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Pilot Study of Sap Properties of Norway Spruce (*Picea abies* (L.) Karst.) Trees Used and Not Used for Sap-Feeding by Three-Toed Woodpeckers (*Picoides tridactylus*)

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Abstract: The ecophysiology of the Norway spruce (*Picea abies* (L.) Karst.) trees that were used by three-toed woodpeckers (TTW) (*Picoides tridactylus*) for their sap-feeding activities were investigated. The pilot study was conducted in southern Finland (61°15′ N, 25°00′ E). During April–June 2015, three different tree categories of Norway spruce were selected for monitoring: trees that were frequently used by the TTWs for phloem sap-feeding for several previous years; trees that were only recently used by TTWs for sap-feeding; and control trees that were not used at all for sap-feeding. Phloem sap and phloem tissue samples were frequently extracted from tree trunks and analyzed for the content and composition of nonstructural carbohydrates, phloem sap osmolality, solute, and water content, as well as for the content and composition of secondary metabolites typical for defense reactions in the phloem. Simple crown characteristics were also measured, including tree height, diameter at breast height, and their ratio (slenderness index). According to our results, the TTWs preferred Norway spruce trees that showed advanced spring phenology to feed on, as evidenced especially by the lower ratio of raffinose (typically high during the winter months) to total soluble sugars of phloem tissues as compared to non-used control trees. The lower slenderness index of the trees chosen by the TTWs indicates low canopy competition pressure with good access to light (i.e., the sun heats the trunks well in spring). There were no differences in the phloem osmolality or solute content between the used or unused control trees. The trees used by the TTWs had significantly higher concentrations of antioxidant phenolic (+)-catechins and stilbene glycosides in phloem tissue, and the stilbene content was also higher in the extracted sap. The phenolics content of the phloem tissue had a clear seasonal trend, being the highest in the early spring and lower towards the onset of the cambial growth processes. The phloem sap is rich in antioxidants and soluble sugars that are potentially beneficial for the TTWs, but more quantitative research is needed to explore the importance of the sap properties to TTWs.

Keywords: antioxidants; bark; calcium; carbohydrates; minerals; Norway spruce; phloem; phloem sap; sap-feeding; three-toed woodpecker

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1. Introduction

The three-toed woodpecker (TTW, *Picoides tridactylus*; Figure 1A) has a broad Eurasian distribution mainly within the boreal forest zones [1,2], where it is adjusted to forest disturbance dynamics that create suitable, spatially, and temporally variable breeding areas with large quantities of dead and damaged trees [3–5]. For most of the year, the TTWs feed predominantly on conifer bark beetle larvae that are found in these dying and dead trees [2,6–8].

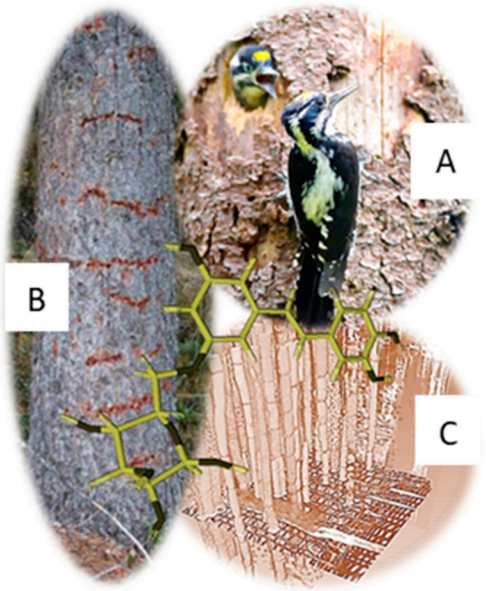

Figure 1. Three-toed woodpecker (*Picoides tridactylus*; **A**) and its sap feeding rows on Norway spruce (*Picea abies* (L.) Karst.) tree trunk (**B**), with inner view on phloem cellular and chemical structures (**C**).

However, in a similar way to several other woodpecker species [9], the TTW has also been found to use the sap of forest trees [1,2]. The species prefers the sap of coniferous trees, especially the Norway spruce (*Picea abies* (L.) Karst. Figure 1B), and phloem sap is used for feeding mainly during the early spring season [2,5,10,11]. According to previous studies, sap-feeding seems to play an important role just before the nesting of the TTWs [10]. However, we do not yet have comparable quantitative data about the different sap ingredients of spruce trees during spring, in relation to the feeding activities of TTWs (i.e., trees used by TTWs and those not used).

The Norway spruce is ecologically and economically one of the most important tree species in the boreal coniferous forest zone. Spruce phloem (Figure 1C) is a living transport tissue located in woody tree parts such as the tree trunk between the outer bark and the cambial layer (i.e., where secondary growth occurs). Phloem can be divided into different zones according to its function: the inner layer, the outermost layer, and the parenchyma cells. The inner layer, only a few hundred micrometers thick, is an active transport tissue (phloem sieve cells), where photosynthetic assimilates and numerous other compounds are transported from leaves to other metabolically active tissues of the tree [12]. The outermost layers of the phloem consist of nonfunctional sieve elements that have already ceased their transport operations. Parenchyma cells in the phloem function as a repository of nutritional and defense compounds such as phenolic stilbene compounds and tannins [13–15]. Outside the phloem, the outermost bark layer is called the periderm and it consists mainly of dead cells, which protect the wood mechanically.

Phloem transport activity depends on the sugar concentration of phloem sap, and the turgor pressure gradient in the phloem, which are affected also by xylem transport, i.e., transport of water from the roots to the leaves in the wood cells, and the tension of the

transported water, i.e., water potential [16,17]. In addition, the composition of the phloem sap changes according to the season and environmental conditions [17,18].

Phloem contains soluble sugars and starch, as well as chemical defense compounds such as phenolic (+)-catechins and stilbene glycosides, which are strong antioxidants and have antibacterial efficacy [14,15,19,20]. The phloem of the Norway spruce is also relatively rich in calcium oxalate (CaOx) crystals, in a similar manner as the secondary phloem of many other conifer tree species [21]. CaOx crystals serve as a constitutive defense of bark, providing a mechanical barrier against bark-boring insects [22].

The phloem sap-feeding of TTWs in southern Finland typically starts as early as late winter, continues into March, and is the most active in late April and early May (Table 1) [10]. It has been suggested that the southern, sun-exposed parts of the tree trunks of Norway spruce with dark bark, warm up by many degrees above ambient conditions during the sunny late winter and early spring days [10]. This may decrease the viscosity of the phloem sap, allowing the TTWs to use the sap. Later in May, when the TTWs start their nesting, phloem sap is used only occasionally, and the TTWs have not been observed feeding their nestlings with the sap [10]. Sap-feeding thus largely coincides with important stages of spring phenology of the trees in southern Finland (Table 1): subcellular changes in shoots and phloem, such as the content of starch and soluble sugars in the inner bark, occur from March towards later spring [23–26]. Fluids and nutrients also begin to flow in the xylem and phloem of tree trunks at the time of frost melt, well before the onset of the cambial growth processes that take place in late-April–early-May in the south of Finland [14,25].

Table 1. Average spring and summer schedules of the three-toed woodpeckers (TTWs) and average spring phenological events of Norway spruce in southern Finland [23–26].

Time	Nesting Cycle	Sap Feeding by TTWs	Norway Spruce Phenology
March	Pairs begin to settle into territories; territorial activity starting to increase	Ongoing, but usually occasional and restricted to warm days and afternoons	Ecodormancy, environmental factors preventing growth: (a) initial events in primordial bud development from mid-March; (b) soluble sugar levels in phloem decline and starch amount increases from mid-winter conditions. Especially raffinose content starts to decline.
1–20 April	Pairs mostly at breeding territories; active territoriality, pair-bonding behavior increases	Ongoing, time used feeding on sap increases	Initial events in primordial bud development depleted with early development of vascular tissue and primordial needles by late-April. Starch and soluble sugar transformation active in phloem. Warm spells enhance tissue-level metabolism and growth.
20 April to 15 May	Cavity excavation or preparation of old cavities for nesting; active territoriality, pair bonding finalized, copulations	Ongoing, peak period with decrease to the end of the period	Peak period for vascular tissue and needle development in primordial buds at the end of April. Peak period for cambial reactivation, first towards phloem. Phloem formation begins by later-April–early-May. Both soluble sugar and starch levels are high in phloem.
15 May to 30 May	Nesting period starts; egg-laying, incubation; some late pairs may still prepare the cavity	Ongoing, turning occasional, clear decrease from the peak period	Bud break by late-May. Cambial divisions towards xylem begin by late-May–early-June. Soluble sugar levels begin to decline, starch content still high in phloem.
June	Nesting period continues; nestlings hatch mostly from the 1st to the 10th, and leave the nest mostly from the 20th to the 30th	Infrequent by adult TTWs, nestlings are fed mostly by insect larvae, sap is not served to nestlings	Peak period of cambial growth. Xylem formation at maximal rate around mid-June. Phloem formation depletes by late June—early-July. Height increment decreases. Soluble sugar amounts at the lowest level and starch content begin to decline in phloem.
July and onwards	Latest nestlings leave the nest during 1st to 10th; nestlings move with adults first in the neighborhoods of the nest cavity, later wider in the nesting territory, brood often divided by the male and female	Infrequent, adults feed young with insects for some weeks, no observations of independent sap feeding of the young birds (this period is generally rather poorly known, occasional observations of sap feeding have been observed in later autumn)	Xylem formation and maturation ongoing. New bud set. Soluble sugar content in phloem increases from August towards winter.

During the sap-feeding of the TTW, usually horizontal sap rings or rows of small holes penetrating the outer bark and the phloem are created on the tree trunks. Moreover, TTWs quite often tend to first peck off the periderm layer, before pecking the sap holes into the inner phloem (Figure 1B) [10]. Because phloem transport is locally blocked over the sap rings, phloem sugars and pressure are suspected to accumulate above the sap rings [27,28]. However, the effects of phloem sap-feeding of TTWs or other woodpecker species on tree defenses have not been studied. The mechanical damage of the bark structure induces anatomical and chemical defense of the phloem and cortex: e.g., wound cells are formed to repair the damage, and the cells synthesize secondary defense compounds (e.g., phenolic secondary metabolites such as (+)-catechin and stilbene glycosides, and resinous compounds such as resin acids) [15].

It is also not known which tree-specific factors make woodpeckers choose certain trees or groups of trees for sap-feeding. Individual tree size, competitive position, vitality, sap availability (e.g., early onset of physiological functions, such as changes in water and solute/sugar content), quality and quantity, and/or bark constitutive (existing) defense compounds have been suggested to have an effect [4,10,27,29,30]. However, individual research results on the effects of tree size, position, and health status are somewhat contradictory. There is very little data on the chemical properties of sap in trees selected by woodpeckers, but trees with high sugar concentrations and low amounts of tannins have been observed to be used [31].

