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Nonstructural carbohydrate dynamics' relationship to leaf development under varying environments

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Summary

- Leaf-out in temperate forests is a critical transition point each spring and advancing with global change. The mechanism linking phenological variation to external cues is poorly understood. Nonstructural carbohydrate (NSC) availability may be key.
- Here, we use branch cuttings from northern red oak (*Quercus rubra*) and measure NSCs throughout bud development in branch tissue. Given genes and environment influence phenology, we placed branches in an arrayed factorial experiment (three temperatures × two photoperiods, eight genotypes) to examine their impact on variation in leaf-out timing and corresponding NSCs.
- Despite significant differences in leaf-out timing between treatments, NSC patterns were much more consistent, with all treatments and genotypes displaying similar NSC concentrations across phenophases. Notably, the moderate and hot temperature treatments reached the same NSC concentrations and phenophases at the same growing degree days (GDD), but 20 calendar days apart, while the cold treatment achieved only half the GDD of the other two.
- Our results suggest that NSCs are coordinated with leaf-out and could act as a molecular clock, signaling to cells the passage of time and triggering leaf development to begin. This link between NSCs and budburst is critical for improving predictions of phenological timing.

Introduction

Phenology, or the timing of recurring life events in organisms, is a locally adapted trait in many species and is being altered by climate change (Parmesan & Yohe, 2003; Visser & Both, 2005; Cook *et al.*, 2012). Over the past century, the phenological timing of leaf-out in plants has advanced 2.8 ± 0.35 d per decade in response to global warming (IPCC, 2014). This early onset of spring creates feedbacks with the climate system, particularly in temperate deciduous forest tree species. Spring leaf-out timing is both altered by warming temperatures and alters global CO_2 and H_2O fluxes via the onset of leaf gas exchange as well as land surface albedo (Schwartz *et al.*, 2006; Penuelas *et al.*, 2009; Richardson *et al.*, 2013). Thus, predicting whether temperate tree leaf-out continues to advance in spring is critical for understanding the degree to which the globe will warm over the next century.

Variation in phenological timing is comprised of both genetic and plastic (environmental) contributions. Plastic variation is key for short-term (within one generation) response of individuals (Valladares *et al.*, 2006; Wortemann *et al.*, 2011; Anderson *et al.*, 2012; Des Marais *et al.*, 2013), whereas genetic variation is central to the long-term, adaptive evolution of populations (Davis *et al.*, 2005; Aitken *et al.*, 2008). The environmental drivers of variation in leaf-out in spring have been well characterized; the length of winter dormancy (chilling), accumulated spring temperatures (warming), and the length of day (photoperiod) are all important and interact (Körner & Basler, 2010; Polgar & Primack, 2011; Cook *et al.*, 2012; Laube *et al.*, 2014). In addition, phenological leaf traits tend to be highly heritable (Alberto *et al.*, 2011; McKown *et al.*, 2014; De Kort *et al.*, 2016), meaning that phenological traits can be passed onto offspring, and thus, natural selection can act to shape the trait over generations. As a result, phenological traits are typically locally adapted, even in wind-pollinated tree species, indicating that some populations are genetically predisposed to leafing out earlier than others depending on their recent evolutionary past, even when grown in the same environment (Hall *et al.*, 2007; Mimura & Aitken, 2010; Keller *et al.*, 2011; Kreyling *et al.*, 2014). Finally, genes and environment can also interact (Des Marais *et al.*, 2013), leading species (e.g. Flynn & Wolkovich, 2018), populations (e.g. De Kort *et al.*, 2016), and individuals (e.g. Cooper *et al.*, 2019) to have different sensitivities to these environmental cues. All three components of trait variation, genetics, environment, and their interaction, are key for predicting how plants and plant populations will – or will not – be able to alter their traits in response to future change.

While genes and environment influence the timing of leaf-out, the exact mechanisms that link phenological variation to external

cues are poorly understood. A recent hypothesis suggests that nonstructural carbohydrate (NSC) availability and concentration may be this key missing piece and play a role in regulating plants' phenological clocks (Klein *et al.*, 2016; Sperling *et al.*, 2017; Tixier *et al.*, 2019). Nonstructural carbohydrates exist as either soluble sugars (largely glucose, fructose, and sucrose) or insoluble starches (Hoch *et al.*, 2003) and readily convert between the two categories on daily (Tixier *et al.*, 2018) and seasonal cycles (Martínez-Vilalta *et al.*, 2016; Furze *et al.*, 2019), and in response to environmental variability (O'Brien *et al.*, 2014; He *et al.*, 2020). Nonstructural carbohydrates are found in the parenchyma cells embedded throughout woody tissues, particularly the xylem (Chapin *et al.*, 1990). They are stored in the order of days to decades and are used in a variety of ways, including to support metabolism when photosynthesis is inhibited (e.g. at night or during dormancy) and are the source of energy and carbon for new leaf development in spring (reviewed in Chapin *et al.*, 1990; Hartmann & Trumbore, 2016).

Recent work on NSC concentrations regarding phenology reveals tight coordination between the two. Observational studies demonstrate correlations between growth, NSC concentrations in branch tissue, and phenological timing in spring (Klein *et al.*, 2016; Tixier *et al.*, 2017; Loiez & Piper, 2022). Carbon used to form leaf tissue in the early stages of development comes from previous seasons' stores (Gaudinski *et al.*, 2009) and the majority of these stores are likely located in the branch adjacent to the bud, rather than having been overwintered in the bud itself (Liu *et al.*, 1999; Klein *et al.*, 2016; Tixier *et al.*, 2017). Interestingly, when these branch NSC concentrations are experimentally manipulated before dormancy, either through defoliation or girdling, leaf-out is delayed (Roxas *et al.*, 2021). One proposed mechanism for the link between NSC and phenology is that NSC may act as a molecular clock (Tixier *et al.*, 2019). Because starch synthesis and degradation enzymes have different temperature sensitivities, the activity levels of enzymes catalyzing starch degradation vs synthesis reverse when a temperature threshold is reached (suggested to be *c.* 5°C; Pollock & Lloyd, 1987), thereby signaling the end of dormancy (Zwieniecki *et al.*, 2015).

Altogether, this recent evidence demonstrates that the amount of NSC (Rosas *et al.*, 2013; Klein *et al.*, 2016; Loiez & Piper, 2022) and the percentage that is soluble (Gibon *et al.*, 2009; Tixier *et al.*, 2018) – particularly in branch tissue proximal to buds – are likely key factors in determining the onset and release of dormancy. While recent studies have done much work to highlight patterns *in situ*, none have yet examined how NSCs vary with phenology under controlled, experimental conditions, thus limiting our ability to model what specific environmental cues are instrumental in initiating the phenological transition. This is a key step in understanding these patterns and eventually unraveling the mechanisms underlying leaf development in spring, as both genotype and environment may influence NSC concentrations (Blumstein *et al.*, 2020; Blumstein & Hopkins, 2021) and phenological timing (Cooper *et al.*, 2019), although more work is needed. Here, we use experimental manipulation of twigs post-bud set in the ring-porous species northern red oak (*Quercus rubra L.*) to examine how sugars and starches vary across genotype, environment, and time, and to test whether the observed patterns are consistent with the hypothesis that differential metabolism of starch may be driving dormancy release.

