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Individual variation in oak (*Quercus robur*) phenology results in increased caterpillar abundance and defoliation for earlier oaks, but no response of antiherbivory defense mechanisms.

Master's Thesis

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Abstract

Oaks vary consistently in budburst phenology, with earlier phenology associated with heightened caterpillar abundance during the caterpillar peak in spring. This variation may lead to differences in herbivory defense mechanisms, such as leaf toughness and phenolic content. In this field study conducted in the Netherlands, we investigated the pedunculate oak (*Quercus robur*). We measured caterpillar abundance, defoliation, budburst date, leaf toughness, and the concentrations of total phenolics and tannins. We hypothesized that trees with earlier budburst would display higher caterpillar abundance and defoliation, increased leaf toughness and a stronger increase in leaf toughness, and elevated concentrations of total phenolics and tannins in response to higher defoliation rates. Our findings confirmed the first hypothesis, partially supported the second, and contradicted the third. Specifically, while earlier trees exhibited higher leaf toughness, the rate of increase was uniform across all trees. Furthermore, the concentrations of total phenolics and tannins were best explained by leaf age, leaf toughness and their interaction, with young leaves with a low leaf toughness displaying the highest concentrations. Our study demonstrates that oak phenology influences leaf traits, affecting herbivore food availability. Additionally, oak phenology influences caterpillar distribution, impacting insectivore food availability. This highlights the importance of considering oak phenology in the context of multitrophic interactions.

1. Introduction

Seasonality in temperate regions determines the optimal time for reproduction and growth of many organisms, which is often determined by periods of increased food availability (Van Asch et al., 2007). For herbivores, food availability and quality is often shaped by the phenology of their host plant, therefore synchronization with their host plant is important (Van Asch et al., 2007, Ekholm et al., 2020). The phenology of plants and their herbivores is influenced by environmental cues, primarily by temperature and photoperiod (Van Asch et al., 2007). The increasing temperatures caused by climate change have resulted in the advancement of the timing of many organisms (Both et al., 2009, Van Asch et al., 2007). Multiple studies show varying degrees of advancement in the phenology of organisms across different trophic levels, resulting in temporal mismatches. These mismatches can impact mortality and reproduction in the species involved (Van Dis et al. 2023, Van Asch et al., 2007; Both et al., 2009 et al., 2008). Therefore, gaining a deeper understanding of these timing-dependent relationships across trophic levels is crucial.

Pedunculate oak (*Quercus robur*) is the second most common deciduous tree in European forests and an important food source for multiple caterpillar species (Schroeder et al., 2021, Ekholm et al., 2020, Van Asch et al., 2007). The palatability of oak leaves is the highest in spring, immediately following budburst, aligning with a corresponding peak in caterpillar abundance (Feeny, 1970; Ekholm et al., 2020, Both et al., 2009, Van Asch et al., 2007). The increase in caterpillar abundance lasts approximately three weeks (Both et al., 2009 et al., 2008, Crawley & Akhteruzzaman, 1988). The number of caterpillars can differ substantially between years, with a maximum of a fifty-fold difference from year to year (Van Asch et al., 2007). This variation is often related to outbreak species, such as the autumnal moth (*Epirrita autumnata*), the gypsy moth (*Lymantria dispar*), the winter moth (*Operophtera brumata*), and the European oak leafroller (*Tortrix viridana*) (Hill et al., 2021, Forkner et al., 2008). Oak trees can become completely defoliated in years with a high caterpillar abundance (Van Asch et al., 2007). However, there is a lot of variation in the proportion in which the oaks are defoliated. At least a part of this variation

has been correlated with between-individual variation in budburst phenology. The variation in budburst between oak trees can be up to five weeks, whereby the same individuals are consistently early or late among years. Trees with an earlier budburst phenology have a heightened caterpillar abundance and defoliation in comparison to trees with a later budburst phenology (Crawley & Akhteruzzaman, 1988, Ekholm et al., 2020, Wesołowski & Rowiński, 2008, Milenin et al., 2023).

It is suggested that the difference in caterpillar abundance between trees is caused by the synchronization between the timing of the budburst of the tree and the hatch date of the principal defoliators of oaks (Crawley & Akhteruzzaman, 1988). Caterpillars on early trees hatch close to the budburst date of the tree, and are met with a high quality food source. Caterpillars on late trees have a higher chance to hatch before the budburst of the tree, when there is no leaf material to feed on yet. This can lead to high mortality (Van Asch et al., 2007, Tikkanen & Julkunen-Tiitto, 2003). When leaf material is scarce, caterpillars have the ability to disperse to trees in the surroundings to find an alternative food source. However, this practice can also result in high mortality, especially with large distances between trees (Van Asch et al., 2007, Sarvašová et al., 2021, Tikkanen & Julkunen-Tiitto, 2003). Both mortality as a result of food scarcity as well as dispersal to earlier trees can increase the difference in abundance between trees with different phenology. Having a late budburst phenology might be a way for trees to cope with herbivory by partly avoiding the peak in caterpillar abundance (Wesołowski & Rowiński, 2008).

The variation in oak phenology has a strong genetic basis and is seen across the whole range of its occurrence, although it is strongest in the southeastern part of its range (Milenin et al., 2023). In studies from eastern Europe the two phenological varieties are treated as separate taxa, namely *Quercus robur* var. *praecox* (early variety) and *Quercus robur* var. *tardiflora* (late variety) (Utkina et al., 2017). The varieties differ in a number of traits, for example in tree morphology, size and tree performance depending on the environmental conditions (Milenin et al., 2023, Utkina et al., 2017, Marchand et al., 2020). Early trees perform better on dry soils than late trees, because early trees have a higher risk of encountering spring frost, while late trees are more vulnerable to summer droughts (Milenin et al., 2023). Early trees also face increased defoliation and late trees have a shorter growing season (Milenin et al., 2023). These different challenges have possibly shaped differences in for example frost resistance, the ability to grow new leaves after defoliation or investment in antiherbivore defense (Wesołowski & Rowiński, 2008).

Trees can employ different mechanisms of defense. They produce specialized (also known as secondary) metabolites, such as phenolics, which serve a broad array of functions to protect plants from stressors. Phenolics have been shown to increase with ultraviolet light, insect feeding, wounding, low soil nitrogen, and pathogen infections (Lauer et al., 2011, Barbehenn et al., 2011, Visakorpi et al., 2020, Madritch et al., 2015). The amount of phenolics varies between trees, different locations within a single individual and different timepoints. The concentration of phenolics also depends on environmental factors, especially increased nutrient availability and increased UV light can lead to an increase in phenolics (Visakorpi et al., 2020, Barbehenn et al., 2011). Tannins are a subgroup of phenolics, which represent the most abundant group of specialized metabolites in plants, normally constituting 5-10% of the plant fresh weight (Barbehenn et al., 2011). They serve a smaller range of functions than total phenolics and are thought to play a larger role in antiherbivore defense (Barbehenn et al., 2011). In *Quercus* species it has been shown that damage caused by insects on growing leaves can strongly increase the amount of tannins (Barbehenn et al., 2011, Visakorpi et al., 2020). The underlying reasons for the observed increase in the concentration of tannins are still a topic of

ongoing discussion, but the main suggestion is that tannins function as a feeding deterrent or cause oxidative stress in the herbivores, depending on the tannin type (Barbehenn et al., 2011). Feeny (1968) found a significant reduction in caterpillar growth rate and pupal weight when oak leaf tannin was added to their diet, but this result is not consistent across studies (Van Asch et al. 2007, Barbehenn et al., 2011). Studies looking at the effect of tannins on herbivory presence have not found consistent results, which can partly be explained by the large effect of environmental factors on the amount of tannins (Barbehenn et al., 2011, Skovmand et al., 2023). An alternative hypothesis suggests that tannins do not affect the herbivores, but instead improve nutrient cycling, which helps trees recover after defoliation (Barbehenn et al., 2011, Madritch et al., 2015).

