 21, 2067-2075.

Sokal RR, Rohlf FJ (1981) Biometry: The Principles and Practice of Statistics in Bological Research, 2nd edn. WH Freeman, San Francisco.

Ungerer MC, Strakosh SC, Zhen Y (2006) Genome expansion in three hybrid sunflower species is associated with retrotransposon proliferation. Current Biology, 16, r872-r873.

Verslues PE, Batelli G, Grillo S, et al. (2007) Interaction of SOS_2 with nucleoside diphosphate kinase 2 and catalases reveals a point of connection between salt stress and H_2O_2 signaling in Arabidopsis thaliana. Molecular Cell Biology, 27, 7771-7780.

Wessler SR (1996) Plant retrotransposons: Turned on by stress. Current Biology, 6, 959-961.

Wettenhall JM, Smyth GK (2004) limmaGUI: a graphical user interface for linear modeling of microarray data. Bioinformatics, 20, 3705-3706.

Zimmermann P, Heinlein C, Orendi G, Zentgraf U (2006) Senescence-specific regulation of catalases in *Arabidopsis thaliana* (L.) Heynh. Plant Cell and Environment, 29, 1049-1060.

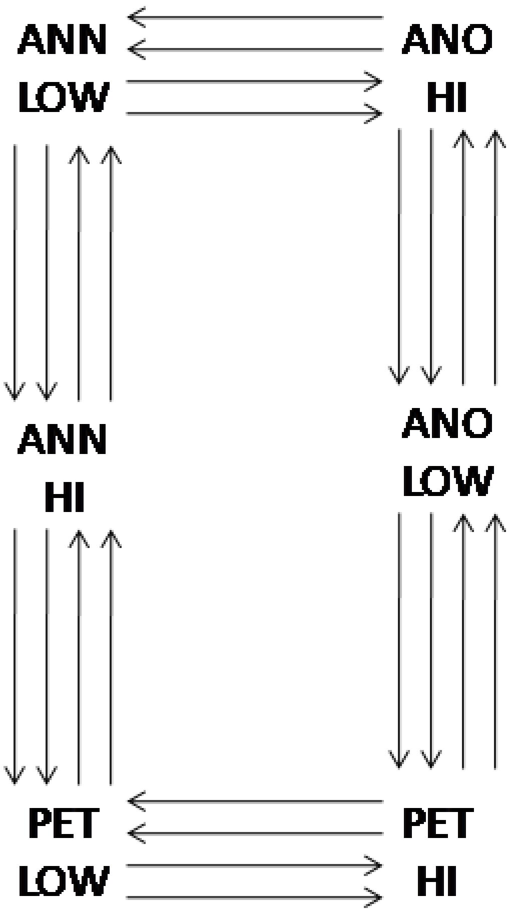

Figure 4.1. Loop hybridization design. Pair wise hybridizations were performed using a loop design as shown. Each arrow represents one hybridization using biological replicates independent of all other hybridiziations. Samples at the arrow head denotes the cy5-labelled sample, with the cy3-labelled sample at the base of the arrow. Species are abbreviated ANN, ANO, and PET for *H. annuus*, *H. anomalus,* and *H. petiolaris*, respectively, and the high nitrogen treatment is abbreviated HI.

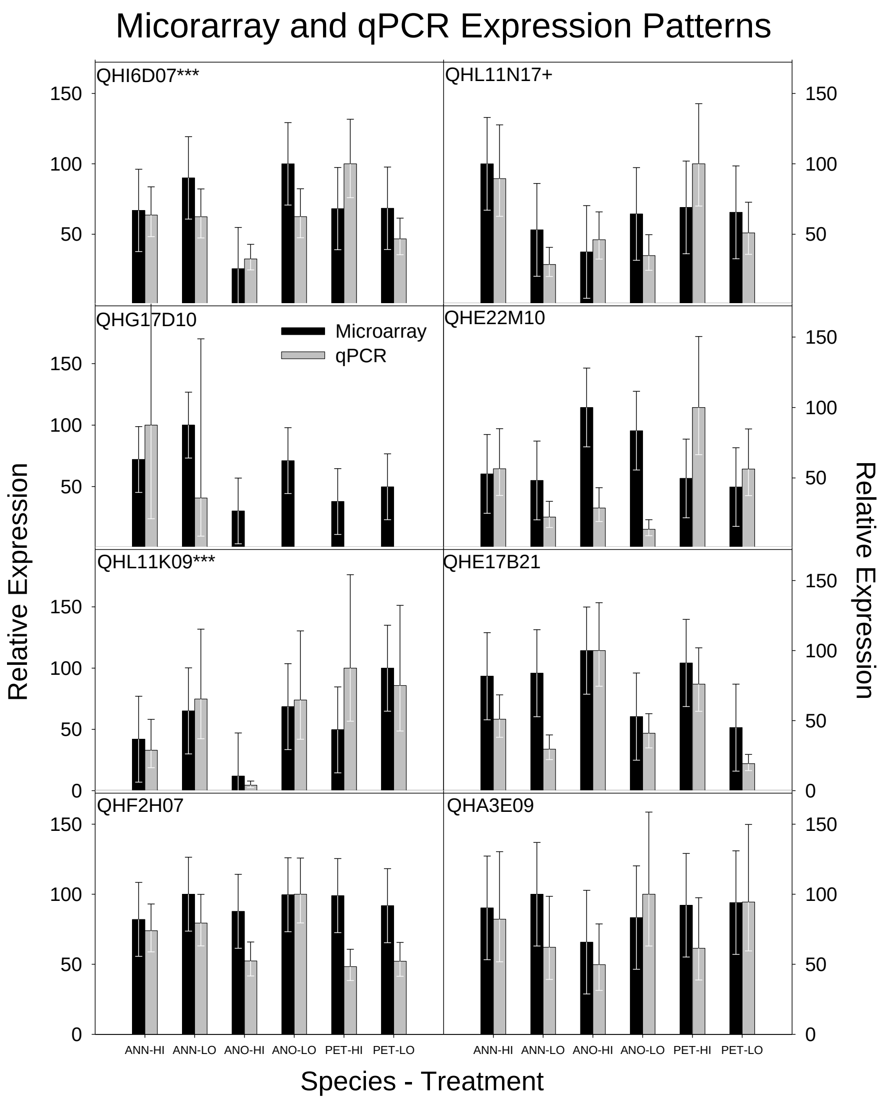

Figure 4.2. Comparison of microarray and qPCR results. Relative expression in microarray and quantitative PCR assays are presented with 95% confidence intervals. QHF3H09 was used as a normalization gene to correct for initial differences in total RNA concentrations. Candidate genes from the microarray analysis were re-evaluated using qPCR data. Symbols “+” and “***” denote candidate genes for which the linear contrast between the *H. anomalus* and parental species was recovered in the qPCR data at the 0.1 and 0.001 levels of significance, respectively.

Table 4.1. Candidate genes. Genes with a significantly different response to nitrogen treatment in *H. anomalus* relative to its parental species are presented here. Given are the express sequence tag (EST) identifications, p-value after Benjamini-Hochberg correction for multiple comparisons, homologue in *Arabidopsis thaliana*, and putative function.

ESTID	Adj. p-value	*Arabidopsis* hits	Description
QHI6D07.yg.ab1	0.00033	At1g07890	ascorbate peroxidase
QHL11N17.yg.ab1	0.00199	At1g20630	catalase 1
QHG17D10.yg.ab1	0.00270	At1g05010	1-aminocyclopropane-1-carboxylate oxidase
QHE22M10.yg.ab1	0.01503	At3g44890	50S ribosomal protein L9, chloroplast precursor
QHL11K09.yg.ab1	0.01639	At2g45180	protease inhibitor / lipid transfer protein family

Table 4.2. Contingency table of GO classifications. Sequence data for all genes on the microarray were compared to *A. thaliana* and classified according to gene ontology (GO). The population of unigenes on the array is compared to the number of genes showing a significantly different response to nitrogen treatment in *H. anomalus* relative to *H. annuus* and *H. petiolaris* (without a correction for multiple comparisons) using a chi-square test. Chi-square statistics for the cellular component, molecular function, and biological process categories are χ^2_{12}=5.43, χ^2_{11}=8.61, and χ^2_{12}=39.46***, respectively. For simplicity, only the contingency table for the biological process category is shown here.

	Differentially Expressed			Not Differentially Expressed		
Gene Ontology Classification	Obs.	Exp.	$(O-E)^2/E$	Obs.	Exp.	$(O-E)^2/E$
other metabolic processes	43	56.45	3.20	743	729.55	0.25
other cellular processes	46	54.36	1.29	711	702.64	0.10
protein metabolism	15	24.27	3.54	323	313.73	0.27
response to abiotic or biotic stimulus	28	15.08	11.07	182	194.92	0.86
unknown biological processes	20	25.06	1.02	329	323.94	0.08
response to stress	22	14.65	3.69	182	189.35	0.29
transport	16	16.88	0.05	219	218.12	0.00
developmental processes	17	9.41	6.13	114	121.59	0.47
other biological processes	14	8.47	3.60	104	109.53	0.28
cell organization and biogenesis	11	9.48	0.24	121	122.52	0.02
signal transduction	10	6.82	1.48	85	88.18	0.11
transcription	8	6.68	0.26	85	86.32	0.02
electron transport or energy pathways	3	5.39	1.06	72	69.61	0.08

CHAPTER 5

VARIATION IN FUNCTIONAL PLANT TRAITS RELATIVE TO NEUTRAL GENETIC VARIANCE IN *HELLIANTHUS ANOMALUS*[1]

---

[1] Brouillette LC, Donovan LA. To be submitteed to *Molecular Ecology*.