Red oak is an ideal study species as it is one of the most abundant forest tree species across its broad eastern North America range by biomass and has been increasing in volume over the past decade (US Forest Service, 2019). As a result, red oak is a key driver of carbon sequestration in eastern forests (Finzi *et al.*, 2020). Twigs were placed in two different photoperiods and three spring warming treatments (six treatments total) and came from eight genotypes of known provenance (spanning a latitudinal transect from Alabama to Massachusetts) growing in a common garden (The Arnold Arboretum of Harvard University in Boston, Massachusetts). We measured sugars (bulk glucose, fructose, and sucrose) and starches in twig tissue proximal to the terminal bud to investigate how carbohydrates varied from dormancy to full leaf-out. We hypothesized:

(**1**) If the timing of leaf-out phenology is adaptive, as has been highlighted by many prior studies (e.g. Alberto *et al.*, 2011; Anderson *et al.*, 2012; De Kort *et al.*, 2016), we expect to find heritable variation in the timing of leaf-out and for this timing to correspond to environmental gradients of temperature and photoperiod.

(**2**) If the amount of NSC in sugar vs starch in spring is governed by enzyme sensitivity to temperature, then we predict each treatment, on average, to reach the same concentrations at the same number of accumulated GDD.

(**3**) If phenology is heritable (i.e. genotypes genetically differ in their timing) and phenological timing is correlated with GDD and starch concentrations, then the molecular clock hypothesis predicts that we will also observe heritable variation in NSC concentrations within each treatment and phenophase, particularly in the transition from dormancy to phase 1.

Because phenology is heritable, the driver of phenological timing must also be heritable. Thus, we would expect heritable differences within the treatment of sugar and starch concentrations if differential enzyme sensitives are driving dormancy release.

Materials and Methods

Experimental design

We placed terminal branches (accessioning described below) of northern red oak (*Quercus rubra L.*) into BioChambers FXC-9 plant growth chambers (BioChambers, Winnipeg, MB, Canada) at six different treatments: three temperature and two photoperiod treatments. Severed branches are frequently used for phenological studies (e.g. Heide, 1993; Caffarra & Donnelly, 2011; Basler & Körner, 2012; Flynn & Wolkovich, 2018), and their phenology closely follows their parent tree when kept in similar conditions to a greater extent than seedlings or saplings (Vitasse & Basler, 2014). The three temperature treatments were cold (4°C : 14°C, night : day), moderate (9°C : 20°C, night : day), and hot (14°C : 26°C, night : day). These temperatures ranges approximate the average for Massachusetts, where the study was conducted, from March to July and approximate a realistic range

of current to future warming for the spring (March–May). The two photoperiod treatments were 10 and 15 h days. The growth chambers have two isolated levels, which allow the daylength on the top level to be shorter than that on the bottom level. We set photoperiod timing so that mid-day aligned between all treatments. Because temperature is uniform in each chamber, we set temperatures to start cooling according to the long day schedule.

We collected all branch tissues for the experiment from the Arnold Arboretum in Boston, MA on 8 January 2022. We selected eight *Q. rubra* genotypes – or genetically distinct individuals – based on provenance records at the Arboretum, with provenances spanning *c.*10 degrees of latitude (Fig. 1). We cut *c.* 50 cm of terminal branch for each sample using a pole pruner. For each treatment, we collected six total terminal branches from each of the eight genotypes, one for each phenophase stage (1–5) that we planned to observe (described below), plus an additional individual in case of mortality. In total, each treatment had six replicates from each of eight genotypes, for a total of 48 branches. In addition, we collected a dormant terminal branch (stage 0) from each genotype which was immediately stored at −70°C for later NSC analysis.

During experimental setup, we cut branch ends underwater and placed them in 500 ml flasks, with four branches to a flask, each from a different genotype. We ensured that each terminal branch had no side branches and removed any side buds, leaving only the terminal buds (typically three to four in a bunch in *Q. rubra*). Each flask contained 400 ml of deionized (DI) water (Flynn & Wolkovich, 2018). Flasks were then randomized and evenly spaced within each treatment. Each week throughout the experiment, we re-cut each branch end underwater, changed the water, and re-randomized the flasks.

Phenology observations and tissue collection

We made phenology observations every other day throughout the experimental period at the mid-day point for the growth chambers. We scored plants on phenology scale from 1 to 5, following a scale used in genetic studies of plants (see Derory *et al.*, 2006; Fig. 1). When a genotype was observed to enter a new phenophase, we destructively harvested the tissue by removing the buds, clipping the outer 4 cm closest to the terminal buds, and flash-freezing this tissue on liquid Nitrogen before transferring the tissue to storage in a −70°C freezer. As a result of destructive harvesting, we have one replicate per phenophase per genotype for NSC analysis and have a decreasing number of replicates across phenophases for phenology observations. For example, all replicates reached stage 1, so we report six phenological observations per genotype per treatment at stage 1, five at stage 2, four at stage 3, and so forth, not accounting for mortality. Across the six treatments and 288 total branches, 40 branches never leafed out (1.4%).

NSC extraction

We freeze-dried frozen branches for 24 h before processing. Branches were first ground using a Wiley Mill, then further ground to a fine powder using a ball-beating machine (FastPrep24 Grinding and Lysis system). We then weighed samples out to an average of 40 mg ± 0.07 mg standard deviation and recorded the amount for each sample. We extracted and read bulk sugars (sucrose, fructose, and glucose) and starch following protocols from Landhausser *et al.* (2018; Protocols S1 and S5). In short, we extracted sugars by