The aim was to study whether there were any differences in the phloem characteristics between the trees that were used by TTWs for sap-feeding and those that were not used. We hypothesized that the trees with early phenological activity are selected by TTWs and that the selected trees show increased amounts of defense compounds as compared to trees not used by TTWs. To test our hypothesis, we monitored tree parameters that are strongly connected to the springtime activation of vascular phloem functioning, namely (1) the decrease in the phloem tissue water content during late spring after the saturation between snowmelt and reactivation of transpiration [32–34]; (2) the decrease in phloem tissue osmolality during late spring/early summer following the winter de-hardening [33]; (3) chemical changes in the content and composition of non-structural carbohydrates (NSC) of phloem tissues; and (4) chemical properties of the phloem sap. Additionally, we studied the (5) variation in concentration of phloem secondary metabolites due to season and between the sample tree groups (i.e., trees with long-term or recently initiated ringing and sap-feeding activity, and control trees with no ringing activity by the TTWs; see below). Concentrations of phenolic defense compounds (i.e., stilbenes and catechins) may either increase as a response to the long-term ringing and sap-feeding activity of the TTWs or, on the other hand, TTWs may also choose trees with higher amounts of primary or secondary metabolites of phloem, potentially beneficial for the diet of TTWs. Finally, we compared tree structure (tree height, diameter at breast height, and tree slenderness defined as height: diameter at breast height) between the sample tree groups to study whether some general characteristics could be linked to the trees used by the TTWs.

2. Materials and Methods

2.1. Research Area and Tree Material

The study was carried out in the Evo—forest area located in southern Finland (61°15′ N, 25°00′ E; Figure 2). The area consists of mainly spruce-dominated coniferous or coniferous-deciduous mixed forests of *Myrtillys* site type [35,36]. The ecology of the TTW has been studied in a study area of 170 km^2 over 30 years [10,35,37]). The sample trees were selected within five long-term TTW territories (Figure 2).

Within each selected TTW territory, we chose typical TTW sap-feeding sites located at the southern or southwestern edges of a mature spruce-dominated forest of the *Myrtillus* site type against clear cuts or saplings [10]. Within these sites, we searched triplets of Norway spruce trees, which (i) were located near each other and at similar distances from the edge of the mature forest, (ii) were mature trees of generally similar size class, and

(iii) contained each of the following cases based on the past and current phloem sap-feeding activity of the three-toed woodpeckers: (1) ringed trees actively used by the birds for several years ("old trees" O); (2) ringed trees with very recently started ringing activity by the birds ("new trees" N); and (3) control trees without visually detectable ringing activity ("control trees" C). Sap rows are typically pecked at heights of 1–15 m in the study area [10], but the trunks of the trees were checked totally from ground to top with the help of binoculars for the possible signs of sap rows. In total, six triplet sites were selected: in four territories, one site, and in one territory, two sites. In one triplet site, no suitable trees of type O were found, and instead, two trees of type N were chosen. Thus, the number of type O trees was five, that of type N was seven, and the number of control trees was six.

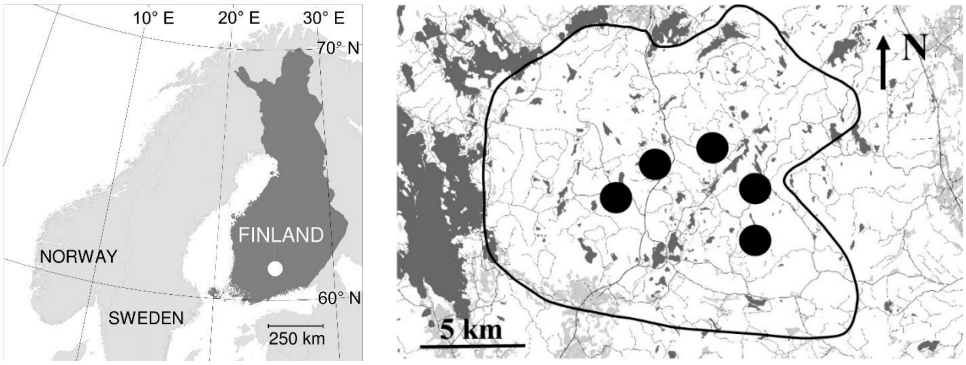

Figure 2. Location of the Evo research area in southern Finland (**left**; white dot) and the five sample sites within the area (**right**; black dots). The border of the long-term study area of the TTW (170 km^2) is marked with black line.

Tree height, tree diameter at breast height (DBH; 1.3 m), and the slenderness index of each sample tree were measured. The slenderness index was calculated as height: diameter at breast height.

The characteristics of the sample trees and sampling dates of phloem are shown in Table 2. The sampling period lasted for nearly two months and covered the peak onset season of the cambial activity of the trees ([24]; see above). Figure 3 shows the weather conditions for the experiment period as well as the long-term climatic conditions measured at the weather station of the Lammi Biological Station of the University of Helsinki located ca. 20 km south of the Evo research area. The climatic factors, especially air temperature, are key drivers for the onset of tree growth processes in the spring.

Table 2. Mean (±standard deviation) characteristics of sample trees, and sampling dates in 2015. Phloem water content, osmolality and solute content were measured on the sampling dates A, chemical properties of phloem sap on the sampling dates B and chemical properties of phloem tissue on the sampling dates C.

	Sample Tree Class		
	Old (O)	**New (N)**	**Control (C)**
Diameter at BH (cm)	35.4 (10.9)	30.8 (7.4)	31.9 (4.9)
Height (m)	26.4 (6.8)	25.6 (4.6)	28.5 (2.3)
Slenderness index (height:diameter at BH)	75.5 (5.9)	84.5 (9.8)	90.3 (9.6)
Sampling dates A (Phloem water content, osmolality and solute content)			
14 April, 30 April, 12 May			
Sampling dates B (Chemical properties of phloem sap)			
12 May, 25 May, 29 May, 4 June			
Sampling dates C (Chemical properties of phloem tissue)			
30 April, 12 May, 29 May, 4 June			

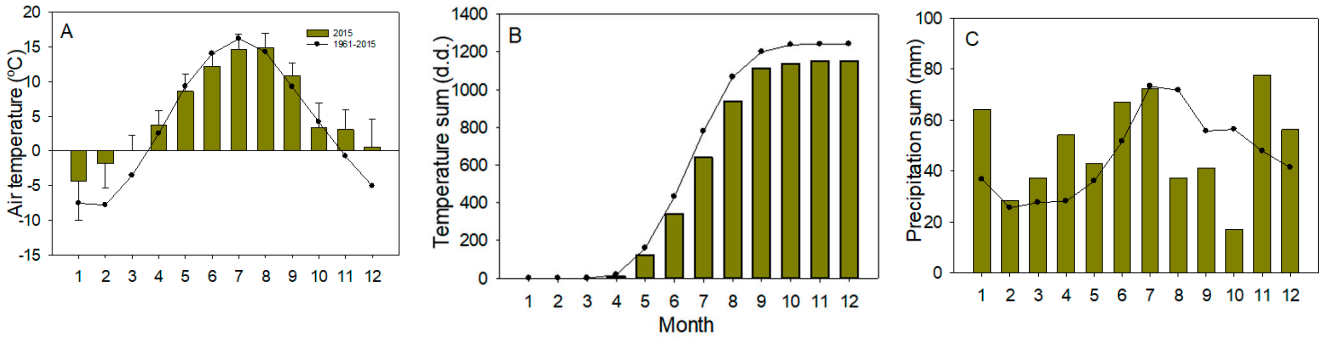

Figure 3. Monthly mean temperature (**A**), temperature sum (**B**), and monthly precipitation (**C**) during the study year (2015, bars) and the long-term average (1961–2015, dotted lines).

2.2. Sampling of Phloem

To analyze osmolality, water content, and the composition of the sugars and extractives in the tissues and sap of phloem, we collected small intact blocks containing bark, cambium, and wood (ca. 2 cm × 2 cm × 2 cm in tangential, radial, and longitudinal directions) twice a month at breast height (BH; at 1.3 m height on stem) from the sample trees (Figure 4, Table 2). The distance between the sample locations, as well as between the sample location and woodpecker sap rows, was at least 10 cm to avoid wounding effects due to sampling on the chemical and anatomical properties of the samples. Additional sample blocks including parts of the woodpecker sap rows were also collected to study the woodpecker sap row structures within the tissues. The sample blocks were immediately frozen in liquid nitrogen (N_2, −195 °C), and transported to the laboratory either in N_2 or in dry ice (−78 °C) and stored at −78 °C until further processing. Outer bark was scraped away with a razor blade. The inner bark (including cambium, and all the tissues from cambium to the innermost periderm) was separated from the xylem. All the separations were performed based on the hardness and color differences between the tissues. The inner bark of the sample blocks was divided into three parallel sub-samples (in radial-longitudinal direction), which were used for the analysis of (1) phloem water content; (2) osmolality and content of NSCs and extractives of phloem sap; and (3) NSC and extractive content of phloem tissue.

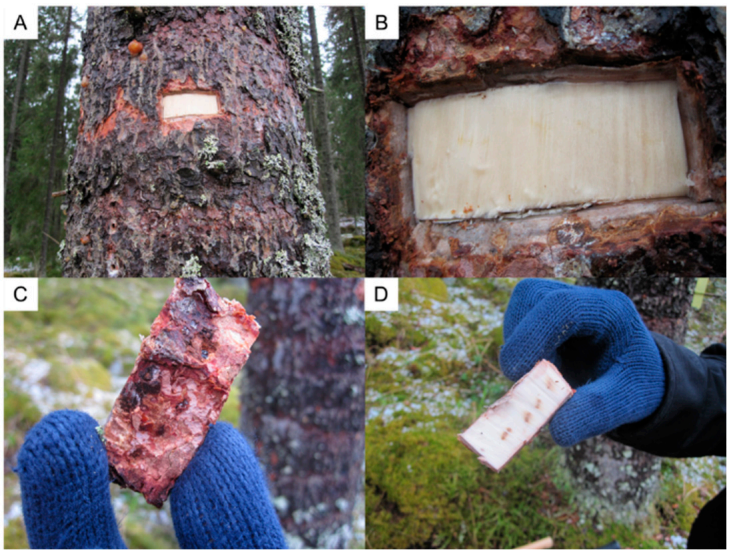

Figure 4. Typical rows made by the TTWs (upper left) on Norway spruce tree actively used by the TTWs for several years ("old" tree O) and example of intact bark sample block collected from Norway spruce tree (**A**,**C**), a surface of lignified sapwood with marks of holes beneath the cambial layer (**B**), sample block with clearly visible rows on outer bark (**C**), and the same rows seen from the inner bark side next to cambial zone (**D**).

2.3. Samples for Phloem 3D Structure

Samples for the cellular level structural analysis for the phloem were obtained from Loppi in southern Finland (60°44′ N, 24°30′ E, 120 m above sea level). For details, see [14,25].