The production of phenolics can be costly, resulting in a trade-off between growth and defense (Madritch et al., 2015 et al., 2015, Ekholm et al., 2020, Crawley & Akhteruzzaman, 1988). According to the growth-differentiation balance hypothesis growth is favoured in conditions with competition, and the production of specialized metabolites, such as phenolics, is favoured in conditions with herbivory (Ekholm et al., 2020, Ekholm et al., 2020, Gaytan et al., 2022, Herms & Mattson, 1992, Riipi et al., 2002; Glynn et al., 2007). Tannins are not expected to contribute to the decrease in leaf palatability for caterpillars in spring, since studies about the increase in tannins during the spring season produced varied results (Salminen et al., 2004, Tikkanen & Julkunen-Tiitto, 2003; Gaytan et al., 2022, Van Asch et al. 2007). A potential reason is that growth is favoured over defense in the summer months (Salminen et al., 2001).

In addition to chemical defense, trees also have structural defenses, such as leaf toughness. Leaf toughness increases during the spring, meaning that the leaves become increasingly more difficult to tear apart. This has been suggested to be one of the main reasons for the decrease of leaf palatability (Feeny, 1970, Tikkanen, Van Asch et al., 2007).

Our aim is to better understand the interplay between oaks differing in phenology and the caterpillars that grow on them. I will look at the differences in caterpillar abundance, the concentrations of total phenolics and tannins, leaf toughness, and defoliation between trees with different phenology. I will be looking at oaks in national park Dwingelderveld in the north of the Netherlands. Based on longitudinal data on oak phenology and caterpillar abundance in this area, we know that oaks can differ in budburst phenology up to three weeks, and trees with an earlier budburst have a higher peak in caterpillar abundance. We expect that trees with an earlier budburst will have a higher caterpillar abundance with a corresponding higher amount of defoliation. Additionally, we expect that earlier trees will have a higher leaf toughness and increase their leaf toughness faster during the spring. Lastly, we expect earlier trees will have a higher concentration of total phenolics and tannins in response to higher defoliation rates.

2. Materials and methods

The study area is located in the north of the Netherlands in the national park Dwingelderveld, composed of mixed forest surrounded by wet heathland (fig.1).

We did our measurements in the spring (April until early June). All dates are in April date, defined as the amount of days since the 1st of April.

2.1 Study area and sample size

In 2023 we selected a study plot of 3 ha. with a relatively high density of pedunculate oak (*Quercus robur*) (coordinates: 5281.8709 N., 00643.2064 E., fig. 1). We assessed the phenology of all oaks in the study plot based on the budburst date (see section 2.2). In the study plot, we selected 20 trees distributed across the study plot with varied phenologies (7 trees from the first tertile, 6 trees from the second tertile and 7 trees from the third tertile of budburst date). We measured caterpillar biomass per m² tree, leaf toughness, the concentration total phenolics, the concentration tannins and defoliation for those 20 trees, from this point on called the sample trees.

From 2007 to 2023, we measured the phenology of 67 trees, of which 15 trees were located in the 2023 study plot. The other 52 trees were located in the area surrounding the 2023 study plot at a maximum distance of 400 meters from the edge of the plot.

From the set of 67 trees, we measured caterpillar abundance for 4 trees from 2007 to 2019 and 11 trees from 2019 to 2023, of which 10 trees were located in the 2023 study plot. In 2023 we measured the caterpillar abundance of an additional 15 trees in the 2023 study plot, resulting in a total of 25 trees, measured in the 2023 study plot.

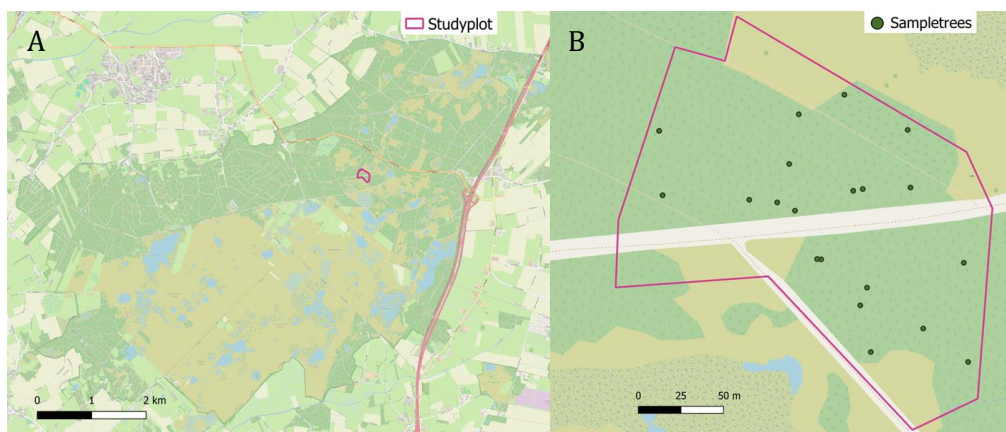


Figure 1: A) A map of the national park Dwingelderveld with location of the study plot in 2023. B) The sample trees in the study plot.

2.2 Tree traits

We measured the circumference of all 206 oak trees in the study plot before leafing (beginning of April) with a soft tape measure at chest height.

We measured the phenology of oaks based on the budburst date. The stage of budburst was scored every 4 days by examining the crown of the tree with a pair of binoculars and scoring the average budburst stage across the crown based on the scoring chart below (fig. 2, Visser & Holleman, 2001). The budburst date was defined as the date that the leaves were fully unfolded, which is stage 3 or stage 3.5 in the scoring chart. In cases where a tree was scored as budburst

stage 2 on one day and then as stage 4 four days later, we considered the day in between as the budburst date.

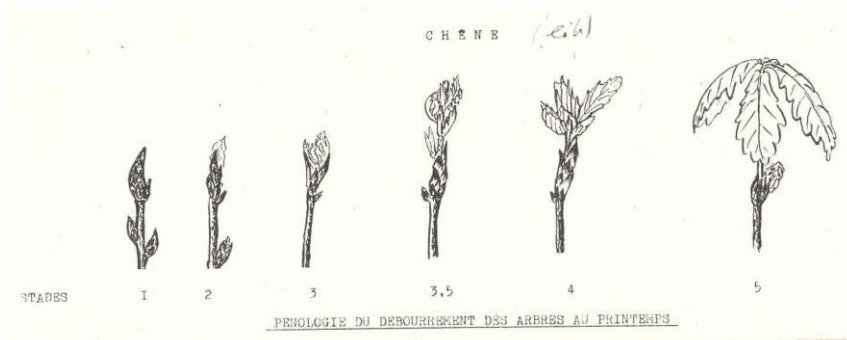


Figure 2: Stages of budburst

2.3 Caterpillar abundance

We placed cloth nets of 0.5 by 0.5 m underneath the oaks to measure the caterpillar abundance per tree. We placed two nets on the 40th of April and 18 nets on the 42th of April, which is one week after the average budburst date. For three trees with a late budburst date we placed the nets on the 45th and for two trees on the 47th of April.

We emptied the nets every 2-4 days. After emptying the nets, the contents were dried for 48 hours at 60 degrees. Then we removed all debris by sieving the contents through sieves of sizes 0.6 mm, 1.0 mm and 2.0 mm. All debris larger than 2.0 mm was removed. For the contents between 1.0 and 2.0mm, 0.6 and 1.0 mm and smaller than 0.6 mm, we visually removed all debris by sliding the materials back and forth on a steel surface. The caterpillar frass moved faster on the surface compared to the debris, effectively separating the two.