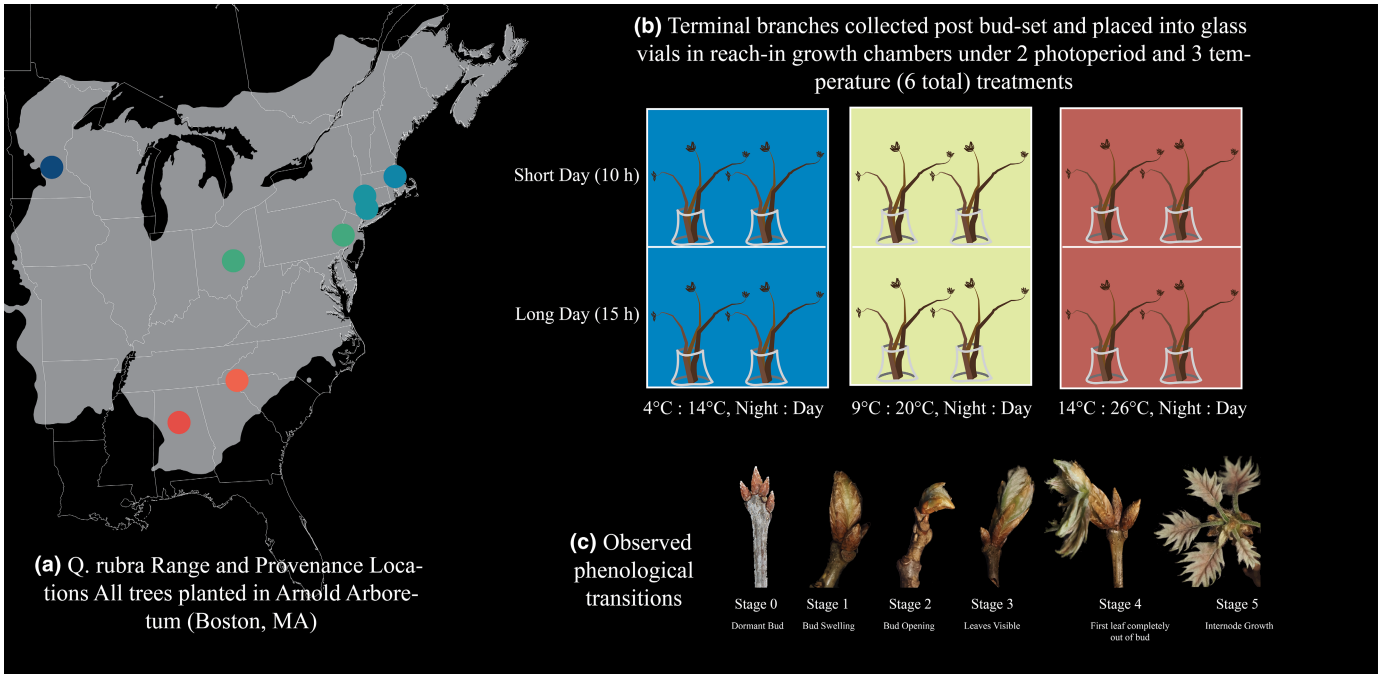

Fig. 1 Experimental design. The (a) provenance of the eight genotypes of *Quercus rubra* chosen from the Arnold Arboretum in Boston, MA for the experiment, the (b) experimental treatments, containing six replicates from each of eight genotypes ($n = 48$), and (c) the phenophases observed during the experiment. We included a total of eight genotypes in the experiment. In each treatment, we had six cuttings from each genotype, for a total of 48 cuttings in each treatment, as well as a stage 0 dormant cutting processed immediately after collection in January.

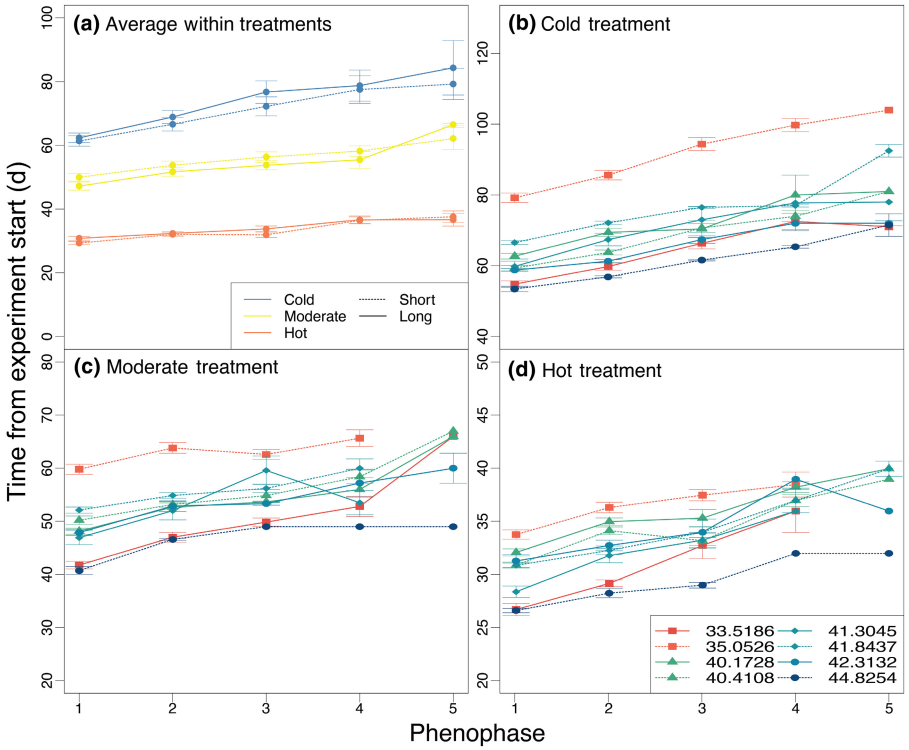

Fig. 2 Leaf development phenology of *Quercus rubra* exhibits clear patterns of heritability. (a) The average number of days individuals in each treatment took to reach each phenophase. The results are also broken down for each genotype (averaged across replicates) within the cold (b), moderate (c), and hot (d) treatments. Genotypes are colored by latitude of provenance and short and long day treatments are averaged together. Lines are connecting observations with standard error bars given for each phenophase. Due to destructive sampling, N at time point 1 is 12 for each genotype in each treatment and is reduced by 2 until $n = 4$; however, due to mortality, we had as few as $n = 0$ by stage 5. Data show clear differences across treatments and genotypes within treatments.

boiling tissues in 80% ethanol, then retaining the supernatant for reading using a colorimetric assay with 2% phenol and 95% sulfuric acid. We then digested starch into soluble glucose from the remaining pellet using the enzymes alpha-amylase and amyloglucosidase. We performed a colorimetric assay using peroxidase-glucose oxidase (PGO) and o-dianisidine dihydrochloride, then 75% sulfuric acid to stop the reaction. We read sugar absorbances at 495 nm and starch as glucose equivalents at 525 nm using a spectrophotometer (Thermo Scientific GENESYS 180 UV–Vis; Waltham, MA, USA). From these measurements, we calculated four metrics of NSC: the total amount of sugar in percent dry weight of tissue (% dw), the total amount of starch (% dw), the total NSC ((sugar + starch) % dw), and the percent starch (starch $\text{NSC}^{-1} \times 100$). Note that because we found no significant difference between photoperiod treatments (see Results section), we only processed short-day treatments for NSC.

Statistical analyses

We performed all statistical analyses in R v.4.1.3 (R Core Team, 2022). All linear models relied on the 'lm' function in base R; for ANOVAs, we used 'aov' in base R, and for linear mixed models, we used the packages LME4 v.1.1–29 (Bates *et al.*, 2015) for formulating the model and MuMIn v.1.46.0 (Barton, 2022) for extracting the R^2.