Abstract

The distribution of variation in quantitative traits (Q_{ST}) relative to analogous measurements of neutral genetic variance within and among populations (F_{ST}) informs our understanding of natural selection in the sampled populations. Here, we examine quantitative trait variation in the annual desert sunflower *Helianthus anomalus* and estimate neutral genetic variation using microsatellite markers. *Helianthus anomalus* is a natural hybrid species endemic to sand dunes in the southwestern United States. The habitat of *H. anomalus* is nutrient poor, and previous studies of the species have pointed toward nitrogen-use traits as being important for success in the field. We sampled from eight natural populations, representing the majority of its narrow island-like geographic distribution. We found that Q_{ST} is generally much greater than F_{ST}, indicating that populations have undergone diversifying selection for those traits. Unexpectedly, physiological traits dealing with both nitrogen and water use showed evidence of diversifying selection. We speculate that this may be due to shifting relative importance of water and nutrient limitation in the populations sampled. Longer leaf lifespan confers greater nitrogen use efficiency and appears to be a key trait that differs between *H. anomalus* and its parental species. In this study, we detected little genetic variation for leaf lifespan, possibly due to strong selection for longer tissue longevity and consequently higher nutrient use efficiency across the sampled populations of *H. anomalus*.

Introduction

The distribution of plant genetic variation within and among populations has been an active area of research for many years (Hamrick & Godt 1996). Characterization of variation in neutral genetic markers has been the most frequently-used method of surveying plant variation, with the expectation that neutral genetic variation is predictably related to variation in functional traits. Among those most interested in this information are conservation biologists interested in preserving populations with high diversity (Knopp et al. 2007). When used in this way, neutral genetic variation is a proxy for variance in plant traits. In general, however, there is less structuring in neutral genetic variance than in functional traits (Leinonen et al. 2007).

The level of population differentiation in neutral genetic variation is commonly reported as F_{ST}, the proportion of total genetic variance that is partitioned among populations. Assuming F_{ST} is measured on markers that are selectively neutral, the statistic provides an estimate of the partitioning of plant variation that is due to genetic drift. This baseline measurement of population differentiation can be compared to an analogous parameter, Q_{ST}, calculated over phenotypic traits (Spitze 1993). The value of Q_{ST} relative to F_{ST} has well-defined interpretations about the action of natural selection on the plant trait (Yang et al. 1996):

1. If Q_{ST} for a trait is not significantly different from $F_{ST,}$ trait differentiation is not distinguishable from drift.
2. If Q_{ST} is greater than F_{ST}, selection acts differently on the trait in different environments indicating populations are locally adapted.
3. If Q_{ST} is less than F_{ST}, selection on the trait is similar across the sampled range.

Therefore, the comparison of the two statistics can be used to infer the action of natural selection on plant traits, allowing tests of hypotheses about selective pressures across the populations sampled.

Here, we present a test of the hypothesis that nitrogen limitation is a unifying selective pressure across the range of *Helianthus anomalus*. The study species is a narrowly-distributed, annual sunflower endemic to sand dunes in Utah and northern Arizona and is a stable hybrid derived from *H. annuus* and *H. petiolaris* (Rieseberg 1991). Because *H. anomalus* grows on active sand dunes, it was originally hypothesized that water limitation was the primary selective force driving the evolution of the species (Thompson et al. 1981, Rieseberg 1991). However, several recent experiments have highlighted the importance of nutrient limitation in the *H. anomalus* habitat. Rosenthal and colleagues (2005) showed that the sand dunes in a representative population of *H. anomalus* store water that is accessible throughout the growing season. Ludwig and others (2006) showed that supplying additional water to field-grown *H. anomalus* does not improve plant performance. A recent review of several phenotypic selection experiments in the *H. anomalus* habitats showed consistent selection for increased foliar nitrogen (L.A. Donovan, in review). Additionally, *H. anomalus* appears to be more tolerant of nutrient stress than *H. annuus* and *H. petiolaris* (Brouillette et al. 2006). In this study, we survey a suite of traits, most of which were used in a previous comparison of *H. anomalus* to its parental species *H. annuus* and *H. petiolaris* (Rosenthal et al. 2002). We use the present analysis to determine whether the subset of traits important in plant nitrogen use show evidence of unifying selection, which would lend support to the hypothesis that nutrient limitation is the primary factor in the ecological speciation of *H. anomalus* (Schluter 2009).

Materials and Methods

Maternal half-sibling families of were collected from eight natural populations of *Helianthus anomalus* between 24 and 26 August 2007 (Table 5.1). Seeds were dried at 40°C and stored at room temperature until use. After removal of blunt ends, seeds were placed in the dark overnight on filter paper moistened with a 0.005% solution of fusicoccin to break dormancy. The following day, seedcoats were removed, and seeds were placed on moist filter paper under grow lights (80-90 μmol m^{-2} s^{-1}) with a 12-hour photoperiod. After developing roots and green cotyledons (3-10 days after seedcoat removal), seedlings were transplanted into 25 cm pots filled with a 3:1 sand-to-Turface mixture (Profile Products, Buffalo Grove, IL) at the UGA Plant Biology Greenhouses in Athens, Georgia. The experimental design was a randomized complete block design with 12 families from each of 8 populations replicated in each of 3 blocks (N = 288), but mortality and poor germination of seeds from White Sands and Jericho populations resulted in an unbalanced design (Table 5.1).

Starting on the last day of transplanting (21 January 2008) and continuing three times each week, plants were fertilized by watering pots to field capacity with half-strength Hoagland's solution (Epstein & Bloom 2005). Plant height was estimated weekly starting 21 January 2008, and the slope of a simple linear regression through the first four heights served as the estimate of relative growth rate for the plant.

Gas exchange measurements were taken on the most recently fully expanded leaf on 18-20 February 2008 (LI-6400, LiCor Biosciences, Lincoln, NE), with one experimental block measured each day. Chamber conditions were set to 380 ppm CO_2, 2000 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), and 30°C block temperature. Chamber relative humidity was adjusted to be slightly above ambient (~50-55%). The morning following gas exchange measurement, when leaves were fully hydrated, the gas exchange leaf was excised, weighed, and scanned at 150 dpi with flat bed scanner. Leaf area and perimeter were obtained with ImageJ freeware (National Institutes of Health, Bethesda, MD). For leaves that did not completely fill the chamber, the corners of the gasket were marked, and the area inside the chamber was estimated using ImageJ. Gas exchange leaves were dried at 60°C, weighed, and ball mill ground to estimate leaf carbon and nitrogen (NA1500, Carlo Erba Strumentazione, Milan, Italy) and stable carbon isotopic ratio (leaf $\delta^{13}C$, continuous flow mass spectrometer, Finnegan, Bremen, Germany). Specific leaf area was calculated as the leaf area divided by the dry mass of the gas exchange leaf, and leaf succulence was calculated as the difference between fresh and dry leaf mass divided by leaf area. Photosynthetic nitrogen use efficiency (PNUE) was calculated after Field & Mooney (1986).

The leaf opposite the gas exchange leaf was tagged and tracked for leaf lifespan. The tagged leaf was observed every other day to determine the date when 25% of the leaf area turned yellow. The number of days between tagging and the leaf color change was used as the estimate of leaf lifespan. The date of the emergence of a terminal flower bud and the date of the first flower were observed at the same time as leaf lifespan. An inflorescence was considered a flower if at least one ligule was fully expanded. After all ligules had expanded, several floral metrics (Table 5.2) were estimated on the first flower.

Leaf toughness and leaf hair density were measured on the most recently fully expanded leaf on 3 April 2008. Leaf hair density was estimated by counting the number of leaf hairs on three regularly-spaced, 1/3 cm^2 areas on the adaxial surface of the leaf. The density of the leaf hairs per cm^2 was calculated as the sum of the three counts. Leaf toughness was measured as the force required to penetrate the leaf (E-DFE-002, Chatillon/Ametek, Largo, FL). Seven measurements were taken on the same leaf, and the average of those was used as the estimate of leaf toughness for the plant.

Plants were harvested 26 April – 1 May 2008. Each plant was partitioned into leaves, stem, reproductive, and root components. Inflorescences were categorized as buds, flowers, or seedheads. Roots were removed from the soil within two weeks of harvest, and a subsample of the fine roots less than 1mm in diameter were collected and stored in water for estimation of specific root length (SRL). The root subsample was stained with toluidine blue O, and the total length of the root sample was estimated using WinRhizo (Regent Instruments, Quebec, QC, Canada). All biomass was dried at 60°C, weighed, and summed to estimate total plant biomass.

Bulk pollen was collected from and reapplied to open flowers three times weekly between 3 March 2008 and 4 April 2008. When all ligules were shed, pollinated inflorescences were bagged with nylon mesh to prevent loss of seeds. Mature seeds were extracted from bagged seedheads, counted, dried, and weighed. Seeds were also scanned at 600 dpi on a flatbed scanner with a black background. Seed images were imported into Tomato Analyzer (Brewer et al. 2006) to estimate the seed shape and seed color parameters (seedshp, seedlm, Table 5.2). Average seed mass was calculated as the mass of the dried seeds divided by the number of seeds.

Leaf tissue from the harvest was coarsely ground (Wiley mill, Thomas Scientific, Swedesboro, NJ) and ashed to estimate foliar concentrations of boron, calcium, magnesium, phosphorus, and potassium (Enviro 36 Inductively Coupled Argon Plasma, Thermo Jarrell Ash Corp., Franklin, MA).

Leaf samples from each plant were collected and placed in polypropylene tubes containing silica gel desiccant and stored at room temperature until use. DNA was extracted from one individual from each of the 97 maternal families using DNeasy kits (Qiagen, Valencia, CA). The concentration and quality of the DNA was determined with a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, Wilmington, DE), and all samples were diluted to 10 ng μL^{-1}. A twelve-locus genotype was obtained for each sample using SSR markers located in intergenic regions of the nuclear genome (Table 5.3) from the Compositae Genome Database (CGP, http://cgpdb.ucdavis.edu). Markers were chosen to be largely unlinked and polymorphic in *H. anomalus* (Lai et al. 2005). Fluorescently-labeled primers (6FAM, NED, HEX, TET) were used to amplify SSRs with a touchdown 58 protocol (Don et al. 1991). Products from the reaction were diluted 1:20 and analyzed using capillary gel electrophoresis (ABI 3730xl, Applied Biosystems, Valencia, CA). Size of fragments was determined using Genescan software (Applied Biosystems).