We weighed the caterpillar droppings on a scale with 0.001 grams accuracy. The sum of caterpillar droppings per day was calculated by dividing the total weight of caterpillar droppings by the amount of hours since the net was last emptied and multiplying that by 24. The sum of caterpillar droppings per day was used to calculate the caterpillar biomass with the following formula (Tinbergen et al., 2023):

$$4 * \text{sum caterpillar droppings per day} + \exp(4.74 - 0.22 * \text{average temperature})$$

Average temperature is the average temperature in Hoogeveen over the days since the net was last emptied.

The peak caterpillar biomass was calculated as the maximum biomass. The caterpillar peak date is the date that the tree reached maximum caterpillar biomass. Three trees were excluded from the calculation, namely the trees with nets placed on April 47th and one tree with less measurements. As a result, the sample size was 22 trees.

The summed caterpillar biomass was calculated by using the area under the curve function of the package bayestestR (version 0.13.1). The area under the curve was calculated for day 45 until day 60. For the trees with nets placed on April 45th and April 47th, these calculations were not performed. Five trees were excluded from the calculation, namely the trees with nets placed after April 45th and one tree with less measurements. As a result, the sample size was 20 trees.

2.4 Leaf traits

The leaf toughness was measured six times in the spring of 2023 with a penetrometer (fruit penetrometer GY-3 from NEWTRY). We measured the leaves every 3-4 days starting the 10th of May until the 31st of May. We measured six leaves per tree from the lower branches, including three leaves at approximately two meters height and three leaves at around five meters height. The leaves were measured immediately in the field by securing them between two plastic plates with a hole in the middle. Subsequently, a part of the leaf without large veins was punctured with the penetrometer. We averaged the six measurements per tree.

We collected leaves for measurements of the concentrations of total phenolics and tannins five times in the spring of 2023 (on the 40th, 49th, 52nd, 56th and 60th of April). We collected the leaves in the afternoon between 13 and 17 at four different collection points per tree: two at a height of two meters and two at a height of five meters, with one set on one side of the tree and the other set on the opposite side. We collected four leaves per collection point, resulting in a total of 16 leaves per sample.

Immediately after collection the lowest leaves were stored in a ziplock bag and the higher leaves were stored in a ziplock bag, resulting in 2 ziplock bags per tree. They were placed in a box of dry ice. On the same day, the leaves were transferred to a -20 degree freezer and stored there until analysis.

For an assessment of the within individual variation, we collected additional leaves from two trees once, whereby we kept the leaves of the four separate locations separate from each other.

The concentrations of total phenolics and tannins were measured using Folin-Ciocalteu Reagent with tannic acid as standard, following chapter 2 and 3 of the protocol of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (2020).

In short, I freeze-dried the samples for 48 hours. From each sample, I randomly selected 8 leaves out of the 16 leaves available and ground those to a fine powder using liquid nitrogen to keep the samples cold. To extract the phenolics, I followed the following steps. I added 10 ml of 70% acetone to the sample. This mixture was placed in an ultrasonic water bath for 20 minutes at room temperature, then centrifuged for 10 minutes at 3000 rpm and 4 degrees Celsius. After the supernatant was removed, the steps were repeated with the pellet. The supernatants of both extraction rounds were added together, resulting in a total volume of 20 ml. For the quantification of the concentration of total phenolics, I added 10 μ L of the supernatant to a test tube and I diluted it with 490 μ L of demi water, 250 μ L of Folin-ciocalteu reagent and 1250 μ L of sodium carbonate. After leaving the sample for at least 40 minutes, I analyzed the sample with a spectrophotometer at 725 nm. With a calibration curve, I calculated the total phenolics in mg per 100 mg dry leaf material.

For the quantification of the concentration of tannins, I added 1 ml of the supernatant, 1 ml of demi water and 100 mg of PVPP to a test tube. After 15 minutes at 4 degrees Celsius, the sample was centrifuged. In a test tube, I added 30 μ L of the supernatant and I diluted it with 490 μ L of demi water, 250 μ L of Folin-ciocalteu reagent and 1250 μ L of sodium carbonate. After leaving the sample for at least 40 minutes, I analyzed the sample with a spectrophotometer at 725 nm. With a calibration curve, I calculated the concentration of phenolics without tannins in the sample. By subtracting the concentration of phenolics without tannins from the concentration of total phenolics obtained earlier, I could calculate the concentration of tannins in mg per 100 mg dry leaf material.

2.5 Defoliation

We estimated the percentage of defoliation six times between the 10th of May and the 30th of May by examining the tree with a pair of binoculars. All estimations were done by the same observer.

2.6 Statistical analysis

I used R (RStudio 2023.09.1) for statistical analysis. The focus of my analysis was on linear models, using the packages lme4 (version 1.1.30) and lmerTest (version 3.1.3). I used linear mixed models with treeID as a random effect when dealing with repeated measurements of the same trees. The model selection process was based on the Akaike Information Criterion (AIC), where the model with the lowest AIC value was considered the best, provided there was a difference of at least 2 points compared to other models. In cases where models showed similar AIC values (within a 2-point range), preference was given to the model with fewer effect terms.

From the variables Aprildate and budburst date, we calculated an additional variable leaf age. The variable leaf age was calculated as the budburst date subtracted from the Aprildate. Measurements that were taken one day apart, were considered to belong to the same sampling point, whereby the collection date of the response variable was used as variable in the model. The defoliation data is proportional, thus we applied an arcsine square root transformation before using it in linear models.

Repeatability of tree budburst date is the percentage of variation in budburst date explained by treeID. Initially, we computed repeatability with a mixed model with treeID and year as random effects. We calculated repeatability a second time with a correction for the variation caused by year. This involved adjusting for the year effect by subtracting the average budburst date of a given year from the corresponding year's observations. The modified budburst date then served as the response variable in a new mixed model with treeID as a random effect.

We analyzed the relationship between caterpillar peak date and budburst date over multiple years with a mixed linear model with caterpillar peak date as response variable, within year variation and between year variation as fixed effects and treeID as random effect (fig. 7, Van de Pol & Wright, 2009). The between year variation is calculated as the average budburst date per year. The within year variation is calculated as the average budburst date in a year subtracted from the budburst date per tree in the same year.

We calculated the relationship between caterpillar abundance and budburst date with a mixed linear model with caterpillar abundance as response variable, within individual variation and between individual variation as fixed effects and treeID as random effect (Van de Pol & Wright, 2009). The between year variation is calculated as the average budburst date per year. The within year variation is calculated as the average budburst date in a year subtracted from the budburst date per tree in the same year.

3. Results

3.1 Tree traits

3.1.1 Tree budburst date in 2023

In 2023, the average budburst date for the observed trees was on 34 April (N = 206, SD: 5.41). The difference between the earliest and latest tree was 30 days (fig. 3).

Trees with an earlier budburst date have a larger circumference (fig. 4, table 1, linear model with budburst date as fixed effect, $\beta = -2.037$, $F_{204,1} = 28.24$, $P = 2.8 \cdot 10^{-7}$).

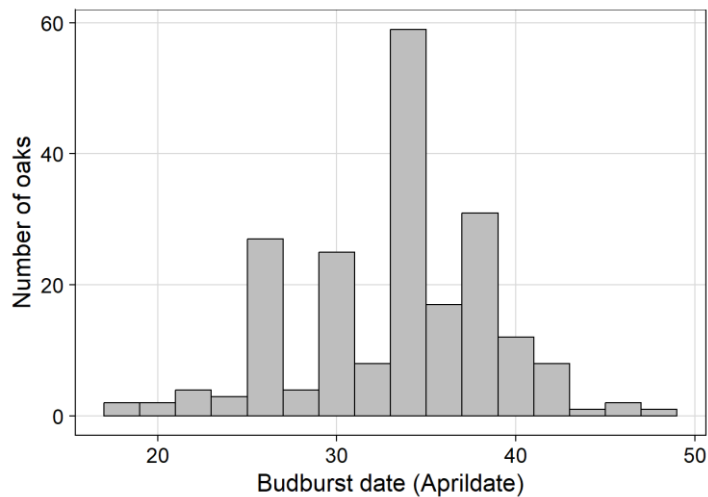


Figure 3: The distribution of the budburst date (in April date) of all oaks in the plot in 2023.