To examine how long each genotype took to reach each phenophase under each treatment, we used a linear mixed model as formulated in Eqn 1:

$$y_{ig} = \beta_0 + \beta_T X_T + \beta_S X_S + \beta_P X_P + \alpha_g + \varepsilon_{ig} \quad \text{Eqn 1}$$

where y is the number of days to reach each phenophase from the experiment's start on 8 January, i is individual, g is genotype, the betas are the fixed effects of temperature (T), phenophase/stage (S), and photoperiod (p) (Fig. 2). α is the random effect of genotype. In all cases, temperature is given by the daytime temperature of the treatment (14°C, 20°C, 26°C).

From Eqn 1, we can use the variance of the random effect of genotype (α_g) to calculate the broad-sense heritability of leaf-out timing in *Q. rubra* using the following equation:

$$H^2 = \sigma^2_{\text{Genotype}} / \left(\sigma^2_{\text{Genotype}} + \sigma^2_{\text{Microenvironment}} \right) \quad \text{Eqn 2}$$

where variation due to microenvironment is assumed to be the residual variation in the model, and variation due to genotype is the variance of the random effect of α_g.

Given that there are two important transitions in our data, the transition from dormancy to active leaf development and the transition from initial stages to the final stage of leaf development, we chose to examine how the NSC metrics (sugar, starch, NSC, and percent starch) vary by phenophase using two different linear models.

$$y_i = \beta_0 + \beta_S X_S + \varepsilon_i; \quad S = [0, 1] \quad \text{Eqn 3}$$

$$y_i = \beta_0 + \beta_S X_S + \varepsilon_i; \quad S = [1, 2, 3, 4, 5] \quad \text{Eqn 4}$$

Here, the betas are fixed effects, where S indicates phenophase. In model one, only samples measured from stage 0 (dormancy) to 1 are included. In the second formulation, only individuals from active development stages are included (1–5; Fig. 3).

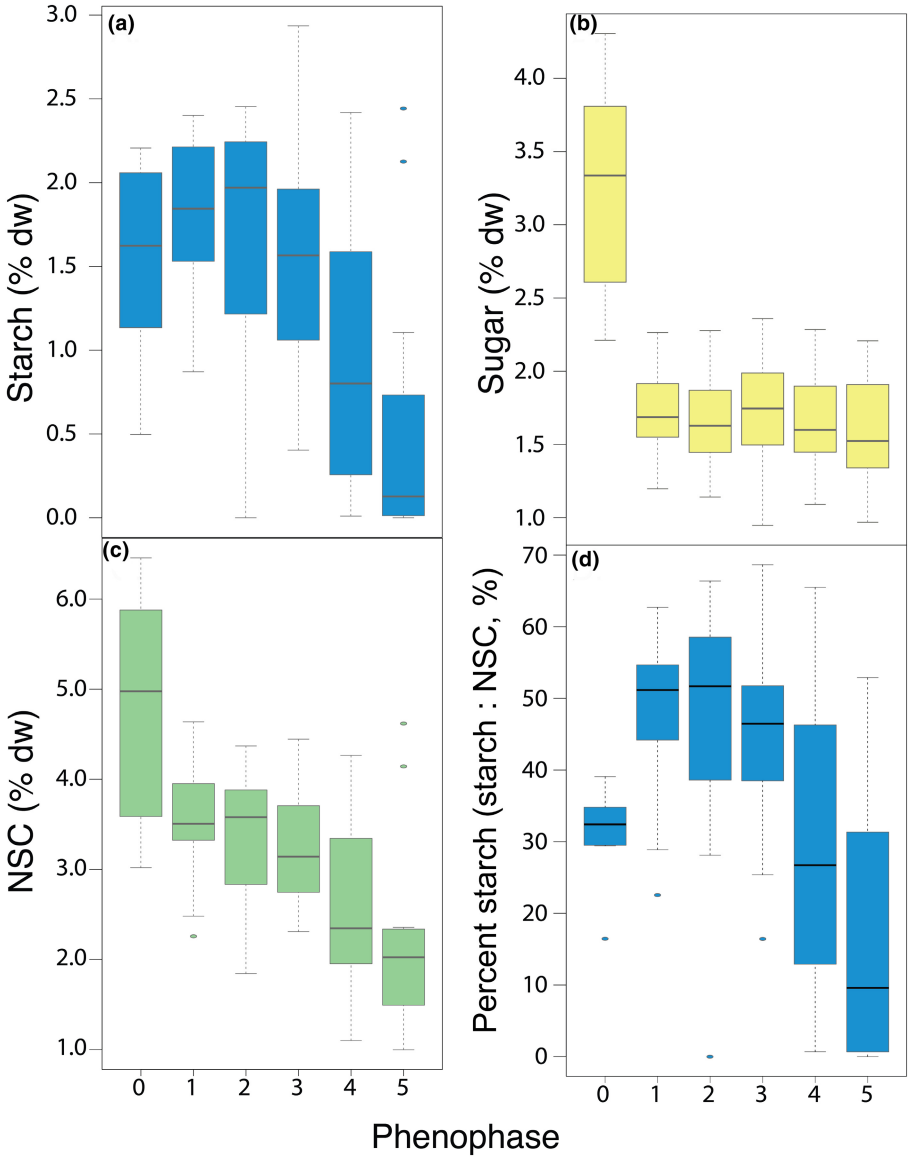

Fig. 3 We found consistent nonstructural carbohydrate (NSC) patterns as *Quercus rubra* leaves develop across treatments. All samples across treatments vs phenophase, broken down by the NSC metrics (a) starch, (b) sugar, (c) NSC, and (d) percent starch. Boxplots show median (middle dark line), 25th and 75th percentile (interquartile range (IQR) – upper and lower edge of boxes) and 1.5× IQR (upper and lower bars) Full results of linear models testing patterns found in S1. Outliers, represented as points, are 3× IQR or greater.

To test if our NSC metrics (sugar, starch, NSC, and percent starch) differ by temperature, we used an ANOVA of NSC metric vs temperature. As our dormant samples were not placed in treatments, they were excluded from this analysis (Supporting Information Fig. S1; Table S1). We further broke down our analysis and performed ANOVAs comparing our NSC metrics by temperature for each individual phenophase (1–5; Fig. S2; Table S2).

In addition to our ANOVAs, we also used linear models to compare our NSC metrics to the average number of GDD attained at each phenophase by treatment to understand how trends varied temporally (Fig. 4). Growing degree days were calculated at the cumulative sum of the average daily temperature of minus 5°C, the hypothesized threshold at which starch synthesis/degradation enzyme activity switches (Pollock & Lloyd, 1987). Similar to our phenophase comparisons, we use a two-step approach, comparing phenophases 0–1, and then phenophases 1–5. However, because phenophase 0 samples were not put into treatments (they are the dormant phase collected on the first day of the experiment), Eqn 5 omits temperature.

$$y_i = \beta_0 + \beta_N X_N + \varepsilon_i; S = [0, 1] \quad \text{Eqn 5}$$

$$y_i = \beta_0 + \beta_N X_N + \beta_T X_T + \varepsilon_i; S = [1, 2, 3, 4, 5] \quad \text{Eqn 6}$$

where y is the response NSC metric, the betas are fixed effects of number of days to each phenophase (N) and the temperature treatment (as a factor; T) given by name (cold, moderate, and hot). We treat treatment, here, as a factor and perform a Tukey *post hoc* test on temperature treatment to determine whether the three treatments differ on average from each other in relation to GDD (Table S4, see later).

