

Temperature influence on willow growth and phenolic production

Final Report

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Background

Following the reintroduction of wolves to Yellowstone National Park willows in various parts of the northern winter range have increased in size and now resemble willow patches not seen since the early 20th century. This has been attributed to the cascading effects of change in elk behavior caused by the wolves (Ripple and Beschta 2004a, 2004b). However, there has been a concomitant phenomenon involving the innate physiology of willows and climate change that may also explain the increase in willow height.

Since 1996 current annual growth (CAG) produced by willows in the northern winter range of Yellowstone National Park and other areas has been greater than ever recorded. Average length of CAG measured in 1987-90 was approximately 12-25 cm (Singer et al. 1994) and 5-23 cm in 1986. (Singer et al. 1996) Measurements taken in 2004 averaged 38-72 cm and many twigs were more than 100 cm long. (Despain 2004 unpublished data). In some places this growth has experienced much lower browsing levels than has occurred in the last 50 or more years and many clumps now exceed 2 m in height.

We hypothesized that two things must have changed. Obviously, conditions for growth changed. But the ability of the willows to protect that increased growth may also have changed. Willows grow in moist places that also produce large amounts of herbaceous winter forage. Elk and bison use these areas intensively. Tracks, feeding craters and bedding craters are widespread and common in these areas throughout the winter and all the willow clumps show at least some browsing by large ungulates. It does not appear that behavioral changes caused by the reintroduction of wolves have been sufficient to keep elk from using the willow patches. However, climate has been shown to have changed in the recent past in biologically significant ways (Walther et al. 2002, Allen and Beeley 2005).

Growth depends on availability of carbohydrates produced by photosynthesis, but is mediated by a group of plant hormones (auxins) which are produced in expanding leaves and buds. Recent research on auxin initiation and transport has illuminated processes in members of the Salicaceae. Several days with minimum temperature $>0^{\circ}\text{C}$ are needed to stimulate the genes that produce the proteins needed to begin growth and control auxin transport. Buds begin to swell producing auxins that are then transported to the growing region of the stems. The transport process is very sensitive to temperature. If minimum daily temperature drops below freezing, the auxin transport is negatively affected (Schrader et al. 2003). Growth is also affected by temperature. Willow seedlings grown at 20°C then transferred to 10°C produce only 15% of the growth achieved by controls (Hjelm and Ögren

2003). Photosynthesis on the other hand is not affected as much as growth. Mature willow leaves grown in warm conditions have been shown to acclimate to cool temperatures and increase their photosynthetic capacity (Hjelm and Ögren 2003).

Stem growth is also regulated by day-length (Howe et al. 1995, Olsen et al. 1995), thus growth ends about midsummer, long before photosynthesis stops. Storage carbohydrates can then build up in the stems and roots.

Defensive chemicals are also synthesized from carbohydrates but must wait until growth needs are met (Herms and Mattson 1992). Defensive chemicals in the bark of willows begin to increase after leaf fall and continue to increase until March (Thieme 1965). The synthesis of these compounds is carried out during the cold part of the year apparently from carbohydrate reserves in the stems.

From the above physiological responses of willows and their close relatives it would seem that climate could have a significant effect on both growth and production of defensive chemicals. We hypothesize that sensitivity of the auxin transport system to minimum daily temperatures would make growth of willows sensitive to the number of days in the spring with minimum daily temperature $>0^{\circ}\text{C}$ and that temperatures in the late summer and fall would influence the amount of carbohydrates stored; allowing for the protection of the current annual growth.

Climatic records from Northeast Entrance from 1985-2004 were examined for the periods of 1985-1996 relative to 1997-2004. The number of days with daily minimum temperature $>0^{\circ}\text{C}$ for the period of May-July increased by 12 days (23%); for the fall period of August-October the increase was 13 days (37%). Overall, the growing season, by this definition, has increased by 29%.

These observations suggest a possible mechanism that could explain the phenomenal growth of willows on the northern winter range. The longer spring season allows for longer growth of the current year leaders. The cessation of growth imposed by day-length response and the longer fall season allows for more carbohydrates to be used for defensive chemicals and reserves for increased growth the following spring. Variation in microclimates, genetic variation among the species as well as intraspecific variation could explain the variation in willow response to browsing seen throughout the winter range.

Objectives

The objectives of this project were to; 1) measure the change in stem concentration of nonstructural carbohydrates and selected phenolic compounds related to ungulate herbivory and daily temperature variation for a two year period, 2) identify some of the phenolic compounds involved in the ungulate/willow interactions, 3) determine if phenolics are produced during the willow dormant period, 4) determine if changes in phenolic concentrations are directly related to carbohydrate levels, 5) identify the relationship between date of bud break and number of days with minimum temperature above 0°C and 6) determine the timing of the end of willow growth period related to day-length.