Statistical Analysis

Variance for all traits was partitioned into population, family nested within population, and error components using a completely random model (PROC MIXED, SAS 9, Cary, NC). The variance component for populations was used as the estimate of between population variance (σ^2_b). The within population genetic variance (σ^2_w) was estimated as four times the family within population variance component because half-siblings were used (Lynch & Walsh 1998, Chenoweth & Blows 2008). Q_{ST} was calculated after Spitze (1993). Standard error for the mean Q_{ST} value of each trait was estimated as the standard deviation of Q_{ST} estimates from 1,000 iterations bootstrapped over all samples.

Broad-sense heritabilities (H^2) were estimated using separate completely random models fit using a restricted maximum likelihood algorithm for each population (PROC MIXED, SAS 9). Trait variation was modeled as a function of family and residual, the variance estimates of which were used as estimates of heritable and non-heritable variance, respectively. Broad-sense heritability was calculated as four times within family variance component divided by the sum of family and residual variance components (Becker 1984). Because JER and WHS were represented by few individuals in the study, heritabilities from those populations fluctuated widely. Only estimates from the other six populations were used to calculate average broad-sense heritability for each trait.

F_{ST} was calculated using GDA (Weir & Cockerham 1984, Lewis & Zaykin 2001), with bootstrapped confidence intervals estimated over 1,000 iterations.

Results

F_{ST} was estimated to be 0.1837 which is similar to allozyme-based estimates of F_{ST} for other composites (Hamrick & Godt 1996). The value is higher than was found in a previous study, though *H. anomalus* was pooled with its parents in that analysis (Schwarzbach & Rieseberg 2002). In general, Q_{ST} was much greater than F_{ST} for most traits (Figure 5.1), with the average of all estimable Q_{ST} values at 0.4307. This indicates that most traits are under diversifying selection in the different populations sampled.

Discussion

Eight traits show evidence of unifying selection in this analysis, eight are evolving in a pattern indistinguishable from drift, and twenty-two show evidence of diversifying selection (Figure 5.1). Traits leaf lifespan, PNUE, resorption efficiency, and percentage of red seedcoat area had estimated σ^2_w and σ^2_b estimates both equal to zero, making Q_{ST} undefined.

Unifying selection on a particular trait indicates that similar trait values are selected for across the sampled range. This is of particular interest in *H. anomalus* because the species is likely to have had multiple origins (Schwarzbach & Rieseberg 2002). Traits that show evidence of unifying selection across the sampled range should give some clues into which selective pressures are constant across the species and could have potentially been the driving force in the speciation of *H. anomalus* (Schluter 2009). The traits that show evidence of unifying selection in this study are foliar potassium, calcium, boron, and magnesium, integrated water use efficiency ($\delta^{13}C$), number of flowers at final harvest, petiole length, and relative growth rate. Foliar boron has long been thought to be an important trait in desert habitats (Jefferies et al. 1999, Rosenthal et al. 2002), and it may be the case that tight control of concentrations of several ions is also required or a consequence of regulation of one or a few particular ions. $\delta^{13}C$ shows evidence of unifying selection across the populations sampled. Previous studies have suggested that nutrient limitation is more severe than water limitation in the *H. anomalus* habitat (Rosenthal et al. 2005, Ludwig et al. 2006), with selection for lower water use efficiency measured as more negative $\delta^{13}C$ found in *H. anomalus* (Donovan et al. 2007). Water use efficiency is frequently found to be lower in *H. anomalus* than in parental species when grown in a common environment (Schwarzbach et al. 2001, Brouillette et al. 2006), suggesting that selection over evolutionary time has not favored more efficient water use in the *H. anomalus* habitat. The results of the current analysis further suggest that the pattern in $\delta^{13}C$ found when only one population was used to represent *H. anomalus* is able to be extended to the species as a whole. Seedling RGR under optimum conditions has been used as a rough assessment of the stress tolerance of a wide range of species (Lambers et al. 2008), and it is not surprising that our data show evidence of a reduced seedling relative growth rate across the sampled populations.

It was expected that the largest number of traits would show evidence of diversifying selection as Q_{ST} is frequently greater than F_{ST} (Leinonen et al. 2007). Higher leaf toughness, leaf hair density, and seed luminosity are likely to be advantageous when sand abrasion (Read et al. 2005), herbivory (Mauricio 1998), and seed predation (Nystrand & Granstrom 1997) are strong selective pressures. From rough assessments of the habitats, these environmental variables are likely very different across the populations sampled. Life history traits show particularly strong evidence of diversifying selection, indicating that there are marked differences in flowering time. In the field and glasshouse SOU populations appeared flower much earlier than the other populations (L. C. Brouillette, personal observation). Selection for a faster life cycle may be a function of water availability (Heschel & Riginos 2005). Previous comparisons of *H. anomalus* to another hybrid sunflower species, *H. deserticola*, showed that *H. deserticola* appears to be much more like a typical desert annual than *H. anomalus*, with reproduction initiated before drought becomes too severe (Rosenthal et al. 2002, Gross et al. 2004). Leaf shape is a plant trait that affects water use efficiency (Stiller et al. 2004, Nicotra et al. 2008) and is likely under selection when water limits growth (Robichaux 1984, Friar et al. 2007). Leaf shape shows strong evidence of diversifying selection in this analysis.

Leaf lifespan shows very low broad-sense heritability in this study, with genetics accounting for only about 14% of variance in the trait. In the Q_{ST} analysis, both within and among population variance components were estimated to be zero. While leaf lifespan is a difficult trait to measure, we have found species-level differences in leaf lifespan using the same method, suggesting that there is some genetic component to the trait (L.C. Brouillette & L.A. Donovan, in prep.). Traits expected to be under extremely heavy selection, such as plant fitness, should show low levels of genetic variance (Geber & Griffen 2003 and references therein). Longer leaf lifespan increases nitrogen use efficiency by increasing the length of time nitrogen remains in the plant (Aerts & Chapin 2000). It may be the case that leaf lifespan is under strong selection in all sampled populations due to low soil fertility, causing much of the genetic variation in the trait to be eliminated in *H. anomalus*. Leaf lifespan is longer in *H. anomalus* than in the parental species (L.C. Brouillette & L.A. Donovan, in prep.), and a candidate gene for this adaptive change has been found using a comparative genomic approach (L.C. Brouillette & L.A. Donovan, in prep.). Leaf lifespan is emerging as a key trait in the adaptation of *H. anomalus* to its active sand dune habitat.

We did not observe unifying selection on nitrogen-use traits that we expected from habitat differences and previous studies. It is likely that the relative limitation of nutrients and water varies by population. Previous studies have focused on the WHS and JER populations because of their ease of access and proximity to populations of *H. deserticola* and *H. annuus*. The present results indicate that species-level trait variation is underestimated when only one representative population is used. It is likely that the relative importance of water and nutrient limitation at each population has had some effect on phenology and nitrogen-use traits.

As with any analysis, these results must be interpreted with some caution. Traits that show evidence of selection in this analysis may not be under direct selection and instead may be dragged along due to selection on a correlated trait (Lande & Arnold 1983). It is interesting to note that in phenotypic selection analyses carried out in one of the populations, foliar nitrogen on a mass basis was found to be under direct selection (Ludwig et al. 2004, Donovan et al. 2007). These results taken together give additional confidence in our results. We must also point out that our plants were grown from field-collected seeds. Our analysis may overestimate structuring because variation in the maternal environments could have exaggerated differences between the populations. Because paternity is unknown, it is likely that some of the families contain full and half siblings, artificially decreasing our Q_{ST} estimates and increasing our estimates of heritability. The inclusion of some full siblings would affect heritability and Q_{ST} similarly across all traits, but differences caused by maternal effects would vary from trait to trait. The use of field-collected half sibling families introduced some error in our experiment due to correlated paternity and/or maternal effects, as is evidenced by heritability estimates for phyllary shape and leaf hair density.

In conclusion, we observed selection for local adaptation for most traits in the analysis. We did not find unifying selection for the majority of nutrient use traits such a s foliar nitrogen. This may be a result of a shifting relative importance of nutrients and water as factors limiting growth across the broad geographic range sampled. We found little genetic variance for leaf lifespan, which is emerging as an important trait in the nutrient relations of *H. anomalus*. We speculate that this may be due to strong selection for longer lea lifespan, which could have eliminated much of the genetic variance in the trait.

Acknowledgements

The authors wish to thank C Darragh and S Fatkin for help with measurements and harvests, the Donovan lab group for helpful comments on a previous draft, and the Knapp lab, particularly S Tang and N Kahlilian, for outstanding genotyping training and support. This research was supported by NSF grant #0614739 to LAD and a Garden Club of America Award in Desert Studies to LCB. LCB was supported by a Dissertation Completion Award from the University of Georgia Graduate School.

References

Aerts R, Chapin III FS (2000) The mineral nutrition of wild plants revisited: A re-evaluation of processes and patterns. Advances in Ecological Research, 30, 1-67.

Becker WA (1984) Manual of Quantitative Genetics, 4th edn. Academic Enterprises, Pullman, WA.

Brewer MT, Lang L, Fujimara K, et al. (2006) Development of a controlled vocabulary and software application to analyze fruit shape variation in tomato and other plant species. Plant Physiology, 141, 15-25.

Brouillette LC, Gebremedhin M, Rosenthal DM, Donovan LA (2006) Testing hypothesized evolutionary shifts toward stress tolerance in hybrid *Helianthus* species. Western North American Naturalist, 66, 409-419.

Chenoweth SF, Blows MW (2008) Q_{ST} meets the G matrix: The dimensionality of adaptive divergence in multiple correlated quantitative traits. Evolution, 62, 1437-1449.

Don R, Cox P, Wainwright B, Baker K, Mattick J (1991) Touchdown PCR to circumvent spurious priming during gene amplification. Nucleic Acids Research, 19, 4008.

Donovan LA, Dudley S, Rosenthal DM, Ludwig F (2007) Phenotyic selecton on leaf WUE and related ecophysiological traits for natural populations of desert sunflowers. Oecologia, 152, 13-25.

Epstein E, Bloom AJ (2005) Mineral Nutrition of Plants: Principles and Perspectives, 2nd edn. Sinauer, Sunderland, MA.