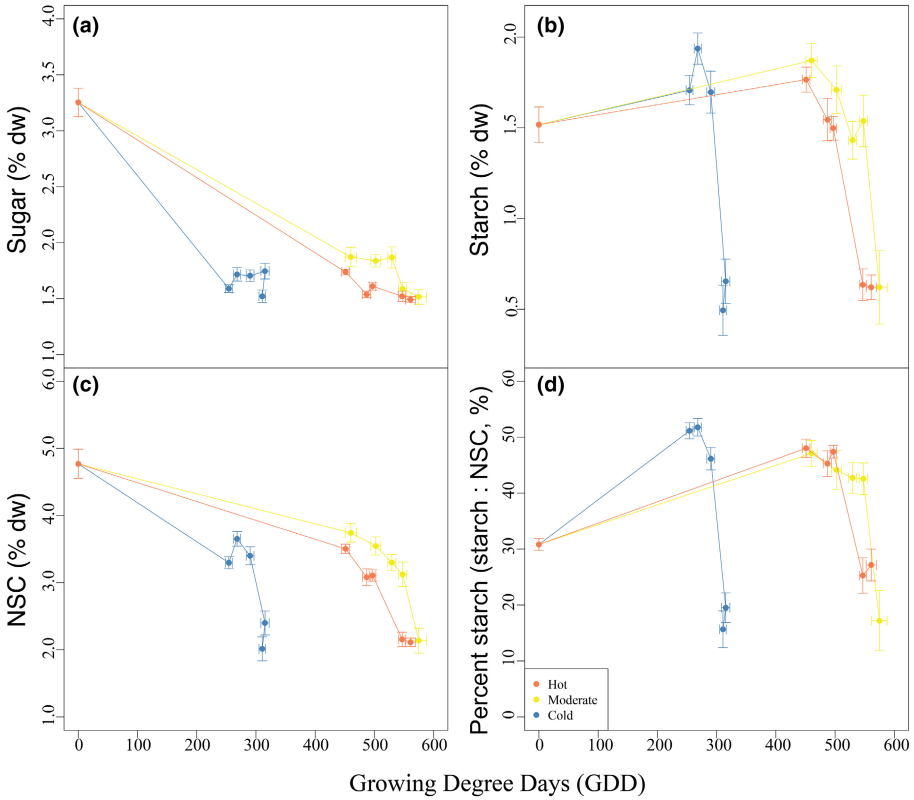

Fig. 4 All three temperature treatments follow similar patterns of nonstructural carbohydrate (NSC), but *Quercus rubra* leaf development is initiated at different growing degree days (GDD) in each treatment. The mean of each NSC metric; (a) sugar, (b) starch, (c) NSC, and (d) percent starch vs the, calculated from the start of the experiment. Each point is the mean of one of the six phenophases (0–5), with standard error bars given for both NSC metric (vertical) and GDD date (horizontal).

Finally, we tested how our NSC metrics varied within each phenophase by calculating their heritability using the following equation:

$$y_{ig} = \beta_0 + \alpha_g + \varepsilon_{ig} \qquad \text{Eqn 7}$$

where α_g is the random effect of genotype. Using the results of Eqn 7, we were able to calculate the broad-sense heritability (H^2, Eqn 2) of each NSC metric in relation to phenophase.

Results

Phenological patterns

Our model examining the number of days as a function of temperature treatment, photoperiod treatment, phenophase, and given the random effect of genotype was significant ($P < 0.001$), with a conditional R^2 of 0.93 (Fig. 2). Temperature treatment and phenophase were significant as main effects (Temperature: $m = -3.10$, $P < 0.001$; Phenophase: $m = 3.48$, $P < 0.001$), but the treatment of photoperiod was not ($m = 0.12$, $P = 0.312$). Using the random effects of genotype and residual variance of the model, we calculated the broad-sense heritability (H^2) of leaf-out progression in *Q. rubra* to be 0.59. This indicates that 59% of variation measured in our model can be attributed to differences between genotypes and that this variation can likely be inherited by offspring.

NSC patterns

Across all treatments and genotypes, NSCs vary significantly in twigs during the transition from dormancy to phenophase 1 (i.e. short description of phenophase 1; Table S3; Fig. 3). Both sugar and total NSC significantly decrease (sugar: $m = -1.53$, $P < 0.001$; NSC $m = -1.25$, $P < 0.001$), while percent of NSC comprised by starch increases significantly ($m = 18.62$, $P < 0.001$). However, there is no significant change in raw starch.

Moving from phenophase 1 through 5, total starch, percent starch, and NSC significantly decrease (Starch: $m = -0.32$, $P < 0.001$; percent starch: $m = -7.8$, $P < 0.001$; NSC: $m = -1.25$, $P < 0.001$; Table S3; Fig. 3). Conversely, there is no significant change in the amount of sugar following the transition from dormancy ($m = -0.03$, $P = 0.288$).

When we stratified the data by temperature treatment and further examined it against the GDD achieved by each phenophase (rather than by phenophase itself), we found that the moderate (9°C : 20°C, night : day) and hot (14°C : 26°C, night : day) treatments follow similar patterns with regard to GDD and NSC, while the cold treatments (4°C :14°C, night : day) transitioned between stages at almost half the GDD of the other two. Sugar and NSC significantly decrease between dormancy (phenophase 0) and phenophase 1 (Sugar: $m = -0.003$, $p \leq 0.001$; NSC: $m = -0.002$, $P = 0.014$), while percent starch significantly increases ($m = 0.033$, $P = 0.001$) and raw starch remains the same ($m \leq 0.001$, $P = 0.126$; Fig. 4; Table S4).