2.4. Analysis of Phloem Water Content

The seasonal changes in the osmolality, content of solutes (p), water content (WC), and relative water content (RWC) of the inner bark tissue were determined by using one sub-sample from each sample block. The fresh weight was first measured, and the samples were then soaked in a water bath for 48 h to measure saturated weight. The samples were then oven-dried at 80 °C for 72 h, the dry weight measured and the WC (g g^{-1} DM) and the RWC determined (as the ratio of water content in the fresh sample to the water content in the water-saturated sample). The content of solutes (p, mol kg^{-1} DM) was calculated as osmolality multiplied by WC.

2.5. Osmolality Analysis

Another sub-sample of each block was cut into small pieces and set in silica-based membrane collection tubes (Gene JET Plasmid MiniprepKit, ThermoScientific, Waltham, MA, USA) into a centrifuge (Heraeus Fresco 17, ThermoScientific, Waltham, MA, USA) at 14,000× g for 10 min [38]. The liquid was collected into osmometer tubes, and the osmolality of the liquid (mol kg^{-1}) was measured with a freezing-point osmometer (Osmomat-030 Freezing point osmometer, Gonotec, Berlin, Germany). We assumed that the ratio of phloem tissue volume compared to the whole inner bark tissue volume was large enough that it is justified to refer to the collected inner bark sap as phloem sap (Lintunen et al. 2016). After the osmolality analysis, the remaining sap was immediately frozen with liquid nitrogen and stored at −78 °C until used for the carbohydrate analysis.

2.6. Carbohydrate Analysis of Phloem Sap

The amounts of mono- and disaccharides, extractives, and non-cellulosic polysaccharides (i.e., hemicelluloses and pectins) of the phloem sap were analyzed with chemical microanalysis methods including gas chromatography (GC) with a flame-ionization detector (FID), as well as GC-mass spectrometry (MS), for quantitative and qualitative analyses, respectively. The remainder of the sap was used for the osmolality analysis. The sap representing different tree groups (O, N, C) on each sampling date was allowed to melt, pooled together, placed into Eppendorf vials, and then immediately freeze-dried. The freeze-dried phloem sap was weighed and extracted in a test tube three times with 3 mL of acetone water (9:1 v/v) at room temperature in total darkness (total extraction time was 24 h). The test tube was periodically agitated. The clear extracts were transferred to another test tube with Pasteur's pipettes, and the solvents from the cumulative extracts were evaporated in a water bath at 40 °C with N_2 flow, and carefully dried in a vacuum desiccator at 40 °C for 1 h in the dark. The dried extractive content was determined gravimetrically, and 10 mL of acetone was added to each test tube to obtain a stock solution of the extractives. An aliquot of the stock solution was placed in separate test tubes to obtain about 1 mg of the extractives on a dry basis, and 0.1 mL of 0.1 mg mL^{-1} xylitol solution (in methanol) was added as the internal standard. To the same test tube, 2 mL of another standard solution containing 0.02 mg mL^{-1} of betulinol in MTBE was also added. The solvents were then evaporated using N_2. Silylation was completed at 70 °C for 40 min with a 4:1:1 ($v/v/v$) mixture of BSTFA-TMCS-pyridine, and the derivatized sample was left overnight in the dark. The silylated samples were then analyzed by GC-FID using a PerkinElmer Clarus 500 gas-chromatograph equipped with HP-1 (25 m × 0.2 mm; 0.11 µm film thickness) and HP-5 (25 m × 0.2 mm; 0.11 µm film thickness) capillary columns, with the temperature programming providing simultaneous analysis of mono-/disaccharides and stilbene glucosides during one GC-run: 100 °C, 8 min hold, 2 °C min^{-1}, 170 °C, 12 °C min^{-1}, 310 °C, 7 min hold. The temperature of the injector and detector was 250 °C and 320 °C, respectively. The sample injection volume was 1 µL and the split ratio was 1:30.

Mono- and disaccharides were quantified against xylitol using correction factors that were determined by the separate analysis of authentic mono-/disaccharides. Stilbene glucosides were quantified against betulinol without using a correction factor (i.e., CF 1.0). After the extraction, the dry weight of each extracted specimen was determined by a microbalance (Mettler Toledo Excellence Plus XP Analytical balance XP205) and the sample was taken for acid methanolysis into a separate test tube. After acid methanolysis, the remaining non-cellulosic polysaccharides (i.e., hemicelluloses and pectins) were decomposed into monomeric sugar units. The contents of the test tube were neutralized with pyridine, the sorbitol and resorcinol standards were added, and solvents were evaporated with N^2. The residues were dried in a vacuum desiccator at 40 °C for 40 min, silylated with a 1:2:1 ($v/v/v$) mixture of HMDS-TMCS-pyridine overnight according to [39], and analyzed by GC.

The TMS-derivatives of the corresponding methyl-glycosides were analyzed with the GC method using a Varian 3400 instrument equipped with a capillary columns HP-1 (25 m × 0.20 mm; film thickness 0.11 µm) and HP-5 (25 m × 0.20 mm; film thickness 0.11 µm). The protocol for the column oven was as follows: starting temperature 100 °C, temperature increase rate 4 °C/min to reach the temperature of 175 °C, then the temperature rate was changed to 12 °C/min, end-temperature 290 °C, hold time 5 min. Hydrogen with a flow rate of 1 mL/min was used as a carrier gas. The injector was a conventional evaporator heated at the temperature of 260 °C. The temperature of the FID was 290 °C. The sample volume was 0.8 µL (split 1:20).

2.7. Carbohydrate Analysis of Phloem Tissue

Non-structural carbohydrates (NSCs, i.e., soluble sugars and starch) of the inner bark were analyzed using a third sub-sample of the inner bark blocks, which were cut into matchstick-sized pieces, freeze-dried for 72 h, and milled with a ball-mill while still frozen. About 50 mg of powder was weighed into glass test tubes and heated to 100 °C to deactivate the enzymes. The soluble sugars were extracted from the samples in a water bath (100 °C) containing 80% ethanol to which m-erythrit (Calbiochem, Merck KGaA, Darmstadt, Germany) was added as an internal standard. The sugar extracts were evaporated to dryness with nitrogen flow, silylated with 20% TMSI-pyridine mixture (1-trimethylsilylimidazole; Sigma-Aldrich, St. Louis, MO, USA), and analyzed with gas chromatography-mass spectrometry (GC–MS; Agilent Hewlett-Packard 6890 GC, equipped with a Zebron ZB-SemiVolatiles column (30 m 9 0.25 mm i.d. 90.25 lm df) and Hewlett-Packard 5973 MSD, EI-MS 70 eV), in which helium was used as a carrier gas (flow 1.5 mL/min). The chromatographic conditions were as follows: initial temperature 110 °C; rate of temperature increase 10 °C min^{-1}; final temperature 320 °C maintained for 14 min; injector temperature 260 °C, and split ratio 1:20. The MS-interface temperature was 300 °C and ion source temperature was 230 °C. The results were calculated using an internal standard and the following external standards: D-fructose (Merck), myo-inositol (Merck), D-glucose (BDH AnalaR, VWR International Ltd., Poole, UK), sorbitol (Fluka, Sigma-Aldrich), sucrose (BDH AnalaR), D-raffinose pentahydrate (Fluka). For pinitol, fructose was used as a standard, and for stachyose, raffinose was used as a standard. The soluble sugar-free samples obtained after the extractions were used for starch analyses with a commercial starch assay kit (Total Starch Assay Procedure, Megazyme International, Wicklow, Ireland). Briefly, starch in residual pellets was hydrolyzed into maltodextrins by adding a-amylase (in MOPS-buffer, pH 7) and incubating for 6 min at 100.5 °C. Next, the samples were suspended in acetate buffer (pH 4.5) and amyloglucosidase was added to hydrolyze maltodextrins into D-glucose by incubating for 30 min at 50.5 °C. The absorbance of the samples was measured colorimetrically (Shimadzu UV-2401 spectrometer at 510 nm) using glucose oxidase and peroxidase. The standard curve was made with D-glucose (BDH AnalaR).

2.8. 3D-Microtomographic Analysis of Phloem Cellular Features

The cellular and tissue-specific features of Norway spruce phloem were analyzed by means of 3D-microimaging. Samples were dried through a super-critical-drying-process

for phase-contrast X-ray microtomography on beamline ID19 at the European Synchrotron Radiation Facility [14]. Phloem cell types, including those with CaOx crystals, were identified from the images across phloem layers [14].

2.9. Statistical Analysis

Values are given as mean ± standard deviation. To analyze the statistical differences in the content and composition of phloem solutes and chemical constituents between sampling times and tree categories, analysis of variance was fitted to the data with the assumption of the normal distribution (tested by Shapiro–Wilk). Hierarchical mixed models were applied for comparing the water content of phloem, osmolality of phloem sap, and phloem tissue chemical constituents between the treatments in each sampling time. Tree was used as a random variable to account for the variation within multiple samples of a single tree, thus avoiding pseudo-replication. In the mixed models, the Bonferroni method was used to counteract the problem of multiple comparisons. The variance analysis of tree slenderness index was simpler having only one main effect in the model (i.e., tree category). The variance analysis of phloem sap chemical constituents was also simplified due to the small data set having only one main effect in the model (i.e., tree category). The method of Tukey was used in pairwise multiple comparisons with a significance level of $\alpha = 0.05$. The residuals were checked for normality using graphical figures. The models were fitted by using the MIXED procedure in SAS (SAS Institute, Cary, NC, USA) and one-way ANOVA—and MIXED procedures of IBM SPSS STATISTICS (v. 26.0) (IBM, Armonk, NY, USA).

3. Results

3.1. Visual Appearance of Bark Used by the TTWs for Phloem Sap Feeding

Frequent, destructive sampling of bark tissues (Figure 4) was conducted for analysis of the chemical properties of phloem (both sap and tissues), as well as water content during spring. The collected sample blocks were also visually studied for the appearance and structure of the sap rows made by the TTWs. The TTWs pecked small punctures through the outer bark layers, reaching the inner phloem and soft cambial zone. However, the holes did not pass beyond the cambium to hard xylem layers i.e., lignified, mature tree-ring formed in the previous growing season.

3.2. Cellular Structures of Norway Spruce Phloem

The phloem of the Norway spruce consists of conducting and non-conducting layers (Figure 5). The conducting layer is composed of only a narrow strip of sieve cells close to the cambium, along with albuminous cells and ray and axial parenchyma cells, of which the latter also include CaOx crystals (Figure 5). The amount of CaOx increased as a function of distance from the cambial layer towards the outer layers of the phloem.