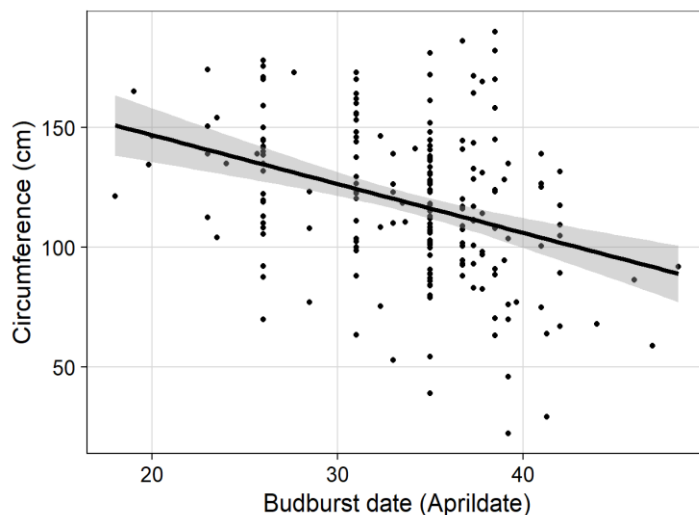


Figure 4: The relation between budburst date (in April date) and circumference (in cm).

3.1.2 Repeatability of budburst date among years

Analysis of the budburst date of 67 trees from 2007 until 2023 shows that budburst date is mainly explained by year and treeID, giving a total repeatability of 0.88 (mixed model with year and treeID as random effects, repeatability year = 0.65, 95% CI: 0.57 - 0.77 , repeatability treeID = 0.23, 95% CI: 0.16 - 0.24, number of observations = 916). This entails that 88% of the variation in tree ID is explained by the variables year and individual. After adjusting for the yearly effect by subtracting the mean budburst per year from each observation, the repeatability of treeID is 0.65 (mixed model with treeID as a random effect, 95% CI = 0.58 - 0.70).

3.2 Caterpillar biomass

3.2.1 Caterpillar biomass in 2023

The average peak in caterpillar biomass in 2023 was at 48.20 April (fig. 5, sd = 2.37, N = 22). The caterpillar peak date was 4 days earlier in the earliest tree compared to the latest tree, with a difference in budburst date of 30 days (linear model with budburst date as fixed effect, $\beta = 0.13$, $F_{2,20} = 4.552$, $P = 0.045$).

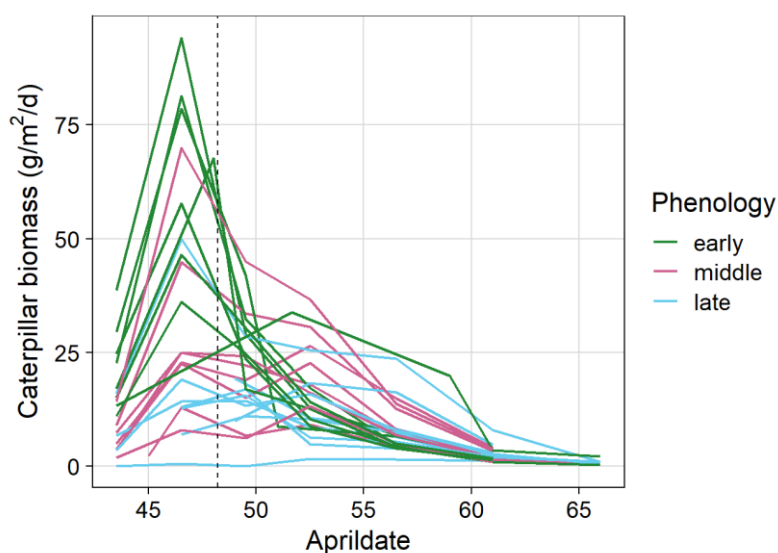


Figure 5: The caterpillar biomass (in $g/m^2/day$) per tree during the spring of 2023 with the average peak in caterpillar biomass as a dashed line and tree phenology in colour. The trees are categorised by budburst date as follows: early (range: 18.00 - 32.33 April, N = 68), middle (32.33 - 36.75 April, N = 83) and late (range 36.75 - 48.40 April).

The caterpillar peak biomass per m^2 tree was four times as high in the earliest trees compared to the latest ones (fig. 6, linear model with budburst date as fixed effect, $\beta = -2.47$, $F_{2,20} = 19.55$, $P = 0.00026$). The summed caterpillar biomass was twice as high in the earliest trees compared to the latest ones (fig. 6, linear model with budburst date as fixed effect, $\beta = -9.29$, $F_{2,18} = 5.304$, $P = 0.033$).

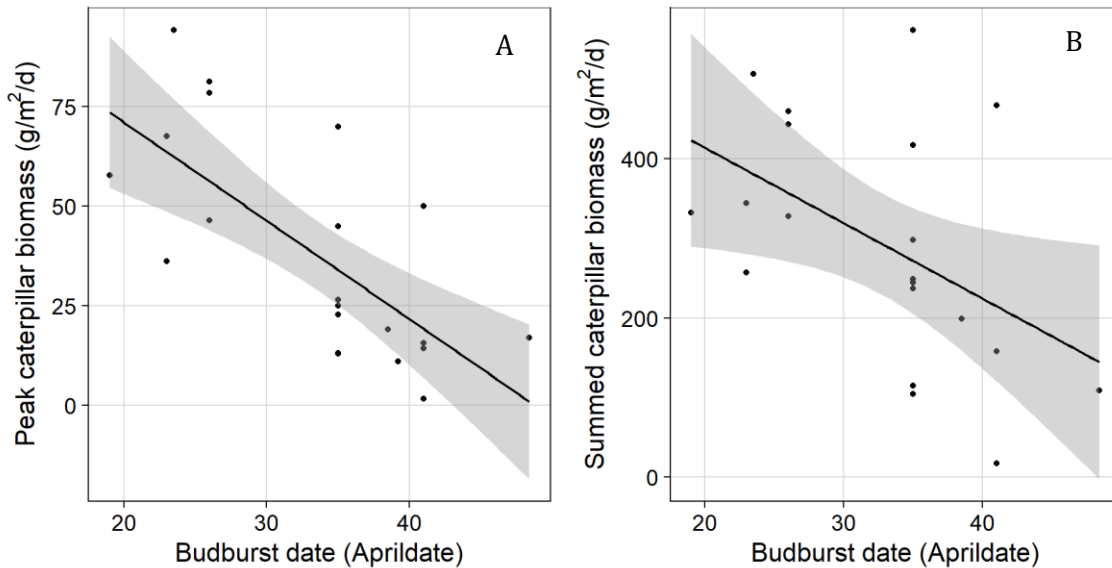


Figure 6: The relation between caterpillar biomass and budburst date. A) Peak caterpillar biomass. B) Summed caterpillar biomass for the days 47 until 62.

3.2.2 Caterpillar biomass across multiple years

The variability in caterpillar biomass across years had a standard deviation of 16.01 g/m²/day on a mean caterpillar peak biomass of 15.87 g/m²/day (fig. 7). In 2024, the caterpillar biomass was particularly high, averaging 38 g/m²/day at the caterpillar peak.