Methods

We collected temperature and stem tissue samples from 3 willow communities, dominated by Booth's willow (*Salix boothii*), along stream drainages across Yellowstone's northern range: 1) Blacktail Deer Creek, (elevation 6656 ft, approximately 3100 ft upstream from the Grand Loop Road crossing); 2) Crystal Creek (elevation 6193 ft, approximately

200-600 ft upstream from the Northeast Entrance Road crossing); and 3) Soda Butte Creek (elevation 6598 ft, just downstream from the livestock trail head to the upper Lamar River). We deployed small temperature data loggers (i-button recorders, ca 2 cm dia. X 0.7 cm) in selected willow clumps. The temperature recorders were placed at 10 cm below ground surface, 30 cm above the surface, and within the top one third of the crown in each of three clumps at each site. Subsurface data loggers were programmed to record temperature every 4 hrs, and data were downloaded approximately every nine months. Aerial data loggers were programmed to record temperature data every ½ hr and were downloaded every month. Data were downloaded from ibuttons onto handheld Palm units (m500 series, Palm Inc.) under field conditions, and transferred onto PC computers (Palm Desktop software, version 4.0 for Windows) in the office.

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We also collected monthly samples of the current annual growth from *Salix boothii* for analysis of phenolic concentrations and carbohydrate levels. A 10-gram sample was collected from each of 10 different willow clumps (5 releasing and 5 short) at each site. Samples from the releasing (e.g., tall) willow clumps were generally collected from unbrowsed growth produced during the previous growing season, while current annual growth samples from the short (e.g., repeatedly browsed) willow clumps were obtained from stems browsed the previous winter. Samples were collected each month from September 2005 through mid August 2007 for a total of (10 samples x 12 months x 3 sites x 2yrs =) 720 samples.

Collection and sample preparation

Current year's growth of willow twigs was collected (3-5 twigs each about 6-8 inches in length), placed in a labeled plastic bag, and put immediately on dry ice in the field. Frozen samples were then shipped to the Chromatography Center at Brigham Young University. Upon arrival within 24 hrs, samples were frozen at least to - 80 C, and then freeze dried for 48 hours.

Sample extraction and analysis

Monomeric phenolics ... Willow stems were frozen in a -80 ultra-low (Queue Cryostar, Parkersburg, West Virginia), freeze dried (Labconco Lyph-Lock 12, Kansas City, MO), and then 200 mg dried tissue was ground to a fine power capable of passing through a 0.5 mm screen. The ground sample was transferred to a 16 mm x 125 mm test tube to which 3 ml 50/50 dichloromethane/methanol were added, sonicated (Cole-Parmer International, Chicago, IL) for 5 minutes, and then centrifuged (Fisher Scientific model 225) for 10 minutes. The supernatant was pipetted into a 3 dram vial, and the sample was extracted twice more using the same procedure. Of the combined 9 ml of extract, 1 ml solution was placed into an autosampler vial (Sigma-Aldrich, Milwaukee, WI) and analyzed by high pressure liquid chromatography (HPLC Agilent model 1100, Santa Clara, CA). The HPLC was equipped with a Luna (2) 150 mm x 4.6 mm, 3 um particle size, C₁₈ reverse phase column (Phenomenex, Torrance, CA). Solvents were: A = water/CH₃COOH (98/2); B = MeCN/CH₃COOH (98/2). The HPLC gradient program was: 0 min A = 5%, B = 95%; 8 min A = 10%, B = 90%; 13 min A = 25%, B = 75%; 19 min A = 50%, B = 50%. The UV detector was set at 280 nm and injection volume was 20 ul.

Soluble carbohydrates ... 200 mg freeze dried tissue was finely ground, placed into a test tube, and 5 ml 70/30 methanol/water were added. The mixture was sonicated for 5 min, centrifuged for 10 minutes, and the supernatant then pipetted into a 3 dram vial. 5 ml were again added to the sample and the procedure was repeated once more. All 10 ml were combined, mixed well, then 1 ml of the extract was placed in a low-volume autosampler vial, and the extract blown dry using nitrogen gas to prevent oxidation. Once dry 50ul DMF and 50ul BSTFA (Pierce Biotechnology, Chicago, IL) were added and the vial capped. The sample was heated at 70 C for 15 min and then analyzed on a HP Gas Chromatograph (HP model 5890) equipped with a RTX-1 30m x 0.32 mm x 0.25 um column. Set point temperature was 145 C. Level 1 ramping rate was 10 C / min, final temperature 175 C, final time 5 min; level 2 ramping rate was 5 C /min, final temperature 195 C, final time 0 min; level 3 was 10 C/min, final temperature 275 C, final time 4 min. Injection volume was 3ul, inlet temperature 300 C, FID detector temperature 300 C (HP model 5890).