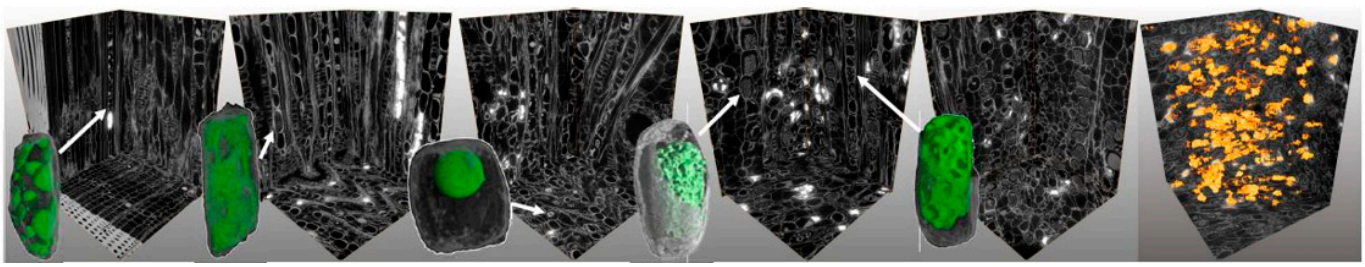

Figure 5. Axial, radial, and tangential cross-sections of *Picea abies* phloem, sampled from the xylem (**left**) to near the outer bark (**right**). In the rightmost image, the 3D renderings show the accumulation of calcium oxalate crystals (seen in white in the 2D cross-sections) in the phloem. The insets show various identified types of axial parenchyma cells, with the cell contents rendered in green and the cell walls in gray.

3.3. Phloem Water Content and Osmolality of Phloem Sap

Phloem relative water content was ca 0.48–0.53 (Figure 6A). The differences between tree categories and sampling dates in the spring were not statistically significant (Table 3). Phloem water content was 1.5–1.7 g g^{-1} DM (Figure 6B, Table 3). The differences between the categories of trees were not significant, but phloem water content was significantly different between the sampling times, being higher in mid-April than in mid-May (Table 3).

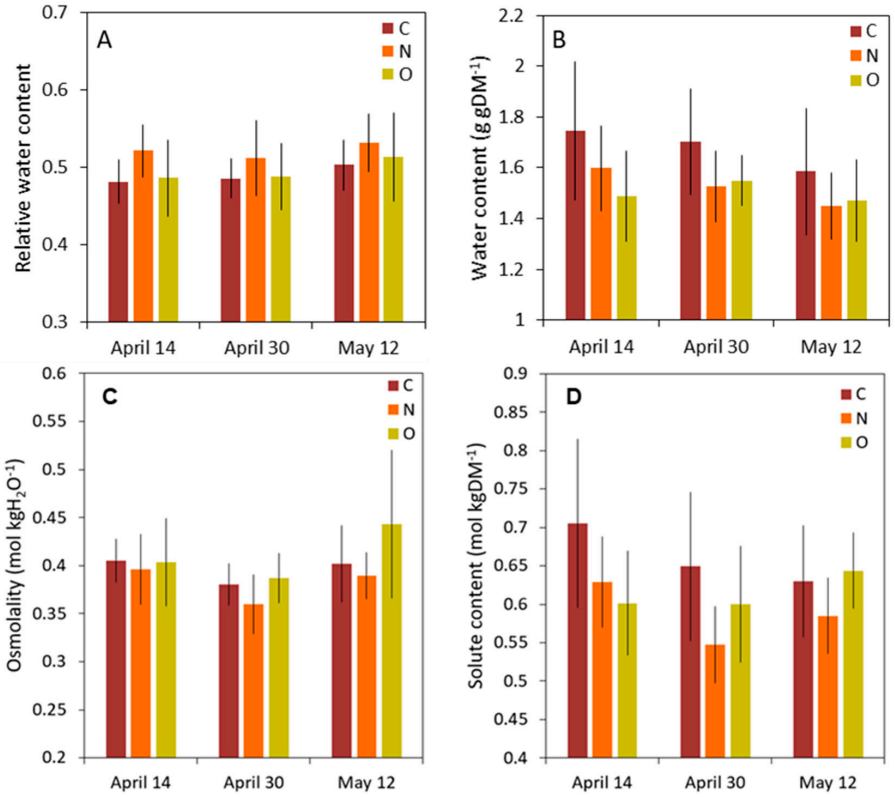

Figure 6. Phloem relative water content (**A**), phloem water content (**B**), phloem osmolality (**C**), and phloem tissue solute content (**D**) in trees used by TTWs for several years ("O"), trees with newly started sap-feeding activity ("N") and control trees ("C"). Means and standard deviations are shown. Note: the units and value scale in the y-axis differ between sub-figures.

Table 3. Mixed model results from testing the differences in phloem relative water content (RWC), water content (WC, g g^{-1} DM), osmolality of phloem sap (mol kg^{-1}), phloem sap solute content (p, mol kg^{-1} DM) as well as tree slenderness index of Norway spruce between tree categories (T) (i.e., trees used by birds for several years, trees with newly started bird activity and control trees) and sampling times (S) in spring 2015.

Dependent Variable	Fixed Effect	*F*-Value	*p*-Value
RWC	T	2.00	0.153
	S	1.29	0.290
	T*S	0.23	0.919
WC	T	1.53	0.232
	S	3.65	**0.038**
	T*S	0.84	0.509
Osmolality	T	0.29	0.752
	S	4.84	**0.015**
	T*S	1.05	0.401
p	T	2.13	0.138
	S	2.16	0.134
	T*S	2.13	0.102
Slenderness	T	2.97	*0.082*

"*": Interaction between fixed factors of the statistical model. Significant factors are shown in bold ($p \leq 0.05$). Nearly significant factors are shown with italics ($p \leq 0.10$).

To better understand the different growth vigor as well as the stability of the trees in the different treatments, we used the slenderness index (Table 2). The differences between tree categories were nearly statistically significant (p = 0.08; Table 3), the average slenderness being lower in the control trees than the trees being used by birds for phloem sap feeding. Phloem osmolality was ca 0.4 mol kg^{-1} (Table 3). No differences between tree categories were found (Figure 6C; Table 3). Phloem osmolality was the lowest in late April and highest in May, and the differences between the dates were significant (Table 3). Phloem solute content was ca. 0.6–0.7 mol kg^{-1} DM (Table 3, Figure 6D). No statistically significant differences were found between tree categories and sampling dates (Table 3).

3.4. Chemical Properties of Phloem Sap

The solid content of phloem sap of the Norway spruce was 12.8% (±0.50%), with no clear seasonal trends between the sampling dates. From the analyzed compounds of sap solids, the majority were non-structural sugars, mainly di- and monosaccharides (ca. 60% and ca. 10%, respectively), lipophilic extractives (ca. 10%), and hemicelluloses and pectins (ca. 30%) (Figures 7 and 8).

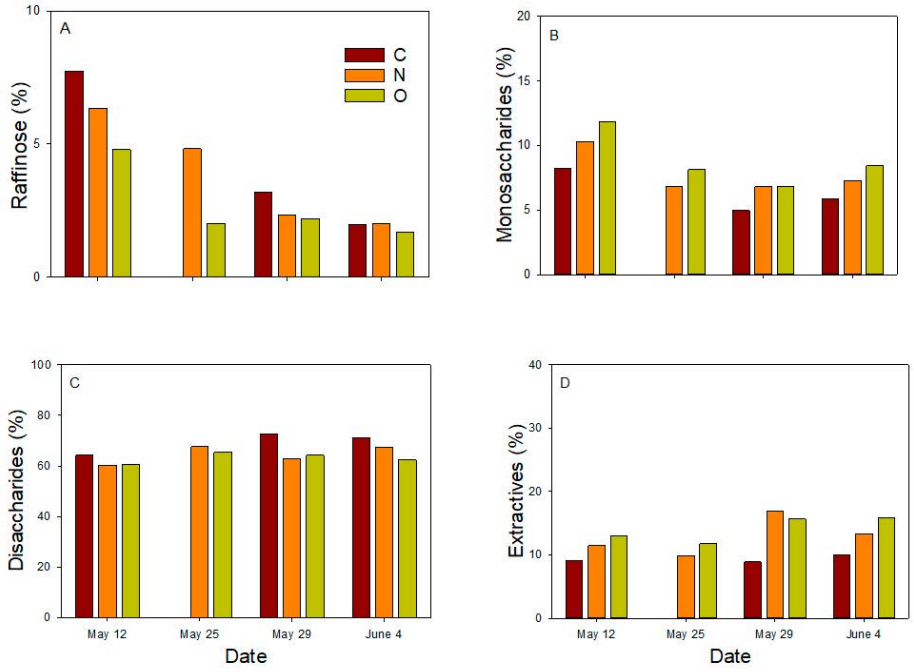

Figure 7. Ratio of raffinose (**A**), monosaccharides (**B**), disaccharides (**C**), and total extractives (**D**) to total content of solids in phloem sap (%) in trees used by TTWs for several years ("O"), trees with newly started sap feeding activity ("N"), and control trees ("C").

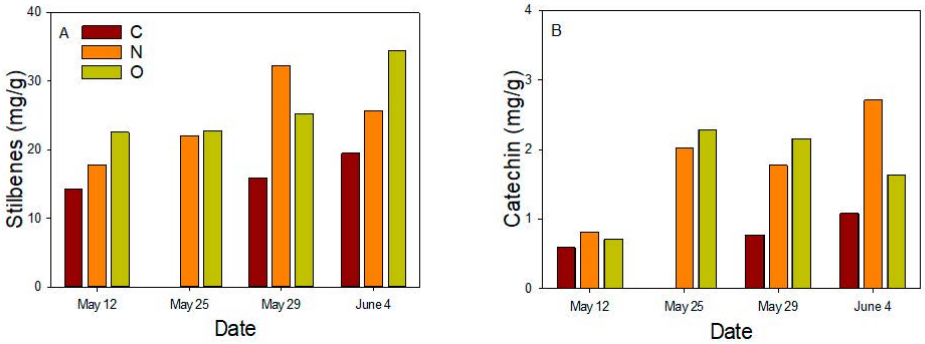

Figure 8. Content of stilbenes (**A**) and catechins (**B**) in the phloem sap (mg/g dry matter) in trees used by TTWs for several years ("O"), trees with newly started sap feeding activity ("N"), and control trees ("C").

The ratio of raffinose to all sap solids was the highest at the beginning of the sampling period in late-April and steadily decreased to the lowest level in early-June. In April, the average daily air temperature was below +5 °C, i.e., the thermal growing season began at the end of April, coinciding with the decline in phloem sap raffinose level. The raffinose content during the first sampling (30 April) did not differ between control trees and trees that were made use of by TTWs ($p = 0.538$; Figure 8A). No differences ($p > 0.05$) between the groups of trees were found in the content of monosaccharides (Figure 7B) or disaccharides (Figure 7C).

Furthermore, the trees the TTWs made use of showed the highest lipophilic extractive content (Figure 7D). The total amount of polyphenolic stilbene glycosides was not higher in the trees with woodpecker sap-feeding rings ($p = 0.095$; Figure 8) than in control trees. The content of monomeric tannin structures, that is (+)-catechins, was neither different in woodpecker sap-feeding trees as compared to control ones ($p = 0.163$).

