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Resource allocation for acorn production: A comparison across species pairs of oaks with contrasting acorn production patterns and water use strategies

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RESOURCE ALLOCATION FOR ACORN PRODUCTION: A COMPARISON
ACROSS SPECIES PAIRS OF OAKS WITH CONTRASTING ACORN
PRODUCTION PATTERNS AND WATER USE STRATEGIES

by

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Kyle A. Funk, Ph.D.

University of Nebraska, 2017

Advisor: Johannes M. H. Knops

Mast seeding, or masting, is a phenomenon where inter-annual seed production by individuals is synchronized across a population of plants. Masting is hypothesized to confer a selective advantage to plants by increasing rates of pollination or by decreasing rates of seed predation. Masting can also play a crucial role in ecosystem functioning as fluctuations in annual seed crops correspond with fluctuations in seed predator populations, which in turn have consequences that ripple throughout food webs. The mechanism(s) that causes masting is unresolved, but the high variability in seed production of masting plants is hypothesized to be caused, in part, by resource limitation. One hypothesized mechanism for masting that has gained support in recent years is known as the resource budget model (RBM). The RBM hypothesizes that plants store up resources across years until a threshold is reached, after which they flower and set seed, which depletes resources. It is still unknown how common the RBM is in masting species, and it is unknown whether the RBM describes a pattern of resource allocation

that is distinctive of masting species, or if non-masting plants exhibit similar patterns. In this dissertation, we seek to resolve some of this uncertainty by comparing patterns of resource allocation and seed production among four species of California oak trees and shrubs. In Chapter 1, we test predictions of the RBM in two shrub species, one masting and one non-masting, in the Klamath Mountains of northern California. In chapter 2, we explore the carbon demands for acorn development of the masting species used in Chapter 1, in order to learn more about whether carbohydrates are limiting for seed production in this species. In Chapter 3, we use two masting tree species in central coastal California with contrasting water use strategies to test for resource limitation to flowering and seed production, and to compare which nutrients are the most important for each species. Overall, we found evidence for resource-limited reproduction in all three masting species. Furthermore, differences in patterns of seed production and in species traits matter for how resources are used for seed production.

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Chapter 1: Introduction

The production of seeds is crucial to life on many levels. For plants that reproduce sexually, seeds are necessary for future generations. For seed-eating animals, they provide energy necessary for survival. The struggle to produce the most offspring has led to the evolution of a diverse array of reproductive strategies, but environmental conditions are often limiting and producing seeds can be costly (Obeso 2002). Plants with different strategies face different challenges. For an annual plant, everything must be sacrificed to reproduce before the growing season ends, but perennial plants can afford to forego reproduction in some years when it is necessary to ensure survival. Some plants may even gain a reproductive advantage by producing large crops of seeds in some years, while abandoning reproduction entirely in other years (Janzen 1976, Silvertown 1980, Kelly and Sork 2002). When this phenomenon of boom and bust cycles of seed production is synchronized across a population, it is known as ‘mast seeding’ or simply ‘masting.’

Construction costs of seeds in mast years can be substantial. For example, in a mast year for *Fagus sylvatica* fruits used 40 to 50 times more carbohydrates and proteins than leafy branches (Kozlowski and Keller 1966). In the past it was thought that masting plants manage this burden by switching resource allocation from growth to reproduction (Harper 1977, Koenig and Knops 1998), but some research has shown that an apparent trade-off between growth and reproduction can be mediated by the environment rather than the intrinsic costs of seed production (Knops et al. 2007). Storage (acquired resources that can be remobilized) has also been hypothesized to trade off with

reproduction in masting plants. This hypothesis has been formalized by a series of theoretical models where reproduction only occurs after storage has accumulated beyond a certain threshold (Isagi et al. 1997, Satake and Iwasa 2000). These models make very clear predictions that stored resources should accumulate between years with large reproductive efforts, and then be depleted following the large reproductive effort. A fair amount of research has tested these predictions in both cultivated and wild populations but evidence has been mixed.

Part of the controversy has derived from the fact that the importance of various currencies of storage depends on the system in question. For example, stored carbohydrates are the important currency in flux in the models (Isagi et al. 1997), but nitrogen could be more limiting in some temperate systems (Miyazaki et al. 2014), and phosphorous might be more important in some tropical systems (Ichie and Nakagawa 2013). In wild populations of masting trees, there has been more evidence against the importance of stored carbohydrates for seed production (Ichie et al. 2005, Hoch et al. 2013, Ichie et al. 2013) than in support (Miyazaki et al. 2002). Evidence for the importance of nitrogen and/or phosphorous has been much more positive (Han et al. 2008, Han et al. 2011, Sala et al. 2012, Ichie and Nakagawa 2013, Han et al. 2014, Miyazaki et al. 2014), even if the number of species investigated remains few. Plant traits seem to be as important as biome in determining which resources are most limiting to reproduction. Stored carbohydrates were depleted by fruiting in an herbaceous legume (Crone et al. 2009) and a small tropical shrub (Marquis et al. 1997). Likewise, stored carbohydrates were depleted by production of citrus (Goldschmidt and Golomb 1982), prunes (Weinbaum et al. 1994), and persimmons (Choi and Kang 2007), but not olives

(Bustan et al. 2011). The hypothesis that storage is important for mast seed production is corroborated in many cases, but its generality is still limited.

Research on more species of wild plants is clearly warranted, but there are two more ways in which the understanding of resource allocation by masting plants needs to be improved. For one, individual variation in resource allocation within populations of masting plants is under-studied. Within populations of masting plants, even highly synchronous populations with strong bimodality in inter-annual patterns of seed production, there can be a lot of individual variation in seed production (Koenig et al. 2003). We have little knowledge of whether the same factors that drive individual-level variation in seed production within a year also drive population-level variation in seed production across years. Another knowledge gap exists in the relationship between masting and non-masting species. If masting is driven by a special resource allocation strategy, then species with more consistent patterns of seed production should have markedly different resource allocation patterns. In other words, is there an intrinsic quality to the way species allocate resources that leads to highly variable patterns of seed production, or would other similar species be masting if only they had slightly different reproductive traits and could get in synch? The best way to test this would be with common gardens, reciprocal transplants, and fertilization experiments, but long generation times and large plants can be prohibitive to this type of approach. An alternative approach is to compare sympatric, related species with similar growth forms but different patterns of seed production. My research aims to increase knowledge in these areas using two species of oak trees and two species of oak shrubs in California.

California oak trees are a classic system in which to do mast research (Kelly 1994, Koenig et al. 1994). Moreover, the Fagaceae family, and especially the *Quercus* (oak) genus, can be very useful for studying mast. *Quercus* species are widespread in terms of geography and habitat types, they grow as both trees and shrubs, and they exhibit a wide range of variation in inter-annual patterns of seed production. Additionally, patterns of growth and reproduction in some populations have been well documented for a long time. This system is well suited to address the research questions I am trying to ask.

Oak woodlands support impressive numbers of species and acorn production patterns have cascading effects on wildlife populations (Jones et al. 1998, Koenig and Knops 2005, Clotfelter et al. 2007). Indeed, mast systems in general can have large effects on ecosystems (Ostfeld and Keesing 2000). The effects of mast may change, though, as patterns of mast have changed over the past 30 to 60 years, with mast events becoming more frequent (Övergaard et al. 2007) or mean seed production increasing (Richardson et al. 2005). Climate is hypothesized to be responsible for the altered patterns via increasing temperatures or atmospheric carbon dioxide concentration. How these changes will affect wildlife populations or plant recruitment is yet to be seen, but seasonal variation in temperature, carbon dioxide concentration, and precipitation patterns all have an impact on plant resources. Reaching a greater understanding of how mast plants allocate resources for reproduction can help us predict how robust mast systems will be to future changes.

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Chapter 2: Testing the resource budget model for masting using two sympatric oak shrubs with contrasting annual seed production

Abstract

Mast seeding, or masting, is a phenomenon where highly variable inter-annual seed production is synchronized across a population of plants. The highly variable seed production patterns of masting plants are hypothesized to be driven, in part, by accumulation and depletion of stored resources. This hypothesis has been formalized into a resource budget model which proposes that plants store up resources until a threshold is reached. At resource levels above the threshold, plants flower, set seed, and the reproductive effort of producing seeds depletes storage below the threshold which starts the cycle anew. The resource budget model has received support in several masting species, but it is still unknown how widespread this mechanism is among masting taxa. Furthermore, the resource budget model has rarely been tested in both masting and non-masting species, and therefore it is unknown whether the hypothesized pattern of resource allocation that produces masting is qualitatively different from how non-masting plants allocate resources for reproduction. The resource budget model makes three predictions: (1) Storage will be high at the beginning of years with high acorn production, and low at the beginning of years with low acorn production; (2) storage during floral initiation will positively relate to flowers in the following spring; (3) stored resources will be depleted more in plants that produce more seeds. We tested these predictions of the resource budget model in two sympatric, related oak shrubs with contrasting patterns of

inter-annual seed production: one masting species, *Quercus vaccinifolia* and one non-masting species, *Notholithocarpus densiflorus*. We measured flower production, acorn production, and resources over three years, including two years of high seed production for the masting species. The resources we measured were trunk non-structural carbohydrates (NSC), branch NSC, branch nitrogen (N), and leaf N. We sample twice each year: before acorns start developing and after they reach full size. We found no support for any of the predictions in the non-masting species. In the masting species, branch NSC followed the predicted annual pattern; branch NSC, branch N, and leaf N during flower initiation had positive effects on flowering; and trunk NSC and branch NSC declined more in plants that produced more acorns. Our results offer support for the resource budget model in the masting species, and indicate that these two species with contrasting inter-annual patterns of seed production have qualitatively different patterns of resource allocation for reproduction.

Introduction

Mast seed production, or masting, is a population-level phenomenon in which seed production by individuals is highly variable from year to year, and this variability is synchronized across the population. Masting is an important phenomenon from the perspective of plants as well as the animals that consume their seeds. For masting plants, the strategy is hypothesized to confer increased fitness either via increased rates of flower fertilization and/or decreased rates of seed predation in mast years (Kelly and Sork 2002). For seed predators, population numbers rise and fall depending on the size of the seed

crop (Elkinton et al. 1996, Ostfeld and Keesing 2000, Koenig and Knops 2005). The pattern of intermittent and synchronous seed production has intrigued naturalists for quite a long time, but mechanisms that produce it remain a matter of debate.

One hypothesized mechanism for masting that has gained considerable traction over the last two decades is known as the resource budget model (Isagi et al. 1997, Satake and Iwasa 2000, Crone and Rapp 2014, Abe et al. 2016, Pesendorfer et al. 2016). This model assumes that multiple years are required to accumulate enough resources for a mast seed production year, and proposes that masting plants accumulate stored resources during years with low seed crops. Once a threshold level of storage is reached, then plants flower. If no other trees flower, flowers are aborted and since flower production is relatively cheap, resources are not depleted and an individual will flower again in the next year. If a sufficient number of individuals flower within a population, trees produce seeds, which depletes storage and causes the cycle to start over again (Isagi et al. 1997). When plants are obligate outcrossers and thus depend on their flowering neighbors for pollen, then the pattern of individual accumulation and depletion of storage eventually synchronizes throughout the population (Satake and Iwasa 2000). In short, resource allocation dynamics are hypothesized to produce inter-annual variability in seed production, and pollen limitation is hypothesized to produce spatial synchrony. Elements of this model have been supported in species as disparate as *Fagus crenata* (Miyazaki et al. 2014), *Astragalus scaphoides* (Crone et al. 2009), and *Chionochloa pallens* (Rees et al. 2002). Here we focus on the resource allocation aspect of the resource budget hypothesis.

Several questions regarding this resource budget model mechanism remain to be answered. First, the specific currency or currencies of stored resources that limit seed production by masting species do not appear to be consistent across species. The evidence concerning carbon storage as a limiting resource for masting has been mixed, with more evidence against (Hoch 2005, Ichie et al. 2005, 2013, Hoch et al. 2013, Abe et al. 2016) than for (Miyazaki et al. 2002, Crone et al. 2009, Rapp and Crone 2015). While fewer studies have examined nutrients, the accumulation and depletion of nitrogen (Han et al. 2008, 2011, 2014, Sala et al. 2012, Miyazaki et al. 2014) and phosphorous (Sala et al. 2012, Ichie and Nakagawa 2013) in relation to masting has been demonstrated in a few species of wild plants. In sum, evidence from empirical research that tests the resource budget model indicates that stored resources are likely to be important for masting, but the specific currency of storage apparently varies by species (Crone and Rapp 2014).

A second issue is the implicit assumption that inter-annual fluctuation of stored resources, a key part of the resource budget model, is not related to reproduction in species with more regular patterns of seed production (i.e. a ‘non-masting’ species). For simplicity, we call this “masting” versus “non masting” species, but note that masting and non-masting represent ends of a continuum rather than a dichotomy (Kelly 1994, Herrera et al. 1998). Consequently, mechanisms for masting are likely to represent a continuum as well, with increasing levels of expression being associated with increasing variability in annual seed production. Evaluating how patterns of resource use, storage and accumulation differ between masting and non-masting species, will add rigor to tests of the resource budget model. If the resource budget model is a mechanism that produces

masting patterns, then it follows that the mechanism should not be present in non-masting species. As yet, few studies have taken a comparative approach to validating hypotheses for proximate causes of masting (Ichie et al. 2013, Kelly et al. 2013, Funk et al. 2016).

We hypothesize that testing the resource budget model in masting species vs. non-masting species can yield three possible outcomes. First, the inter-annual fluctuations of resources predicted by the resource budget model may be occurring in many species, whether they mast or not, but lead to different patterns of seed production. For example, perhaps sympatric populations of masting and non-masting species show the same pattern of fluctuation in stored resources across years, but different patterns of annual seed production. This would suggest that stored resource dynamics are not driving annual patterns of seed production, thus countering the resource budget model. Second, stored resources may be important for reproduction in many species, but especially important for masting species. For example, many species may experience stored resource depletion with seed production, but the stored resources of masting species may be depleted to a greater degree than those of non-masting species, thus leading to more pronounced inter-annual fluctuations in stored resources (Satake and Iwasa 2000). This would support the resource budget model, but would not suggest that it is an intrinsic quality of masting species. Instead, it would suggest that the resource budget model only produces masting patterns in concert with key species traits that may affect resource levels, such as large fruit or slow growth. The third possible outcome is that the resource dynamics predicted by the resource budget model occur in masting species, but not in non-masting species. For example, stored resources may correlate with flowering and seed production in

masting species, but not in non-masting species. This would support the resource budget model and suggest that it may be an intrinsic quality of the species.