Starch analysis ... In order to determine how much soluble carbohydrate is found in willow starch, 100 mg of freeze dried willow stems were ground to a fine powder capable of passing through a 0.5 mm screen and placed in a 16 mm x 120 mm test tube. 5 ml of aqueous methanol (80% v/v) were added to the sample and incubated at 85 C in a water bath for 5 min. The contents were then thoroughly stirred with a vortex mixer, another 5 ml of aqueous methanol were added, and the sample again vortexed. The sample was centrifuged for 10 min, the supernatant discarded, and the pellet was re-suspended in 10 ml 80% aqueous ethanol. The sample was stirred using a vortex mixer, centrifuged as above, and the supernatant carefully poured off. 3 ml of thermostable alpha-amylase (Total starch kit K-TSTA, Megazyme International Ireland, Ltd, Wicklow, Ireland) in MOPS solution (sodium salt, Sigma-Aldrich M-9381, St. Louis, MO) buffer (50 mM, pH 7 using 1M HCL, plus 5 mM calcium chloride, and 0.02% sodium azide w/v) (Oakton pH 510 Meter, Fisher Scientific Co., Houston, TX) were added which were stirred vigorously using a vortex mixer and then incubated in a boiling water bath for 6 min (tube stirred vigorously after 2 min and 4 min). The sample in the tube was placed in a 50 C water bath and 4 ml sodium acetate buffer (200mM at pH 4.5 plus 0.02% w/v sodium azide) followed by 0.1 ml amyloglucosidase were added. The sample was then stirred using a vortex mixer and incubated at 50 C for 30 min. The contents of the tube were transferred to a 25 ml volumetric flask using a wash bottle to rinse tube contents thoroughly, adjusted to volume with distilled water, centrifuged for 10 min, and 0.1 ml duplicate aliquots of diluted solution transferred to the bottom of each duplicate glass test tube. 3.0 ml GOPOD reagent (Megazyme, AOAC Method 996.11, AACC Method 76.13, ICC Standard Method No. 168) was added to each test tube (including the reagent blanks consisting of 0.1 ml H₂O and 3 ml GOPOD reagent) and the tubes were incubated at 50 C for 20 min. Spectrophotometric absorbance (Milton Baby Spectronic 1001 Plus) was set at 510 nm, and the glucose control was read against the reagent blank.

Identification of willow phenolics ... A liquid chromatography/mass spectrometry detector (Agilent model 1100/6100 Series Qudarupole System, Santa Clara, CA) equipped with a Luna (2) C₁₈, 4.6 mm x 150 mm with a particle size of 3 um was used to identify individual peaks resolved in the sample chromatograms. For the HPLC, the mobile phase was A: HPLC water/acetic acid (98%/2%); B: MeCn/acetic acid (98%/2%). The gradient program was at time "0" min 95% A/ 5% B; time 8 90% A/ 10% B; time 13 0% A/100% B; time 25 0% A/100% B. Column flow was 1ml/min, injection volume was 20 ul, and the

UV detector (G1315A, Agilent 1100 System) was set at 280 nm. The protocol for the mass spectroscopy detector was: Ionization mode: MM-ESI, negative polarity, gas temperature at 350 C, vaporizer set at 200 C, drying gas at 5.0L / min, and nebulizer pressure at 40 psi. The VCap was negative 2000V and the VCharge negative 2000V.

Results

Objective 1-: Measure the change in stem concentration of nonstructural carbohydrates and selected phenolic compounds related to ungulate herbivory and daily temperature variation for a two year period.

A total of 720 willow samples were collected. Two samples were misplaced between collection and sample preparation and two samples were not sufficiently large to permit both phenolic and carbohydrate analysis, so the carbohydrate analysis is missing four samples. The rest have been collated into single files for both phenolics and carbohydrates.

Temperatures: Temperature data was downloaded monthly from the respective ibuttons and stored individually as files labeled for site/plant/crown position/date. For analysis, files were appended for each crown position (n=3) in each plant (n=3) at each site (n=3) to create a continuous record for each of the 27 site/plant/crown position combinations. During analysis, it became apparent that the ibuttons were not accurately either storing or transferring data, especially when recorded temperature data were compared to high/low temperature readings from nearby established weather stations. The manufacturer of the ibutton technology was contacted to assist with troubleshooting the discrepancy, but as of this writing could not resolve the issue. Apparently the software allowing for memory overwrite was faulty in some as-yet unexplained way, and we are continuing to work with the technology manufacturer to overcome this problem.

As an alternative, we used climatic data available from the NOAA weather stations Yellowstone National Park (at Mammoth Hot Springs), Tower Ranger Station, Cooke City and the NRCS SNOTEL site at Northeast Entrance. These stations are distributed across the study area with Mammoth at 6230 ft. on the west side and Northeast Entrance and Cooke City at the east side at 7400 and 7460 ft. elevation respectively. Tower Ranger Station is near the center at 6266 ft.

Blacktail is about 6 miles south and west of Mammoth and 450 ft higher, Crystal Creek is about 5 miles east of Tower and just 86 ft. lower. Soda Butte Creek is about 12 miles southwest of Northeast Entrance and 770 ft. lower. Minimum temperatures at Tower are more similar to those of the two higher stations even though they are 1100 and 1200 ft. higher, obviously not following the adiabatic lapse rate. Average maximum temperature was similar to Mammoth. This is probably due to cold air drainage forming a large pocket in the basin behind the constriction of the downstream canyon. There were more days with a minimum temperature above freezing in 2007 than in 2005 and 2006 at both Cooke City and Tower (Table C1A). But at Mammoth and Northeast Entrance there were fewer. Cook City and Northeast Entrance are only about 1.5 miles apart and only 60 ft. different in elevation. These observations indicate significant variations even within short distances in

this mountain environment. Unfortunately the i-buttons were not able to help us know just what the temperatures in the study sites were.