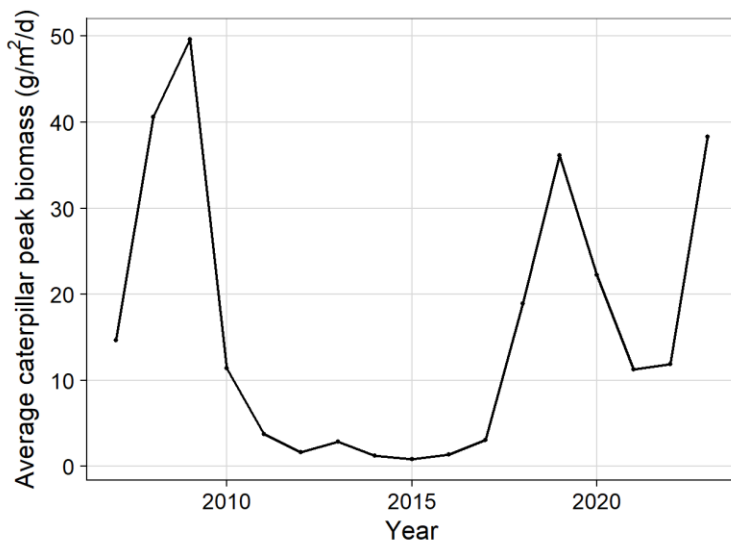


Figure 7: The average caterpillar peak biomass per year.

While examining the relationship between the caterpillar peak date and the budburst date across years, we separated the variation in budburst date into variation within years and between years. We find that the caterpillar peak date was significantly earlier for earlier trees within the same year, although the effect is small (fig. 7, table 1). The caterpillar peak date is significantly earlier for years with an earlier average budburst date. For each day the budburst date is earlier, the caterpillar peak date is nearly one day earlier as well (fig. 7, table 1).

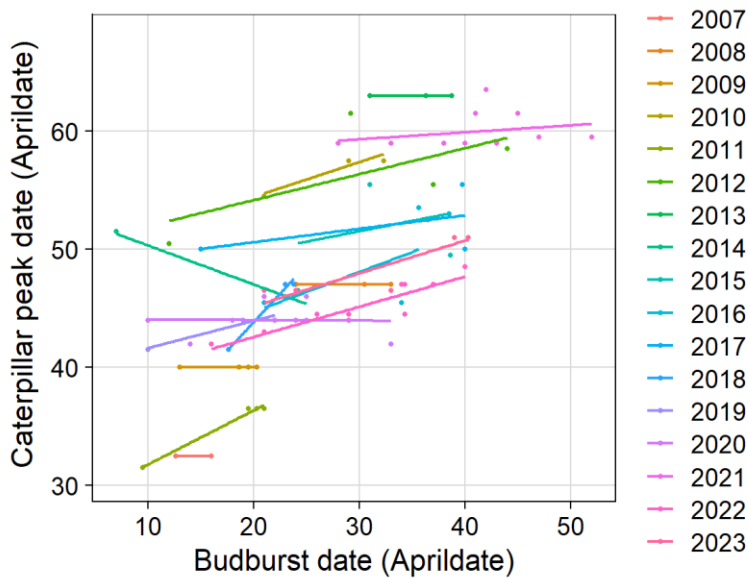


Figure 8: The relation between the budburst timing and the timing of the caterpillar peak for the years 2007 - 2023.

To examine the relationship between the caterpillar peak biomass and the budburst date across years, we separate the variation in budburst date into variation within individuals and between individuals. Trees with an on average earlier budburst date had a significantly higher log caterpillar biomass, but within an individual an earlier budburst date in a given year does not correlate with a higher log caterpillar peak biomass in that same year (table 1).

3.3 Defoliation

The amount of defoliation increased linearly during the spring of 2023, with early trees experiencing twice as much defoliation as late trees (fi. 9, table 1). The maximal defoliation was three times higher for the tree with the highest caterpillar peak biomass compared to the tree with the lowest caterpillar peak biomass (fig. 10, table 1, linear model with caterpillar peak biomass as fixed effect, $t = 4.52$, $P = 0.00035$, $n = 18$).

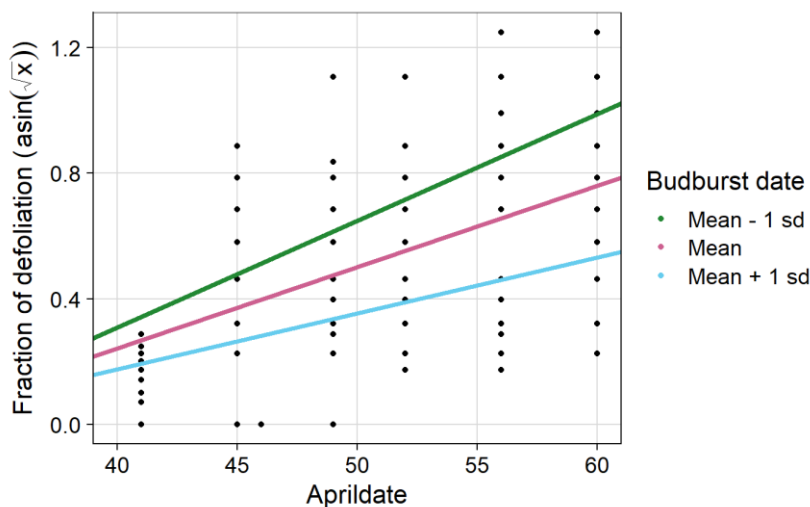


Figure 9: Fraction of defoliation per tree ($\text{asin}(\sqrt{x})$) over time (in Aprildate) with the linear regression lines for three values of budburst date.

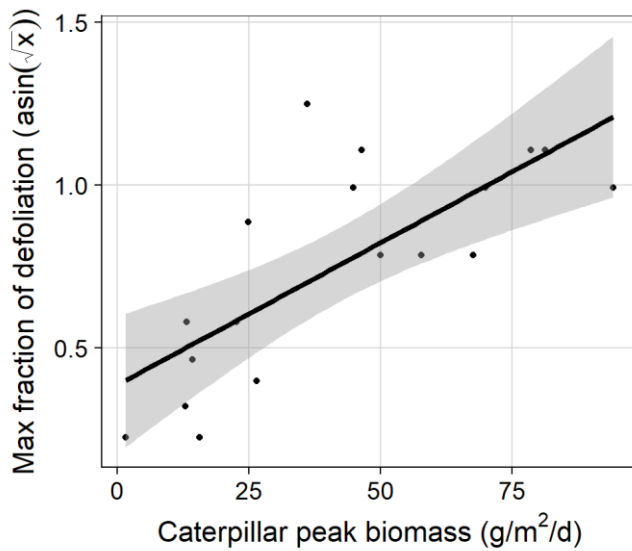


Figure 10: The relation between the maximal fraction of defoliation ($\text{asin}(\sqrt{x})$) and the caterpillar peak biomass (in $\text{g}/\text{m}^2/\text{day}$) per tree.

3.4 Leaf traits

3.4.1 Leaf toughness

Leaf toughness increased during the spring of 2023, and was higher for earlier trees (Fig. 11, table 1).