Using a comparative approach to test hypothesized mechanisms for masting can be especially powerful when comparing similar or related species that occur in the same environment (Ichie et al. 2013, Funk et al. 2016). This helps to hold constant the abiotic conditions experienced by the plants as well as some functional traits that affect resource acquisition and allocation. It is interesting that similar and even related species occur in the same forests and yet have markedly different patterns of annual seed production. It inspires the question, why does one species mast and the other does not?

Here we used syntopic populations of two similar, related oak shrubs (Family Fagaceae) with contrasting patterns of annual seed production to test the resource budget model for masting. Oak shrubs are ideal study species for two reasons. First, they are related to other well-studied masting species in the *Quercus* and *Fagus* genera, which helps to put our results in context of an established body of knowledge. Second, the small stature of shrubs allows us to count all the flowers and acorns on individuals instead of just subsets. Our study species, *Quercus vacciniifolia* Kellogg (huckleberry oak) and *Notholithocarpus densiflorus* var. *echinoides* (R. Br. Ter) Manos, C.H. Cannon & S. Oh (shrub tanoak), are sympatric evergreen shrub species that also grow in syntopy. *Quercus* exhibited highly variable and synchronous seed production during the three-year study period and will be referred to as a ‘masting’ species in this study. *Notholithocarpus* had less variable and synchronous seed production during the study. Other *Notholithocarpus* populations with longer term data have also shown less variable annual seed production compared to other sympatric oak species (W. D. Koenig and J. M. H. Knops, unpublished

data), so we refer to it as a ‘non-masting’ species in this study. The resource storage pools that we sampled were trunk non-structural carbohydrates (NSC), branch NSC, branch nitrogen (N), and leaf N.

Many field studies of the resource budget model for masting tend to aggregate data at the population-level in order to compare treatment groups in an experiment or to compare resource concentration at different phenological stages (Crone et al. 2009, Sala et al. 2012, Ichie and Nakagawa 2013). There is, however, often considerable individual variation within populations of masting plants, both within and across years, that may be used to apply additional tests of hypotheses for masting (Koenig et al. 2003, Minor and Kobe 2017). We take advantage of within-year individual variation to apply a novel approach to testing key predictions of the resource budget model for masting.

The resource budget model makes three clear predictions about the relationships among stored resources and flowers or seeds. First, stored resources are high at the beginning of years where there is high seed production, and low at the beginning of years with low seed production (Table 1, Prediction 1). This is the resource accumulation part of the model. Second, concentration of stored resources during floral initiation in buds are positively correlated with the number of flowers subsequently produced (Table 1, Prediction 2). This is the threshold part of the model. Finally, levels of seed production are negatively correlated with changes in resource storage during the growing season, with individuals with higher seed production experiencing a greater loss in storage (Table 1, Prediction 3). This is the depletion part of the model.

Methods

Study Species and Field Site

Huckleberry oak, *Quercus vacciniifolia* Kellogg (Fagaceae), is a shrub that occurs throughout much of the high Sierra Nevada Mountains, Cascade Mountains, Inner North Coast Mountains, and Klamath Mountains in northern California and southern Oregon, USA (Jepson Flora Project). Shrub tanoak, *Notholithocarpus densiflorus* var. *echinoides* (R. Br. Ter) Manos, C.H. Cannon & S. Oh (Fagaceae), is a shrubby variety which occurs in the Klamath Mountains, Cascade Mountains, and Sierra Nevada Mountains in northern California and southern Oregon, USA. Both shrubs are monoecious, evergreen, and produce biennial fruit (i.e. flowers come out and are pollinated in June or July in one year, and develop into acorns in August of the following year). Female flowers of both species are quite small when they emerge (less than 3 mm wide for *Q. vacciniifolia* and less than 4 mm wide for *N. densiflorus*; K. Funk, personal observation). Female flowers of both species grow a little bit after pollination, but the vast majority of growth occurs in the following year when they mature in acorns. Acorns of *N. densiflorus* are larger on average than acorns of *Q. vacciniifolia* (1.4 g dry mass vs. 0.4 g dry mass, respectively; K. Funk, unpublished data). Male and female inflorescences of *Q. vacciniifolia* are typical of the anemophilous *Quercus* genus. *N. densiflorus* inflorescences are either androgynous, with pistillate flowers growing at the proximal base of staminate catkins, or strictly male. There is evidence for insect pollination in other *N. densiflorus* populations based on observations and insect exclusion experiments (Wright and Dodd 2013). In our

study population we observed putative pollination by beetles, but we also observed abundant *N. densiflorus* pollen in pollen traps which leads us to suspect that *N. densiflorus* is ambophilous (K. Funk, unpublished data). In 2011, we tagged 30 individuals of each species in Shasta-Trinity National Forest in the Klamath Mountains in northern California, USA (41.24°N, 122.69°W). Both species form multi-stemmed patches and the experimental unit we used was one stem (or trunk). In order to maintain independence of data, we only used trunks that were spaced apart by greater than 5 m and did not appear to be part of the same genet. Trunk diameter at ground height (DGH) was measured in 2013 with a diameter tape measure. DGH of huckleberry oaks ranged from 2.60 cm to 8.05 cm, with a mean of 4.21 cm and a standard deviation of 1.14 cm. DGH of tanoaks ranged from 2.56 cm to 5.71 cm, with a mean of 3.82 cm and a standard deviation of 0.67 cm.

The study population was distributed along a forest service road on south and southeast facing slopes, north and upslope of Tangle Blue Creek, between 1200 m and 1350 m elevation. The dominant overstory vegetation is composed of ponderosa pine (*Pinus ponderosa*), sugar pine (*P. lambertiana*), douglas fir (*Pseudotsuga menziesii*), white fir (*Abies concolor*), and incense cedar (*Calocedrus decurrens*), with the understory dominated by huckleberry oak and tanoak, frequently growing in adjacent patches. During 2011 to 2013, the mean minimum temperature was 3.9 °C, the mean maximum temperature was 14.3 °C, and the mean annual precipitation was 960 mm (PRISM Climate Group). Most precipitation occurred between the months of October and May. Total precipitation in 2011 was 1035 mm, in 2012 it was 1464 mm, and in 2013 it was 381 mm.

Sampling Protocol

In 2011, 2012, and 2013, we counted all female flowers and acorns on all 30 plants of each species. We quantified vegetative growth as well, but do not report on it here. The ranges and means \pm standard deviations of flowers and acorns for each species over the study period were as follows. *Q. vaccinifolia* female flowers: (0 – 616; 51.8 ± 108.7). *Q. vaccinifolia* acorns: (0 – 150; 7.7 ± 22.0). *N. densiflorus* female flowers: (0 – 106; 15.7 ± 20.4). *N. densiflorus* acorns: (0 – 56; 5.7 ± 9.1).

In order to later measure nonstructural carbohydrates (NSC) and nitrogen (N), we harvested tissues at two key phenological stages: in mid-June after the plants had grown new shoots and flowers, right before acorns start developing, and in mid-August after acorns are full sized. We collected trunk cores at ground height and branches from sunny parts of the crown from 10 plants of each species in 2011 and 2012, and all 30 plants of each species in 2013. Some samples were lost between harvest and analysis, and final sample sizes for each assay are listed in Table 2. We harvested four branches from each plant including three years of growth. When plants had acorns in August, we harvested two branches with acorns and two without. Tissues were always harvested in the afternoon between 1300 and 1700 hours. On the same day of harvesting we microwaved the tissues for 90 seconds at 600 watts in order to denature enzymes that could alter the carbohydrate chemistry of the wood (Popp et al. 1996). Later, we dried the plants at 65 °C for three days.

Non-Structural Carbohydrates Assays

All the plant tissues for chemical assays were ground to a fine powder using a two-step process. First, we ground them in a Wiley mill (Thomas Scientific, Swedesboro, New Jersey, USA) until they passed through a 0.85 mm mesh. Next, we crushed them in a ball mill (Cianflone 2601, Pittsburgh, Pennsylvania, USA).

We measured NSC in branches and the outer 1 cm of xylem in the trunk cores. First, we extracted 20 mg (\pm 1 mg) of tissue per sample in sodium acetate. Then we removed an aliquot to analyze for soluble sugars (sucrose, fructose, and glucose). We used the remaining fraction to analyze for starch by first digesting with α -amylase from *Bacillus licheniformis* (Bio-Cat, inc.), and then with amyloglucosidase from *Aspergillus niger* (Sigma A9913). Prior to the assay, we dialyzed the amyloglucosidase in sodium acetate to remove any glucose from the enzyme solution. After this step, both starch and soluble sugar fractions received the same treatment. Invertase (Sigma I4504) was used to digest sucrose, before putting aliquots of each fraction of each sample into wells of 96 well microplates to be measured colorimetrically. In the microplates we digested glucose and fructose with a glucose assay reagent (Sigma G3293) and phosphogluco-isomerase (Sigma P5381), respectively. Following this final digest, we measured the absorbance of each well at 340 nm using a spectrophotometer (Cary 50, Varian Inc., Palo Alto, California, USA). The absorbance reading was directly proportional to the concentration of glucose in the well. We calculated the concentrations of soluble sugars and starch for each sample using a glucose standard curve and dilution factors. Total NSC is the sum of the concentrations of the soluble sugars and starch fractions. We included standards of

starch, sucrose, glucose, and fructose in each batch of samples assayed in order to confirm the effectiveness of each enzyme digest. Our threshold for soluble sugar recovery was 95%, and starch was consistently recovered at 90%.

Nitrogen Assay

To analyze plant tissues for total N, we packed 3 to 4 mg of ground up samples into tin capsules, which were analyzed at the Ecosystem Analysis Lab at University of Nebraska – Lincoln, using a Costech Analytical ECS 4010 (Costech Analytical Technologies Inc., Valencia, California, USA) to measure the amount of N.

Data Analysis

We tested each of the three predictions about stored resources made by the resource budget model (Figure 1) for each species and for each pool of stored resources that we sampled (trunk NSC, branch NSC, branch N, and leaf N). We performed all statistical analyses in R 3.2.3 (R Core Team 2015). To test the first prediction (Table 1), we used linear mixed models with the particular stored resource concentration (from the early season sampling period) as the response variable, year as a categorical fixed effect, and individual ID as the random effect to account for repeated sampling of individuals. Models used the identity link function and a Gaussian error distribution. We fit all mixed models using the ‘lme4’ package (Bates et al. 2015). We evaluated model significance with Wald type II χ^2 tests using the ‘car’ package (Fox and Weisberg 2011). If models

were significant, we conducted multiple comparisons of means using the “multcomp” package (Hothorn et al. 2008).

To test the second prediction (Table 1), we used linear mixed models with log-transformed flower counts ($\ln(\text{flowers} + 1)$) as the response variable, the particular stored resource concentration and trunk diameter (DGH) as fixed effects, and individual ID as the random effect. In these models, the stored resource concentrations were of samples from the summer in the year prior to the flower count in the response. Floral initiation in oaks is hypothesized to occur in spring or summer of the year before flowers emerge (Merkle 1980), and therefore this is the key phenological stage when stored resources can affect the subsequent flower crop. We included DGH in the models as an indicator of plant size because we had considerable variation in plant size within both populations. We did not include the interaction term between the fixed effects of storage and DGH because we could not come up with biologically meaningful explanations for all of the potential outcomes. Again, we fit these models using lme4 and evaluated significance of fixed effects with Wald type II χ^2 tests.

We used different approaches to testing the third prediction (Table 1) in each species because of their different annual patterns of acorn production. *Q. vaccinifolia* produced acorns in 2011 and 2013. However, only two out of the ten plants from which we harvested tissues in 2011 produced more than one acorn, and thus we only used the 2013 data to test prediction three in this species. As such, we used linear models with log-transformed acorn counts ($\ln(\text{acorns} + 1)$) as the response variable, and the particular stored resource concentration and DGH as independent variables.

To test the third prediction in *N. densiflorus*, we used linear mixed models with log-transformed acorn counts ($\ln(\text{acorns} + 1)$) as the response variable, and the particular stored resource concentration and DGH as fixed effects, and individual ID as the random effect. We included all three years of data in these models because the *N. densiflorus* population, including most of the individuals from which we harvested tissues, produced acorns in all three years. As above, we fit these models using lme4 and evaluated significance of fixed effects with Wald type II χ^2 tests.

For all of these analyses, we used linear regression with the log-transformation yielded because they fit the data the best. The results were robust across the different statistical methods.

Results

Quercus vaccinifolia, the ‘masting’ species, produced acorns in 2011 and 2013, but had a total crop failure in 2012 (Figure 1a). *N. densiflorus*, the ‘non-masting’ species, produced acorns in all three years, but had a smaller acorn crop in 2013 (Figure 1b).

Prediction 1: The only stored resource that showed the annual dynamics predicted by the resource budget model in *Q. vaccinifolia* was branch NSC ($\chi^2 = 35.5$, $df = 2$, $P < 0.0001$; Figure 2). The only other significant model for *Q. vaccinifolia* was for leaf N, but it was in the opposite direction of the prediction ($\chi^2 = 35.9$, $df = 2$, $P < 0.0001$; Figure 2). The annual dynamics of branch NSC in *N. densiflorus* were similar to those of *Q. vaccinifolia* despite having a different pattern of annual acorn production. The models for branch N ($\chi^2 = 68.4$, $df = 2$, $P < 0.0001$; Figure 2) and leaf N ($\chi^2 = 11.3$, $df = 2$, $P <$

0.01; Figure 2) in *N. densiflorus* also showed differences between years, but these were more consistent with prediction 1, with levels of stored resources lower in the lowest acorn production year of 2013.