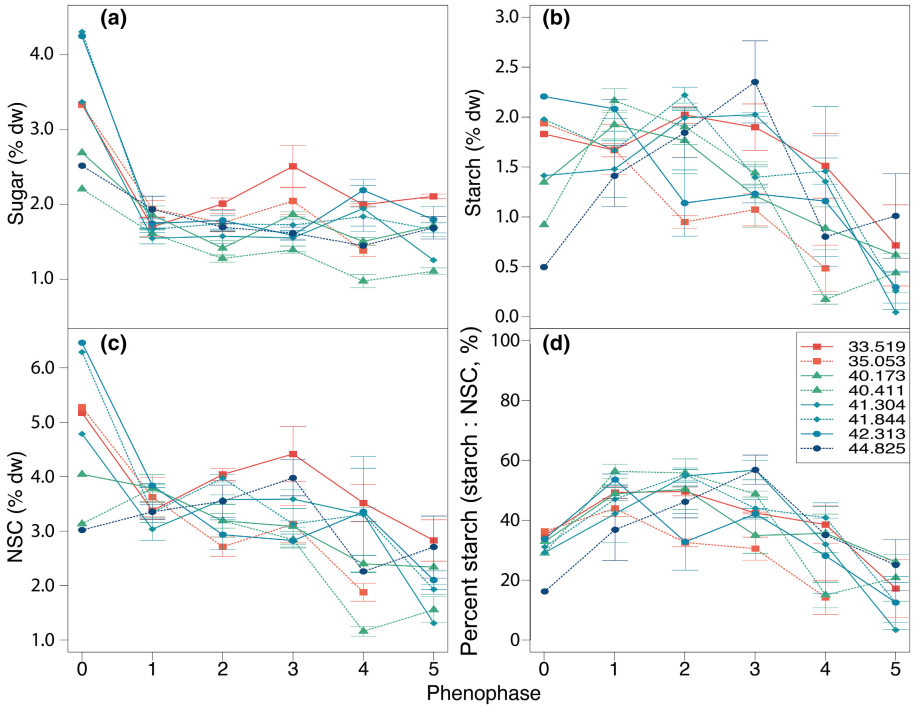

Fig. 5 While *Quercus rubra* phenology is heritable, genotypic level patterns in nonstructural carbohydrate (NSC), are less clear. NSC metric (a) sugar, (b) starch, (c) TSC, and (d) percent starch by phenophases. Each line is a genotype, colored by latitude, with standard error bars across replicates. Data demonstrate while some genotypes differ from others in their NSC amounts at any given phenophase, there are no clear discernable patterns in NSC heritability across phenophases or treatments.

Across the leaf development phenophases (1–5), all measured carbohydrates except sugar significantly decrease as phenophases progress and GDD increases (Sugar: $m \leq 0.001$, $P = 0.166$; Starch: $m = -0.008$, $P \leq 0.001$; NSC: $m = -0.009$, $P \leq 0.001$; percent starch: $m = -0.166$, $P \leq 0.001$; Fig. 4; Table S4). Interestingly, in all cases where an NSC metric varied significantly with GDD (i.e. not sugar), a Tukey *post hoc* test revealed that the cold treatments significantly differed from the moderate and hot treatments, respectively (Table S4). Conversely, the moderate and hot treatments did not significantly differ from each other, except by a modest amount for TNC (moderate–hot contrast: −0.52, $P = 0.025$; Table S4). This analysis demonstrates that while each treatment follows a similar pattern across phenophases in relation to GDD, the cold treatments begin advancing through phenophases and depleting reserves at a much lower GDD than the other two treatments, which begin advancing through phenophases at approximately the same GDD.

We further examined patterns within phenophases by genotype, treating the three temperature treatments as replicates (Eqn 7). Within phenophases, the amount of variation explained by genotype varies widely (Table S5; Fig. 5). At phase 0, the amount of sugar and total amount of NSC is heritable, that is these traits differ significantly among genotypes, while the amount of starch and thus percentage NSC comprised of starch does not (H^2_{sugar}: 0.76 & H^2_{TNC}: 0.45 vs H^2_{starch} & $H^2_{\text{Per.Starch}} = 0$; Table S5). By stage 1, there are no heritable differences between genotypes, indicating that all individuals are behaving similarly with respect to NSC consumption by the transition to phenophase 1 (Table S5; Fig. 5). As phenophases progress, heritable variation fluctuates, with genotype explaining more variance in starch in phases 2 and 3 (Phase 2 $H^2_{\text{starch}} = 0.18$, Phase 3 $H^2_{\text{starch}} = 0.24$), while variation in sugars is not explained by genotype again until stages 4 and 5 (Phase 4 $H^2_{\text{sugar}} = 0.47$, Phase 5 $H^2_{\text{sugar}} = 0.63$; Table S5; Fig. 5).

Discussion

Here, we tested three related hypotheses seeking to better understand the relationship between stored carbohydrates and spring leaf development in a temperate deciduous species. Using excised branch cuttings from mature trees and a factorial of warming and photoperiod treatments in controlled growth chambers, we observed five stages of leaf development and concurrently measured NSC in the proximal branch tissue. Critically, we conducted this experiment with eight genotypes with genetic replicates in each treatment and phenophase, allowing us to test whether patterns in phenology and NSC are inherited, a crucial baseline for the carbohydrate signaling hypothesis to be true.

Hypothesis 1: *If the timing of leaf-out phenology is adaptive, we expect to find heritable variation in the timing of leaf-out and for this timing to correspond to environmental gradients of temperature and photoperiod.*

We examined how the timing of *Q. rubra* bud development in spring is impacted by genotype and environment, using a factorial experiment comprising two photoperiod and three temperature treatments (Fig. 1). Overall, plants in the different temperature treatments developed as expected, with plants in the coldest treatment leafing out the latest and those in the hottest treatment leafing out the earliest (Fig. 2). Somewhat surprisingly, photoperiod did not significantly impact bud phenology, despite another study conducted using cuttings sourced from the Arnold Arboretum finding it to be an important factor in red oak (Flynn

& Wolkovich, 2018). This could be the result of differences between years in accumulated warming and chilling during dormancy *in situ* before twig collection, which may have downstream effects on bud development timing (Laube *et al.*, 2014; Flynn & Wolkovich, 2018).

Within treatments, variation among individuals is partly explained by genotype (Fig. 2). This observation of high heritability in spring bud phenology is consistent with previous studies that reported high heritability in phenological traits in plants within common gardens (Alberto *et al.*, 2011; McKown *et al.*, 2014; De Kort *et al.*, 2016). This is critical as a high proportion of the total variance can be inherited by offspring and thus shaped by natural selection. Contrary to expectations, however, the provenance patterns in bud development do not consistently follow latitude of origin. While most of the genotypes fall as we would expect, with genotypes originating from latitudes closer to the equator leafing out earlier relative to genotype originating further north, two genotypes stand as outliers to this; the one from North Carolina (leafs out much later than we would expect) and the one from Minnesota (leafs out much earlier). This mismatch could be due to the fact that all of the individuals included in this study were collected as seed, not cuttings, before being planted out in the Arnold Arboretum. Thus, individuals may not actually be locally adapted to their environment of origin (i.e. they may not have established had they germinated at the place of origin). There may also be maternal effects on phenology that is passed onto offspring (Rohde & Junttila, 2008); however, our observation that only two of the eight genotypes studied herein do not follow the expected latitudinal cline suggests that maternal effects are not pervasive for this trait. Finally, the genotype from North Carolina comes from a southerly latitude, but at a higher elevation (greater than 1250 m), thus explaining, in part, why it leafs out much later than we might expect.