Field F, Mooney HA (1986) The photosynthesis-nitrogen relationship in wild plants. In: On the Economy of Plant Form and Function (ed. Givnish TJ), pp. 25-55. Cambridge University Press, Cambridge.

Friar EA, Cruse-Sanders JM, McGlaughlin ME (2007) Gene flow in *Dubautia arborea* and *D. ciliolata*: the roles of ecology and isolation by distance in maintaining species boundaries despite ongoing hybridization. Molecular Ecology, 16, 4028-4038.

Geber MA, Griffen LR (2003) Inheritance and natural selection on functional traits. International Journal of Plant Sciences, 164, S21-S42.

Gross BL, Kane NC, Lexer C, et al. (2004) Reconstructing the origin of *Helianthus deserticola*: survival and selection on the desert floor. American Naturalist, 164, 145-156.

Hamrick JL, Godt MJ (1996) Effects of life history traits on genetic diversity in plant species. Philosophical Transactions of the Royal Society of London, Series B, 351, 1291-1298.

Heschel MS, Riginos C (2005) Mechanisms of selection for drought stress tolerance and avoidance in *Impatiens capensis* (Balsaminacea). American Journal of Botany, 92, 37-44.

Jeffries SP, Barr AR, Karakousis A, et al. (1999) Mapping of chromosome segments conferring boron toxicity tolerance in barley (*Hordum vulgare* L.). Theoretical and Applied Genetics, 98, 1293-1303.

Knopp T, Cano JM, Crochet PA, Merilä J (2007) Contrasting levels of variation in neutral and quantitative genetic loci on island populations of moor frogs (*Rana arvalis*). Conservation Genetics, 8, 45-56.

Lai Z, Livingstone K, Zou Y, et al. (2005) Extensive chromosomal repatterning and the evolution of sterility barriers in hybrid sunflower species. Genetics, 171, 291-303.

Lambers H, Chapin III FS, Pons TL (2008) Plant Physiological Ecology, 2nd edn. Springer, Berlin.

Lande R, Arnold SJ (1983) The measurement of selection on correlated characters. Evolution, 37, 1210-1226.

Leinonen T, O'Hara RH, Cano JM, Merilä J (2007) Comparative studies of quantitative trait and neutral marker divergence: a meta-analysis. Journal Evolutionary Biology, 21, 1-17.

Lewis PO, Zaykin D (2001) Genetic Data Analysis: Computer program for the analysis of allelic data. Version 1.0 (d16c). Free program distributed by the authors over the internet from http://lewis.eeb.uconn.edu/lewishome/software.html.

Ludwig F, Jewett RA, Donovan LA (2006) Nutrient and water addition effects on day and night-time conductance and transpiration in a C3 desert annual. Oecologia, 148, 219-225.

Ludwig F, Rosenthal DM, Johnston JA, et al. (2004) Selection on leaf ecophysiological traits in a desert hybrid *Helianthus* species and early generation hybrids. Evolution, 58, 2682-2692.

Lynch M, Walsh B (1998) Genetics and Analysis of Quantitative Traits, 2nd edn. Sinauer, Sunderland, MA.

Mauricio R (1998) Costs of resistance to natural enemies in field populations of the annual plant *Arabidopsis thaliana*. American Naturalist, 151, 20-28.

Nictra AB, Cosgrove MJ, Cowling A, Schlichting CD, Jones CS (2008) Leaf shape linked to photosynthetic rates and temperature optima in South African *Pelargonium* species. Oecologia, 154, 625-635.

Nystrand O, Granstrom A (1997) Post-dispersal predation on *Pinus sylvestris* seeds by *Fringilla spp.*: Ground substrate affects selection for seed color. Oecologia, 110, 353-359.

Read J, Sanson GD, BB Lamont BB (2005) Leaf mechanical properties in schlerophyll woodland and shrubland on contrasting soils. Plant and Soil, 276, 95-113.

Rieseberg LH (1991) Homoploid reticulate evolution in *Helianthus* (Asteraceae): Evidence from ribosomal genes. American Journal of Botany, 78, 1218-1237.

Robichaux RH (1984) Variation in the tissue water relations of two sympatric Hawaiian *Dubautia* species and their natural hybrid. Oecologia, 65, 75-81.

Rosenthal DM, Schwarzbach AE, Donovan LA, Raymond O, Rieseberg LH (2002) Phenotypic differentiation between three ancient hybrid taxa and their parental species. International Journal of Plant Sciences, 163, 387-398.

Rosenthal DM, Ludwig F, Donovan LA (2005) Plant responses to an edaphic gradient across an active sand dune/desert boundary in the Great Basin Desert. International Journal of Plant Sciences, 166, 247-255.

Schwarzbach AE, Donovan LA, Rieseberg LH (2001) Transgressive character expression in a hybrid sunflower species. American Journal of Botany, 88, 270-277.

Schwarzbach AE, Rieseberg LH (2002) Likely multiple origins of a diploid hybrid sunflower species. Molecular Ecology, 11, 1703-1715.

Schluter D (2009) Evidence for ecological speciation and its alternative. Science, 323, 737-741.

Spitze K (1993) Population structure in Daphnia obtuse: Quantitative genetic and allozymic variation. Genetics, 135, 367-374.

Stiller WN, Reid PE, Constable GA (2004) Maturity and leaf shape as traits influencing cotton cultivar adaptation to dryland conditions. Agronomy Journal, 96, 656-664.

Thompson TE, Zimmerman DC, Rogers CE (1981) Wild *Helianthus* as a genetic resources. Field Crops Research, 4, 333-343.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution, 38, 1358-1370.

Yang RC, Yeh FC, Yanchuk AD (1996) A comparison of isozyme and quantitative genetic variation in *Pinus contorta* ssp. *latifolia* by F_{ST}. Genetics, 142, 1045-1052.

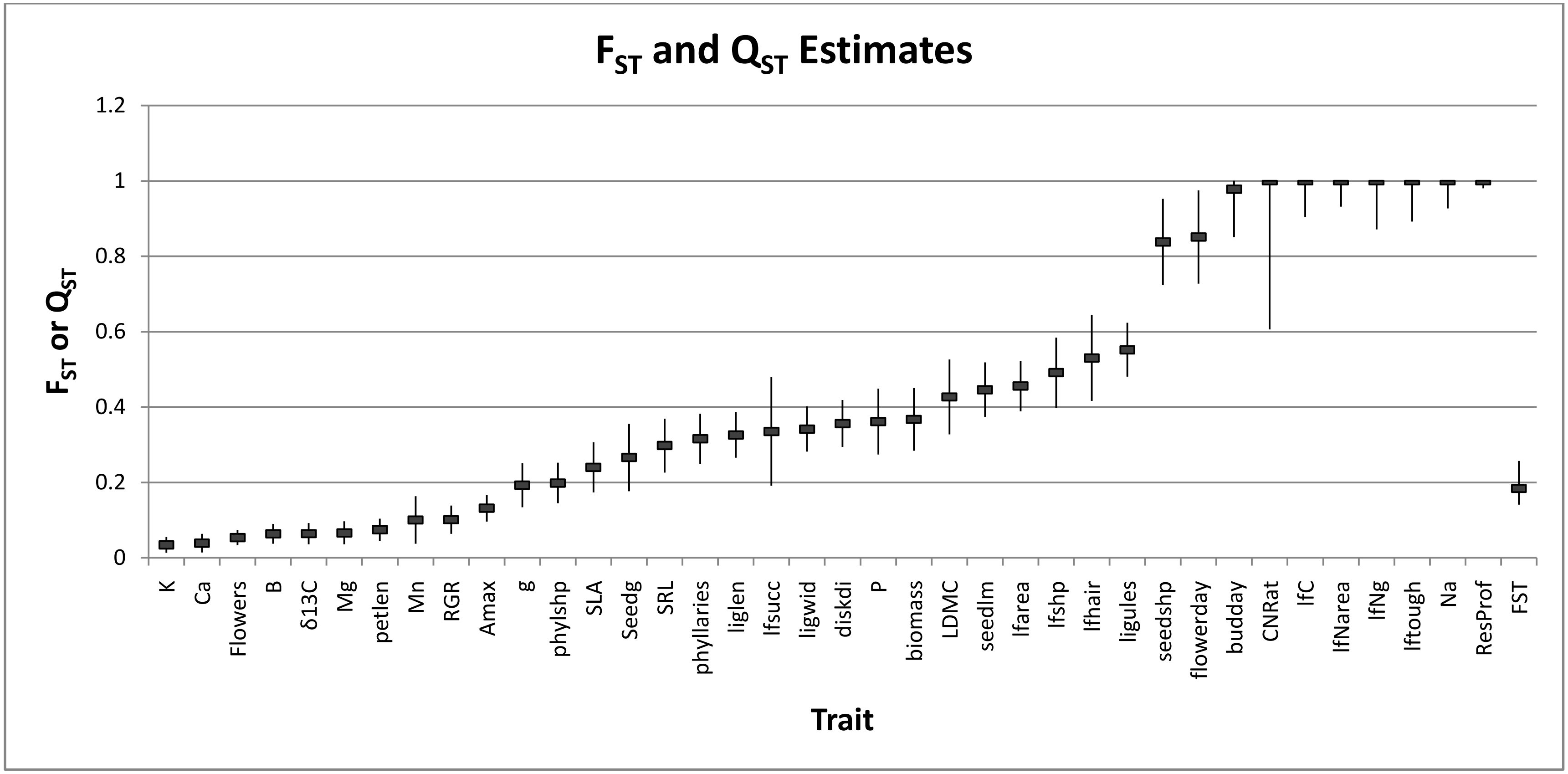

Figure 5.1. F_{ST} and Q_{ST} estimates. Point estimates and bootsrapped 95% confidence interval for F_{ST} and Q_{ST} for traits measured. Values represent the proportion of total heritable variance that is partitioned among populations.

Table 5.1. Populations Sampled. Names and abbreviations for populations are presented along with their GPS coordinates, and sample sizes. The number of half-sibling families sampled is given in parentheses next to the total sample size from that population.