Prediction 2: This predicts a positive relationship between stored resources during floral initiation and the number of flowers produced in the following year. In *Q. vaccinifolia*, we found significant positive relationships between branch NSC, branch N, and leaf N measured in summer of one year, and the number of flowers produced in the following year (Table 3; Figure 3). There were no significant effects of trunk diameter (DGH) on flower counts in *Q. vaccinifolia* (Table 3). In contrast, we found no effects of stored resources on flower counts in *N. densiflorus* (Table 3; Figure 4). There were, however, weak positive relationships between DGH and flower counts in all four models for *N. densiflorus* (Table 3).

Prediction 3: This prediction concerns the resource depletion aspect of the resource budget hypothesis. The prediction is that plants that produce more acorns will also accumulate less storage (or experience more storage depletion) during acorn development, than plants that produce less acorns. The changes in trunk NSC and branch NSC in *Q. vaccinifolia* were significantly negatively related with acorn production in 2013 (Table 4; Figure 4). The effect the change in branch N during acorn development on acorn production bordered on statistical significance. There were significant positive effects of DGH on acorn counts that were mostly driven by two large plants that also produced many acorns (Table 4; Figure 4). In *N. densiflorus*, there were no significant effects of changes in any of the stored resources during acorn development or plant size on acorn production (Table 5; Figure 4).

Discussion

Our results indicate that these two similar and related species with contrasting annual patterns of acorn production have qualitatively different patterns of resource allocation with respect to stored resources and reproduction. We found no support for any of the predictions of the resource budget model in the ‘non-masting’ *N. densiflorus*, but predictions 2 and 3 were supported in the ‘masting’ *Q. vaccinifolia* (Table 1). Specifically, N and NSC appear to be important for flowering in *Q. vaccinifolia*, and NSC appears to be important for acorn maturation. This adds support to the resource fluctuation part of the resource budget model for masting and suggests that it may be a distinctive property of masting species. Therefore, if components of the hypothesized mechanism have been under natural selection, then they could have played a role in the evolution of masting.

The results for prediction 1 (Table 1) of the resource budget model were more complex to interpret than the other results. In the masting *Q. vaccinifolia*, there was support for prediction 1 only with measurements of branch NSC. That is, we found higher concentrations of branch NSC at the beginning of the years with acorn production and lower concentrations in the year with no acorn production. There were no annual differences in trunk NSC or branch N, and leaf N exhibited the opposite of the predicted pattern. We might have interpreted the results for *Q. vaccinifolia* branch NSC as support for prediction 1, except that the branch NSC in *N. densiflorus* followed the same annual pattern, with higher branch NSC in 2011 and 2013 and lower branch NSC in 2012.

Acorn production by *N. densiflorus* was relatively high in both 2011 and 2012, and relatively low in 2013. This indicates that branch NSC in *N. densiflorus* apparently fluctuates independently of acorn production patterns. The fact that branch NSC in both species followed the same annual pattern in spite of different annual patterns in acorn production suggests that population-level storage dynamics across years may be driven by variability in abiotic conditions. Consequently, we are unable to conclude that annual fluctuations in branch NSC of the *Q. vaccinifolia* population are driving masting behavior in this system.

In *Q. vaccinifolia*, there was support for the threshold part of the resource budget model (prediction 2, Table 1) in which plants need to reach a certain concentration of stored resources before they flower (Isagi et al. 1997). However, a strict threshold would produce a different shape of the relationship between stored resources and flowers than what we observed. In a strict threshold model, plants with low stored resource concentrations would be in a straight line at zero flowers until the threshold resource concentration, after which there would be a positive relationship. Instead, the relationship we observed looks relatively linear, which is similar to the relationship observed in *Fagus crenata* between N concentration and gene expression for flowering (Miyazaki et al. 2014).

Given that flowering can be an important limiting stage for mast seeding, it is surprising how few studies on masting and the resource budget model have investigated whether stored resource levels during floral initiation relate to future flowering (Crone and Rapp 2014). However, there is other evidence linking flowering to resources, including studies that manipulated available resources to plants (Cunningham 1997,

Miyazaki et al. 2009, Montesinos et al. 2012) or that measured stored resources before and after flowering (Marquis et al. 1997). Previous research has typically implicated carbon reserves or mineral nutrients separately, so it is interesting that we found both NSC and N to have significant effects on flowering. Unfortunately, we are unable to disentangle the relative importance of these two resources for flowering, and the issue is further complicated by the fact that leaf N and branch NSC are correlated.

In support of prediction 3, we found that seasonal changes in NSC declined with acorn production in *Q. vaccinifolia*. The values for change in trunk NSC were both positive and negative, which indicates that acorn producing plants may not actually experience depletion of trunk NSC, but simply less accumulation. In contrast, most of the values for change in branch NSC were negative, which indicated that this pool of stored resources was depleted during acorn production. It is unclear how long-lasting the stored resource depletion effects may be. Population-level data used to test prediction 1 indicate that there may be longer term effects in branch NSC (Figure 2). It is just as likely, however, that the effects are transient, representing a temporary shift in carbon allocation priority from storage to acorn development. *Q. vaccinifolia* individuals that produced zero acorns had values for storage that nearly covered the entire range of variation in the study population. This may indicate that we included several non-reproductive ramets in the study population. In support of this possibility, we found that five out of the nine plants that produced zero acorns in 2013 also produced zero acorns in 2011. None of the plants produced acorns in 2012.

Carbon storage depletion or the competition between storage and developing seeds for current photosynthesis, as observed in *Q. vaccinifolia*, is an uncommon result

in large masting trees (Hoch et al. 2013, Ichie et al. 2013, Crone and Rapp 2014).

Depletion of carbon reserves with reproduction has, however, been observed in other smaller masting plants like shrubs and forbs (Marquis et al. 1997, Miyazaki et al. 2002, Crone et al. 2009). This may reflect that larger trees have larger carbon budgets (Stephenson et al. 2014). Perhaps smaller plants are more likely to have carbon-limited reproduction. Indeed, we found positive effects of stem diameter on acorn production (Table 4). The carbon demands of acorn development in *Q. vaccinifolia* are explored more in another study, which found that acorn bearing branches had more leaves than vegetative branches, and drew photo-assimilates from neighboring vegetative branches (Chapter 2).

This study highlights the value of evaluating data at both the individual and the population level to evaluate hypothesized mechanisms for masting. If we had only evaluated population mean storage across years, then we would have concluded there was weak or no support for the resource budget model because the non-masting species showed the same resource storage dynamics across years. Instead, we found support for relationships between resources and flowering and fruiting that extends our understanding of how resource allocation relates to masting. Analyzing within-year individual variability can improve our understanding of processes that occur over longer time scales.

Likewise, using a comparative approach added inferential power to our study. If we had only evaluated the masting species, we would have interpreted the pattern of branch NSC across years as support for the resource budget model, instead of acknowledging the distinct possibility that branch NSC is merely tracking annual

differences in resource availability. Wherever it is possible, testing hypothesized mechanisms for masting in multiple sympatric species that span a range of variation in annual seed production will add rigor to our interpretations (Ichie et al. 2013, Funk et al. 2016).

Using a comparative approach will also help inform research into the evolutionary origins of masting. We found that *Q. vacciniifolia*, a species with variable annual seed production, and *N. densiflorus*, a species with more regular annual seed production, had qualitatively different patterns of resource allocation with respect to reproduction. The resource allocation patterns of *Q. vacciniifolia* provide support for the resource budget model for masting, particularly for the prediction that flowering is linked to resource concentrations. Genes that control the mechanisms regulating resource allocation for reproduction should be investigated as potentially responsible for leading to the evolution of mast seed production (Miyazaki et al. 2014).

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Tables

Table 1. Predictions of the resource budget model and a summary of support found for each species and resource sampled. A tick indicates that the graphically represented prediction was observed for the particular resource and species; a dash indicates that the graphically represented prediction was not observed for the particular resource and species.

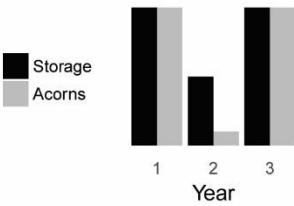
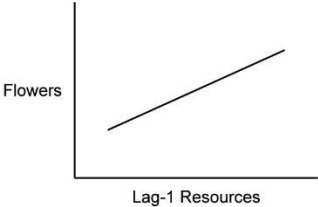
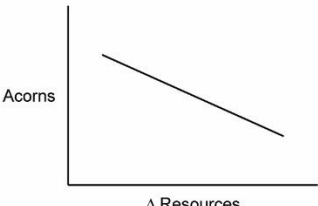
Prediction		Trunk NSC	Branch NSC	Branch N	Leaf N	Species
 <p>1: Storage will be high at the beginning of years with high acorn production and low at the beginning of years with low acorn production</p>		-	✓	-	-	<i>Q. vaccinifolia</i> ("masting")
		-	-	-	-	<i>N. densiflorus</i> ("non-masting")
 <p>2: Storage during floral initiation will positively relate to flowers in the following spring</p>		-	✓	✓	✓	<i>Q. vaccinifolia</i> ("masting")
		-	-	-	-	<i>N. densiflorus</i> ("non-masting")
 <p>3: Greater acorn production will be associated with lower, or more negative, seasonal change in resources during acorn development (Δ = final - initial)</p>		✓	✓	-	-	<i>Q. vaccinifolia</i> ("masting")
		-	-	-	-	<i>N. densiflorus</i> ("non-masting")

Table 2. Sample sizes for each species, assay, and year.

Species	Assay	2011	2012	2013
<i>Q. vaccinifolia</i>	Trunk NSC	6	10	28
	Branch NSC	10	10	30
	Branch N	10	10	26
	Leaf N	10	10	30
<i>N. densiflorus</i>	Trunk NSC	6	7	26
	Branch NSC	8	9	29
	Branch N	9	10	28
	Leaf N	10	10	27

Table 3. Fixed effects on log-transformed flower counts (2011 – 2013) from linear mixed models. Significant effects at $P < 0.05$ are in bold with corresponding significance codes.

Species	Main Effect	Effect size \pm SE	Co-variate	Effect size \pm SE
<i>Q. vaccinifolia</i>	Trunk NSC (%)	-0.103 \pm 0.164	Stem diameter	0.702 \pm 0.457
	Branch NSC (%)	0.713 \pm 0.172***	Stem diameter	0.322 \pm 0.343
	Branch N (%)	8.83 \pm 2.78**	Stem diameter	0.378 \pm 0.318
	Leaf N (%)	8.60 \pm 2.08***	Stem diameter	0.446 \pm 0.292
<i>N. densiflorus</i>	Trunk NSC (%)	-0.209 \pm 0.118	Stem diameter	0.869 \pm 0.429*
	Branch NSC (%)	-0.393 \pm 0.252	Stem diameter	0.776 \pm 0.467
	Branch N (%)	-1.32 \pm 4.32	Stem diameter	0.878 \pm 0.502
	Leaf N (%)	-1.27 \pm 3.10	Stem diameter	0.909 \pm 0.514
Significance codes ($x < P < x$): 0 '***' 0.001 '**' 0.01 '*' 0.05				

Table 4. Effects on log-transformed acorn counts (2013) in *Q. vaccinifolia* from linear models. Significant effects at $P < 0.05$ are in bold with corresponding significance codes.

Main effect	Effect size \pm SE	Co-variate	Effect size \pm SE	Model F-value (df)	Model <i>P</i> -value	Adjusted R ²
Δ Trunk	-0.358 \pm	Stem	0.548 \pm	4.84 (2 and	0.017	0.222
NSC (%)	0.160 *	diameter	0.206 *	25)		
Δ Branch	-0.673 \pm	Stem	0.551 \pm	4.89 (2 and	0.015	0.212
NSC (%)	0.304 *	diameter	0.198 **	27)		
Δ Branch N	-5.820 \pm	Stem	0.420 \pm	2.90 (2 and	0.075	0.132
(%)	3.010	diameter	0.214	23)		
Δ Leaf N	-3.734 \pm	Stem	0.468 \pm	3.17 (2 and	0.058	0.130
(%)	2.702	diameter	0.201 *	27)		

Significance codes ($x < P < x$): 0 '****' 0.001 '***' 0.01 '**' 0.05

Table 5. Effects on log transformed acorn counts (2011 – 2013) in *N. densiflorus* from linear mixed models. Significant effects at $P < 0.05$ are in bold, with corresponding significance codes.

Main Effect	Effect size \pm SE	Co-variate	Effect size \pm SE
Δ Trunk NSC (%)	0.034 \pm 0.088	Stem diameter	-0.198 \pm 0.271
Δ Branch NSC (%)	-0.124 \pm 0.082	Stem diameter	-0.037 \pm 0.276
Δ Branch N (%)	-1.08 \pm 0.994	Stem diameter	-0.048 \pm 0.270
Δ Leaf N (%)	-2.17 \pm 1.44	Stem diameter	-0.121 \pm 0.257
Significance codes ($x < P < x$): 0 '***' 0.001 '**' 0.01 '*' 0.05			

Figures

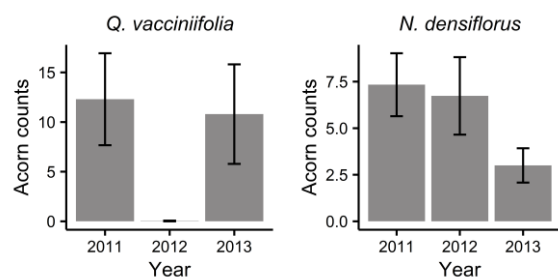


Figure 1. Annual mean (\pm standard error) acorn production for *Q. vaccinifolia* and *N. densiflorus*.