3.5. Chemical Properties of Phloem Tissue

Phloem tissue had a high content of non-structural carbohydrates (NSC; 177 mg g^{-1} DW), from which 72% was starch and 28% free sugars, mainly sucrose (i.e., 19% of total NSC and 68% of free sugars was sucrose; Figures 9 and 10). In the amount of starch, free sugars, and phenolic secondary metabolites, a statistically significant seasonal variation was observed from early spring (30 April) to early summer (June 4) in all tree-categories. By the last sampling date (4 June), the total amount of free sugars lowered significantly from that of the first three sampling dates (Figure 9). Starch and raffinose content declined significantly from late-April–mid-May to late-May–early-June (Figure 9).

For raffinose, there was also a significant interaction between the main effects of the tree-category and the sampling date, i.e., trees used by birds for phloem sap-feeding reached a steady state in raffinose content earlier than control trees.

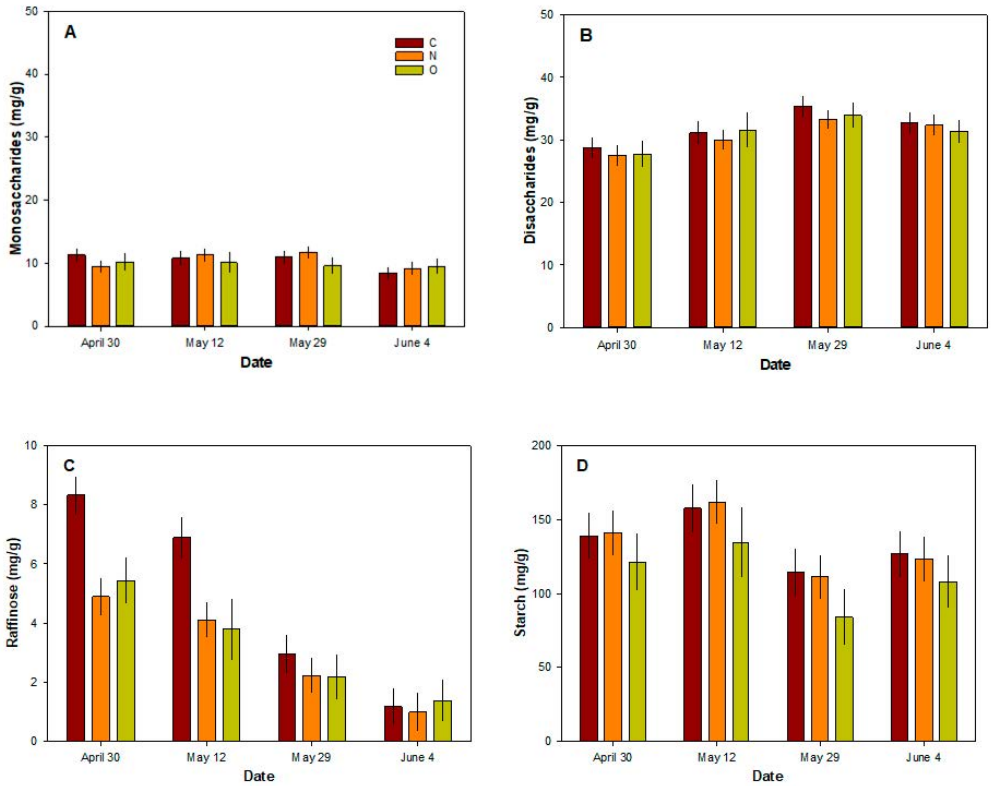

Figure 9. Content of monosaccharides (**A**), disaccharides (**B**), raffinose (**C**), and starch (**D**) in the phloem tissue (mg/g dry matter) in trees used by TTWs for several years ("O"), trees with newly started sap feeding activity ("N"), and control trees ("C").

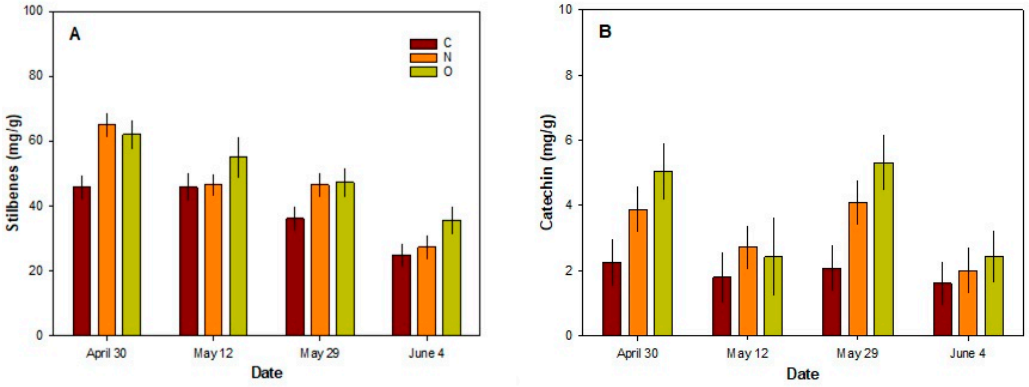

Figure 10. Content of stilbenes (**A**) and catechins (**B**) in the phloem sap (mg/g dry matter) in trees used by TTWs for several years ("O"), trees with newly started sap feeding activity ("N"), and control trees ("C").

The concentration of phenolic stilbene glycosides declined significantly from late-April to early-June (Figure 10). A significantly higher (21%–32%) stilbene content was found for sap-feeding trees than control ones (46, 50, and 38 mg g^{-1} DW for "N", "O" and "C" trees, respectively). For (+)-catechins, tree-category differences were also observed: the phloem tissue of trees the TTWs made use of had 100% ("O") and 68% ("N") higher content of (+)-catechins than the control trees (Figure 10).

4. Discussion

Based on the findings of this pilot study and the existing literature, we discuss the puzzling phenomenon of TTWs sap-feeding and highlight the future research needs in boreal Europe in the following sections (Figure 11).

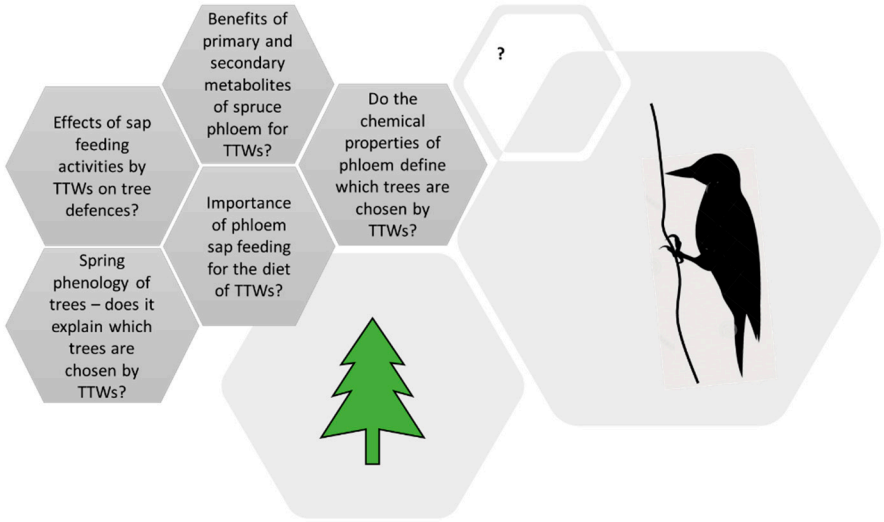

Figure 11. Puzzling phenomenon of three-toed woodpecker phloem sap-feeding on Norway spruce trees: still more questions than answers.

4.1. Patterns of Phloem Sap Feeding in the Three-Toed Woodpecker

Phloem sap-feeding, mainly on deciduous trees, has been observed in several woodpecker and bird species around the world [9,27,30,40–44]. Based on current knowledge [1,2,10], the TTW seems to be the only European woodpecker species, which uses tree sap regularly and in considerable amounts in boreal forests. However, the sap-feeding of the TTW is predominantly restricted to spring, before the nestling period [10]. To our knowledge, there are no confirmed observations of TTWs collecting sap for nestlings, which is a common phenomenon, e.g., among North American sapsuckers [9,27,43].

In the long-term study of TTW in southern Finland within the area of this study [4,10,40], sap trees were found in all detected TTW territories, and they contained both fresh (i.e., trees with newly started sap-feeding activity) and longer-used trees (oldest of which were observed to be used during several decades). The sap trees (mostly Norway spruces, commonly Scots pines *Pinus sylvestris*, occasionally aspens *Populus tremula* and birches *Betula* spp.) were found especially at the southern edges of mature forests, with the most abundant sap rows located on the southern and southwestern, sun-exposed sides of the trunks of these trees [10].

In the sap-feeding of the TTW, usually horizontal rows of small holes are created on the tree trunks. Moreover, TTWs quite often tend to first peck off the outermost bark layer, before pecking the sap holes [10]. We observed that TTWs pecked punctures through the outer bark layers, reaching the inner phloem and soft cambial zone, but the holes did not pass beyond the cambium. Based on long-term monitoring of sap trees within the study area, the pecking of sap rows by TTWs relatively often induced some resin flow from the sap holes of the coniferous trees used for sap-feeding. However, the sap rows did not increase the mortality of the respective tree individuals compared to nearby control trees (T. Pakkala, unpubl. data).

The mechanisms of bird phloem sap-feeding are not well understood. Phloem is well protected, and thus the study of the phloem composition is challenging (see Section 4.3 below). It has been speculated that the saliva of woodpeckers could act as a kind of anticoagulant that would inhibit the self-defense function of the phloem cells and ensure the flow of the phloem sap [27]. The stiff hairy structures on the surface of the tongue of some sapsucker species have been suggested to act as capillary tubes in feeding and to promote access to phloem sap [45,46].

In contrast, the tongue of TTWs does not have the structural adaptations like those in the tongue of typical sapsuckers, but instead its tongue structure is like other European woodpecker species having small bristles (T. Pakkala, unpubl. data), which are supposed to help in handling the food items, e.g., insect larvae [9]. TTW has a relatively slender and sharp bill [1,2], and the sap holes are usually small and well-defined [10]. Moreover, old sap holes are often used and "reopened" [10]. These features, together with field observations of the sap-feeding TTWs, indicate that TTWs may "lick" the sap with their tongue. However, the phloem tissue is evidently also damaged in making the sap holes, and it is probable that TTWs obtain small pieces of tissue material along with the sap. These phloem tissue materials may also be important (see Section 4.4.); they include e.g., antibacterial, antiviral, and antioxidant substances (i.e., catechins, stilbene derivatives, and condensed tannins) as well as minerals, such as CaOx crystals [14,15]. The North American sapsuckers actually "eat" a combination of phloem sap and inner bark, and several herbivorous mammals, from large ruminants to small rodents, strip the bark of trees to get minerals [47].