Table CIA. Climatic Station temperature and days >0 degrees Centigrade

	Elevation	Average Minimum Temperature	Average Maximum Temperature	1971-2000 Average annual Temperature	Average growing season Temperature	2005 d>0	2006 d>0	2007 d>0
	(ft.)	(°F)	(°F)	(°F)	(°F)			
Mammoth	6230	27.7	51.9	40.5	54.0	158	154	140
Tower	6266	19.4	52.7	35.7	49.6	96	86	109
Northeast	7400	20.9	47.4	33.4	45.8	124	126	115
Cooke City	7460	20.4	47.4	33.5	46.6	89	90	106

Carbohydrates: The concentrations (mg/g) of seven different compounds were isolated as available sugars: D-glucose1, D-glucose2, fructose1, fructose2, galactose, maltose, and sucrose. Additionally, starch as a stored form of sugar, was also isolated and treated independently from the available sugars. The compounds are singularly addressed below, then pooled for reanalysis. To account for within-year variation and trends in production of the different compounds, paired-sample t-tests were used to test for differences in site (Blacktail, Crystal, and Soda Butte creeks) and treatment (short vs. releasing willow clumps) effects. Additionally, paired-sample t-tests were used to assess differences, by month for each year (year1 vs year 2) , for each site/treatment combination. All concentration values analyzed are the average of the 5 samples (short and releasing each) collected per site per sampling date except where noted above.

D-glucose1: On average, D-glucose1 accounted for < 2% of the total available sugar content sampled. Concentrations were generally low throughout the year (Fig. 1). Peak values occurred during the month of June and decreased to slightly elevated levels from July through September. Peak concentrations were higher in 2006 than 2007. Significant differences ($p < 0.05$) were noted only among sites for only the releasing willow clumps, where concentrations were significantly greater at Blacktail than at either Crystal Creek or Soda Butte Creek.

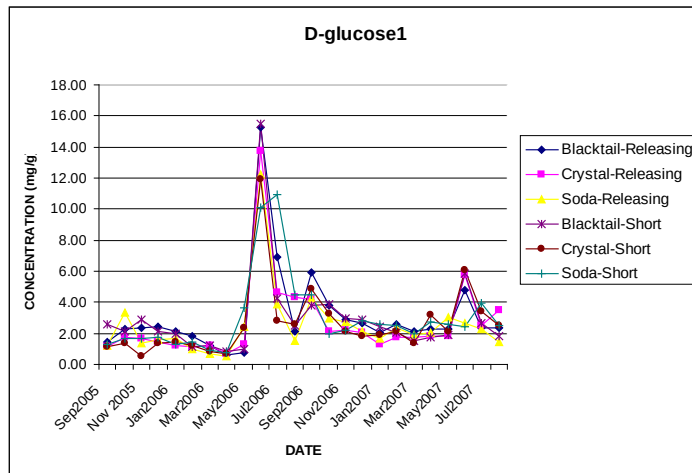


Figure 1: Average concentration of D-glucose1 extracted from monthly samples of willow tissue (n=5) collected from 2 different willow heights at 3 different sites.

D-glucose2: Concentrations of D-glucose2 (Fig 2) were very similar to D-glucose1, where overall concentrations were generally low but peaked during July, decreased but remained somewhat elevated through September, and continuously decreased to their lowest annual levels during April/May. Again, peak concentrations were higher in 2006 than 2007. Significant differences ($p < 0.05$) were noted only among sites for only the releasing willow clumps, where concentrations were significantly greater at Blacktail than at either Crystal Creek or Soda Butte Creek. Like D-glucose1, D-glucose2 on average accounted for < 2% of the total available sugar content sampled.

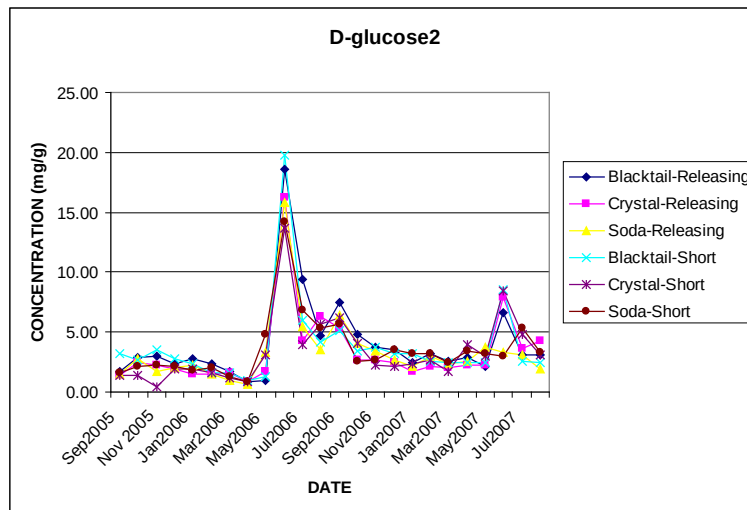


Figure 2: Average concentration of D-glucose2 extracted from monthly samples of willow tissue (n=5) collected from 2 different willow heights at 3 different sites.