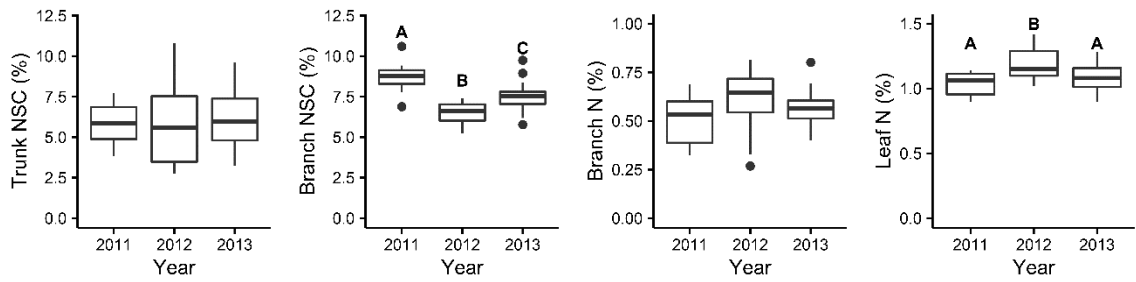
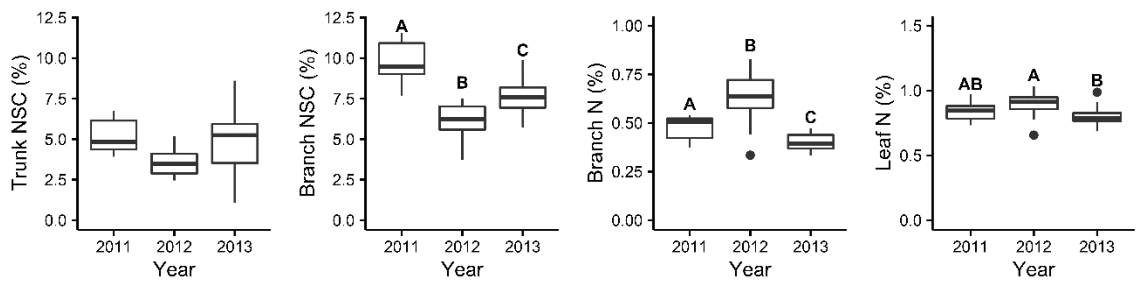
(a) *Q. vaccinifolia*(b) *N. densiflorus*

Figure 2. Test of prediction 1. Annual early season storage for *Q. vaccinifolia* (a) and *N. densiflorus* (b). Boxes include data between the 25th and 75th percentile, or the interquartile range (IQR); bold lines represent the median; whiskers extend to 1.5 * IQR; points outside that range are represented as dots.

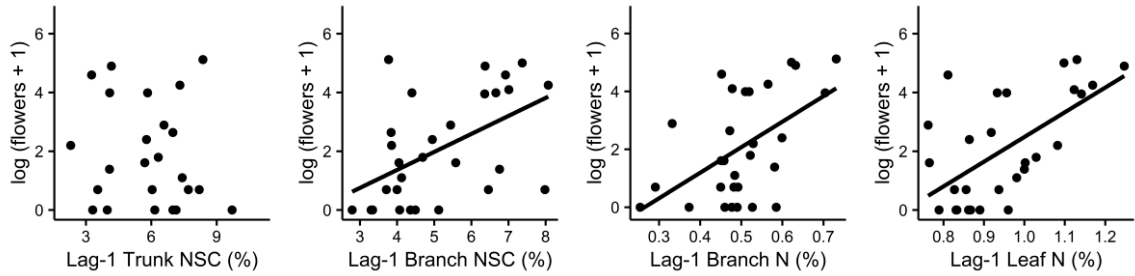
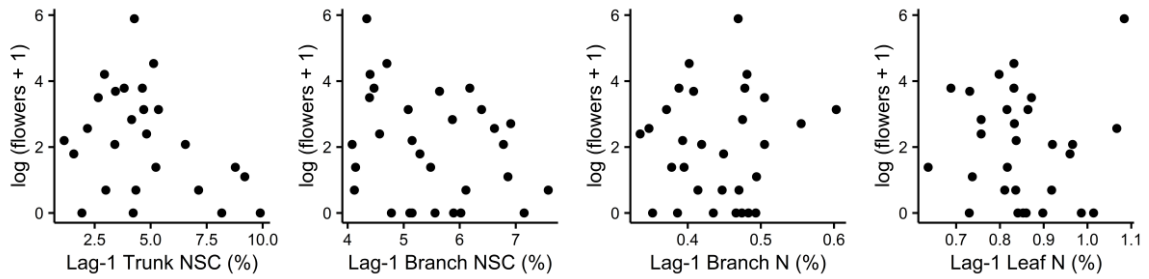
(a) *Q. vaccinifolia*(b) *N. densiflorus*

Figure 3. Test of prediction 2. Natural log-transformed flower counts vs. storage in the previous year for *Q. vaccinifolia* (a) and *N. densiflorus* (b). Lines are plotted for models where lag-1 storage had a significant effect on natural log-transformed flower counts ($P < 0.05$).

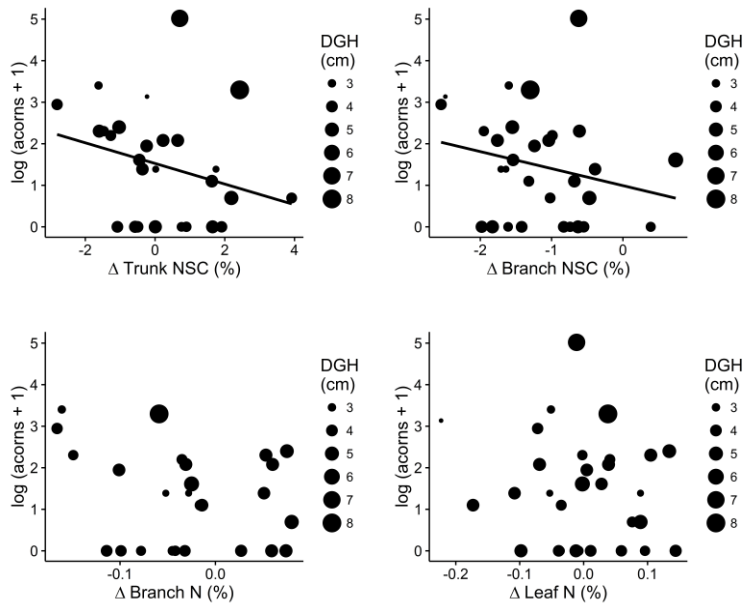
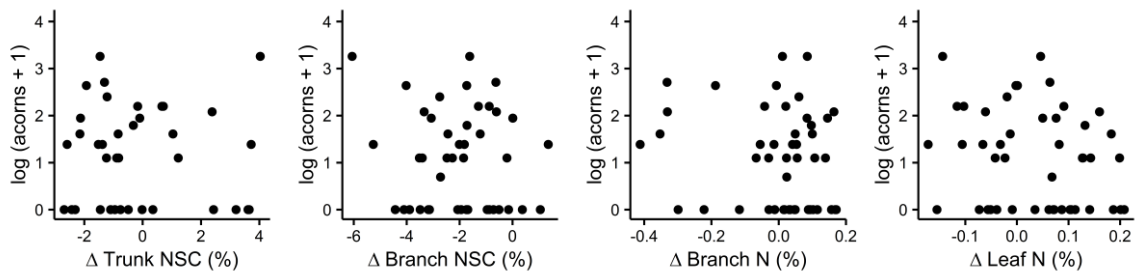
(a) *Q. vaccinifolia*(b) *N. densiflorus*

Figure 4. Test of prediction 3. Natural log-transformed acorn counts vs. the change in storage during acorn development (final – initial; Δ storage) for *Q. vaccinifolia* (a) and *N. densiflorus* (b). Size of points for *Q. vaccinifolia* figures correspond to the stem diameter at ground height in cm. Lines are plotted for models where delta storage had a significant effect on natural log-transformed acorn counts ($P < 0.05$).

Chapter 3: Carbohydrate demands of acorn development in the huckleberry oak
(*Quercus vacciniifolia*), a masting oak shrub

Abstract

There is controversy over whether carbon is a limiting resource for reproduction in woody plants with synchronous and variable annual seed production (i.e. “masting” behavior). Life history theory predicts that when resources are limited, there will be trade-offs in resource allocation between major life history functions such as growth, reproduction, and storage. Resource allocation trade-offs can be difficult to detect, especially in long-lived trees, because they occur over various time scales and plants may compensate by diverting resources from other organs. It is, however, possible to test corollary hypotheses. If carbon is a limiting resource for reproduction, then we expect that developing seeds should be demanding sinks for carbohydrates. We assessed the extent of carbohydrate demands during acorn maturation in the huckleberry oak *Quercus vacciniifolia*, an evergreen shrub that requires two growing seasons to mature acorns. Carbohydrate demand was measured at three levels: size and leaf number of acorn-bearing branches, carbohydrate sharing with neighboring branches, and the seasonal change in the pool of branch nonstructural carbohydrates. We tested whether acorns grow on branches with greater resource acquisition potential, as indicated by branch size and leaf number. Acorns developed on shoots that grew in the previous year, thus minimizing competition for carbohydrates between shoot growth and acorn growth, given that there was little new growth in the year of acorn production. Therefore, if acorns are expensive

to produce in terms of carbohydrates, we predicted that acorns would be found on branches that were larger and had more leaves than vegetative branches, which is what we observed. Using stable isotope labeling with $^{13}\text{CO}_2$, we found that acorn-bearing branches drew carbohydrates from adjacent vegetative branches, but not from adjacent acorn-bearing branches. We also found a weak negative relationship between the flux in mass of the branch nonstructural carbohydrate pool during the acorn maturation period and the number of acorns produced by a plant. This suggests that storage and fruit development may be competing sinks for carbohydrates. Overall, our findings suggest that carbohydrates may be a limiting resource for acorn production by *Q. vaccinifolia*. This result suggests that smaller masting plants, such as shrubs, are more likely than bigger trees to have carbon-limited reproduction.

Introduction

Carbohydrates are units of chemical energy produced by photosynthesis that plants can invest in growth, reproduction, or storage. Once stored, non-structural carbohydrates (NSC) can be remobilized to support growth and reproduction (Chapin III, Schulze & Mooney 1990), but energy invested in growth and reproduction cannot be remobilized. Storage of NSC is essential for plants because the supply of carbohydrates from photosynthesis and the demand for carbohydrates for growth and other metabolic processes are often asynchronous. For example, stored carbohydrates are used to support new growth in spring (Chapin III 1980), or to recover following disturbances such as defoliation or fire (Canadell & López-Soria 1998; Mcpherson & Williams 1998; Bell &

Ojeda 1999). Life history theory predicts that as long as the supply of energy is limited, there will be trade-offs in allocation of carbohydrates among the life history functions of growth, storage, and reproduction (Reznick 1985; Obeso 2002).

Resource trade-offs have been hypothesized to lead to irregular annual patterns of seed production that are synchronized population-wide, or “masting” behavior. Specifically, masting is hypothesized to be driven by a trade-off between current storage and future reproduction (Sork, Bramble & Sexton 1993). The “resource budget model” hypothesizes that storage reserves in masting plants are accumulated across years until a threshold level is reached, after which plants flower and produce seeds, which in turn depletes storage (Isagi *et al.* 1997).

Research that tests the resource budget model has been limited to only a handful of study systems so far, but the preponderance of evidence suggests that seed production by at least some masting plants is resource-limited (Rees, Kelly & Bjornstad 2002; Crone & Rapp 2014; Funk, Koenig & Knops 2016; Pesendorfer *et al.* 2016). Empirical evidence on the role of stored resources in mast seed production diverges when it comes to which specific storage compound is most important, with support for carbohydrates in some systems (Miyazaki *et al.* 2002; Crone, Miller & Sala 2009; Rapp & Crone 2015), and support for nitrogen and/or phosphorous in other systems (Sala *et al.* 2012; Ichie & Nakagawa 2013; Miyazaki *et al.* 2014).

Although stored carbohydrates appear to be limiting for seed production in some species, other studies have shown that some tree species have ample storage that is not depleted by masting (Ichie *et al.* 2005, 2013; Hoch *et al.* 2013). An abundance of stored carbohydrates has been interpreted to indicate that trees are not limited by carbon (Körner

2003). More evidence that suggests carbohydrates are not limiting for seed production comes from studies that shade or defoliate branches while still being able to mature seeds (Chapin III & Wardlaw 1988; Hoch 2005). When photosynthetic rates are lowered via shading or defoliation, plants can compensate either by drawing upon stored carbohydrates, increasing photosynthesis of remaining green tissues, including the fruit itself, and/or importing carbon from neighboring branches (Bazzaz, Carlson & Harper 1979; Sprugel, Hinckley & Schaap 1991; Kozlowski 1992; Lacointe *et al.* 2004). Taken together, there is currently no consensus within long-lived trees as to whether seed production is limited by carbohydrates.

Trees and large shrubs have a modular architecture where branches are the basic units of carbon resource acquisition that repeat throughout the plant. Branches are necessarily integrated with the rest of the plant for uptake of water and nutrients from the soil, but they may be independent from each other for carbon acquisition and allocation, depending on the species and environmental context (Sprugel *et al.* 1991; Lacointe *et al.* 2004). Whether or not fruit-bearing branches are autonomous for carbohydrate supply varies by species, just as do the results for plant-wide carbon dynamics (Hasegawa *et al.* 2003; Hoch 2005; Miyazaki, Hiura & Funada 2007). To advance our understanding of what limits seed production, it is necessary to consider carbon acquisition and carbon allocation not only at the plant-wide level, but also at the local branch level where seeds are growing.

The goal of this study was to determine the sources of carbohydrates used by and affected by developing seeds in a plant species with intermittent and synchronized annual seed production. We used an evergreen shrub, the huckleberry oak *Quercus vacciniifolia*

Kellogg (Fagaceae), a slow-growing, montane, wind-pollinated species that produces "two-year" acorns (female flowers pollinated during the spring of one year develop into acorns during the summer of the following year, Fig. 1). We hypothesized that if carbohydrates are limiting for life history functions then developing acorns will be expensive in terms of carbohydrates, which will be indicated by the sources from which carbohydrates are drawn during acorn maturation. Potential sources of carbohydrates for acorn maturation include photosynthesis in the acorn itself, leaves on the acorn-bearing shoot, leaves on neighboring shoots, and stored carbohydrates. If acorn development is carbohydrate intensive for the particular shoot on which it is growing, then we predicted that acorn-bearing shoots will be larger and have more leaves than vegetative shoots, thus indicating greater potential for resource acquisition. If photosynthesis from the acorn bearing branch is insufficient for acorn development, then we predicted that carbohydrates will be drawn from neighboring vegetative branches. If acorn production is a carbohydrate intensive process at the level of the whole plant, then we predicted that plants with greater acorn production will experience greater depletion in the total pool of branch non-structural carbohydrates than plants with fewer acorns.