Population	Abbrev.	GPS - N	GPS - W	N(families)
Hanksville airport	AIR	38°26.996'	110°39.383'	44(14)
Goblin Valley	GOB	38°36.889'	110°34.606'	45(14)
Hall's Crossing	HAL	37°24.305'	110°26.864'	40(12)
Junction	JCT	38°50.516'	110°22.975'	41(13)
Jericho	JER	39°41.278'	112°22.175'	15(8)
North of Hanksville	NTH	38°35.321'	110°35.520'	43(14)
South of Hanksville	SOU	38°01.218'	110°33.642'	38(13)
White Sands	WHS	39°44.397'	112°19.153'	22(9)

Table 5.2. Traits measured. Trait names, abbreviations, means, standard deviations, sample size, and average broad-sense heritabilities are presented. Broad-sense heritabilities (H^2) were estimated using separate analysis of variance models for each population, with heritability calculated as four times the variance due to family divided by the sum of family and residual variances. The average of broad-sense heritability for all populations except WHS and JER is presented here, with each population receiving equal weight. For ease of comparison, traits are ordered as they appear in Figure 5.1, except the final three traits for which Q_{ST} was undefined.

Variable	Abbrev.	Units	N	Mean	Std Dev	H^2
Foliar potassium	K	ppm	276	13022	2085	0.7176
Foliar Calcium	Ca	ppm	276	25792	4730	0.9665
Number of flowers at harves	Flowers	count	276	21.34	8.97	0.3731
Foliar boron	B	ppm	276	0.0729	0.0177	0.6163
Foliar carbon isotopic ratio	δ^{13}C	‰	281	-31.61	0.60	0.4475
Foliar Magnesium	Mg	ppm	276	1106000	255000	0.4773
Petiole length	petlen	cm	281	11.83	6.59	0.8791
Foliar Manganese	Mn	ppm	276	7639	4767	0.3255
Relative growth rate	RGR	cm day^{-1}	287	0.1658	0.0693	0.4887
Instantaneous photosynthetic rate	Amax	µmol m^{-2} s^{-1}	280	35.89	3.42	0.3331
Instantaneous stomatal conductance	G	µmol$_{CO2}$ m^{-2} s^{-1}	280	1.926	0.439	0.408
Phyllary shape	phylshp	mm mm^{-1}	281	7.195	2.119	1.3297
Specific leaf area	SLA	cm^2 g^{-1}	281	252.4	25.2	0.3671
Seed mass	Seedg	g	231	9.844	2.650	0.4664
Specific root length	SRL	m g^{-1}	276	111.8	32.0	0.4444
Number of phyllaries	phyllaries	count	281	17.73	3.13	0.5626
Ligule length	liglen	Mm	281	29.03	5.50	0.3187
Leaf succulence	lfsucc	g$_{H20}$ cm^{-2}	280	0.0457	0.0055	0.3136
Ligule width	ligwid	mm	281	13.56	2.36	0.561
Flower disk diameter	diskdi	mm	281	14.54	2.81	0.2927
Foliar phosphorus	P	ppm	276	73.01	17.11	0.5034
Total biomass at harvest	biomass	g	276	53.31	15.44	0.5521
Leaf dry matter content	LDMC	mg g^{-1}	280	80.76	7.86	0.355
Seed luminosity	seedlm	relative scale	230	90.66	10.11	0.4216
Leaf area	lfarea	cm^2	281	21.84	8.00	0.3874
Leaf shape	lfshp	cm^2 cm^{-1}	281	0.8818	0.1926	0.4696
Leaf hair density	lfhair	count cm^{-1}	280	19.50	12.41	1.1494
Number of ligules	ligules	count	281	10.93	2.11	0.2712
Seed shape	seedshp	cm^2 cm^{-1}	230	0.0627	0.0079	0.4275
Days to first flower	flowerday	days	281	55.58	6.54	0.2743
Days to budding	budday	days	281	36.98	5.48	0.3014
Foliar carbon-to-nitrogen ratio	CNRat	% %$^{-1}$	281	5.898	0.833	0.338
Foliar carbon	LfC	%	281	34.88	1.92	0.4089
Foliar nitrogen on an area basis	lfNarea	mmol m^{-2}	281	170.0	20.7	0.6781
Foliar nitrogen on a mass basis	lfNg	%	281	5.967	0.529	0.6781
Leaf toughness	lftough	g	280	56.20	11.49	0.1791
Foliar sodium	Na	ppm	276	87.59	39.86	0.1116
Leaf resorption proficiency	ResProf	%	280	1.024	0.771	0.1822
Leaf lifespan	lflife	days	281	32.76	7.59	0.1389
Photosynthetic nitrogen use efficiency	PNUE	µmol$_{CO2}$ mol$_{N}$ $^{-1}$s^{-1}	280	2130	251	0.4216
Leaf resorption efficiency	ResEff	% %$^{-1}$	279	83.01	12.46	0.1742

Table 5.3. SSR Markers. Marker names, sample size (N), number of alleles (A), expected heterozygocity (H_e), and observed heterozygocity (H_o).

Locus	N	A	H_e	H_o
ORS229	94	15	0.851	0.617
ORS297	96	13	0.7872	0.6771
ORS511	82	2	0.2061	0.0122
ORS588	77	9	0.1967	0.039
ORS618	96	8	0.558	0.4375
ORS844	92	15	0.8331	0.6087
ORS896-1	97	5	0.3953	0.2165
ORS896-2	81	12	0.8528	0.284
ORS1008-1	80	9	0.5961	0.2875
ORS1008-2	82	11	0.7983	0.061
ORS1017	92	17	0.8749	0.2935
ORS1141	92	9	0.5416	0.5

CHAPTER 6

GENETIC ARCHITECTURE OF LEAF ECOPHYSIOLOGICAL TRAITS IN *HELIANTHUS*[1]

---

[1] Brouillette LC, Rosenthal DM, Rieseberg LH, Lexer C, Malmberg RL, Donovan LA. 2007. Journal of Heredity. 98: 142-146. Reprinted here with permission of publisher.

Abstract

We investigated quantitative trait loci (QTL) for several leaf chemistry traits in early generation hybrids between *Helianthus annuus* and *H. petiolaris*, the parental species of the ancient diploid hybrid sunflower species *H. anomalus*, *H. deserticola*, and *H. paradoxus*. We grew individuals of a second-generation backcross (BC_2) toward *H. petiolaris* under optimum conditions in a glasshouse experiment. Trait values were measured once for each individual. In addition, genotypic data previously determined for each individual were employed for composite interval mapping of QTL. We detected QTL for leaf carbon concentration, leaf nitrogen concentration, leaf nitrogen per unit area, and photosynthetic nitrogen use efficiency. Leaf carbon isotope discrimination ($\delta^{13}C$) and leaf nitrogen isotopic composition ($\delta^{15}N$) were analyzed, but no significant QTL were found for these traits. Interestingly, two neighboring loci explained a relatively large percentage of the variation in leaf nitrogen per unit area. This was notable because leaf nitrogen has been shown to strongly affect the fitness of early generation sunflower hybrids in the *H. anomalus* habitat, and QTL of large effect are expected to respond relatively quickly to selection. We speculate that the genetic architecture underlying leaf nitrogen may have facilitated the colonization of active desert sand dunes by *H. anomalus*.

Introduction

Studies of the genetic architecture of ecologically-important traits shed light on the evolution of those traits in natural populations. Studies of genetic architecture of complex traits are important because they can predict whether a phenotypic transition involved major leaps or occurred more smoothly (Burke et al. 2002). Also, alleles with large effects will fix more rapidly than will those of very small effect (Barton & Keightley 2002). An historical illustration of genetic architecture is the study by Beadle examining the inheritance of maize domestication traits. Using a primitive landrace of maize and a teosinte accession, Beadle observed the segregation of the maize and teosinte phenotypes at a 1:500 ratio, indicating that four or five genes of major effect were responsible for the bulk of changes from teosinte to maize (Beadle 1972). This demonstrated that the phenotypic transition from wild progenitor to primitive maize likely occurred in major leaps. This contrasts with sunflower, where domestication appears to have involved many small QTL, suggesting a smoother and more gradual transition to the domesticated form (Burke et al. 2002).

Phenotypic differences between wild sunflower species (*Helianthus*) also seem to be controlled by a large number of loci, each with a relatively small contribution to the phenotype (Kim & Rieseberg 1999, Rieseberg et al. 2003, Lexer et al. 2005). Two *Helianthus* species that have been the targets of previous genetic study, *H. annuus* and *H. petiolaris*, are of particular interest because they hybridized to form three stable, ancient, diploid hybrid species *H. anomalus*, *H. deserticola*, and *H. paradoxus* (Rieseberg et al. 1990, Rieseberg 1991), which occupy extreme habitats compared to the parents: active sand dunes, dry desert floors, and brackish salt marshes, respectively. The range of phenotypes observed in the hybrid species is also more extreme than those of the parental species (Schwarzbach et al. 2001, Rosenthal et al. 2002).

Evolution of the hybrid sunflower species appears to have occurred quickly (Ungerer et al. 1998, Buerkle & Rieseberg 2008), and two of the hybrid species may have multiple origins (*H. anomalus,* Schwarzbach & Rieseberg 2002 and *H. deserticola*, Gross et al. 2003). Additionally, phenotypes of the stable hybrid species are present in populations of early hybrids between *H. annuus* and *H. petiolaris* (Rosenthal et al. 2005b). If the pre-adaptive phenotypic differences important for colonizing the extreme habitats of the hybrid species are controlled by multiple genes of small effect, then the rate of fixation of individual QTL alleles is likely to be slow and the sizes of parental chromosomal blocks in the hybrid species should be smaller than when loci of large effect control the phenotype (Barton & Keightley 2002). It seems paradoxical that most of the QTL in *Helianthus* tend to be of small effect but the hybrid species genomes seem to have stabilized relatively quickly (Ungerer et al. 1998, Buerkle & Rieseberg 2008). It may be the case, however, that variation in some of the traits that are important to the survival of early generation hybrids in *Helianthus* are controlled by large-effect QTL. Also, we know that pollen sterility QTL, which also affect hybrid genomic composition (Rieseberg et al. 1996, Karrenberg et al. 2007), are controlled by major QTL (Lai et al. 2005). Here, we examine leaf ecophysiological traits, some of which greatly affect fitness of plants in the wild.