Fructose1: In terms of average available sugar content sampled, fructose1 ranked second highest and accounted for roughly 25% of the concentration of available sugar present in willow tissue (Fig 3). Peak values generally occurred during May/June and decreased sharply during July, but late/summer early fall trends thereafter differed by year. During the first year (September 2005 to August 2006) concentrations alternately fluctuated at relatively low levels throughout the fall and winter prior to peaking the following spring. In the second year of the study (September 2006 to August 2007), however, concentrations more or less increased over the winter months leading up to the May/June peak. Concentrations were lowest during August (2006) of the first year and January (2007) of the second year. Pairwise significant differences ($p < .05$) were noted only among sites for only the short willow clumps, where average concentrations at Blacktail were significantly greater than at Crystal Creek or Soda Butte Creek. No significant differences in concentrations were seen between paired releasing and short willow clumps at each site, or between releasing willow clumps among sites. Furthermore, pairwise significant differences in concentrations between years were seen among all clumps except for releasing willows at Soda Butte Creek ($p = .06$). The concentration of fructose1 was consistently greater during year 2 than year 1 of the study.

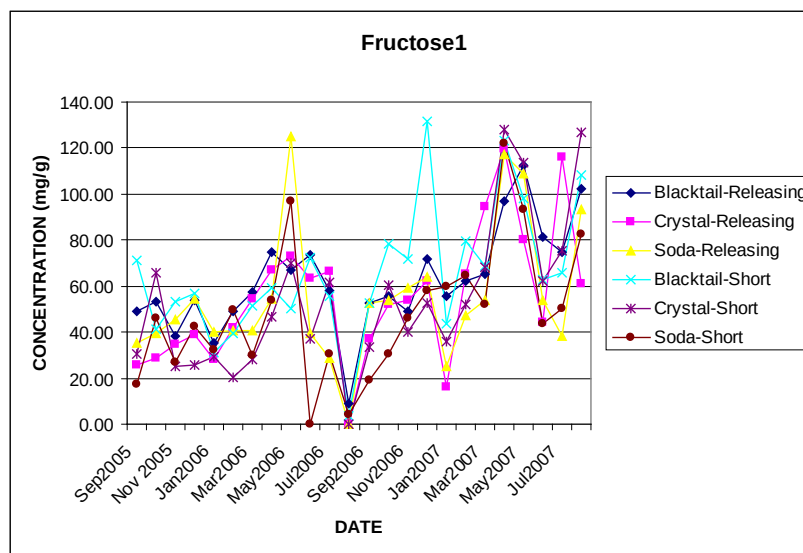


Fig. 3: Average concentration of fructose1 extracted from monthly samples of willow tissue (n=5) collected from 2 different willow heights at 3 different sites.

Fructose2: Concentrations were much unlike fructose1, but comparable to both amount and trend as reported for D-glucose1 and D-glucose2 (Fig 4). On average, fructose2 accounted for < 2% of the total available sugar content sampled. Concentrations were generally low throughout the year (Fig. DD). Peak values occurred during the month of June and decreased to slightly elevated levels from July through September. Peak concentrations were higher in 2006 than 2007. Significant differences ($p < 0.05$) were noted only among sites for only the releasing willow clumps, where concentrations were significantly greater at Blacktail than at either Crystal Creek or Soda Butte Creek.

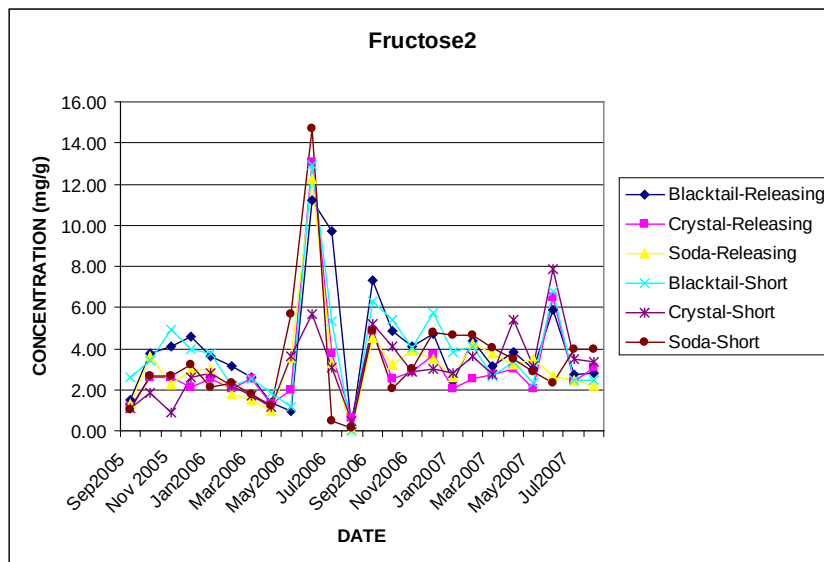


Fig. 4: Average concentration of fructose2 extracted from monthly samples of willow tissue (n=5) collected from 2 different willow heights at 3 different sites.