4.2. Resilient Trees: Growth Vigor and Defense Metabolites

The sap trees of the TTWs tended to be less slender than control trees, but the result is only suggestive because the sample size was small. Tree slenderness has been typically thought to indicate tree growth vigor and tree stability [48] as less slender trees have more secondary growth and higher xylem area per tree height than slender trees. A lower slenderness index also indicates a high living crown ratio (ratio of living crown length to tree height [49]) and low canopy competition pressure [48,50] with good access to light (i.e., the sun can be assumed to warm the trunks in spring more than in trees with high competition pressure). Several studies of woodpeckers have stressed the common use of injured trees or trees in poor health in sap-feeding [4,10,27,29,41]. However, the suggested link between tree vigor and woodpecker sap-feeding requires further studies.

Based on our analysis and previous studies, phloem is especially rich in soluble sugars and starch, as well as phenolic secondary metabolites as compared to outer bark or wood. The (+)-catechins showed higher content in TTW sap trees as compared to control trees, indicating that bird activity may have resulted in induced phloem defense reactions

and the synthesis of phenolic compounds by axial phloem parenchyma cells [15]. The phloem of sap-feeding trees was also richer in stilbene glycosides than that in control ones. However, based on our study design, the effect of sap-feeding by TTWs on the chemical properties of phloem cannot be resolved, as TTWs may also have chosen trees with originally higher content of primary and/or secondary metabolites in phloem as compared to control trees. Due to their long life, conifers have evolved a variety of defense mechanisms to shield themselves against pests and pathogens. Norway spruce integrates preformed (constitutive) and inducible defense compounds and mechanical (structural) barriers against biotic invaders and abiotic stressors. In addition to *trans*-stilbene glucosides, *cis*-stilbenes, dimers, and larger structures have been detected in e.g., pathogen-attacked bark [51–59]. The flow of oleoresin compounds (i.e., resin acids such as abietic acid) also typically occurs after wounding of the bark or fungal infection after bark beetle attacks [59,60]. Biological factors may thus play a significant role in modifying bark properties during the long lifespan of trees, reaching up to 60 to 90 year-rotation-lengths in northern commercial forestry.

4.3. The Phenology of Trees and Seasonal Variation in Sap Chemistry and Availability

The boreal forest ecosystem is characterized by strong seasonality, which also affects both the availability and relative importance of sap for TTWs (see Table 1). The most active sap-feeding period of the TTW is during springtime [2,5,10,11], and it has been hypothesized that the high viscosity of phloem sap in low temperatures may restrict its availability for the TTWs [10]. Therefore, thermally beneficial, southern forest edges and southern sides of the tree trunks should be preferred when selecting suitable sap-feeding locations [10]. In addition, it could also be advantageous to prefer trees in early spring phenology to increase sap-feeding efficiency.

The seasonal variation in spruce wood growth has been monitored in different places from southern to northern Finland [24]. In contrast, the connections between phloem formation, chemistry, and structure have been studied much less by different methods [15,25]. The amounts of non-structural carbohydrates i.e., starch and soluble sugars, in inner bark have been shown to have significant seasonal variation like that observed in this study [25]. The starch content markedly increased from the dormant season in March to the secondary growth onset in May in southern Finland. The starch content was then shown to steadily decline as growth processes progressed. The total amount of soluble sugars has been shown to have a contrary trend: the highest amount has been detected during the rest and dormant condition, and the lowest level was observed during the time of the highest cambial growth in mid-summer [25].

Research data on the chemical composition of pure phloem sap and its seasonal variations are limited, especially for large conifers. Phloem sap is very well protected in the tree—the phloem defends itself against damage immediately, both chemically and structurally. Phloem cells produce a variety of proteins and a sugar polymer called callose that clogs the damage and prevents leakage of phloem fluids [61]. Therefore, the study of the phloem—especially the phloem of large trees—has been challenging, and it is difficult to obtain a clean sample of phloem sap without causing damage and thus changing the composition of the phloem. Studies on the composition of phloem sap have largely been performed on herbaceous plants [62]. The prevailing knowledge is that only a few animal species, mainly some aphids, could break down the chemical defense of the phloem of plants and trees and thus use phloem sap as their food [63].

For the above-mentioned reasons, we collected intact block samples of phloem and analyzed both the tissue and centrifuged phloem sap chemical content and compositions. Our results indicated that woodpeckers made use of trees that were ahead in their phenological spring development as they foraged on trees with lower raffinose. However, there were no differences in the osmolality of the phloem of the control trees vs. the trees utilized by the woodpeckers. It might be that our sample size, sampling frequency, and time span were too small to detect these differences.

4.4. Possible Benefits of Sap for Three-Toed Woodpeckers

This is the first time (to the best of our knowledge) that the properties of phloem sap of the TTW sap trees were studied, although phloem sap has earlier been speculated to have a high content of free sugars and defense compounds presumably beneficial for the general condition of TTWs before breeding [10]. Phloem sap-feeding has also been proposed to be an important way of obtaining energy and minerals for the TTWs [10]. Although our methods did not permit quantifying the exact amounts of nutrients consumed by TTWs, the literature regarding the positive effects of some of these nutrients analyzed in phloem tissue allows speculation about some benefits from sap-feeding.

Outside the nesting phase, adult TTWs, like other birds, consume nutrients to fulfill the maintenance of normal body functions, which include fluid and electrolyte balance, acid-base regulation, and thermoregulation, for example. Although crucial to TTW survival, such functions, do not require large amounts of nutrients, and, therefore, nutritional needs are usually fulfilled by the consumption of a variety of prey, especially bark beetles. During the nesting season, however, numerous modifications take place in the physiology and behavior of TTW, which in turn, affect either the requirements or the partition of nutrients in the TTW body. Regarding behavioral aspects, the acquisition and defense of territory, nest excavation and construction, and mate guarding might last weeks and therefore be physically strenuous [64,65]. Consequently, the nutritional requirements of TTW are increased, exceptionally for energy, and individuals must develop nutritional strategies to survive and successfully reproduce. On the other hand, there may be a shortage of bark beetle individuals in late springtime, at the beginning of nesting season, right before the development of new bark beetle generations [7,10]. Sap could then be an important additive or alternative food source for TTWs. We noticed that the phloem sap of trees that were preferred by TTWs contained higher concentrations of monosaccharides and lower concentrations of raffinose compared to control trees. Whereas monosaccharides can be easily absorbed by the intestinal mucosa and used as a source of energy to support reproduction, for example, raffinose has been negatively correlated with the utilization of energy in birds. Such oligosaccharides cannot be digested into smaller mono- and disaccharides due to the absent activity of endogenous α-1,6 galactosidase in the bird intestine [66]. Parsons et al. [67] reported a negative correlation between the concentration of raffinose in different varieties of soybean and the digestibility of energy in birds. This supports our finding that TTWs seem to prefer sap trees that are ahead in spring phenology in terms of their phloem carbohydrate metabolism.

However, there might be geographical variation in the relative importance of phloem sap as an alternative diet for the TTW, e.g., in a central European study [68], the percentage of foraging time the TTWs allocated to sap-feeding was only 1.2% during the nesting season, whereas in our study area it was 19.5% for the respective season [10].

The calcium content of Norway spruce phloem varies depending on the location within the phloem. As high as 5% of all the phloem axial parenchyma cells may contain CaOx crystals in the outer parts of the phloem [14]. Typically, coniferous trees have CaOx in the phloem as a structural barrier against invaders, such as bark beetles [21]. For female TTWs, in particular, Ca is perhaps the mineral of greatest importance since it plays a crucial role in the calcification of the eggshell [11] and participates in the regulation of the reproductive hormones and ovary growth [61]. There are, so far, very few studies about the possible differences between sexes in woodpecker sap use. In an earlier study within our study area [10], it was observed that sap use was important both for females and males, but there were no significant differences in the proportions of sap-feeding times between sexes during the total pre-nesting or nesting seasons. However, in another long-term study at the sap tree sites of our study area, female TTWs were observed to visit sap trees significantly more often compared to males (T. Pakkala, unpubl. data). The TTW has relatively strong sexual dimorphism [69,70] with larger, socially dominant males choosing the more productive feeding microhabitats than females [68,71,72] although the sexes have quite similar preferences in foraging and diet [8,68,72]. Pechacek [68]

and Zhu et al. [73] found no significant differences in the sap use between TTW sexes, and Imbeau and Desrochers [74] between sexes of the American three-toed woodpecker (*Picoides dorsalis*). Moreover, Pasinelli [75] observed that the Medium Spotted Woodpecker (*Dendrocopos medius*) females used significantly more time on sap use compared with males during the pre-nesting season. In another quantitative study of sap use in woodpeckers, separate results for males and females were not presented, but Kozma [28] detected that the white-headed woodpecker (*Picoides albolarvatus*) males were excavating new sap wells more frequently than females in Washington, USA. Thus, the results of the few quantitative studies are relatively incoherent, and more effort is needed to study the possible differences between sexes in woodpecker sap-feeding.

In this study, the trees used by TTWs had a higher content of catechins in phloem as compared to control trees. Based on our data, we cannot draw conclusions, about whether TTWs select trees with originally higher levels of defense chemicals or whether their sap-feeding results in induced defenses and higher catechin content. However, catechins comprise a broad group of flavonoid compounds largely distributed in nature [76], which has lately become of special interest in female breeder nutrition, mainly due to their strong antioxidant activity.

Previous studies using female Quail (*Coturnix coturnix japonica*) breeders indicate that the enrichment of feeds with catechins increased female fertility and the hatchability of fertile eggs compared with females fed unsupplemented control feeds [77]. Yet, the same authors reported a depression in the concentration of malondialdehyde (MDA) in the yolk of catechin-fed quail. Similarly, Kara et al. [78] noticed that catechin dietary supplementation elicited a decrease in the concentration of MDA in the plasma, liver, and yolk of female quail breeders. Malondialdehyde is a product of lipid peroxidation, and the concentration of malondialdehyde is correlated with the oxidative status of tissues. The depression on MDA concentration in tissues and eggs as a response to dietary catechin concentration confirms, indeed, the biological activity of these polyphenols as free radical scavengers. By quenching oxidants and decreasing MDA content in eggs, catechins might play an important role in maintaining the quality of polyunsaturated fatty acids (PUFA) in the yolk, which can be utilized by the offspring during embryonic development, for example. Chen et al. [79] reported that green tea, whose concentration of catechin may reach 30% of total polyphenols, increased the concentration of PUFA in the yolk of domestic hens (*Gallus gallus domesticus*). An important implication of the enrichment of eggs with PUFA is that the early exposure of broiler chicks (*Gallus gallus domesticus*) to such fatty acids during the embryonic phase has a positive effect on the immune and inflammatory responses of birds during adult life, i.e., growth phase [80]. Furthermore, the benefits of catechins are not restricted to female breeders only: improvements in sperm quality parameters have been reported in cockerels fed with diets containing green tea [81]. Although publications have addressed the effects of catechins on birds reared in intensive systems, their benefits for natural populations of birds are poorly understood and, to our knowledge, have not been studied.