Hypothesis 2: *If the amount of NSC in sugar vs starch in spring is governed by enzyme sensitivity to temperature, then we predict that each treatment, on average, reaches the same concentrations at the same number of accumulated growing degree days.*

While the plasticity of phenological timing to accumulated cold during dormancy and subsequent accumulated warming in spring is well documented (Polgar & Primack, 2011; Richardson *et al.*, 2013; Flynn & Wolkovich, 2018), it is unclear how plants sense this passage of time and trigger a response when some critical threshold is met. A recent hypothesis suggests that differential sensitivities of starch synthesis and degradation enzymes to temperature could be a mechanism by which plants track the gradual change in temperatures: once temperatures cross a certain threshold (suggested to be *c.* 5°C; Pollock & Lloyd, 1987), the activity levels of enzymes catalyzing starch degradation vs synthesis reverse (Zwieniecki *et al.*, 2015). This gradual shift in the relative amount of insoluble vs soluble carbohydrates may then signal to the plant that it is 'safe' to initiate leaf development. The processes initiated could include the clearing of calloses and building of new vasculature needed to support bud growth (Savage & Chuine, 2021), or more direct triggering of cell division and expansion in buds as either signal or by providing energy or the substrate for growth (Tixier *et al.*, 2019). Work in *Arabidopsis* has shown that carbohydrates can act as signaling molecules for growth (Rolland *et al.*, 2006; Gibon *et al.*, 2009; Bolouri Moghaddam & Van den Ende, 2013) and even entrain circadian clocks (Dalchau *et al.*, 2011). Furthermore, on diurnal time scales, the conversion between sugars and starch is known to be important for the onset and release of dormancy (Gibon *et al.*, 2009; Tixier *et al.*, 2018).

According to Hypothesis 2, we expected NSC stores to be comprised principally as sugar in the dormant phase at the onset of our experiment, as starch degradation enzymes are more active than starch synthesis enzymes under cold (< 5°C) temperatures (Pollock & Lloyd, 1987). We did, in fact, find that a majority of NSC stores – *c.* 68% – resided in sugars rather than starch at the onset of our experiment (8 January 2022; Stage 0). This finding is in line with several studies that have examined NSC patterns over seasonal time scales and found starch concentrations to be lowest in winter (Klein *et al.*, 2016; Martínez-Vilalta *et al.*, 2016; Furze *et al.*, 2019) and when temperatures reach extreme minimums (Blumstein & Hopkins, 2021; Blumstein *et al.*, 2022).

Following dormancy, regardless of treatment, the proportion of starch in woody tissues increased from *c.* 32% in dormancy to *c.* 52% by the start of bud development, through a combination of a modest, statistically insignificant increase in starch and a large, significant drop in sugar. In fact, by the start of leaf development (phenophase 1), sugars were already reduced in the stem to a threshold of *c.*1.6% dw, a level at which they remained throughout development (Fig. 1b). This value is very similar to a minimum whole plant NSC threshold found for Red Oak mortality in other studies, suggesting twig sugars were drawn down to their lower limit in our study (Wargo, 1981; Barker Plotkin *et al.*, 2021). This sugar may be used for more than just leaf development, as red oak is a ring-porous species, it may also have gone toward new wood growth, which occurs early on in the spring in oak. As the phenological stages progressed, starch was depleted in – and likely relied upon as an energetic, osmotic, and/or carbon substrate for – the twig and/or developing leaf, as has been shown in other studies in wild populations (Gaudinski *et al.*, 2009; Klein *et al.*, 2016).

As predicted, these patterns in NSC variation in relation to phenophase held across temperature treatments and were reached at the same number of GDD in the moderate and hot treatments (e.g. both treatments reached phenophase stage 1 at 450 GDD and had an average of 40% of NSCs in starch). This is interesting, as GDD reflect accumulated warming experienced by the plant, and not just elapsed time or instantaneous temperatures. For example, while the hot and moderate treatments both reached stage 1 at *c.* 450 GDD, the hot treatment reached this point 20 calendar days earlier than the moderate treatment (Fig. S2). However, while the cold treatment followed similar patterns of NSC concentrations with phenophase progression, it reached each stage at half the number of GDD of the other two treatments (Fig. 4).

Altogether, our NSC, phenophase, and GDD data suggest that the NSC in twigs proximal to buds are sufficient to trigger or support the progression through bud developmental stages during leaf-out. However, the specific physiological mechanism driving changes in NSC is still unknown. The hypothesis that enzymes with differential temperature responses could be involved in sensing accumulated warming, which in turn signal or impact development, is not entirely supported by our observation that plants in our cold treatment still underwent bud development despite accumulating much fewer GDDs than plants the other two treatments. Despite this difference in GDD among treatments, plants in the cold treatment still reached the same NSC concentrations as plants in the warmer treatments at each phenophase (Fig. 4). This observation could simply be a result of the prolonged time in each phase, and thus more time spent respiring, compared to the other treatments, or possibly a result of other enzymes putatively involved in the dormancy break process (Schrader *et al.*, 2003; Bhalerao & Fischer, 2014).

The degree to which NSC stores reside as starch in woody plants is largely dependent on the enzymes involved in starch synthesis vs degradation and their sensitivities to the external environment (Thalmann & Santelia, 2017). Enzymes required for starch degradation at night are similar to those utilized in response to cold stress (e.g. glucan water dikinase (GWD) and beta-amylase 3 (BAM3)), but differ from enzymes that break down starch in response to osmotic stress (e.g. alpha-amylase 3; AMY3; Thalmann & Santelia, 2017). While NSC concentrations could be shifting purely in response to temperature, given that bud and stem vasculature may not yet be fully developed (Savage & Chuine, 2021), enzymes could also be shifting activity to degrade starch in response to limited water. This is not wholly unexpected as soil moisture and water availability have been demonstrated to impact spring bud phenological timing (Zelikova *et al.*, 2015; Yun *et al.*, 2018). Moreover, in drought-deciduous trees it is the cycling of low and high soil water potentials – rather than temperature – that triggers phenological transitions (Jolly & Running, 2004; Bart *et al.*, 2017). Our study reveals some intriguing patterns that do not rule out the role of enzymes as environmental sensors that regulate NSCs and thus impact phenological timing. Rather, our data are suggestive of a role for cellular mechanisms beyond temperature sensitivity.

Hypothesis 3: *For the molecular clock hypothesis to be true, we predict to see heritable variation in NSC concentrations within each treatment and phenophase, particularly in the transition from dormancy to phase 1.*

While we found heritable variation in the timing of bud development and consistent patterns in NSC concentrations across phenophases, there is no consistent explanation for variation within phenophases in NSC concentrations. We had predicted that if NSC availability is the driver of phenological variation, then heritable variation in NSCs would correspond to heritable variation in phenological timing – meaning genotypes that leaf out earlier will also have a different NSC concentration at each phenophase than those that leaf out later. We found that variation in NSC was heritable in some stages and for some metrics, but that there were no consistent patterns across the treatments and traits (Table S5; Fig. 5).