Leaf nitrogen is likely to be ecologically important because it roughly estimates investment in nitrogen-rich photosynthetic enzymes, with investment per unit leaf area influencing photosynthetic capacity (Field & Mooney 1986). Foliar nitrogen is positively transgressive in the hybrid sunflower species *H. anomalus* (Rosenthal et al. 2002) and has been shown to impact survival in its native habitat (Ludwig et al. 2004). Despite being an important physiological trait, leaf nitrogen has only recently received attention in QTL analyses (Ishimaru et al. 2001, Hall et al. 2005, Takai et al. 2006, Weih et al. 2006). Other leaf chemistry traits measured in this study assess important aspects of plant physiology that are related to nitrogen allocation. Leaf carbon is a rough assessment of investment in carbon-rich structural molecules such as cellulose and correlates with construction cost of leaves (Nagel 2002). Carbon isotopic ratio ($\delta^{13}C$) is often used as a proxy for water-use efficiency in C_3 plants (Farquhar et al. 1989, Ehleringer et al. 1992). Differences in leaf nitrogen isotopic composition ($\delta^{15}N$) for plants grown under similar conditions imply differences in nitrogen uptake or use (Evans 2001).

Photosynthetic nitrogen use efficiency (PNUE) is the instantaneous rate of photosynthesis per unit leaf nitrogen, and may be under selection in nutrient-poor or water-limited habitats (Field & Mooney 1986, Fredeen et al. 1991, Wright et al. 2002). Here we report the results of QTL analyses for these ecologically-important, physiologically-based leaf traits.

Materials and Methods

The plants used in this study were those of Rieseberg et al. (2003). The bulk of the phenotypic traits were summarized in the previous study, but trait data reported here were not available at the time of the previous study. Second-generation backcross hybrids between *Helianthus annuus* and *H. petiolaris* were generated as described previously in Rieseberg *et al.* (2003). Briefly, plants were grown from achenes collected from a population of *H. annuus* growing near Hanksville, UT (ANN 1295) and a population of *H. petiolaris* growing near Page, AZ (PET 1277). F_1 seed was produced by crossing a single individual of *H. annuus* with a single individual of *H. petiolaris*. F_1 individuals were backcrossed with a second individual of *H. petiolaris* (PET 1277) to produce BC_1 seeds. Because of low seedset, each of the 38 BC_1 individuals was then backcrossed to a third *H. petiolaris* individual, producing BC_2 plants used for QTL analysis.

A total of 384 plants were germinated according to Schwarzbach et al. (2001) and transferred to 25 cm pots containing a 3:1 mixture of sand and baked clay (Turface, Profile Products, Buffalo Grove, IL) in the University of Georgia Plant Biology greenhouses in Athens, GA. Pots were watered to field capacity twice daily using an automated drip irrigation system, and nutrients were added as time-release fertilizer with macro- and micronutrients (Osmocote Plus, Scotts-Sierra Horticultural Products, Marysville, OH).

A fully expanded leaf was used to calculate specific leaf area (SLA = leaf area / leaf dry mass). Leaves were harvested in the morning when fully hydrated, and area was measured using a LI-3100 area meter (LiCor Biosciences, Lincoln, NE). Leaves were weighed after drying at 60°C. Photosynthesis was measured starting 47 days after planting using a LI-6400 portable gas exchange system between 10:30 and 16:30 (LiCor Biosciences). Chamber conditions were 360 ppm CO_2, 26°C, and 1500 µmol m^{-2} s^{-1} photosynthetically active radiation. Measured leaves were collected, dried at 60°C, ground, and analyzed for nitrogen concentration (mg N / g biomass), carbon concentration (mg_C / $g_{biomass}$, respectively, NA1500, Carlo Erba Strumentazione, Milan, Italy), leaf carbon and nitrogen isotopic composition ($\delta^{13}C$ and $\delta^{15}N$, continuous flow mass spectrometer, Finnegan, Bremen, Germany).

Photosynthetic nitrogen-use efficiency (PNUE) was calculated using photosynthetic rate, leaf N, and specific leaf area after Field and Mooney (1986). Specific leaf area was also used to convert nitrogen concentration on a weight basis into leaf nitrogen on an area basis.

Plants were genotyped for a total of 76 simple sequence repeat and 20 amplified fragment length polymorphism markers (for details, see Lexer et al. 2003). Markers were chosen from genetic maps of *H. annuus* (Burke et al. 2002, Tang et al. 2002) or *H. petiolaris* (Burke et al. 2004) to give relatively complete and even coverage of the linkage groups.

Phenotypic variables were corrected for block using a one-way ANOVA implemented in JMP (SAS Institute, Cary, NC). The trait $\delta^{15}N$ was non-normal, and those data were Box-Cox transformed in JMP. Coded marker data and corrected phenotypic data were imported into Mapmanager QTX version b20 (Manly et al. 2001). Linkage maps were assembled using the Kosambi mapping function as described previously (Lexer et al. 2003). Composite interval mapping of the traits was carried out using QTL Cartographer (Basten et al. 1996). Composite interval mapping (CIM) was run at 2 cM resolution with 1000 permutations to determine Log Ratio Score (LRS) threshold value for the 0.05 level of significance.

Using Mapmanager QTX, data were analyzed for epistatic interactions for each of the traits with default p=0.00001 significance threshold, which may be liberal given the number of possible pairwise comparisons between markers. The average effect and the partial r-squared for significant interactions were estimated using the general linear model procedure in SAS (PROC GLM, SAS 8, Cary, NC). Partial r-squared was calculated as the sum of squares for the interaction term divided by the total sum of squares where variation in the trait was explained by the genotype at the two loci and their interaction.

Results

Significant additive QTL were found for all traits except $\delta^{13}C$ and $\delta^{15}N$ (Figure 6.1; Table 6.1). Especially noteworthy is the pair of QTL on linkage group 5/7, which together account for 24.6% of the variation in leaf nitrogen on an area basis.

Results for epistatic QTL were less strong (Table 6.2). Only two traits had loci with significant interactions: leaf nitrogen on a weight basis and leaf carbon. For both, two significant marker pairs were found. The largest of these interactions explained approximately 2% of the variation in leaf carbon.

Discussion

For the majority of traits assessed in this study, the genetic architecture of leaf chemical traits appears to be largely influenced by multiple loci of small effect. A relatively small number of QTL were detected, some of which were close to the detection limit (r^2 = 0.04-0.06), suggesting that there are a larger number of QTL with small effects controlling these traits, with a subset being detected in our mapping study. One notable exception is leaf nitrogen on an area basis, where two QTL account for nearly a quarter of the observed phenotypic variation. These results support the idea that leaf nitrogen is an important trait in the habitat of the stable hybrid species *H. anomalus* (Ludwig et al. 2004, Rosenthal et al. 2005a, Donovan et al. 2007).

Using phenotypic selection analysis on early generation hybrids between *H. annuus* and *H. petiolaris,* Ludwig et al. (2004) showed that some ecophysiological traits are under selection (*sensu* Lande & Arnold 1983) in the *H. anomalus* habitat. In other words, early generation hybrids with higher foliar nitrogen were significantly more successful in the field, taking into account variation in correlated traits that also affected fitness. Because of the large effect of the two loci associated with leaf nitrogen, fixation of more fit genotypes could proceed more quickly (Barton & Keightley 2002). Also, because the variability in this interspecific cross is so large, the percentage of variation explained is likely an underestimate of the effect of the QTL in natural populations of the *Helianthus* species used (Lexer et al. 2005). In natural populations of *H. anomalus*, the genomic regions where the QTL localize are fixed for the *H. annuus* genotype as one would expect if there were heavy selection on foliar nitrogen in early hybrids (Rieseberg et al. 2003).

It is also noteworthy that no significant QTL were found for $\delta^{13}C$, since the trait is known to be heritable and early marker regression studies found significant genetic correlations in tomato (Martin et al. 1989). In other studies, Handley et al. (1994) localized $\delta^{13}C$ loci on chromosome 4 of barley, Mian et al. (1996) found 4 unlinked restriction fragment length polymorphism markers associated with $\delta^{13}C$ in soybean, and Juenger et al. (2005) detected five loci in *Arabidopsis thaliana*. However, work by Teulat and colleagues (2002) demonstrated the QTL for $\delta^{13}C$ are environment specific. Moreover, a larger number of QTL were found in stressed than unstressed environments. It is possible then that no significant QTL for $\delta^{13}C$ were found because our plants were not stressed. It is also worth noting, as a general caveat, that a second-generation backcross population was used instead of an F_2 population in order to generate an appropriate number of progeny for QTL analysis. This may have hindered our ability to detect weak QTL, but those loci are difficult to detect in any analysis (Mauricio 2001).

In addition to providing some information about an economically-important crop species, QTL studies in *Helianthus* may provide information about the evolution of hybrid species. Of particular interest is whether additive effects explain the bulk of the extreme phenotypic divergence seen in the hybrid sunflower species, as is suggested by previous work (Rieseberg et al. 2003) or whether non-additive effects such as epistasis have a prominent role in trait evolution (Malmberg & Mauricio 2005). We detected four epistatic interactions, with the largest of these explaining only 2% of the phenotypic variation in leaf carbon. In every case, the effect of the interaction was in the opposite direction of the main-effect QTL. This counterbalancing of the additive effects, coupled with the lack of significant interaction terms for most of the traits surveyed, lends support to the standing hypothesis that transgressive segregation accounts for the extreme phenotypes of the hybrid species (Rieseberg et al. 2003).

Further studies will focus on examining QTL in early generation hybrids planted in the field. It is likely that additional QTL will be found when an analysis is done on field-grown plants (Paterson et al 1991, Tetulat et al. 2002).

Acknowledgements

We would like to thank A Howard, C Gormally, S Gevaert, and two anonymous reviewers for comments on an earlier draft of the manuscript. This work was supported by NSF grant IBN-0131078 to LAD and NIH grant GM59065 to LHR.