5. Conclusions

This pilot study shed new light on the phloem sap-feeding phenomenon of the three-toed woodpeckers (TTWs) in the boreal area. We concentrated on the Norway spruce-dominated forest area in southern Finland, where TTWs foraged on the phloem of spruce trees. The trees preferred by TTWs showed advanced spring phenology, as evidenced by the lower ratio of raffinose (typically high during the winter months) to total soluble sugars of phloem as compared to control trees. Furthermore, the phloem of trees actively used by the TTWs for their sap-feeding activities was more abundant in antioxidative compounds (catechins and stilbene glycosides). Our study suggests that phloem sap rich in antioxidants and soluble sugars, as well as calcium, is potentially beneficial to the TTWs, but more research is needed to explore the importance of the sap properties to the TTW.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Glutz von Blotzheim, U.N.; Bauer, K.M. *Handbuch der Vögel Mitteleuropas*; Akademische Verlagsgesellschaft, Frankfurt a.M.: Leipzig, Germany, 1980; Volume 9.
2. Cramp, S. (Ed.) *Handbook of the Birds of Europe, the Middle East and North Africa*; Oxford Univ. Press: Oxford, UK, 1985; Volume 4.
3. Pakkala, T.; Hanski, I.; Tomppo, E. Spatial ecology of the three-toed woodpecker in managed forest landscapes. *Silva Fenn.* **2002**, *36*, 279–288. [CrossRef]
4. Pakkala, T.; Kouki, J.; Piha, M.; Tiainen, J. Phloem sap in damaged Scots pine trees provides instant foraging opportunities for Three-toed Woodpeckers *Picoides tridactylus*. *Ornis Svec.* **2017**, *27*, 144–149. [CrossRef]
5. Versluijs, M.; Eggers, S.; Mikusiński, G.; Roberge, J.-M.; Hjältén, J. Foraging behavior of the Eurasian Three-toed Woodpecker (*Picoides tridactylus*) and its implications for ecological restoration and sustainable boreal forest management. *Avian Conserv. Ecol.* **2020**, *15*, 6. [CrossRef]
6. Hogstad, O. On the ecology of the three-toed woodpecker *Picoides tridactylus* (L.) outside the breeding season. *Nytt. Mag. Zool.* **1970**, *18*, 221–227.
7. Fayt, P. Available insect prey in bark patches selected by the three-toed woodpecker *Picoides tridactylus* prior to reproduction. *Ornis Fenn.* **1999**, *76*, 135–140.
8. Pechacek, P.; Krištín, A. Comparative diets of adult and young three-toed woodpeckers in a European alpine community. *J. Wildl. Manag.* **2004**, *68*, 683–693. [CrossRef]
9. Winkler, H.; Christie, D.A. Family Picidae (Woodpeckers). In *Handbook of the Birds of the World*; Del Hoyo, J., Elliott, A., Sargatal, J., Eds.; Jacamars to woodpeckers; Lynx Edicions: Barcelona, Spain, 2002; Volume 7, pp. 296–555.
10. Pakkala, T.; Piiroinen, J.; Lakka, J.; Tiainen, J.; Piha, M.; Kouki, J. Tree sap as an important seasonal food resource for woodpeckers: The case of the Eurasian three-toed woodpecker (*Picoides tridactylus*) in southern Finland. *Ann. Zool. Fenn.* **2018**, *55*, 77–92. [CrossRef]
11. Versluis, M.; Mikusiński, G.; Roberge, J.-M. Foraging behaviour of the Eurasian Three-toed Woodpecker *Picoides tridactylus* in its peak abundance after wildfire. *Ardea* **2020**, *110*, 75–88. [CrossRef]
12. Jyske, T.; Hölttä, T. Comparison of phloem and xylem hydraulic architecture in Norway spruce stems. *New Phytol.* **2015**, *205*, 102–115. [CrossRef]
13. Jyske, T.; Laakso, T.; Latva-Mäenpää, H.; Tapanila, T.; Saranpää, P. Yield of stilbene glucosides from the bark of young and old Norway spruce stems. *Biomass Bioenergy* **2014**, *71*, 216–227. [CrossRef]
14. Jyske, T.; Kuroda, K.; Suuronen, J.-P.; Pranovich, A.; Roig Juan, S.; Aoki, D.; Fukushima, K. In planta localization of stilbenes within *Picea abies* phloem. *Plant Physiol.* **2016**, *172*, 913–928. [CrossRef]
15. Jyske, T.; Kuroda, K.; Keriö, S.; Pranovich, A.; Linnakoski, R.; Hayashi, N.; Aoki, D.; Fukushima, K. Localization of (+)-Catechin in *Picea abies* Phloem: Responses to Wounding and Fungal Inoculation. *Molecules* **2020**, *25*, 2952. [CrossRef] [PubMed]
16. Hölttä, T.; Vesala, T.; Sevanto, S.; Perämäki, M.; Nikinmaa, E. Modeling xylem and phloem water flows in trees according to cohesion theory and munch hypothesis. *Trees* **2006**, *20*, 67–78. [CrossRef]
17. Paljakka, T.; Jyske, T.; Lintunen, A.; Aaltonen, H.; Nikinmaa, E.; Hölttä, T. Gradients and dynamics of inner bark and needle osmotic potentials in Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.). *Plant Cell Environ.* **2017**, *40*, 2160–2173. [CrossRef]
18. Lintunen, A.; Paljakka, T.; Jyske, T.; Peltoniemi, M.; Sterck, F.; Von Arx, G.; Cochard, H.; Copini, P.; Caldeira, M.C.; Delzon, S.; et al. Osmolality and non-structural carbohydrate composition in the secondary phloem of trees across a latitudinal gradient in Europe. *Front. Plant Sci.* **2016**, *7*, 726. [CrossRef]
19. Raitanen, J.-E.; Järvenpää, E.; Korpinen, R.; Mäkinen, S.; Hellström, J.; Kilpeläinen, P.; Liimatainen, J.; Ora, A.; Tupasela, T.; Jyske, T. Tannins of Conifer Bark as Nordic Piquancy—Sustainable Preservative and Aroma? *Molecules* **2020**, *25*, 567. [CrossRef] [PubMed]
20. Välimaa, A.L.; Raitanen, J.E.; Tienaho, J.; Sarjala, T.; Nakayama, E.; Korpinen, R.; Jyske, T. Enhancement of Norway spruce bark side-streams: Modification of bioactive and protective properties of stilbenoid-rich extracts by UVA-irradiation. *Ind. Crops Prod.* **2020**, *145*, 112150. [CrossRef]

21. Hudgins, J.W.; Krekling, T.; Franceschi, V.R. Distribution of calcium oxalate crystals in the secondary phloem of conifers: A constitutive defense mechanism? *New Phytol.* **2003**, *159*, 677–690. [CrossRef]
22. Krokene, P. Conifer defense and resistance to bark beetles. In *Biology and Ecology of Native and Invasive Species*; Vega, F.E., Hofstetter, R.W., Eds.; Elsevier Academic Press: San Diego, CA, USA, 2015; pp. 177–207.
23. Sutinen, S.; Partanen, J.; Viherä-Aarnio, A.; Häkkinen, R. Development and growth of primordial shoots in Norway spruce buds before visible bud burst in relation to time and temperature in the field. *Tree Physiol.* **2012**, *32*, 987–997. [CrossRef]
24. Jyske, T.; Mäkinen, H.; Kalliokoski, T.; Nöjd, P. Intra-annual xylem formation of Norway spruce and Scots pine across latitudinal gradient in Finland. *Agric. For. Meteorol.* **2014**, *194*, 241–254. [CrossRef]
25. Jyske, T.; Suuronen, J.-P.; Pranovich, A.; Laakso, T.; Watanabe, U.; Kuroda, K.; Abe, H. Seasonal variation in formation, structure, and chemical properties of phloem in *Picea abies* as studied by novel microtechniques. *Planta* **2015**, *242*, 613–629. [CrossRef]
26. Mäkinen, H.; Jyske, T.; Nöjd, P. Dynamics of diameter and height increment of Norway spruce and Scots pine in southern Finland. *Ann. For. Sci.* **2018**, *75*, 1–11. [CrossRef]
27. Eberhardt, L.S. Use and selection of sap trees by yellow-bellied sapsuckers. *Auk* **2000**, *117*, 41–51. [CrossRef]
28. Kozma, J.M. Characteristics of Trees Used by White-Headed Woodpeckers for Sap Feeding in Washington. *Northwestern Nat.* **2010**, *91*, 81–86. [CrossRef]
29. Turček, F. The ringing of trees by some European woodpeckers. *Ornis Fenn.* **1954**, *31*, 33–41.
30. Mancuso, K.; Nol, E.; Burke, D.; Elliot, K. Effect of selection logging on Yellow-bellied Sapsucker sap-feeding habits in Algonquin Provincial Park, Ontario. *Can. J. For. Res.* **2014**, *44*, 1236–1243. [CrossRef]
31. Núñez Montellano, M.G.; Blendinger, P.G. Selection of plants for sap feeding by the white-fronted woodpecker Melanerpes cactorum in Chaco dry forest, Argentina. *Acta Ornithol.* **2016**, *51*, 105–122. [CrossRef]
32. Kozlowski, T.T.; Winget, C.H. Diurnal and seasonal variation in radii of tree stems. *Ecology* **1964**, *45*, 149–155. [CrossRef]
33. Gross, K.; Koch, W. Water relations of Picea abies I. Comparison of water relations parameters of spruce shoots examined at the end of the vegetation period and in winter. *Physiol. Plant* **1991**, *83*, 290–295. [CrossRef]
34. Young-Robertson, J.M.; Bolton, R.; Bhatt, U.S.; Cristóbal, J.; Thoman, R. Deciduous trees are a large and overlooked sink for snowmelt water in the boreal forest. *Sci. Rep.* **2016**, *6*, 29504. [CrossRef]
35. Pakkala, T.; Tiainen, J.; Piha, M.; Kouki, J. Nest tree characteristics of the old-growth specialist Three-toed Woodpecker *Picoides tridactylus*. *Ornis Fenn.* **2018**, *95*, 89–102.
36. Cajander, A.K. Forest types and their significance. *Acta For. Fennica* **1949**, *56*, 1–71. [CrossRef]
37. Pakkala, T.; Tiainen, J.; Piha, M.; Kouki, J. Three-toed Woodpecker cavities in trees: A keystone structural feature in forests shows decadal persistence but only short-term benefit for secondary cavity-breeders. *For. Ecol. Manag.* **2018**, *413*, 70–75. [CrossRef]
38. Devaux, M.; Ghashghaie, J.; Bert, D.; Lambrot, C.; Gessler, A.; Bathellier, C.; Ogee, J.; Loustau, D. Carbon stable isotope ratio of phloem sugars in mature pine trees throughout the growing season: Comparison of two extraction methods. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 2511–2518. [CrossRef] [PubMed]
39. Sundberg, A.; Sundberg, K.; Lillandt, C.; Holmbom, B.R. Determination of Hemicelluloses and Pectins in Wood and Pulp Fibres by Acid Methanolysis and Gas Chromatography. *Nord. Pulp Pap. Res. J.* **1996**, *11*, 216–219. [CrossRef]
40. Pakkala, T.; Lakka, J.; Nurmi, T.; Piha, M.; Piiroinen, J.; Vepsäläinen, V. *Pohjantikat Radioseurannassa A Radio-Tracking Project of Three-Toed Woodpeckers in southern Finland*; Technical Report; University of Helsinki: Helsinki, Finland, 2005.
41. Kilham, L. The relations of breeding yellow-bellied sapsuckers to wounded birches and other trees. *Auk* **1964**, *81*, 520–527. [CrossRef]
42. De Kiriline, L. A Comparative Life-History Study of Four Species of Woodpeckers. *Ornithol. Monogr.* **1967**, *5*, 1–156. [CrossRef]
43. Tate, J., Jr. Methods and Annual Sequence of Foraging by the Sapsucker. *Auk* **1973**, *90*, 840–856. [CrossRef]
44. Schlatter, M.; Vergara, P. Magellanic Woodpecker (*Campephilus magellanicus*) sap feeding and its role in the Tierra del Fuego forest bird assemblage. *J. Ornithol.* **2005**, *146*, 188–190. [CrossRef]
45. Howell, T.R. Natural history and differentiation in the Yellow-bellied Sapsucker. *Condor* **1952**, *54*, 237–282. [CrossRef]
46. Oliver, W.W. Sapsucker damage to ponderosa pine. *J. For.* **1968**, *66*, 842–844.
47. White, T.C.R. The cause of bark stripping of young plantation trees. *Ann. For. Sci.* **2019**, *76*, 105. [CrossRef]
48. Ruiz-Peinado, R.; Pretzsch, H.; Löf, M.; Heym, M.; Bielak, K.; Aldea, J.; Barbeito, I.; Brazaitis, G.; Drössler, L.; Godvod, K.; et al. Mixing effects on Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.) productivity along a climatic gradient across Europe. *For. Ecol. Manag.* **2020**, *482*, 2021. [CrossRef]
49. Temesgen, H.; LeMay, V.; Mitchell, S.J. Tree crown ratio models for multi-species and multi-layered stands of southeastern British Columbia. *For. Chron.* **2005**, *81*, 133–141. [CrossRef]
50. Kantola, A.; Mäkelä, A. Development of biomass proportions in Norway spruce (*Picea abies* [L.] Karst.). *Trees* **2006**, *20*, 111–121. [CrossRef]
51. Viiri, H.; Annila, A.; Kitunen, V. Induced responses in stilbenes and terpenes in fertilized Norway spruce after inoculation with blue-stain fungus Ceratocystis polonica. *Trees* **2001**, *15*, 112–122. [CrossRef]
52. Lieutier, F.; Brignolas, F.; Sauvard, D.; Yart, A.; Galet, C.; Brunet, M.; van de Sype, H. Intra- and inter-provenance variability in phloem phenols of Picea abies and relationship to a bark beetle associated fungus. *Tree Physiol.* **2003**, *23*, 247–256. [CrossRef]
53. Franceschi, V.R.; Krokene, P.; Christiansen, E.; Krekling, T. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytol.* **2005**, *167*, 353–376. [CrossRef]