Galactose: On average, galactose accounted for roughly 4% of the total available sugar content sampled (Fig 5), but was only measureable during spring/early summer. Concentrations were very low for a majority of the year, often undetectable or less than 1mg/g. A dramatic 40- to 100-fold peak occurred in June, sharply declined to low levels in July, and was again hardly detectable during the remainder of the year. No pairwise significant differences in concentrations were observed among sites or willow height status. It appears, however, that peak concentrations were much greater in releasing willow clumps at Blacktail and Soda Butte creeks compared to the respective short willow clumps, but were generally similar for releasing and short willow clumps at Crystal Creek.

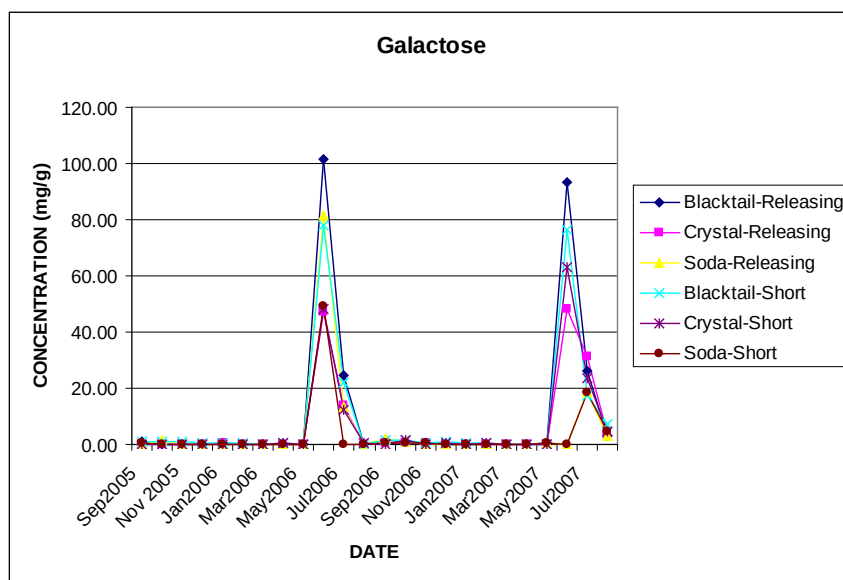


Fig. 5: Average concentration of galactose extracted from monthly samples of willow tissue (n=5) collected from 2 different willow heights at 3 different sites.

Maltose: On average, maltose generally accounted for < .05% of the total available sugar content sampled (Fig 6), and median concentrations were below 1 mg/g. Maltose was consistently measureable but at low concentrations low throughout the sampling period. The timing of peak concentrations was inconsistent among and between sites, and more variation in average monthly concentrations appeared during the second year of the study compared to the first year. Significant ($p < .05$) pairwise differences were evident among releasing willow sites, where concentrations were lower at Crystal Creek than either Blacktail or Soda Butte Creek.

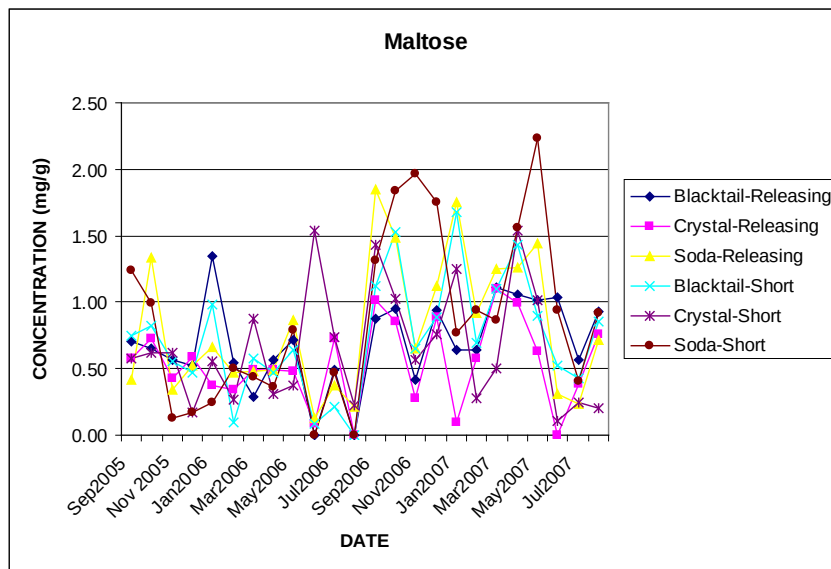


Fig. 6: Average concentration of maltose extracted from monthly samples of willow tissue (n=5) collected from 2 different willow heights at 3 different sites.

Sucrose: Sucrose was by far the most prevalent sugar found in the current annual growth of willow tissue and accounted for roughly 66% of the total available sugar content sampled (Fig. 7). No clear annual trend in tissue concentration, however, is evident. Peak concentrations in year 1 of the study occurred in the fall and spring, while peak concentrations occurred during the summer months in year 2. Significantly higher concentrations ($p < .05$) occurred during year 2 of the study compared to year 1. No pairwise differences in concentration were observed among tall and short willow clumps within each site, but concentrations in releasing willow clumps at Blacktail were significantly greater than releasing clumps at either Crystal or Soda Butte creeks. Additionally, short willow clumps at Blacktail had significantly higher concentrations ($p < .05$) than at Crystal Creek.