Materials and Methods

STUDY SPECIES AND FIELD SITE

Quercus vacciniifolia is a monoecious, evergreen shrub. It has a single flush of new shoots and leaves each year occurring in late spring. Its range covers much of the

high Sierra Nevada Range, Cascade Range, Inner North Coast Range, and Klamath Range in northern California and southern Oregon, USA (Jepson Flora Project). The study population is distributed along a forest service road in the Shasta-Trinity National Forest in the Klamath Range in northern California, USA (41.24°N, 122.69°W, between 1200 m and 1350 m elevation). The dominant overstory vegetation was composed of *Pinus ponderosa*, *Pinus lambertiana*, *Pseudotsuga menziesii*, *Abies concolor*, and *Calocedrus decurrens*, with the understory frequently occupied by patches of *Notholithocarpus densiflorus* var. *echinoides* and *Q. vacciniifolia*. This area experiences a Mediterranean climate, with most precipitation falling during the winter, while summers are hot and dry with infrequent rain. The plants in the study population produced acorns in 2011 and 2013, but had total crop failures in 2012 and 2014 (K. Funk, unpublished data). Thus, acorn production was variable and synchronous over the study period. Here, however, we investigated the carbohydrate dynamics within a single growing season.

BRANCH SIZE

To investigate the relationships between branch size and leaf number with acorn production, we harvested four vegetative and four reproductive branches, each with several years of growth (Fig. 2) from 20 plants in July 2013. We separated branches into cohorts based on the year in which they grew which was clearly demarcated by bud scars. For each branch in each cohort we counted the number of leaves, measured the length, and measured the width between the proximal bud scar and first leaf. Length and width

were measured with calipers and recorded to the nearest 0.1 mm. Branches from the 2011 cohort were classified as either purely vegetative or reproductive based on whether or not there were acorns developing. Branches from the 2012 cohort, on which acorns were developing in 2013, were classified as vegetative, reproductive, or vegetative adjacent to reproductive (Fig. 2a). Because there was little new growth in 2013, we classified branches that developed in that year as either vegetative or reproductive.

CARBON AUTONOMY

To test whether acorn-bearing branches draw carbon from adjacent vegetative branches, we used a stable isotope labeling experiment. The stable isotope carbon-13 (^{13}C) is approximately 1.1% as common as carbon-12 (^{12}C) in atmospheric CO_2 (Nier 1950; Schlesinger 1997). Exposing photosynthesizing plants with ^{13}C -enriched carbon dioxide ($^{13}\text{CO}_2$) causes a pulse in the relative concentration of the stable isotope in the photosynthates produced during the exposure, relative to pre-exposure levels. This label can then be traced by harvesting tissues and comparing their ^{13}C concentrations to pre-exposure concentrations.

We initiated the experiment in July 2013, when acorns were approximately half size. On each of eight plants, we selected four “Y” shaped branches, where two branches from the 2012 cohort grew out of one branch from the 2011 cohort. Each plant received four treatments, in which two of the Y systems had an acorn on one side, one of the Y systems had acorns on both sides, and one of the Y systems had no acorns. In each treatment we applied the labeled gas to one branch of the Y, setting up an acorn \times label

factorial design (Fig. 3a). We removed a 1 cm strip of bark and phloem from the base of each Y system in order to limit carbon import and export between the experiment and the rest of the plant.

To label the branches, we placed a clear plastic bag around one side of each Y system, flushed the air out of it, and sealed it around the base of the twig with putty. We then punctured a small hole in the bag with the nozzle of the two-stage regulator attached to the gas cylinder of 99% $^{13}\text{CO}_2$. After releasing approximately 8 ml of $^{13}\text{CO}_2$ into the bag, we promptly re-sealed the hole in the bag with putty. We gassed the branches between the hours of 1000 and 1400 and then removed the bags just before the following dawn. We removed bags in the dark before dawn with the assumption that leaves would be exchanging negligible amounts of gas with the atmosphere and thus any leftover $^{13}\text{CO}_2$ would diffuse away without the occurrence of unintended labeling. To verify the effectiveness of the labeling procedure, we measured the baseline isotopic signature of branches from each experimental plant once prior to labeling. We harvested all of the experimental branches in mid-August when acorns were full-sized and dried them for several days at 70° C.

All plant tissues for isotopic analysis were ground to a fine powder using a two-step process. First, we ground samples in a Wiley mill (Thomas Scientific, Swedesboro, New Jersey, USA) until they passed through a 0.85 mm mesh. Then we crushed samples into a fine powder in a ball mill (Cianflone 2601, Pittsburgh, Pennsylvania, USA). We packed 1.5 – 2 mg of each sample into tin capsules and samples were analyzed for ^{13}C at the UC Davis Stable Isotope Facility (Davis, California, USA).

ACORN PRODUCTION AND CARBON STORAGE

In order to investigate the hypothesized trade-off between acorn production and carbon storage, we counted the number of acorns and estimated the standing mass of non-structural carbohydrates (NSC) in branches of 30 plants in 2013. These plants were not those used for measuring branch size or for the stable isotope labeling experiment, but they grew at the same study site.

To measure acorn crops we counted all acorns on each plant in mid-August 2013 when acorns were full-sized. To estimate the standing mass of NSC, we first estimated the standing woody biomass in branches of each plant using the following formula:

$$\begin{aligned} & \textit{Count of 2011 branches} * \textit{mean dry mass of 2011 branches} \\ & + \textit{Count of 2012 branches} * \textit{mean dry mass of 2012 branches} \end{aligned}$$

In 2011 and 2012 we counted all new shoots that each plant produced as a metric for growth. We counted new shoots in 2013 as well, but there was very little growth that year, which left us with a small sample size from which to estimate mean mass. Including estimates from 2013 shoots changed the overall estimates by an average of 1.3%, but did not change the statistical analyses. Consequently, we decided to omit the 2013 shoots from our analyses. Mean dry mass of 2011 and 2012 cohorts of branches came from the 20 plants used for the ‘Branch Size’ part of this study. The mean mass for 2011 branches, based on 160 branches, was 0.247 g. The mean mass for 2012 branches, based on 582

branches, was 0.068 g. Therefore, the standing woody biomass of branches from the previous two years of growth for a given plant i (*Branch biomass_i*) was estimated as:

$$\text{Branch biomass}_i = \text{count of 2011}_i * 0.247 \text{ g} + \text{count of 2012}_i * 0.068 \text{ g}$$

After estimating the standing branch woody biomass for each plant, we multiplied values by the NSC concentration in branches measured for each plant to derive an estimate of standing mass of NSC. We harvested branches to be used in NSC assays twice in 2013. The first harvest was in June, before acorns started developing, and the second was in August, after acorns were fully developed. This provided data on the change in branch NSC during acorn development. For each harvest and plant, we cut four branches with three years of growth on each. For the August harvest we took two branches without acorns and two branches with acorns from each plant that had produced acorns. For data analysis, we used the flux in NSC during acorn development, calculated as the NSC in June subtracted from the NSC in August. Thus, both developing acorns and NSC were quantified as fluxes in biomass.

NON-STRUCTURAL CARBOHYDRATES ASSAYS

Branches for NSC assays were ground to a fine powder using the same two-step process described above. We conducted the assay on 20 mg (± 1 mg) of tissue per sample. First, we extracted the sample in 0.1 M sodium acetate, and then we removed an aliquot for soluble sugar (sucrose, fructose, and glucose) analysis. In the remaining

fraction, we digested starch first with α -amylase from *Bacillus licheniformis* (Bio-Cat, inc.), and then with amyloglucosidase from *Aspergillus niger* (Sigma A9913). Prior to the assay, we dialyzed the amyloglucosidase in sodium acetate to remove any glucose from the enzyme solution. From this step, the starch and soluble sugar fractions were treated the same. We used invertase (Sigma I4504) to digest sucrose, and then we put aliquots of each fraction of each sample into wells of 96 well microplates to be measured colorimetrically. The final digest occurred in the microplates with a glucose assay reagent (Sigma G3293) and phosphogluco-isomerase (Sigma P5381) to digest glucose and fructose, respectively. After this final digest, we used a spectrophotometer (Cary 50, Varian Inc., Palo Alto, California, USA) to measure the absorbance of each well at 340 nm. The absorbance reading was directly proportional to the concentration of glucose in the well. Using a glucose standard curve and dilution factors we calculated the concentrations of soluble sugars and starch for each sample, the sum of which represents total NSC. In each batch of samples assayed we analyzed standards of starch, sucrose, glucose, and fructose to confirm the effectiveness of each enzyme digest. The acceptable threshold for soluble sugar recovery was 95%, while starch was consistently recovered at 90%.

DATA ANALYSIS

Analyses were performed in R (R Core Team 2015). To investigate the relationship between branch size and acorn production, we first compared branch sizes and leaf number of the 2012 cohort of branches (where acorns were developing) among

vegetative ($N = 279$), reproductive ($N = 101$), and vegetative adjacent to reproductive shoots ($N = 202$; Fig. 2a). We fit generalized linear mixed models (GLMMs) with a log link function and a Gaussian error distribution using the “lme4” package (Bates *et al.* 2015). We verified the assumption of homogeneity of variance by plotting residuals against fitted values. In each model, individual plant ID was included as a random effect in order to account for the pseudoreplication caused by measuring multiple branches on each of the 20 plants (see BRANCH SIZE above). We constructed models with shoot length, width, volume, and number of leaves as response variables. Volume was estimated by treating the branch as a cylinder, so $\text{volume} = \text{length} * \pi * (0.5 * \text{width})^2$. In each model, the fixed effect was the categorical shoot type (i.e. vegetative, reproductive, vegetative adjacent to reproductive). Models were evaluated with Wald type II χ^2 tests using the “Anova” function in the “car” package (Fox and Weisberg 2011). If models were significant, we used the “multcomp” package (Hothorn, Bretz & Westfall 2008) to conduct multiple comparisons of means on the categorical fixed effect. To evaluate whether there was more new growth (2013 cohort) on reproductive branches versus vegetative branches, we used an unpaired t -test comparing the shoot volumes of the two shoot types.

To evaluate whether we succeeded in labeling branches with $^{13}\text{CO}_2$, we used an unpaired t -test comparing the ^{13}C atoms per mil of the baseline branches and the labeled branches. To evaluate differences in ^{13}C atoms per mil among the four different acorn treatments of unlabeled branches, we used GLMMs as described above, again with individual plant ID as a random effect, followed by a Wald type II χ^2 test and post-hoc multiple comparisons of means as described above.

We tested the relationship between acorn production and branch NSC with a Pearson correlation test, where one variable was the log-transformed number of acorns on a plant and the other was the change in branch NSC mass during acorn development.

Results

BRANCH SIZE

In the 2012 cohort of branches, shoot types had a significant effect on length ($\chi^2 = 49.0$, $df = 2$, $P < 0.001$), width ($\chi^2 = 44.8$, $df = 2$, $P < 0.001$), and volume ($\chi^2 = 61.6$, $df = 2$, $P < 0.001$; Fig. 2b) of shoots. In each model, there was no difference between the two types or vegetative shoots ($P > 0.25$ for all 3 models), and reproductive shoots were the largest ($P < 0.001$ for all 3 models). Shoot type also had a significant effect on the number leaves ($\chi^2 = 15.6$, $df = 2$, $P < 0.001$; Fig. 2c), with reproductive shoots having about 1 more leaf on average than either type of vegetative shoots ($P < 0.01$ for both comparisons). Again, there was no difference between the two types of vegetative shoots ($P = 0.97$). There was no statistical difference between volumes of reproductive shoots and vegetative shoots from the 2013 cohort ($t = -1.58$, $df = 11.3$, $P = 0.14$), but this test was compromised by extremely poor vegetative growth that year, with only 19 new shoots on any of the 582 shoots from the 2012 cohort that we measured.

CARBON AUTONOMY

Labeled branches had significantly greater ^{13}C atoms per mil than the baseline branches by a mean difference of 0.56 ^{13}C atoms per mil ($t = -8.12$, $df = 7$, $P < 0.001$). Of the unlabeled branches, the unlabeled acorn-bearing branch paired with a labeled vegetative branch (treatment 2) had significantly higher ^{13}C atoms per mil than any of the other unlabeled treatments ($\chi^2 = 24.7$, $df = 3$, $P < 0.001$; $P < 0.05$ for all post-hoc comparisons with treatment 2; Fig. 3b). None of the comparisons among unlabeled branches from treatments 1, 3, and 4 were significant ($P > 0.5$ for all comparisons).

CARBON STORAGE

The estimated mass of NSC stored in branch wood sampled at the beginning of the season ranged from 1.3 g to 13.1 g, with a mean (\pm standard deviation) of $4.9 \text{ g} \pm 2.9 \text{ g}$. The early season mean concentration of NSC in branches was $9.74 \% \pm 0.82\%$. Log-transformed acorn counts were weakly correlated with the flux in NSC mass in branches during acorn development ($r = -0.35$, $P = 0.06$; Fig. 4). The seasonal fluxes in branch NSC were largely driven by changes in starch concentration, rather than changes in soluble sugars (sucrose, fructose, and glucose). The mean change in starch concentration was -1.6% , whereas the mean change in soluble sugar concentration was $+0.4\%$.

Discussion

BRANCH SIZE

We found that acorn-bearing branches were larger and had more leaves than vegetative shoots, which supports the hypothesis that acorn development is a carbohydrate-intensive process. A similar pattern has been observed in branches of three other species of oaks, where it has been hypothesized to be due to a trade-off between growth and reproduction (Sánchez-Humanes, Sork & Espelta 2011; Alla *et al.* 2012). Specifically, these authors proposed that the increased carbon sink strength of acorn maturation either suppressed the growth of vegetative branches or incidentally enhanced the growth of acorn-bearing branches.