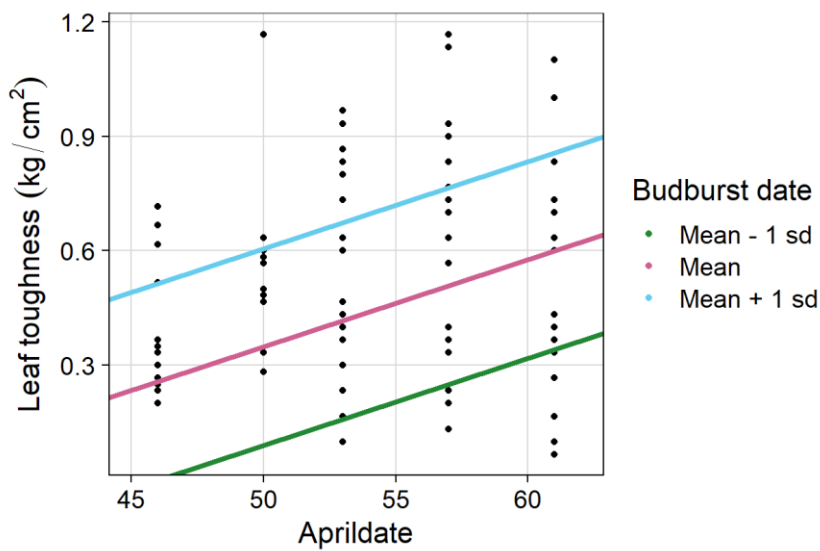


Figure 11: The leaf toughness (in kg/cm^2) per tree over time (in April date) with the linear regression lines for three values of budburst date.

3.4.2 Phenolics

The concentration of total phenolics ranged from 5.26 mg/100 mg dry leaf material to 21.92 mg/100 mg dry leaf material. On average, 89% of phenolics consisted of tannins with little variation (fig. 12, sd: 6.67%). The mean concentration of total phenolics of all samples was 13.75 mg/100mg with a standard deviation of 3.62 mg/100mg.

In leaves with a low to intermediate leaf toughness, the concentration of total phenolics and tannins decreases with leaf age. This trend is not visible in leaves with a high leaf toughness (Fig. 13a and b, table 1).

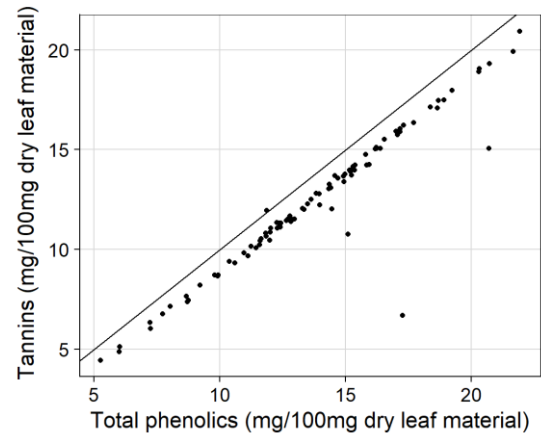


Figure 12: The relation between the concentration of tannins (mg/100mg dry leaf material) and the concentration of total phenolics (mg/100mg dry leaf material).

Leaf toughness • Mean - 1 sd • Mean • Mean + 1 sd

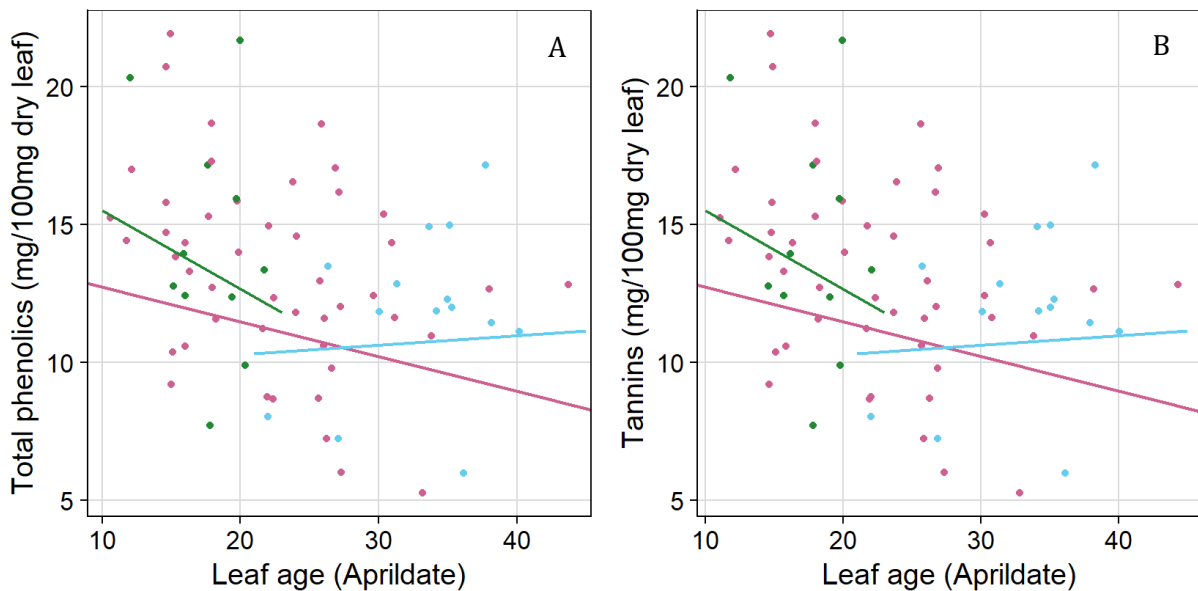


Figure 13: The relation between the concentration of phenolics and leaf age (in April date) with three linear regression lines for different levels of leaf toughness. A) Concentration of total phenolics (mg/100 mg dry leaf material). B) Concentration of tannins (mg/100 mg dry leaf material)

There is an insignificant trend that trees with an earlier budburst date have a lower mean concentration of total phenolics and tannins (Fig. 14, table 1).

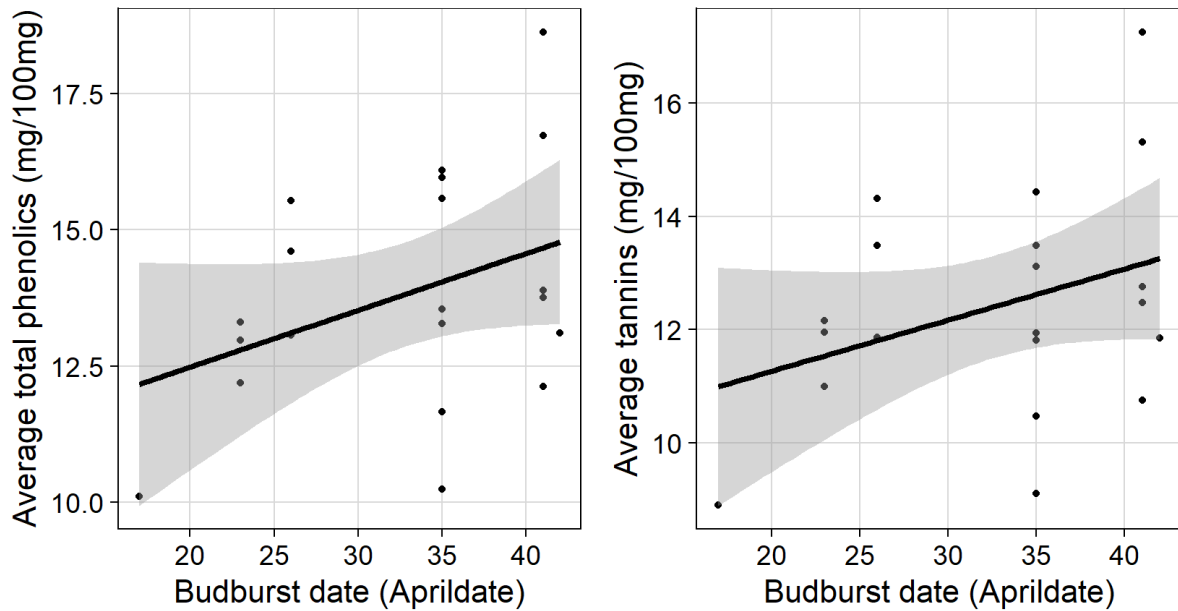


Figure 14: Average concentration of total phenolics and tannins per tree in relation to budburst date in Aprildate. A) Total phenolics (mg/100 mg dry leaf material). B) Tannins (mg/100 mg dry leaf material).