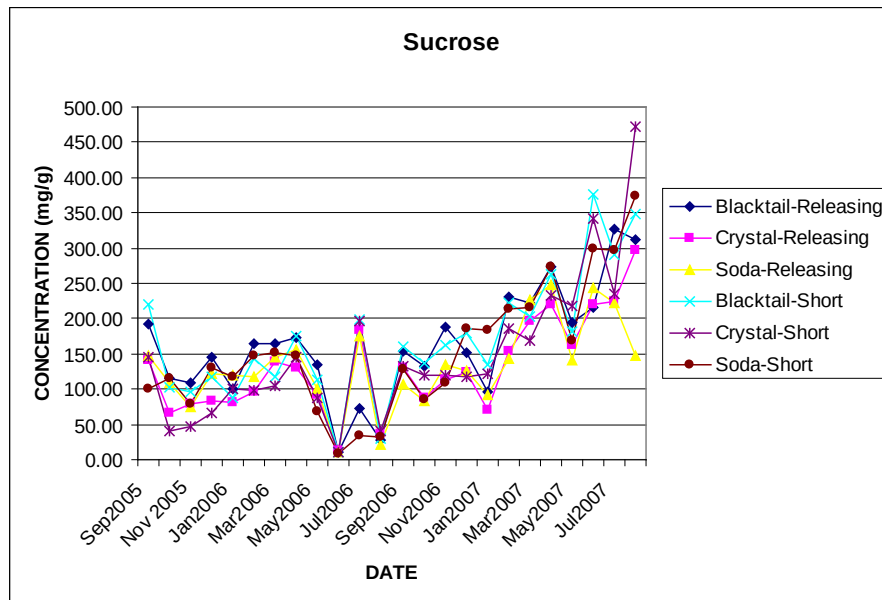


Fig. 7: Average concentration of sucrose extracted from monthly samples of willow tissue (n=5) collected from 2 different willow heights at 3 different sites.

Total concentration summed across all sugars, by month: The summed monthly average for all sugars sampled generally showed lowest concentrations in the fall of the year, and gradually increasing overwinter and into the growing season (Fig. 8). Not surprisingly, the total concentration of all sugars mirrored those of sucrose and fructose1 which together accounted for (66% + 25% =) roughly 90% of the concentration of total available sugar content sampled here. There was no evidence of differences in sugar concentration, either individually or in total, between paired short vs. releasing willow clumps within each site. There were, however, measureable site differences, with Blacktail appearing to be the more productive of the 3 study sites. Releasing willow clumps at Blacktail consistently produced significantly higher sugar concentrations (for D-glucose1, D-glucose2, Fructose2, sucrose, and total sugar content) than releasing willow clumps at either the Crystal Creek or Soda Butte Creek sites. Releasing willows at both Blacktail and Soda Butte Creek produced significantly higher concentrations of maltose than releasing willow at Crystal Creek. Similarly, short willow clumps at Blacktail produced significantly more fructose1 and total sugars than short willows at both Crystal and Soda Butte creeks, and significantly more sucrose than short willows at Crystal Creek. Moreover, yearly differences in the concentrations of the major sugars were evident. All of the willow height classes experienced significantly higher monthly fructose1, sucrose, and total sugar concentrations during the second year of the study (September 2006 to August 2007) compared to the first year (September 2005 to August 2006). Only the concentration of fructose1 in releasing willows at Soda Butte Creek was marginally insignificant ($p = .059$) between years.

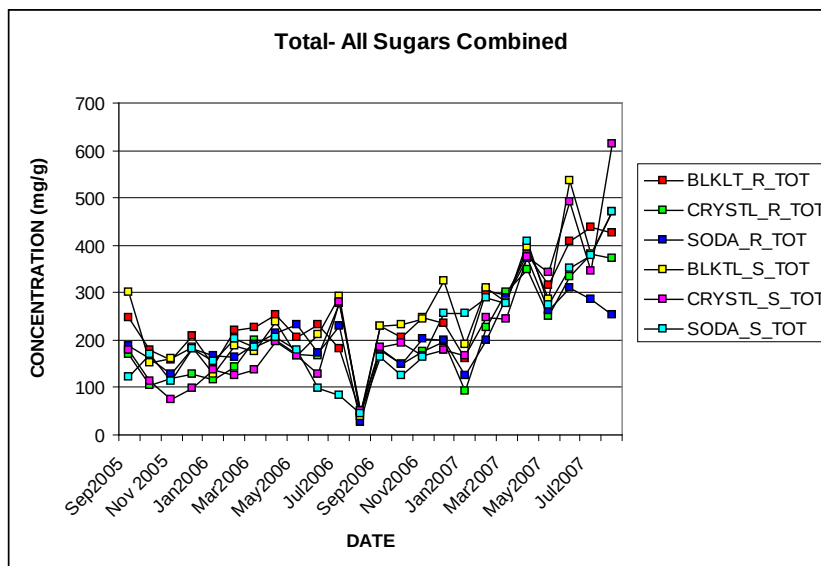


Fig. 8: Sum of the average concentration of all sugars extracted from monthly samples of willow tissue (n=5) collected from 2 different willow heights at 3 different sites.