At the onset of our experiment, the majority of NSC was in sugars (Fig. 1), and these sugars exhibited heritable differences before being almost completely depleted in every treatment by phenophase 1. At phenophase 1, we found no heritable variation in any of the metrics, suggesting a tightly controlled response to environment at the species level; stabilizing selection likely removes genetic variants in the control of NSC at this stage. To some degree, this makes sense, as mortality sets in regardless of other pressures after sugars reduce past a theoretical minimal level (Wargo, 1981; Barker Plotkin *et al.*, 2021) and reliance for core metabolic activities is shifted to the small amount of starch that remains. By phenophases 2 and 3, differences in starch can be observed among genotypes ($H^2_{\text{Phase2}} = 0.18$, $H^2_{\text{Phase3}} = 0.24$; Fig. 5) largely but not exclusively following the rank order of heritable phenological variation (Fig. 2). By stages 3 and 4, starch is once again depleted relative to total stores and variation is heritable, but variation in sugar stores does begin to increase to fairly high levels. We should note, however, that by phenophase 4, we had few replicates for each genotype due to sample mortality, particularly in the cold treatment.

Caveats and conclusions

Together, these experimental data provide preliminary evidence that NSC availability is one potential factor driving bud phenological development. However, our study has several limitations and thus should be considered in this context. First, we only included eight genotypes and, while they represent a large geographic diversity, they were collected from seed so we cannot be sure they were locally adapted to their provenance. Thus, we may not have captured a representative sample of the genetic diversity for phenological transitions in *Q. rubra*. We note, however, that our design benefits from our sampling of twigs from individuals essentially growing in a common garden (the Arnold Arboretum), thus likely minimizing differences due to macroclimate. Future work should include more genotypes within treatments to quantify heritability more accurately and consider the potential impact of maternal or epigenetic effects, which we were unable to do here (Rohde & Junttila, 2008). In practice, our small sample size likely means that our heritability estimates are reduced, rather than inflated, as we pooled across temperature treatments to get replicates and thus our denominator in the heritability equation (representing total phenotypic variance, which includes environmental variance) is likely quite high.

A notable possible limitation of our study is our use of twigs disconnected from the rest of the tree, placed in water, and used to approximate mature tree phenology. Trees are very large, slow-growing organisms that make experimental work challenging; thus, for practical reasons, we chose to use mature cuttings. This is a frequently adopted method (e.g. Heide, 1993; Caffarra & Donnelly, 2011; Basler & Körner, 2012; Flynn & Wolkovich, 2018) and has been demonstrated repeatedly to capture *in situ* processes accurately. Vitasse & Basler (2014) found that twigs

excised from mature trees closely follow the phenology of the parent plant when grown under similar conditions, indeed even more so than do seedlings or saplings. Additionally, Laube *et al.* (2014) found that the 36 species in their study observed *in situ* and in a contrasting growth chamber environment followed the same rank order, despite differing conditions.

Despite the likelihood that phenological timing is well approximated, we could still be introducing experimental artifacts in our NSC data given prior evidence that hormone signaling or NSC translocation from distant sinks may also be factors in triggering leaf-out (e.g. Sperling *et al.*, 2017; Tixier *et al.*, 2017). While long-distance transport might be important in some species, Klein *et al.* (2016) found that up to 90% of NSC used to make new buds in temperate species, including one in *Quercus*, comes solely from nearby branch material. These findings are echoed in a recent *in situ* study examining NSC in *Q. rubra* in all tissues (leaves, branches, stems, and roots) for every month of a year, which found very similar NSC patterns to our branch samples under tightly controlled conditions (Furze *et al.*, 2019). Furze *et al.* also reported very little NSC change over the course of the spring in roots and stems when compared to the high variability observed in branches, suggesting that in *Q. rubra* branches are the main source of NSC at leaf initiation. Together, these *in situ* studies and experimental comparisons of excised tissue make us confident that our results largely reflect what would be observed for leaf initiation *in situ*. While there may be some hormonal signaling or sugar translocation (e.g. Tixier *et al.*, 2017) occurring *in situ* to support leaf-out that is missing from our excised tissue and thus might change the absolute values in our study, we feel our results are particularly useful for comparative patterns (i.e. across genotype or treatment) and our specific hypothesis testing.

Our ability to conduct this experiment with disconnected tissue, and our finding of similar general patterns of NSC vs phenophase to connected tissues, particularly in ring-porous species (Furze *et al.*, 2019), supports a model in which branch NSC stores close to vegetative buds are critical for new leaf development. However, more studies should be done of this nature on other species, functional types (e.g. different wood anatomy, fall vs spring budset), with extreme environmental events, and even incorporating multi-year variations in storage to build a comprehensive model of the role played by variation in NSC and phenology in response to environmental cues. An additional future line of research could assess the relative roles of locally stored sugars – as studied here – and sugars which may have been transported from elsewhere in the plant.

Altogether, these results paint a detailed picture of the relationship between phenological timing, NSC concentrations, and environment, suggesting that sugar concentrations may signal accumulated changes rather than acute environmental thresholds, thus triggering leaf development. This is a critical finding as it brings us closer to understanding the mechanism by which trees can sense the passage of time and accumulation of weather changes. Understanding such mechanisms helps us to better understand plant responses to environmental change and improve our ability to predict phenological timing, a key biosphere transition in global climate models.

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Competing interests

None declared.

Author contributions

MB and DLDM contributed to the conceptualization. MB and MO contributed to the methodology. MB, DLDM, TC-M and MO contributed to the funding Acquisition. MB, TC-M and MO contributed to the data collection and analysis. MB contributed to the writing – original draft. MB, MO, TC-M and DLDM contributed to the writing – review and editing.

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Data availability

All data are available in the main text or the Supporting Information.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 *Quercus rubra* nonstructural carbohydrate metrics plotted by temperature for phenophases 1–5.

Fig. S2 The *Quercus rubra* nonstructural carbohydrate metrics of (A) sugar, (B) starch, (C) NSC, and (D) percent starch vs the number of days from the start of the experiment.

Table S1 ANOVA results comparing nonstructural carbohydrate metrics to temperature, separated by phenophase in *Quercus rubra*.

Table S2 Results of linear models comparing *Quercus rubra* nonstructural carbohydrate metrics to the number of days to reach each phenophase and temperature treatment.

Table S3 Results of linear models comparing *Quercus rubra* nonstructural carbohydrate metrics by phenophase.

Table S4 Results of linear models comparing *Quercus rubra* nonstructural carbohydrate metrics to growing degree days.

Table S5 Heritabilities of *Quercus rubra* nonstructural carbohydrate metrics by phenophase.

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