Table 1: Overview of all linear models and linear mixed effect models with the estimate (β), test statistic, degrees of freedom (df), P-value and number of observations (N). Linear mixed effect models are identifiable by the addition of a random effect as predictor.

Response variable	Predictor	β	Test statistic	P	df	N
Budburst date	Circumference	-2.037	F = 28.24	<0.001	1	204
Caterpillar peak date 2023	Budburst date	0.13	F = 4.552	0.045	2	22
Caterpillar peak biomass 2023	Budburst date	-2.47	F = 19.55	<0.001	1	22
Summed caterpillar biomass 2023	Budburst date	-9.29	F = 5.304	0.033	2	20
Caterpillar peak date 2008-2023	Within year variation in budburst date	0.15	t = 1.93	0.134	8.14	88
	Between year variation in budburst date	0.82	t = 12.67	<0.001	84.04	
	Random effect treeID					
Log caterpillar peak biomass 2008-2023	Within individual variation in budburst date	-0.02	t = -1.324	0.341	83.30	88
	Between individual variation in budburst date	-0.05	t = -2.559	<0.001	69.51	
	Random effect year					
Defoliation	Aprildate	0.062	t = 6.21	<0.001	98	120
	Budburst date	0.035	t = 2.16	0.033	115.4	
	Aprildate X Budburst date	-0.0010	t = -3.67	<0.001	98	
	Random effect treeID					
Defoliation	Caterpillar peak biomass	0.0087	4.39	<0.001	16	18

Leaf toughness	Aprildate	0.076	t = 5.61	<0.001	19.63	85
	Budburst date	0.67	t = 2.13	0.036	69.10	
<i>Random effect treeID</i>						
Concentration total phenolics	Leaf age	-0.42	t = -2.76	0.0075	67.95	72
	Leaf toughness	14.96	t = -2.64	0.01	67.42	
	Leaf age X Leaf toughness	0.53	t = -2.65	0.013	67.62	
<i>Random effect treeID</i>						
Concentration tannins	Leaf age	-0.43	t = -2.89	0.0052	67.91	72
	Leaf toughness	-14.51	t = -2.64	0.010	67.56	
	Leaf age X Leaf toughness	-0.53	t = -2.59	0.012	67.51	
<i>Random effect treeID</i>						
Mean concentration total phenolics	Budburst date	0.10	F = 2.96	0.10	2	20
Mean concentration tannins	Budburst date	0.09	F = 2.50	0.13	2	20

4. Discussion

Here I examined how pedunculate oaks with varying budburst phenology were affected by, and defended themselves against caterpillars. In our rather small study area, oaks differed in budburst phenology up to 30 days, which was repeatable among years. Oaks with an earlier budburst phenology had a larger circumference, a higher amount of caterpillar biomass per m² tree, a higher percentage of defoliation, a higher leaf toughness and, although insignificant, a lower concentration of total phenolics and tannins.

4.1 Tree budburst date and circumference

The continuous variation in budburst phenology with a maximal difference of 30 days in our data was in line with previous studies (Crawley & Akhteruzzaman, 1988, Wesołowski & Rowiński, 2008, Utkina et al., 2017, Van Dongen et al., 1997 et al. 1997, Milenin et al., 2023). We found that the difference in budburst phenology between oaks was repeatable among years, which is consistent with other studies (Van Asch et al., 2007, Crawley & Akhteruzzaman, 1988, Wesołowski & Rowiński, 2008, Van Dongen et al., 1997 et al. 1997). The repeatability can be explained by a strong genetic basis for budburst phenology. Evidence suggests a strong genetic influence on budburst phenology in deciduous trees (Ueno et al., 2011, Lesur et al., 2015; Pellis et al., 2004; Rousi & Pusenius, 2005; Ghelardini et al., 2006), with molecular studies confirming genetic differences between early and late oaks (Chokheli et al., 2016, Pirko et al., 2018). Furthermore, in our study, neighboring oaks could show large differences in budburst phenology, which argues against a large influence of microclimate. Nevertheless we acknowledge a potential influence of microclimate on budburst phenology as well.

Oaks with an earlier budburst date had a larger circumference, consistent with findings of Visser et al. (2006). This observation could be explained by two hypotheses: either oaks advance their budburst timing with age, or those with an earlier budburst grow more. We cannot definitively dismiss either explanation, and both could be true simultaneously. In Augspurger & Bartlett (2003), they observe that juvenile trees exhibit an earlier budburst to

precede the budburst of taller trees that would overshadow them. However, this finding contradicts our results. Additionally, the evidence for different genetic varieties of oak with different phenology argues against large changes in budburst with age (Chokheli et al., 2016, Pirko et al., 2018). The second hypothesis is supported by the fact that oaks with different phenology have different preferences for environmental conditions (Utkina et al., 2017, Milenin et al., 2023). Earlier oaks are more susceptible to spring frost, and are less affected on dry soils (Milenin et al., 2023). Later oaks have their growth season more shifted to summer, and are thus more susceptible to summer droughts, especially on dry soils (Milenin et al., 2023). Earlier oaks thus tend to prevail on dry soils over later oaks. Additionally, earlier oaks have the advantage of a longer growing season. However, there seems to be a positive correlation between the onset of leaf senescence in autumn and the budburst date in spring, thereby reducing the difference in growth season (Marchand et al., 2020, Crawley & Akhteruzzaman, 1988). Earlier trees face a disadvantage during years with high caterpillar abundances, as they experience greater defoliation as found in our data and in line with literature (Sarvašová et al., 2021, Kulfan et al., 2018, Ekholm et al., 2020, Wesołowski & Rowiński, 2008). In years with low caterpillar abundances, the difference in defoliation between earlier and later trees is not evident (Wesołowski & Rowiński, 2008). Ultimately, we cannot definitively conclude whether the combination of environmental factors in Dwingelderveld would promote growth in earlier trees, but it appears to be a plausible scenario.

4.2 Caterpillar abundance

Various explanations can be given for a higher caterpillar biomass in earlier oaks. In our methodology, it's important to acknowledge that tree shape and size may affect the amount of caterpillar frass found in the nets. Larger trees or trees with dense foliage could accumulate more caterpillar frass even with the same caterpillar density. We do not have measurements of tree height or leaf density, but we have found that earlier trees have both a larger circumference as well as a higher caterpillar biomass. A larger circumference is an indication of a larger and potentially taller tree, which could lead to a greater leaf surface area above the caterpillar frass net. Our findings indicate that circumference is not the best explanatory factor for the difference in caterpillar biomass, but budburst date is the best explanatory factor, and we also find significant differences in defoliation. Nevertheless, it's possible that there is an underlying effect of size on the caterpillar biomass found by us. The difference in caterpillar biomass per m² tree between earlier and later oaks may be smaller than initially estimated.

After this consideration, there are also several ecological reasons for the difference. The relationship between caterpillars and oaks is dependent on the caterpillar species (Van Asch et al., 2007). We have a limited understanding of the composition of the caterpillar community in our trees, and several hypotheses for the difference in caterpillar biomass could be true simultaneously, or different hypotheses could be true in different years depending on the caterpillar community.