The species used in these earlier studies produce one-year acorns that develop on shoots grown during the same growing season and thus there is the potential for temporal overlap in these putatively competing carbon sinks. In contrast, *Q. vaccinifolia* produces two-year acorns that develop on shoots mostly grown in the year prior to acorn maturation; that is, all of the primary growth (length), some of the secondary growth (width), and all of the leaf growth occurs in the year prior to acorn maturation. As a result, the potential for carbon sink competition between shoot growth and acorn growth is reduced compared to one-year acorn species. If there is a trade-off between shoot growth and acorn growth in *Q. vaccinifolia*, it is likely to be manifesting across years rather than in the same year.

An alternative explanation for acorn-bearing branches being larger than vegetative branches is that greater size is needed for structural support for the weight of the acorns. We think this is unlikely because acorns are relatively small (less than 1 gram, dry) and we never observed shoots sagging or breaking as a result of the acorn crop (pers. obs.).

CARBON AUTONOMY

In addition to being larger and having more leaves than vegetative branches, acorn-bearing branches drew carbohydrates from adjacent vegetative branches, as revealed by the $^{13}\text{CO}_2$ pulse labeling experiment. Unlabeled branches that had acorns and were paired with a labeled vegetative branch had significantly higher ^{13}C signatures than the unlabeled treatments. When unlabeled acorn-bearing branches were paired with labeled acorn-bearing branches, there was no indication of carbohydrate sharing, which suggests that the sink strengths of the developing acorns on each branch effectively cancelled each other.

We assumed that girdling blocked import of stored carbohydrates from other parts of the plant and export of photosynthates from the experimental branches, which could have diluted the isotopic label. This procedure may, however, have made our experiment less realistic, as girdling has been shown to reduce photosynthesis (Lu & Chacko 1998; Zhou & Quebedeaux 2003). If photosynthesis of leaves on fruit-bearing branches was down-regulated due to the girdling treatment, then it is possible that ungirdled fruit-bearing branches could mature fruit autonomously. Evidence countering this possibility comes from research showing that the increased sink activity of fruit development mitigates down-regulation of photosynthesis due to girdling (Proietti & Tombesi 1990; Urban, Léchaudel & Lu 2004). Additionally, if the high carbon demands of acorn development indicated by our experimental results were an artifact of girdling, then we

would not have expected the observed relationship between carbon storage and acorn production.

CARBON STORAGE

The negative relationship between acorn production and flux in branch NSC pools further supports the conclusion that the carbon demands of acorn development are substantial. We found that plants with greater acorn production also tended to experience greater NSC depletion during acorn development. Plants that produced no acorns were distributed across the range of values for the seasonal flux in NSC. This may indicate that something other than acorn production is driving the changes in NSC, or it could be that there are different storage dynamics in plants that do not produce acorns. We used a single stem as our fundamental experimental unit for comparisons between individuals, but *Q. vaccinifolia* can have many stems and it is possible that we accidentally selected some ramets that do not commonly reproduce.

Depletion of storage associated with seed production is a key component of the resource budget model for masting, which hypothesizes that masting plants must store up resources in years leading up to a large seed production event until a threshold is met. When the threshold is met, plants flower intensely and the resulting effort to produce seeds is so energy intensive that storage pools are left depleted (Isagi *et al.* 1997). Carbohydrates were thought to be the vital energy currency when the models were first published (Isagi *et al.* 1997; Satake & Iwasa 2000), but few studies have actually found depletion of carbon storage in wild populations of masting plants; instead, nitrogen

and/or phosphorous have more often been implicated as the important resource currency for masting (Sala *et al.* 2012; Ichie & Nakagawa 2013; Crone & Rapp 2014; Miyazaki *et al.* 2014). Still, it is curious that the carbon storage dynamics of trees appear to be minimally affected by such a large biomass investment as a mast seed crop. Perhaps reproduction is more likely to be limited by carbohydrates in shrubs than in tall, canopy forming trees, because carbon accumulation increases with size (Stephenson *et al.* 2014).

In our study system, we do not know whether the long term dynamics of carbon storage are affected by the temporary dip in NSC associated with acorn production. It is possible that acorns are not using stored carbohydrates for development of acorns, but instead storage and reproduction are competing sinks for carbon that is assimilated during the acorn maturation period. This explanation would be consistent with observations that many masting species do not use stored carbohydrates in fruit construction (Hoch *et al.* 2013; Ichie *et al.* 2013).

CONCLUSION

We have shown that the carbon demands of acorn development in *Q. vaccinifolia* can be extensive. Acorn-bearing branches are larger than, have more leaves than, and draw photosynthates from vegetative branches. Furthermore, plants that produce more acorns experience greater depletion to their branch NSC pools during the acorn maturation period. Our data can aid in interpretation of purported branch level trade-offs faced by other oak species (Sánchez-Humanes *et al.* 2011; Alla *et al.* 2012). Rather than acorn-bearing branches causing vegetative branches to be smaller, our data indicate that

smaller branches are unable to meet the immediate carbon demands of acorn maturation. This interpretation is consistent with the hypothesis from other oak research that trade-offs faced by oaks manifest over multiple years rather than within a single year (Knops, Koenig & Carmen 2007; Barringer, Koenig & Knops 2013).

Our results also have implications for understanding the resource limitations of acorn production, especially in the context of masting. Few other studies of species with synchronous and variable reproduction have demonstrated carbon storage depletion associated with seed production. Although we did not demonstrate that carbon is limiting for acorn production over multiple years, our data indicate that carbon is limiting for acorn production within a year.

If acorn production is resource limited, then it may be sensitive to climate change, especially if the limiting resource is carbon. Patterns of seed production by populations of masting plants have been changing in both intensity and frequency (Richardson *et al.* 2005; Övergaard, Gemmel & Karlsson 2007; Redmond, Forcella & Barger 2012). Understanding the mechanisms that produce variability in seed production is important for predicting future responses to climate change. This is particularly consequential for oaks because the magnitude and frequency of acorn crops have cascading effects throughout food webs (Elkinton *et al.* 1996; Ostfeld & Keesing 2000; Koenig & Knops 2005; Bogdziewicz *et al.* 2016).

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Figures

Fig 1. Female flowers (left, next to arrow) are pollinated during in the late spring of one year, and acorns (right) develop during the summer of the next year.

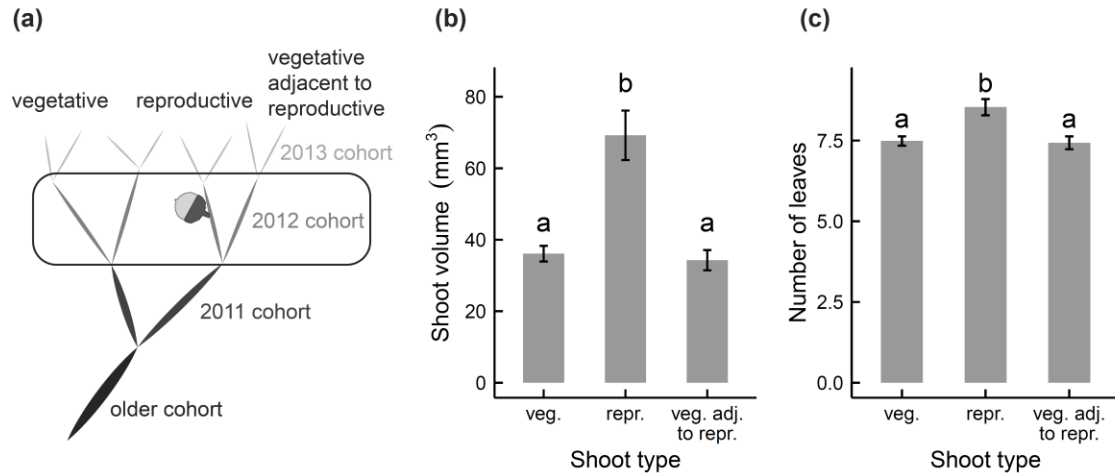


Fig. 2. (a) Sampling schematic and terminology for 'Branch Size' part of the study. (b) Means \pm standard errors of branch volume and (c) number of leaves on the 2012 cohort of shoots, as measured in 2013. Sample sizes are listed on the bars, and letters above the bars indicate significant differences from post-hoc tests.

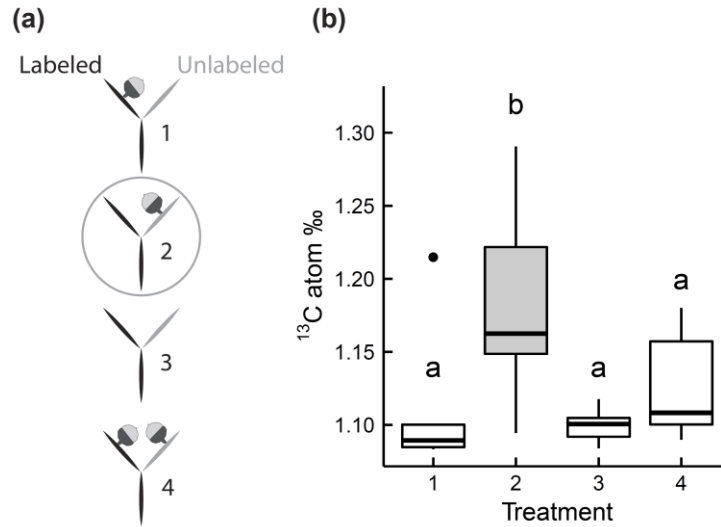


Fig. 3. (a) Illustration of treatments for the carbon autonomy experiment. Branches were girdled at the base of the 'Y.' Branches on the left side of the drawing were pulse labeled with 8 mL of $^{13}\text{CO}_2$. Treatment 2 is circled because interpretation of the results hinges upon this treatment. If acorn-bearing branches use carbon from adjacent vegetative branches, then we expect the unlabeled branch from Treatment 2 will have a greater ^{13}C signature than unlabeled branches from the other treatments. (b) ^{13}C atoms per mil in unlabeled branches. The grey box plot corresponds to the circled treatment. Different letters above boxes indicate significant differences from post-hoc tests.

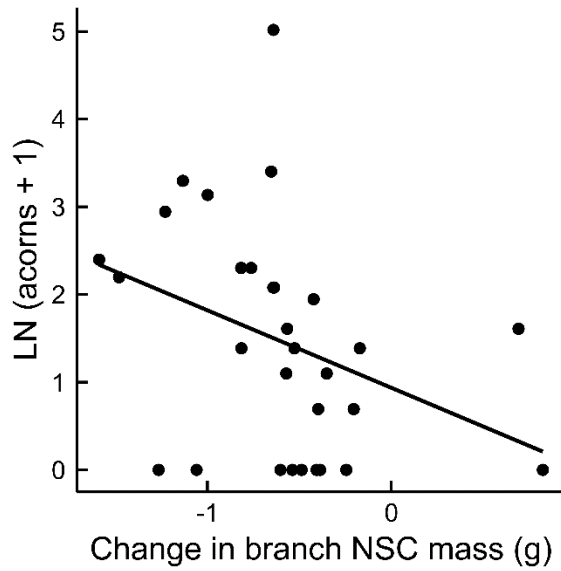


Fig. 4. Log-transformed acorn counts versus the seasonal change in estimated non-structural carbohydrate mass in branches during acorn development (final – initial).

Chapter 4: Constraints on acorn production in two oak species with contrasting water use strategies

Abstract

Highly variable and synchronous seed production, or ‘masting’, is hypothesized to be driven, in part, by resource limitation. The resources that are most relevant for limiting seed production differ across studies, however, and studies that include multiple masting species studied within the same ecosystem are rare. We studied two closely related, sympatric, masting oaks (*Quercus lobata* and *Quercus douglasii*) with contrasting water use strategies. *Q. lobata* avoids drought stress during the driest months of the growing season and is able maintain photosynthesis, whereas *Q. douglasii* tolerates drought stress during the driest months of the growing season and is able to survive with reduced photosynthesis. Our objectives were to test: (1) whether resources had effects on flowering, fruit set, or acorn crop; and (2) whether these two species differed in which resources were most relevant for reproduction. We measured non-structural carbohydrates (NSC) in trunks and branches, and nitrogen (N) in branches and leaves in June and September over a two-year period. In the drought avoiding *Q. lobata*, trees that retained more leaf N during flower initiation produced more flowers in the following spring, and trees with larger acorn crops also experienced more N depletion in branches during acorn maturation. There were no effects of resources on fruit set. In the drought tolerant *Q. douglasii*, trees with greater acorn crops accumulated less branch NSC. There were no effects of resources on flowers. Overall, we found some support for resource-

limited seed production in both masting species, however there were different resources associated with reproduction. We interpret this as evidence that species functional traits, such as water use efficiency, are potentially important drivers of differences in limiting resources among masting species.

Introduction

Mast-seeding, also known as masting, is an emergent ecological pattern where high inter-annual variability in individual seed production is synchronized across a population of plants. The high inter-annual variability in seed production of masting plants is often hypothesized to be driven, in part, by resource limitation (Sork, Bramble & Sexton 1993; Kelly & Sork 2002; Crone & Rapp 2014; Pearse, Koenig & Kelly 2016). Evidence for resource-limited seed production comes from commonly observed patterns of strong negative autocorrelation in annual seed production of masting populations (Sork *et al.* 1993; Kelly 1994; Koenig *et al.* 1994b; Koenig & Knops 2000). This has led researchers to hypothesize that large crops of seeds produced by masting plants deplete internal reserves, which then require more than one year to be replenished (Sork *et al.* 1993; Koenig *et al.* 1994b). This hypothesis was formalized mathematically as a ‘resource budget model’ which, in conjunction with spatially autocorrelated weather patterns and density dependent pollen limitation, can produce the variable and synchronous patterns of seed production characteristic of masting plants (Isagi *et al.* 1997; Satake & Iwasa 2000, 2002). Support for resource budget models has been recently

reviewed, and many questions remain about their generality (Crone & Rapp 2014; Pearse *et al.* 2016).