References

Barton NH, Keightley PD (2002) Understanding quantitative genetic variation. Nature Reviews Genetics, 3, 11-21.

Basten CJ, Weir BS, Zeng Z-B (1996) QTL Cartographer: A manual and tutorial for QTL mapping. Department of Statistics, North Carolina State University, Raleigh, NC.

Beadle GW (1972) The mystery of maize. Field Museum of Natural History Bulletin, 43, 2-11.

Buerkle CA, Rieseberg LH (2008) The rate of genome stabilization in homoploid hybrid species. Evolution, 62, 266-275.

Burke JM, Lai Z, Salmaso M, et al. (2004) Comparative mapping and rapid karyotypic evolution in the genus *Helianthus*. Genetics, 167, 449-457.

Burke JM, Tang S, Knapp SJ, Rieseberg LH (2002) Genetic analysis of sunflower domestication. Genetics, 161, 1257-1267.

Donovan LA, Dudley SA, Rosenthal DM, Ludwig F (2007) Phenotypic selection on leaf water use efficiency and related ecophysiological traits for natural populations of desert sunflowers. Oecologia, 152, 13-25.

Ehleringer JR, Phillips SL, Comstock JP (1992) Seasonal variation in the carbon isotopic composition of desert plants. Functional Ecology, 6, 396-404.

Evans RD (2001) Physiological mechanisms influencing plant nitrogen isotope composition. Trends in Plant Science, 6, 121-126.

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology, 40, 503-537.

Field C, Mooney HA (1986) The photosynthesis-nitrogen relationship in plants. In: On the Economy of Plant Form and Function (ed. Givnish TJ), pp. 25-55. Cambridge University Press, Cambridge.

Fredeen AL, Gamon JA, Field CB (1991) Responses of photosynthesis and carbohydrate-partitioning to limitations in nitrogen and water availability in field-grown sunflower. Plant Cell and Environment, 14, 963-970.

Gross BL, Schwarzbach AE, Riesberg LH (2003) Origin(s) of the diploid hybrid species *Helianthus deserticola* (Asteraceae). American Journal of Botany, 90, 1708-1719.

Hall NM, Griffiths H, Corlett JA, et al. (2005) Relationships between water-use traits and photosynthesis in *Brassica oleracea* resolved by quantitative genetic analysis. Plant Breeding, 124, 557-564.

Handley LL, Nevo E, Raven JA, et al. (1994) Chromosome 4 controls potential water-use efficiency ($\delta^{13}C$) in barley. Journal of Experimental Botany, 45, 1661-1663.

Ishimaru K, Kobayashi N, Ono K, Yano M, Ohsugi R (2001) Are contents of rubisco, soluble protein and nitrogen in flag leaves of rice controlled by the same genetics? Journal of Experimental Botany, 52, 1827-1833.

Juenger TE, McKay JK, Hausmann N, et al. (2005) Identification and characterization of QTL underlying whole-plant physiology in *Arabidopsis thaliana*: $\delta^{13}C$, stomatal conductance and transpiration efficiency. Plant Cell and Environment, 28, 697-708.

Karrenberg S, Lexer C, Rieseberg LH (2007) Reconstructing the history of selection during homoploid hybrid speciation. American Naturalist, 169, 725-737.

Kim SC, Rieseberg LH (1999) Genetic architecture of species differences in annual sunflowers: Implications for adaptive trait introgression. Genetics, 153, 965-977.

Lai Z, Nakazato T, Salmaso M, et al. (2005) Extensive chromosomal repatterning and the evolution of sterility barriers in hybrid sunflower species. Genetics, 171, 291-303.

Lande R, Arnold SJ (1983) The measurement of selection on correlated characters. Evolution, 37, 1210-1226.

Lexer C, Rosenthal DM, Raymond O, Donovan LA, Rieseberg LH (2005) Genetics of species differences in the wild annual sunflowers, *Helianthus annuus* and *H. petiolaris*. Genetics, 169, 2225-2239.

Lexer C, Welch M, Durphy JL, Rieseberg LH (2003) Natural selection for salt tolerance QTL in wild sunflower hybrids: Implications for the origin of *Helianthus paradoxus*, a homoploid hybrid species. Molecular Ecology, 12, 1225-1235.

Ludwig F, Rosenthal DM, Johnston JA, et al. (2004) Selection on leaf ecophysiological traits in a desert hybrid *Helianthus* species and early generation hybrids. Evolution, 58, 2682-2692.

Malmberg RL, Mauricio R (2005) QTL-based evidence for the role of epistasis in evolution. Genetical Research, 86, 89-95.

Manly KF, Cudmore RH Jr., Meer JM (2001) Mapmanager QTX, cross-platform software for genetic mapping. Mammalian Genome, 12, 930-932.

Martin B, Nienhuis J, King G, Schaefer A (1989) Restriction fragment length polymorphisms associated with water-use efficiency in tomato. Science, 243, 1725-1728.

Mauricio R (2001) Mapping quantitative trait loci in plants: uses and caveats for evolutionary biology. Nature Reviews Genetics, 2, 370-381.

Mian MAR, Bailey MA, Ashley DA, et al. (1996) Molecular markers associated with water use efficiency and leaf ash in soybean. Crop Science, 36, 1252-1257.

Nagel JM, Griffin KL, Schuster WS, et al. (2002) Energy investment in leaves of red maple and co-occurring oaks within a forested watershed. Tree Physiology, 22, 859-867.

Paterson AH, Damon S, Hewitt JD, et al. (1991) Mendelian factors underlying quantitative traits in tomato: Comparison across species, generations, and environments. Genetics, 127, 181-197.

Rieseberg LH (1991) Homoploid reticulate evolution in *Helianthus* (Asteraceae): Evidence from ribosomal genes. American Journal of Botany, 78, 1218-1237.

Rieseberg LH, Carter R, Zona S (1990) Molecular tests of the hypothesized hybrid origin of two diploid *Helianthus* species (Asteraceae). Evolution, 44, 1498-1511.

Rieseberg LH, Raymond O, Rosenthal DM, et al. (2003) Major ecological transitions in wild sunflower facilitated by hybridization. Science, 301, 1211-1216.

Rieseberg LH, Sinervo B, Linder CR, Ungerer M, Arias DM (1996) Role of gene interactions in hybrid speciation: Evidence from ancient and experimental hybrids. Science, 272, 741-745.

Rosenthal DM, Ludwig F, Donovan LA (2005a) Plant responses to an edaphic gradient across an active sand dune/desert boundary in the Great Basin Desert. International Journal of Plant Sciences, 166, 247-255.

Rosenthal DM, Rieseberg LH, Donovan LA (2005b) Recreating ancient hybrid species phenotypes from early generation synthetic hybrids. American Naturalist, 166, 26-41.

Rosenthal DM, Schwarzbach AE, Donovan LA, Raymond O, Riseberg LH (2002) Phenotypic differentiation between three ancient hybrid taxa and their parental species. International Journal of Plant Sciences, 163, 387-398.

Schwarzbach AE, Donovan LA, Rieseberg LH (2001) Transgressive character expression in a hybrid sunflower species. American Journal of Botany, 88, 270-277.

Schwarzbach AE, Rieseberg LH (2002) Likely multiple origins of a diploid hybrid sunflower species. Molecular Ecology, 11, 1703-1717.

Takai T, Fukuta Y, Sugimoto A, Shiraiwa T, Horie T (2006) Mapping of QTLs controlling carbon isotope discrimination in the photosynthetic system using recombinant inbred lines derived from a cross between two different rice (*Oryza sativa* L.) cultivars. Plant Production Science, 9, 271-280.

Tang S, Slabaugh MB, Shintani DK, Knapp SJ (2002) Simple sequence repeat map of the sunflower genome. Theoretical and Applied Genetics, 105, 1124-1136.

Teulat B, Merah O, Sirault X, et al. (2002) QTLs for grain carbon isotope discrimination in field-grown barley. Theoretical and Applied Genetics, 106, 118-126.

Ungerer MC, Baird S, Pan J, Rieseberg LH (1998) Rapid hybrid speciation in wild sunflowers. Proceedings of the National Academy of Sciences, USA, 95, 11757-11762.

Weih M, Ronnberg-Wastljung AC, Glynn C (2006) Genetic basis of phenotypic correlations among growth traits in hybrid willow (*Salix dasyclados* x *S. viminalis*) grown under two water regimes. New Phytologist, 170, 467-477.

Wright IJ, Westoby M, Reich PB (2002) Convergence towards higher leaf mass per area in dry and nutrient-poor habitats has different consequences for leaf life span. Journal of Ecology, 90, 534-543.

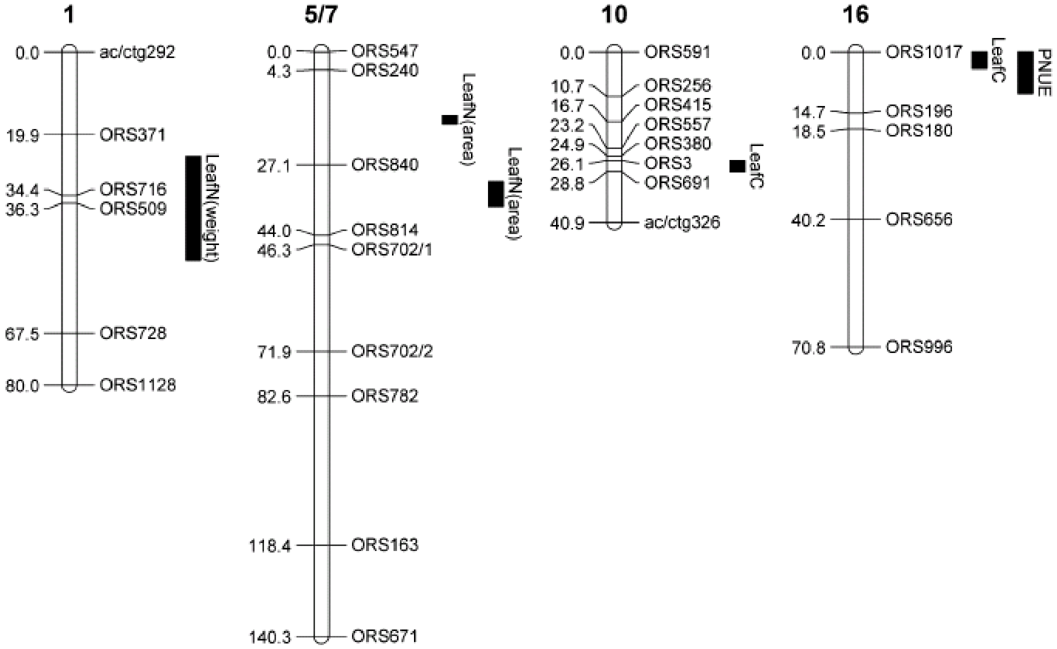

Figure 6.1. QTL map. Composite interval mapping results for each of the seven leaf chemistry traits examined, five of which yielded significant QTL. Significance threshold for each trait was determined through resampling the data, with 1000 repetitions, and chromosome walk speed was set at 2 cM. Shown are the 4 out of 17 linkage groups of the *Helianthus* genetic map where significant QTL localized. Genetic distances (in cM) and marker names are provided.