The main hypothesis proposed for the difference in caterpillar biomass is that the caterpillar hatch date is best synchronized with the budburst date of early oaks (Wesołowski & Rowiński, 2008, Crawley & Akhteruzzaman, 1988, Ekholm et al., 2020). This is in contrast with the hypothesis that caterpillars adapt to a tree with a certain budburst date, or have an equal amount of phenological variety as the oaks. In such scenarios, we would expect to find a later caterpillar peak date on later trees. However, we found that the timing difference in caterpillar peak date between early and late trees was minimal, suggesting that the hatch dates of a large part of the caterpillars are more synchronized with earlier trees. Caterpillars that hatch early on

late trees, hatch before budburst and encounter a food shortage (Van Asch et al., 2007). In this case caterpillars can move to different trees, but this practice can result in high mortality, especially with large distances between trees (Van Asch et al., 2007, Sarvašová et al., 2021, Tikkanen & Julkunen-Tiitto, 2003). Both the mortality of caterpillars on later oaks by food shortages as well as the dispersal of caterpillars from later to earlier trees can add to a higher caterpillar biomass on earlier trees than on later trees. A different way to cope with the problems of hatching early on a late tree would be if moths and butterflies would select earlier oaks for oviposition, which would also increase the caterpillar biomass on early oaks. Since a timing mismatch between the host tree and the caterpillars can lead to high mortality, this would be a beneficial adaptation (Van Asch et al., 2007). However, it has been shown that for two outbreaking caterpillar species of oak, *O. brumata* and *T. viridana*, that they do not select certain trees for oviposition (Sarvašová et al., 2021, Tikkanen & Julkunen-Tiitto, 2003).

In the study of Wesołowski & Rowiński (2008), they have observed that in years with low caterpillar abundances the difference in caterpillar abundance between earlier and later trees was hard to detect or even opposite (Wesołowski & Rowiński, 2008). Our data was mostly collected in years with high caterpillar abundances, namely 2019 to 2023. The increase in outbreaking species in years with high caterpillar abundance might contribute to the difference between earlier and later trees found by us. Outbreak species have been found to hatch earlier and are more synchronized with the budburst date than other species, making them more vulnerable to food shortages when they hatch on late oaks (Kulfan et al., 2018, Ekholm et al., 2020). It has been shown for multiple outbreak species that they have a higher abundance on earlier trees than later trees (Ekholm et al., 2020, Kulfan et al., 2018). Additionally, they can form a large part of the caterpillar community, as seen in Kulfan et al. (2018), where the outbreak species *A. leucophaearia*, *O. brumata* and *T. viridana* constituted more than 85% of the larvae recorded on oak in an outbreak year. As a result, they could be the main cause of the differences in caterpillar abundance between trees with different phenology. *O. brumata* is also an outbreak species, but for *O. brumata* there is proof that they have the opportunity to adapt to a specific tree due to very limited dispersal of both males and females (Van Dongen et al., 1997). Therefore, it is possible that in years where *O. brumata* is dominant, a different result is observed, wherein peak caterpillar dates coincide with the oak budburst dates. However, Kulfan et al. (2018) found that *O. brumata* occurs more on earlier trees than later trees, disregarding the hypothesis that *O. brumata* adapts to the budburst phenology of a specific host tree.

4.3 Leaf traits

After controlling for date, trees with an earlier phenology had a higher leaf toughness, which aligns with our expectations. A possible explanation is that leaves of earlier trees are older and thus more developed. We find no difference in the rate at which the leaf toughness increases between trees with a different phenology, thus we do not find proof that earlier trees increase their leaf toughness more in response to defoliation. A reason can be that trees are restricted in their resources in the early growth season, which constrains a faster increase in leaf toughness (Gaytan et al., 2022). Alternatively, we also do not find evidence that later trees more quickly defend their leaves by having a faster rate of toughening their leaves.

The concentrations of total phenolics and tannins were best explained by leaf toughness and leaf age, even though we expected herbivory to have an influence on the concentrations of total phenolics and tannins. Experimental studies have found an increase in tannins after defoliation and mechanical damage (Hunter & Schultz, 1995; Rossi et al., 2004; Schultz & Baldwin, 1982), but field studies relating caterpillar abundance and performance to phenolic compounds have

produced mixed results (Barbehenn et al., 2011 et al., 2011, Forkner et al., 2008, Visakorpi et al., 2020). Also, there is still a discrepancy in the literature on which phenolic compounds are related to herbivory, even within the subgroup of tannins (Barbehenn et al., 2011 et al., 2011, Visakorpi et al., 2020, Skovmand et al., 2023). If there is a response to herbivory by some compounds, this result might be shielded by measuring the complete group of phenolics and tannins. Additionally, we measured the concentrations of total phenolics and tannins in dry leaf material. The water content in leaves can fluctuate, as has been found by Feeny (1970), thus the trends found in the concentrations in dry leaf material might not directly translate to the concentrations encountered by caterpillars. Potentially, this masks a relation between phenolic content and caterpillar biomass, although in Feeny (1970) the water content is consistent in the first three weeks after budburst. An additional challenge in examining phenolics in a field study is that they are highly influenced by environmental factors, such as nutrient availability, UV light temperature and the presence of pathogens (Salminen, Visakorpi et al., 2020, Dudt & Shure, 1994, Lauer et al., 2011, Barbehenn et al., 2011 et al., Madritch et al., 2015). As a result, high variation in the concentrations of total phenolics and tannins is found within and between individuals (Visakorpi et al., 2020, Covelo & Gallardo, 2004). To counter this, we measured neighbouring trees in, what we expect to be, similar environments, but we still found high variation within and between trees.

Nevertheless, we found a correlation between the concentrations of total phenolics and tannins and leaf age, comparable to the trend found in Salminen et al. (), as well as a relation between leaf toughness and phenolics. The leaves immediately following budburst have a high concentration of total phenolics and tannins, accompanied by a low leaf toughness (Covelo & Gallardo, 2004, Salminen). It has been found that the concentration of phenolics is already high in the buds (Covelo & Gallardo, 2004). The high concentrations in the leaf buds and young leaves can be explained by a need to protect these vulnerable leaves, which have a high protein content but a low leaf toughness (Covelo & Gallardo, 2004, SOURCES). The production of phenolics is costly and resources are limited at the start of the growth season, but the phenolics in the buds of trees could come from stored reserves (Covelo & Gallardo, 2004). Interestingly, even with their high phenolic contents, the youngest leaves are the highest quality food source available for caterpillars due to their combination of high nutritional quality and low leaf toughness (Ekholm et al., 2020). Caterpillars that exploit this high quality food source could have adapted to overcome the high phenolic content, or the phenolics in the young leaves serve a different function than herbivory protection (Ekholm et al., 2020).

We find that the concentrations of total phenolics and tannins decrease with leaf age while the leaf toughness increases, especially in young leaves with a low leaf toughness. This trend disappears in older leaves with a high leaf toughness. Both growth, including increasing leaf toughness, as well as the production of specialized metabolites, including phenolics, are costly, thus it is thought that there is a trade-off between these two factors (Madritch et al., 2015, Gaytan et al., 2022, Herms & Mattson, 1992, Riipi et al., 2002; Glynn et al., 2007). Especially at the start of the growth season, when resources are limited, this trade-off could be the cause of the observed trend (Madritch et al., 2015). The trend disappears after approximately three weeks, both in our results as in Salminen et al., (2004).

We analyzed leaves of the lower branches of the tree, as well as salminen et al. (2004). Feeny (1970) found an increase in total tannin content from April to September, which is opposite from our results. Feeny (1970) analyzed leaves from the top part of the canopy, where leaves could be more influenced by UV light among other potential differences (Dudt & Schure, 1994).

Conclusion

In conclusion, we show that oaks have not only interindividual variability in phenology, but also in leaf toughness, concentration of total phenolics and tannins, caterpillar biomass and defoliation. At the same time, trees with an earlier phenology have older leaves, which have a higher leaf toughness and higher concentration of phenolics. This influences food distribution for herbivores. Additionally, tree phenology influences the distribution of herbivores, which in turn affects food distribution for insectivores. As a result, the variability in oak phenology is important to take into account when looking at these timing-dependent multitrophic interactions.

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