The specific nutrient (e.g. carbon, nitrogen, or phosphorous) that is the most limiting for reproduction appears to vary considerably across study systems (Crone, Miller & Sala 2009; Sala *et al.* 2012; Ichie & Nakagawa 2013; Miyazaki *et al.* 2014). It is not always clear what is driving these differences in limiting resources, although some are predictable. For example, phosphorous was depleted by fruiting in a tropical tree (Ichie & Nakagawa 2013), and for some temperate tree species seed production either depleted nitrogen or was enhanced by nitrogen fertilization (Smaill *et al.* 2011; Sala *et al.* 2012; Miyazaki *et al.* 2014; Bogdziewicz *et al.* 2016). Carbon reserves appear to be important for masting in some species (Miyazaki *et al.* 2002; Crone *et al.* 2009; Rapp & Crone 2015), but not as frequently as previously predicted (Hoch *et al.* 2013; Ichie *et al.* 2013; Miyazaki 2013). It is thus currently unknown what explains differences in the most relevant limiting resource for mast seed production, or if there are consistent patterns. One way to address this uncertainty is to study mechanisms for masting among multiple species in the same landscape, thus comparing varying species functional traits while habitat and environment remain relatively constant (Ichie *et al.* 2013; Funk, Koenig & Knops 2016).

Here we compare two sympatric, masting oak trees from the same subgenus (*Quercus* section *Quercus*) with contrasting water use strategies to investigate whether carbon reserves and/or nitrogen are important factors in explaining variability in acorn production. Our study site experiences a Mediterranean climate, which is characterized by cool, wet winters and hot, dry summers. *Quercus douglasii* is extremely drought

tolerant and during the dry California summers reaches very low values of both pre-dawn and daytime xylem water potential (Knops & Koenig 1994, 2000). During the driest months, which is also when acorns are developing, leaf gas exchange declines precipitously (Xu & Baldocchi 2003). All of this is in spite of having documented rooting depths of 20 m or more (Lewis & Burgy 1964). *Quercus lobata* is more effective at avoiding drought stress, as indicated by higher pre-dawn and daytime xylem water potentials than *Q. douglasii* (Hollinger 1992; Knops & Koenig 1994, 2000). Throughout its distribution, *Q. lobata* is associated with fertile, alluvial soils and relatively shallow water tables (Jepson 1910), even though *Q. lobata* can have roots up to at least 10 m (Lewis & Burgy 1964). The tendency of *Q. douglasii* to reach very negative water potentials during the summer suggests that carbon reserves are most important for acorn production in this species, while the association with fertile soils suggest that nitrogen is likely to be important for acorn production in *Q. lobata*.

The first goal of this study is to test predictions of the hypothesis that reproduction in these two oak species is resource-limited (Koenig *et al.* 2015; Pearse *et al.* 2016; Pesendorfer *et al.* 2016) by examining the relationship between carbon and nitrogen resources and acorn production. Masting patterns in populations of wind-pollinated species are thought to arise from a combination of individual resource limitation and population-wide abiotic drivers, such as temperature, which affect flowering or fruit maturation success (Abe *et al.* 2016; Pesendorfer *et al.* 2016). In oaks, acorn production can be limited by how many flowers are produced, what proportion are pollinated, and by what proportion of pollinated flowers are successfully matured into acorns ('acorn set') (Pearse *et al.* 2016). *Q. lobata* have been experimentally shown to

experience low levels of pollen limitation that varies among years (Pearse *et al.* 2015). Flowering synchrony, which is thought to drive pollen limitation, therefore has an important influence on acorn production by *Q. lobata* (Koenig *et al.* 2012, 2015; Pesendorfer *et al.* 2016).

To complement these findings on the role of pollination, we therefore investigated three possible indications of resource limitation. First, we tested the prediction that an accumulation of carbon or nitrogen resources during the floral initiation will have a positive effect on the subsequent flower crop. Female flowers in oaks are initiated in buds during summer of the year before flowering (Merkle *et al.* 1980). Next, we tested the prediction that resource abundance will have a positive effect on acorn set (number of acorns per flower). Finally, we tested the prediction that the change in resources during acorn development will have a negative effect on acorn production; that is, that plants producing more acorns will deplete carbon or nitrogen reserves.

Our second goal is to contrast the outcomes of these three predictions among our two study species. *Q. lobata* and *Q. douglasii* co-occur in many places throughout our study site, although *Q. douglasii* is much more common on the drier slopes. We predicted that carbon reserves will be more important than nitrogen for explaining differences in acorn production of *Q. douglasii* because the species experiences low water potentials, indicating water limitation (Knops & Koenig 1994, 2000), with an associated decline in maximum photosynthetic rates during the driest months (Xu & Baldocchi 2003), which is also the main period of acorn development. Therefore, we hypothesized that *Q. douglasii* will either rely on carbon reserves for developing acorns or at least need to decrease allocation of assimilated carbon to reserves during acorn development. We predicted that

nitrogen will be more important than carbon reserves for explaining variation in acorn production of *Q. lobata*. *Q. lobata* has higher water potentials than *Q. douglasii* during the summer (Knops & Koenig 1994, 2000), indicating less water limitation and therefore higher photosynthetic carbon gain (Hollinger 1992). If *Q. lobata* is not limited by carbon, then nutrients from the soil, such as nitrogen, are the next most likely to be limiting.

Methods

Study Site and Species

We studied a population of 36 *Q. lobata* and 23 *Q. douglasii* (Fig. 1) at Hastings Natural History Reservation in central coastal California, which is approximately 40 km inland, and ranges in elevation from 460 to 950 m. The climate is Mediterranean, with hot, dry summers and cool, wet winters. Annual precipitation is highly variable, ranging from 153 to 1131 mm between 1940 and 2012, with a mean (\pm SD) of 526 ± 192 mm. Habitat is mostly oak woodland and savannah, dominated by *Q. lobata*, *Q. douglasii*, and *Q. agrifolia*, with *Q. chrysolepis* and *Q. kelloggii* mixed in at higher elevations.

Both *Q. lobata* and *Q. douglasii* are winter-deciduous, monoecious trees that produce one-year acorns. Female flowers are typically pollinated during leaf-out in March, and acorns are typically ripe in September (Fig. 2). Trunk diameter at breast height (DBH) for *Q. lobata* ranged from 40 to 150 cm with a mean (\pm SD) of 89.1 ± 30.1 cm. *Q. douglasii* trunk DBH ranged from 30 to 130 cm, with a mean of 72.2 ± 24.6 cm. Pre-dawn xylem water potential in our population was measured in 1991 (Fig. 3) and

reported in a previous study (Knops & Koenig 1994). A follow up study demonstrated that water potentials of individuals were highly concordant among years (Knops & Koenig 2000).

Resources

To measure tree resources, we sampled non-structural carbohydrates (NSC) in trunks and branches, and nitrogen (N) in branches and leaves. NSC are a form of carbon reserves that consist of starch and soluble sugars such as glucose, fructose, and sucrose. NSC can be stored and remobilized for use in future biosynthesis (Mooney 1972; Chapin III, Schulze & Mooney 1990). We sampled wood from trunks using an increment borer and assayed the distal 3 cm of sapwood for NSC. From each tree, at each sample period, we harvested four branches from the crown using our hands or a telescoping tree pruner. Harvested branches consisted of two to four years of growth (estimated by bud scars). We harvested tissues in early June and mid-September in 2013 and 2014. In terms of acorn development, these sample dates cover the period of acorn development. In June, female flowers are already pollinated but still very small and in September they are full-sized. In September, if the tree had acorns, we harvested two branches with acorns and two without acorns. We always harvested samples in the afternoon, between 1300 and 1700 hours. After harvesting, we microwaved samples for 90 seconds at 600 watts to denature enzymes that could alter the carbohydrate chemistry of the plant tissues (Popp *et al.* 1996). Following the microwave shock treatment, we dried samples at 50 °C for five to seven days.

All of the plant samples for NSC or N assays were ground to a fine powder using a two-step process. First they were chopped up coarsely in a Wiley mill (Thomas Scientific, Swedesboro, New Jersey, USA) and then we crushed them in a ball mill (Cianflone 2601, Pittsburgh, Pennsylvania, USA). NSC were assayed following the same methods described in Chapters 1 and 2. Samples were extracted, fractionated, and digested with a series of enzymes, until they were measured colorimetrically in the final step. We calculated sample concentrations of starch and soluble sugars (glucose, fructose, and sucrose) using a standard curve and dilution factors. The sum of starch and soluble sugar concentrations yielded total NSC. We verified enzyme activity in every batch of samples using prepared standards of starch, glucose, fructose, and sucrose.

Branch and leaf samples were assayed for total N by the Ecosystem Analysis Lab at University of Nebraska – Lincoln, using combustion gas chromatography with a Costech Analytical ECS 4010 (Costech Analytical Technologies Inc., Valencia, California, USA).

Flowers and Acorns

We estimated flower crops by arbitrarily choosing two branches on each tree and counting all of the flowers and leaves. (Pearse *et al.* 2014). This provides a measure of flower density (flowers/leaves), which is a way to control for sampling different sized branches among trees. We counted flowers and leaves in the late spring, after pollination occurs, marked the sampled branches, and returned in fall of the same year to count acorns to determine acorn set (acorns/flowers). Within trees, data from both sampled

branches were pooled. On *Q. douglasii*, we counted flowers only in 2015. On *Q. lobata*, we counted flowers in 2013, 2014, and 2015.

To measure acorn production, two observers counted acorns on different parts each tree for 15 s. The two counts were summed for a total count per 30 seconds per tree. This method correlates closely with estimates from seed traps, but avoids underestimating due to acorn removal by avian seed predators (Koenig *et al.* 1994a). We counted acorns in all years of the study.

Statistical Analysis

We tested three predictions of the hypothesis that acorn production by *Q. lobata* and *Q. douglasii* is resource-limited. The first prediction was that resource concentrations during floral induction, in year $t - 1$, will have a positive effect on flowers in year t . For each species, we tested the effects of branch NSC, branch N, and leaf N, from both sampling dates, and the difference between the sampling dates (Δ resources: final – initial), for a total of nine statistical models for each species. We did not combine independent variables into the same models because of issues with collinearity, and we did not use a model selection approach because interpreting the results was straightforward. For *Q. lobata*, we used mixed effects generalized linear models (GLMMs) with Poisson distributed error structures and log link functions. In each model, we included a random effect of individual ID on the intercept in order to account for repeated sampling of the same individuals (Zuur *et al.* 2009). Models were overdispersed, so we also added an observation level random effect (OLRE) which had the effect of

correcting the overdispersion and making P -values more conservative (Harrison 2014). In each model, the dependent variable was the pooled number of flowers from both sampled branches of each tree in year t and the independent variable was the resource of interest in year $t - 1$. In each model we included an offset of the number of leaves from both sampled branches of each tree in year t , which effectively scales the number of flowers sampled on each tree to the number of leaves sampled on each tree (Pearse *et al.* 2015). For *Q. douglasii*, we only had one year of flower data, and thus we used generalized linear models (without random effects) with a quasi-Poisson distribution and log link function. Again, we included an offset of the number of leaves sampled on each tree. The dependent variable was the pooled number of flowers from both sampled branches of each tree in 2015 and the independent variable was the resource of interest in 2014.

Second, we tested the prediction that resources early in the growing season will predict acorn set (acorns/flowers) in the fall. We could only test this prediction in *Q. lobata* because we counted flowers in *Q. douglasii* in a year when we did not measure resources. For *Q. lobata*, we tested the effect of trunk NSC, branch NSC, branch N, and leaf N from the June sampling date on acorn set, which gives a total of four models. We used the same methods as for the first prediction: Poisson GLMMs with individual ID and OLRE as the random effects. The dependent variable was acorns pooled from the sampled branches for each tree, the independent variable was the resource of interest, and the offset was the pooled flowers from the sampled branches for each tree.

The third prediction we tested was that the change in resources during acorn development (Δ resources: final – initial) would relate negatively with acorn production. We used linear mixed effects models (LMMs) with individual ID as a random effect, to

test the effect of Δ trunk NSC, Δ branch NSC, Δ branch N, and Δ leaf N on ln-transformed acorn counts, which yielded four models for each species. We obtained P -values from Wald Type II chi-squared tests.

Some samples were lost during analysis, so actual sample sizes for analyses are reported in the results. All analyses were performed with R version 3.3.3 (R Core Team 2017). We fit GLMMs and LMMs using the ‘lme4’ package, version 1.1-12 (Bates *et al.* 2015). We performed Wald Type II chi-squared tests using the ‘car’ package, version 2.1-4 (Fox & Weisberg 2011).

Results

Acorn production

Mean annual ln-transformed acorn production (\pm SD) for our study population of *Q. lobata* from 1980 to 2014 was 1.94 ± 1.18 , with a range of 0.13 to 4.24. In 2013 mean ln-transformed acorn production for *Q. lobata* was 1.67, and in 2014 it was 1.90. Thus, the two years in which we measured resources were close to average in terms of acorn production.

For our study population of *Q. douglasii*, mean annual ln-transformed acorn production (\pm SD) from 1980 to 2014 was 1.88 ± 0.96 , with a range of 0.28 to 3.81. In 2013 mean ln-transformed acorn production for *Q. douglasii* was 1.25, and in 2014 it was 0.73. Thus, the two years in which we measured resources had below average and low acorn production, respectively.

Prediction 1: Resources in during flower initiation (year $t - 1$) will have a positive effect on the number of flowers in year t

In *Q. lobata*, Δ leaf N had a significant positive effect on the relative number of flowers ($n = 62$, $Z = 3.87$, $P < 0.001$; Fig. 4). 80% of the values for Δ leaf N were negative, so *Q. lobata* trees that produced more flowers retained more N in their leaves during the previous summer, when flower initiation starts in buds. No other models of *Q. lobata* flower production had significant effects (Table 1). Likewise, we found no effect of resources on flower production in the drought stress tolerant *Q. douglasii* (Table 2).

Prediction 2: Resources in the spring (after flowers are pollinated, but before they start developing into acorns) will have a positive effect on acorn set (acorns/flowers)

We found no significant effects of resources during the spring on acorn set in *Q. lobata* (Table 3).