54. Li, S.H.; Nagy, N.E.; Hammerbacher, A.; Krokene, P.; Niu, X.M.; Gershenzon, J.; Schneider, B. Localization of phenolics in phloem parenchyma cells of Norway spruce (*Picea abies*). *ChemBioChem* **2012**, *13*, 2707–2713. [CrossRef]
55. Hammerbacher, A.; Ralph, S.G.; Bohlmann, J.; Fenning, T.M.; Gershenzon, J.; Schmidt, A. Biosynthesis of the major tetrahydroxystilbenes in spruce, astringin and isorhapontin, proceeds via resveratrol and is enhanced by fungal infection. *Plant Physiol.* **2011**, *157*, 876–890. [CrossRef] [PubMed]
56. Hammerbacher, A.; Schmidt, A.; Wadke, N.; Wright, L.P.; Schneider, B.; Bohlmann, J.; Brand, W.A.; Fenning, T.M.; Gershenzon, J.; Paetz, C. A common fungal associate of the spruce bark beetle metabolizes the stilbene defenses of Norway spruce. *Plant Physiol.* **2013**, *162*, 1324–1336. [CrossRef]
57. Hammerbacher, A.; Paetz, C.; Wright, L.P.; Fischer, T.C.; Bohlmann, J.; Davis, A.J.; Fenning, T.M.; Gershenzon, J.; Schmidt, A. Flavan-3-ols in Norway spruce: Biosynthesis, accumulation, and function in response to attack by the bark beetle-associated fungus Ceratocystis polonica. *Plant Physiol.* **2014**, *164*, 2107–2122. [CrossRef] [PubMed]
58. Hammerbacher, A.; Kandasamy, D.; Ullah, C.; Schmidt, A.; Wright, L.P.; Gershenzon, J. Flavanone-3-Hydroxylase Plays an Important Role in the Biosynthesis of Spruce Phenolic Defenses Against Bark Beetles and Their Fungal Associates. *Front. Plant Sci.* **2019**, *10*, 208. [CrossRef] [PubMed]
59. McKay, S.A.; Hunter, W.L.; Godard, K.A.; Wang, S.X.; Martin, D.M.; Bohlmann, J.; Plant, A.L. Insect Attack and Wounding Induce Traumatic Resin Duct Development and Gene Expression of (-)-Pinene Synthase in Sitka Spruce. *Plant Physiol.* **2003**, *133*, 368–378. [CrossRef] [PubMed]
60. Christiansen, E. Ceratocystis polonica inoculated in Norway spruce: Blue-staining in relation to inoculum density, resinosis and tree growth. *Eur. J. For. Pathol.* **1985**, *15*, 160–167. [CrossRef]
61. Knoblauch, M.; Mullendore, D.L. Sieve element occlusion. In "Phloem" Eds Thompson GA. *Van. Bel. AJE* **2012**, 141–153. [CrossRef]
62. Atkinks, C.A.; Smith, P.M.C.; Rodriguez-Medina, C. Macromolecules in phloem exudates—A review. *Protoplasma* **2011**, *248*, 165–172. [CrossRef]
63. Douglas, A.E. Phloem-sap feeding by animals: Problems and solutions. *J. Exp. Bot.* **2006**, *57*, 747–754. [CrossRef]
64. Short, L.L. Burdens of the picid hole-excavating habit. *Wilson Bull.* **1979**, *91*, 16–28.
65. Schuppe, E.R.; Rutter, A.R.; Roberts, T.J.; Fuxjager, M.J. Evolutionary and Biomechanical Basis of Drumming Behavior in Woodpeckers. *Front. Ecol. Evol.* **2021**, *9*, 649146. [CrossRef]
66. Gitzelmann, R.; Auriccio, S. The handling of soya alpha-galactosides by a normal and a galactosemic child. *Pediatrics* **1965**, *36*, 231–235. [CrossRef]
67. Parsons, C.M.; Zhang, Y.; Araba, M. Nutritional evaluation of soybean meals varying in oligosaccharide content. *Poult. Sci.* **2000**, *79*, 1127–1131. [CrossRef] [PubMed]
68. Pechacek, P. Foraging behavior of Eurasian three-toed woodpeckers (Picoides tridactylus alpinus) in relation to sex and season in Germany. *Auk* **2006**, *123*, 235–246. [CrossRef]
69. Hogstad, O. Sexual dimorphism and divergence in winter foraging behaviour of Three-toed Woodpeckers Picoides tridactylus. *Ibis* **1976**, *118*, 41–50. [CrossRef]
70. Hogstad, O. Sexual dimorphism in relation to winter foraging and territorial behaviour of the Three-toed Woodpecker Picoides tridactylus and three Dendrocopos species. *Ibis* **1978**, *120*, 198–203. [CrossRef]
71. Hogstad, O. Seasonal change in intersexual niche differentiation of the Three-toed Woodpecker. *Ornis Scand* **1977**, *8*, 101–111. [CrossRef]
72. Hogstad, O. The effect of social dominance on foraging by the Three-toed Woodpecker *Picoides tridactylus*. *Ibis* **1991**, *133*, 271–276. [CrossRef]
73. Zhu, Y.; Lü, N.; Pechacek, P.; Li, J.; Sun, Y.-H. Foraging behavior of the Eurasian Three-toed Woodpecker subspecies *Picoides tridactylus funebris* in southern Gansu, China. *Chin. Birds* **2012**, *2012*, 60–66. [CrossRef]
74. Imbeau, L.; Desrochers, A. Foraging ecology and use of drumming trees by Three-toed Woodpeckers. *J. Wildl. Manag.* **2002**, *66*, 222–231. [CrossRef]
75. Pasinelli, G. Sexual dimorphism and foraging niche partitioning in the Middle Spotted Woodpecker *Dendrocopos medius*. *Ibis* **2000**, *142*, 635–644. [CrossRef]
76. Huang, W.Y.; Cai, Y.Z.; Zhang, Y. Natural phenolic compounds from medicinal herbs and dietary plants: Potential use for cancer prevention. *Nutr. Cancer* **2009**, *62*, 1–20. [CrossRef] [PubMed]
77. Kara, K.; Kocaoğlu Güçlü, B.; Şentürk, M.; Konca, Y. Influence of catechin (flavan-3-ol) addition to breeder quail (*Coturnix coturnix japonica*) diets on productivity, reproductive performance, egg quality and yolk oxidative stability. *J. Appl. Anim. Res.* **2016**, *44*, 436–441. [CrossRef]
78. Kara, K.; Guclu, B.K.; Senturk, M.; Eren, M.; Baytok, E. Effects of catechin and copper or their combination in diet on productive performance, egg quality, egg shelf-life, plasma 8-OHdG concentrations and oxidative status in laying quail (*Coturnix coturnix japonica*). *J. Appl. Anim. Res.* **2021**, *49*, 97–103. [CrossRef]
79. Chen, X.; Li, T.; He, K.; Geng, Z.; Wan, X. Dietary green tea powder supplementation enriched egg nutrients and physicochemical property in an indigenous chicken breed. *Poult. Sci.* **2021**, *100*, 388–395. [CrossRef]

80. Cherian, G. Essential fatty acids and early life programming in meat-type birds. *World's Poult. Sci. J.* **2011**, *67*, 599–614. [CrossRef]
81. Abdo, Z.M.; Hassan, R.A.; El-Salam, A.A.; Helmy, S.A. Effect of adding green tea and its aqueous extract as natural antioxidants to laying hen diet on productive, reproductive performance and egg quality during storage and its content of cholesterol. *Egypt. Poult. Sci. J.* **2010**, *30*, 1121–1149.