Table 6.1. Composite interval mapping results. Markers associated with significant QTL in the CIM analysis were regressed against untransformed trait values, which were corrected for variation due to experimental block. R-squared values from this analysis are used to estimate the percentage of phenotypic variation explained by the QTL determined to have significant in the composite interval mapping (CIM) analysis. The additive effect is the average effect of the maternal (*H. petiolaris*) genotype on the trait, and the mean and standard deviation of the mapping population are given.

Trait [units]	Mean (SD)	Linkage Group	Marker	Additive effect	LOD	R^2
Leaf C [g g^{-1}]	0.4078 (0.0150)	10	ORS3	0.0129	3.6257	0.044
		16	ORS1017	0.0091	3.0715	0.038
Leaf N [mg g^{-1}]	0.0583 (0.0063)	1	ORS716	0.0038	4.4215	0.056
Leaf N [mmol m^{-2}]	136.87 (261.16)	5/7	ORS240	-27.74	2.7721	0.128
		5/7	ORS840	-27.88	3.1554	0.111
PNUE [mmol CO^2 mol N^{-1} s^{-1}]	289.3 (142.44)	16	ORS1017	-79.13	4.2487	0.116

Table 6.2. Epistasis results. Epistatic associations were determined in Mapmanager QTX at the recommended $p=1.0e^{-5}$ level of significance. Provided are the chromosome number and marker for both loci, the log ratio scores (LRS) and mean effects on the phenotype for both main effects and the interaction term. R^2 values from this analysis are used to estimate the percentage of phenotypic variation explained by QTL detected in the CIM analysis.

Trait	Linkage Group 1	Marker 1	Linkage Group 2	Marker 2	Locus 1 LRS	Locus 2 LRS	Interaction LRS	Main Effect 1	Main Effect 2	Interaction Effect	Partial R^2
Leaf C	10	ORS557	16	ORS1017	7.1	11.7	6.8	-0.0070	-0.0060	0.0167	0.0122
	10	ORS3	16	ORS1017	10.6	11.7	10.2	-0.0094	-0.0071	0.0209	0.0201
Leaf N (weight)	1	ORS716	9	ORS887	18	0.6	7.8	-0.0019	-0.0049	0.0030	0.0157
	1	ORS509	9	ORS887	6.9	0.6	18.9	-0.0012	-0.0037	0.0055	0.0131

CHAPTER 7

CONCLUSIONS

In the first chapter, we posed a series of questions to be addressed by studies presented in the dissertation. Here, we summarize our results by revisiting those questions.

Do the hybrid species show a greater tolerance of nutrient stress?

We compared *H. anomalus* and *H. deserticola* to their parental species *H. annuus* and *H. petiolaris* under three nutrient levels. The hybrid species showed a slightly higher tolerance of nutrient stress, measured as a resistance to change in height and stem growth in response to nutrient stress. Both hybrid species appear to maintain investment in photosynthetic enzymes despite nutrient limitation. *Helianthus anomalus* appears to have evolved a classic stress-tolerance phenotype, with longer leaf lifespan and higher leaf toughness.

How does nutrient availability affect the growth and allocation of H. anomalus and its parental species?

Helianthus anomalus has a lower maximum relative growth rate than its parental species. Growth rate was partitioned into its carbon- and nitrogen-use components to determine which parameters were responsible for the lower relative growth rate in *H. anomalus*. With respect to carbon components, *Helianthus anomalus* has a lower specific leaf area. Leaf area is the functional unit of photosynthesis, so per unit of mass invested in leaves, *H. anomalus* is less productive than its parental species. This inverse relationship between SLA and RGR appears to hold across a wide range of plant species (Lambers & Poorter 2004). Looking at the nitrogen components, an increased mean residence time of nitrogen contributes to the reduced relative growth rate of *H. anomalus* relative to its parents.

What effect does nutrient availability have on the gene expression in *H. anomalus*, *H. petiolaris*, and *H. annuus*?

We found five unique genes that show a different response to nitrogen limitation in *H. anomalus* relative to the parental species. One of these genes has a homologue in *Arabidopsis thaliana* that is known to affect leaf lifespan. Genes showing a different response to nutrient stress in *H. anomalus* relative to *H. annuus* and *H. petiolaris* tend to be stress response genes and genes involved in development. It appears that several genes in the generalized stress response of *H. anomalus* are differentially regulated in response to nutrient stress, which was not unexpected. It is also reassuring that we have a candidate gene for the adaptive timing of leaf senescence in *H. anomalus*.

Do traits important in the nutrient stress response of *H. anomalus* show evidence of selection?

We found evidence for selection on a range of morphological and ecophysiological traits. Among the traits showing the strongest evidence of selection was foliar boron concentration, which has long been posited as an important trait in dryland plants (Rosenthal et al. 2002, Jefferies et al. 1999). We also found an interesting pattern for leaf lifespan, where we failed to find heritable variation or variation among populations. This may have been caused by strong selection for increased leaf lifespan in *H. anomalus*, which could have removed much of the genetic variation from the species.

What is the genetic architecture of traits related to nitrogen use?

We found two relatively large-effect quantitative trait loci for foliar nitrogen using an artificial hybrid population between *H. annuus* and *H. petiolaris*. Foliar nitrogen has been shown to be under direct selection in the *H. anomalus* habitat, with plants having a higher concentration of nitrogen in the leaves being larger at the end of the growing season (Ludwig et al. 2004). Because the size of quantitative trait loci is proportional to the rate of fixation of the allele (Orr & Coyne 1992), the fact that there are two large-effect loci underlying the trait may have helped the fixation of the advantageous phenotype.

Future Directions

It was encouraging that four of the five studies presented here point toward leaf lifespan as a potentially important trait in the *H. anomalus* habitat. Increased leaf lifespan is part of a low nutrient stress tolerant syndrome (Aerts & Chapin 2000). Increased leaf lifespan is thought to help the nutrient status of the plant by increasing the efficiency of nutrient use. Longer leaf lifespan requires additional structural investment in leaves (Riech et al. 1997), which comes at a cost of reduced maximum growth rate (Lambers & Poorter 2004). Should further experiments show the importance of longer leaf lifespan in the *H. anomalus* habitat, we may begin to think of the timing of leaf senescence as a critical trait in the ecological speciation of *H. anomalus* (Schluter 2009). With some additional validation, catalase 1 may be thought of as an ecological speciation gene.

Candidate genes for increased leaf lifespan may not be the best genes for use in crop improvement because of the need for increased structural investment and the concomitant reduction in plant size and, presumably, yield. Future studies should determine whether the candidate genes targeted in the microarray study confer greater stress tolerance and if this increased tolerance may be decoupled from reduced growth rates.

Nutrients can and do limit growth in various habitats. In natural habitats, approximately ninety percent of nitrogen inputs come from bacteria or cyanobacteria that are either free-living in the soil or part of root nodules within compatible plants such as legumes (Schlesinger 1997). In agricultural settings within the developed world, nitrogen limitation is overcome by the use of chemical fertilizers. Fixation of nitrogen for use in soil amendment is typically by the Haber-Bosch process that requires large amounts of energy (Taiz & Zeiger 2006). As energy continues to become more expensive, chemical fertilizers may need to be used less frequently in favor of increased tolerance of low soil fertility. This research program may provide some insight into plant adaptations to low nutrient conditions, which may be used to reintroduce stress tolerance to crop plants.

References

Aerts R, Chapin III FS (2000) The mineral nutrition of wild plants revisited: A re-evaluation of processes and patterns. Advances in Ecological Research, 30, 1-67.

Jefferies SP, Barr AR, Karakousis A, et al. (1999) Mapping of chromosome regions conferring boron toxitity tolerance in barley (*Hordeum vulgare* L.). Theoretical and Applied Genetics, 98, 1293-1303.

Lambers H, Poorter H (2004) Inherent variation in growth rate between higher plants: A search for physiological causes and consequences. Advances in Ecological Research, 34, 283-362.

Ludwig F, Rosenthal DM, Johnston JA, et al. (2004) Selection on leaf ecophysiological traits in a desert hybrid *Helianthus* species and early-generation hybrids. Evolution, 58, 2682-2692.

Orr A, Coyne JA (1992) The genetics of adaptation revisited. American Naturalist, 140, 725-742.

Reich PB, Walters MB, Ellsworth DS (1997) From tropics to tundra: Global convergence in plant functioning. Proceedings of the National Academy of Sciences, USA, 94, 13730-13734.

Rosenthal DM, Schwarzbach AE, Donovan LA, Raymond O, Rieseberg LH (2002) Phenotypic differentiation between three ancient hybrid taxa and their parental species. International Journal of Plant Sciences, 163, 387-398.

Schlesinger WH (1997) Biogeochemistry: An Analysis of Global Change, 2nd edn. Academic Press, San Diego, CA.

Schluter D (2009) Evidence for ecological speciation and its alternative. Science, 323, 737-741.

Taiz L, Zeiger E (2006) Plant Physiology, 4th edn. Sinauer, Sunderland, MA.