Prediction 3: Δ resources (the change in resources from June to September) will vary negatively with acorn production

In *Q. lobata*, Δ branch N had a negative effect on ln-transformed acorn counts ($n = 61$, $\chi^2 = 4.73$, $P = 0.03$; Fig. 5a). 70% of values for Δ branch N were negative, so trees that produced more acorns also experienced more depletion in branch N during the

summer, when acorns were developing. No other resources had significant effects on ln-transformed acorn counts (Table 4).

In *Q. douglasii*, Δ branch NSC had a negative effect on ln-transformed acorn counts ($N = 21$, $\chi^2 = 7.37$, $P = 0.007$; Fig 5b). All but one of the values for Δ branch NSC were positive, so trees that produced fewer acorns accumulated more NSC in branches during the growing season. No other resources had significant effects on ln-transformed acorn counts (Table 4).

Discussion

Our results suggest that different resources are important for these two closely related oak trees with different water use strategies. In drought avoiding *Q. lobata*, the change in leaf N during flower initiation had a positive effect on flowering, and the change in branch N during acorn maturation varied negatively with acorn production. In the drought tolerant *Q. douglasii*, there was no effect of the resources we measured on flowering, but the change in branch NSC during acorn maturation varied negatively with acorn production. Collectively, this evidence suggests that N and NSC are important for explaining variation in acorn production for *Q. lobata* and *Q. douglasii*, respectively. Furthermore, there is support for some predictions of the hypothesis that acorn production in these two species is resource limited.

Based on what we know about the water relations of these trees, the differences in the key nutrients for reproduction is perhaps unsurprising. *Q. douglasii* reaches very low water potentials during the summer (Knops & Koenig 1994, 2000), which is likely to

limit rates of carbon assimilation (Xu & Baldocchi 2003). There is likely to be a cost to other functions for *Q. douglasii* to develop large crops of acorns during this period of relative carbon famine. Our data suggest that one cost may be to branch carbon reserves. This is an unusual result for masting trees, as only two prior studies have found effects of fruiting on carbon reserves (Miyazaki *et al.* 2002; Rapp & Crone 2015).

Q. lobata does not reach such low water potentials as *Q. douglasii* (Knops & Koenig 1994, 2000), and thus is not likely to experience the same constraints on carbon assimilation during the dry months. Therefore, the next most likely limiting factors are nutrients from the soil. Indeed, soil N was important for flowering and fruiting in several other temperate masting tree species (Han, Kabeya & Hoch 2011; Smaill *et al.* 2011; Sala *et al.* 2012; Miyazaki *et al.* 2014; Bogdziewicz *et al.* 2016). There is a general association of *Q. lobata* with fertile soils (Jepson 1910), but that is unlikely to driving the species differences we observed, as a previous study at our site found no difference in soil fertility between *Q. lobata* and *Q. douglasii* (Knops & Koenig 1997). The same study did, however, find that *Q. douglasii* retranslocated more N from its leaves before abscission in the fall than did *Q. lobata*. Consequently, differences in limiting nutrients between our two study species are likely to be driven by functional traits such as water use efficiency and nitrogen use efficiency, rather than differences in habitat.

Although we found support for predictions of the hypothesis that acorn production in these species is resource limited, we were unable to conclusively demonstrate resource limitation for several reasons. For one, we did not manipulate resource availability. Previous studies on masting trees have successfully increased seed production by adding fertilizer to the soil (Smaill *et al.* 2011; Bogdziewicz *et al.* 2016),

but it would be difficult to manipulate these large trees' carbon budgets. Furthermore, we are unable to reveal anything about the longer term dynamics of the resources we measured. The effects we observed could be transient during the dry growing season, and resource levels may rebound when temperatures cool and rain returns in the fall. Lastly, a mast year did not occur during our study period. Although, we had some individuals with very high seed production, resource allocation patterns may be qualitatively different in years where most individuals have large seed crops.

We found that water potential correlated with some of the resources we measured, but in unexpected ways. First, the resources that most correlated with water potential in each species were reversed compared to the patterns observed for acorn production. NSC correlated with water potential in *Q. lobata* and N correlated with water potential in *Q. douglasii*. Moreover, the direction of the correlation was surprising: drier plants had more resources. This is intriguing, especially in light of previous research that showed that water potential correlated positively with acorn production in both of our study species (Barringer, Koenig & Knops 2013). All things considered, trees with less access to water may be generally more conservative with their resources than trees with greater access to water. Alternatively, resources may build up in trees with less access to water due to growth ceasing in response to drought stress faster than photosynthesis does (Muller *et al.* 2011).

In conclusion, we found support for patterns predicted by the hypothesis that seed production is resource-limited in masting plants. We also found that two closely related plants living in the same habitat contrasted in which resources were most relevant for reproduction. This suggests that in addition to the abiotic environment, species functional

traits are important for determining which resources are most relevant for limiting reproduction in masting species. This is germane for future research on proximate mechanisms for masting because it implies that there may not be a widespread evolutionarily conserved mechanism for masting. Even between these two sympatric and closely related taxa, we found evidence for differences in resource limitation, which is hypothesized to be a fundamental part of the mechanism driving masting (Sork *et al.* 1993; Kelly & Sork 2002; Crone & Rapp 2014; Pearse *et al.* 2016).

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Tables

Table 1. Model results for prediction 1 in *Q. lobata* for each resource analyzed. $b \pm SE$ is the unstandardized regression coefficient \pm standard error.

Resource	$b \pm SE$	z	P
Δ Branch NSC	-0.04 ± 0.22	-0.16	0.87
Δ Leaf N	2.93 ± 0.76	3.87	0.0001
Δ Branch N	-0.27 ± 1.63	-0.16	0.87

Table 2. Model results for prediction 1 in *Q. douglasii* for each resource analyzed. $b \pm SE$ is the unstandardized regression coefficient \pm standard error.

Resource	$b \pm SE$	z	P
Δ Branch NSC	-0.72 ± 0.31	-2.31	0.05
Δ Leaf N	-2.69 ± 1.52	-1.77	0.09
Δ Branch N	2.00 ± 2.20	0.91	0.37

Table 3. Model results for prediction 2 in *Q. lobata* for each resource analyzed. $b \pm$ standard error is the unstandardized regression coefficient \pm standard error.

Resource	$b \pm SE$	z	P
Δ Trunk NSC	-0.02 ± 0.06	-0.33	0.74
Δ Branch NSC	-0.16 ± 0.33	-0.48	0.63
Δ Leaf N	-1.17 ± 1.1	-1.16	0.25
Δ Branch N	2.06 ± 2.07	1.00	0.32

Table 4. Model results for prediction 3 in *Q. lobata* and *Q. douglasii* for each resource analyzed. $b \pm$ standard error is the unstandardized regression coefficient \pm standard error.

Species	Resource	$b \pm$ SE	χ^2	P
<i>Q. lobata</i>	Δ Trunk NSC	-0.06 ± 0.06	0.97	0.33
	Δ Branch NSC	0.10 ± 0.25	0.17	0.68
	Δ Leaf N	0.09 ± 0.84	0.01	0.92
	Δ Branch N	-4.10 ± 1.89	4.73	0.03
<i>Q. douglasii</i>	Δ Trunk NSC	0.12 ± 0.09	1.78	0.18
	Δ Branch NSC	-0.48 ± 0.18	7.37	0.007
	Δ Leaf N	-1.34 ± 1.63	0.68	0.41
	Δ Branch N	0.52 ± 0.63	0.67	0.41

Figures



Figure 1. Left, *Q. lobata* leaves and acorns in September. Right, *Q. douglasii* leaves and an acorn in early development, center, in June.

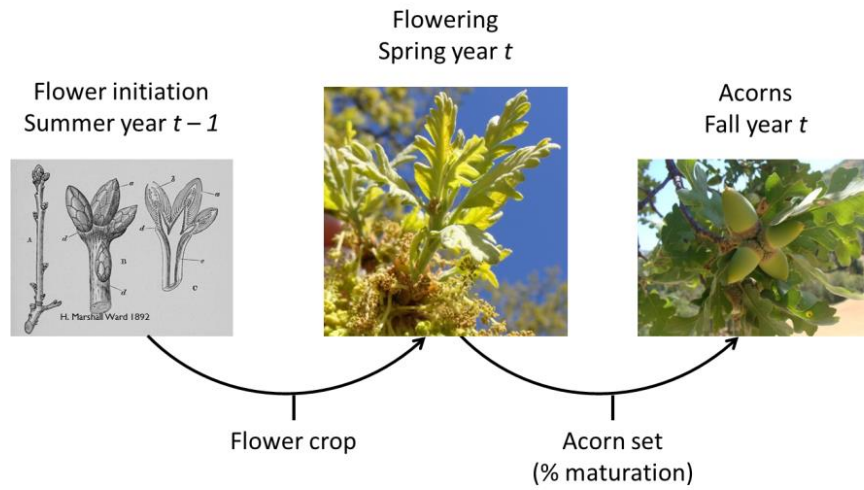


Figure 2. Reproductive phenology of *Q. lobata* and *Q. douglasii*, and potential stages at which acorn production can be limited.

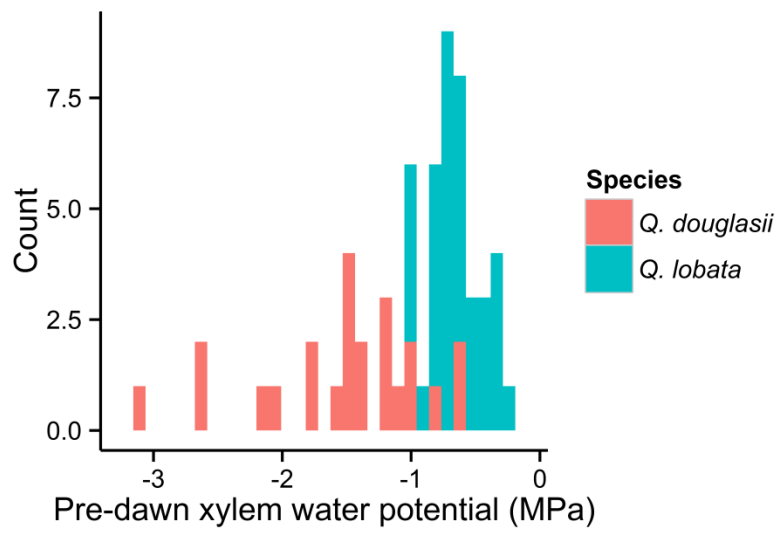


Figure 3. Histogram of pre-dawn xylem water potential of *Q. douglasii* (red) and *Q. lobata* (blue) in our study population (data from Knops and Koenig 2000).

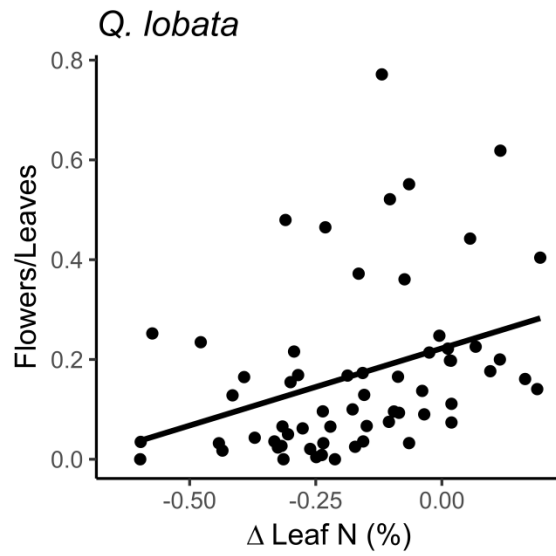


Figure 4. Relationship between the change in leaf nitrogen concentration from June to September and relative flower production in sampled branches of *Q. lobata*.

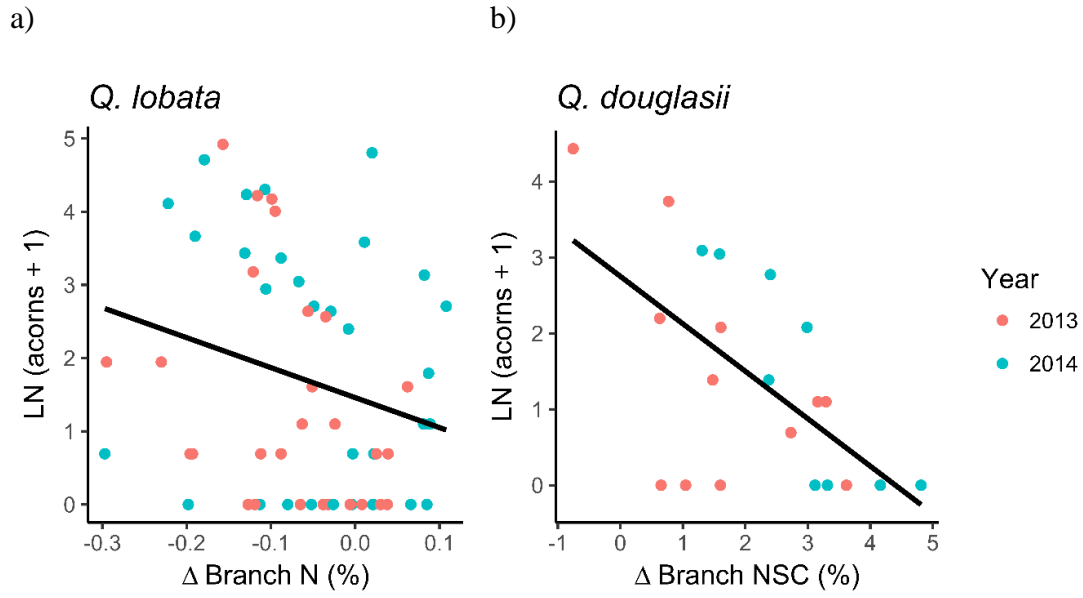


Figure 5. Relationship between the change from June to September in either branch nitrogen concentration (a; *Q. lobata*) or branch non-structural carbohydrates concentration (b; *Q. douglasii*) and natural log-transformed acorn counts. Points are colored according to year of sampling.