Starch: Concentrations alternate between biannual peaks during spring and fall, and relatively low to trace concentrations during summer and winter (fig 9). Unlike sugars, there were significant ($p < .05$) differences in starch concentrations between short and releasing willow clumps with each site, but the differences were not consistent. Releasing willow clumps at Blacktail had significantly higher concentrations than short willows, but the trend was reversed at Crystal and Soda Butte creeks where short willows had higher concentrations than releasing willows. A site effect was also observed among releasing willow clumps where concentrations were higher at Blacktail than either Crystal or Soda Butte creeks. No site effect of concentrations in short willow clumps was observed. Also unlike sugars, there was no measureable effect of year on starch concentrations.

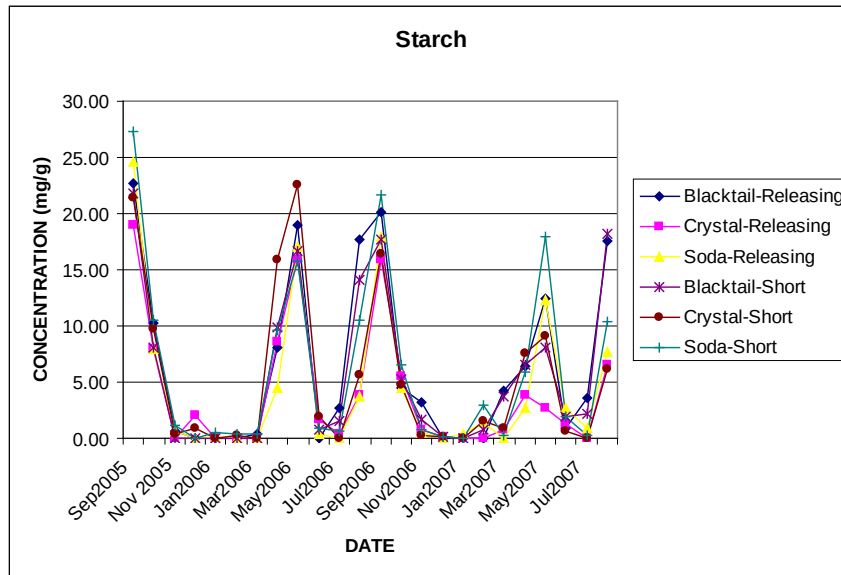


Fig. 9: Sum of the average concentration of starch extracted from monthly samples of willow tissue (n=5) collected from 2 different willow heights at 3 different sites.

Phenolics: The phenolic analysis showed that there were two dominant peaks (Fig. 10), one at approximately 7.8 minutes which accounted for about 40 percent of the total phenolic content. A second peak at about 15.7 minutes is associated with a group of smaller peaks which accounted for most of the rest.

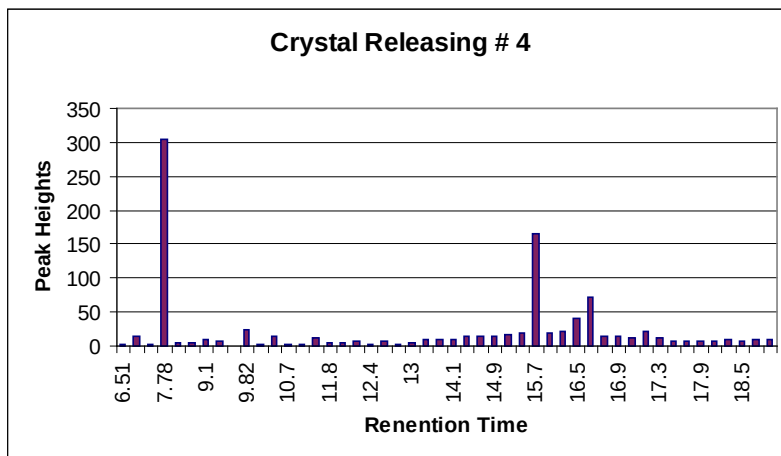


Figure 10. Phenolic analysis of sample #104 taken on 15 Dec 2005 from clump #4 at the Crystal Creek site

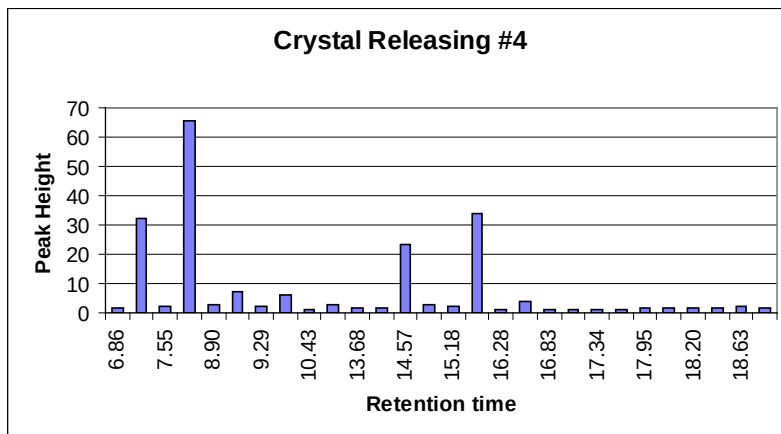


Figure 11. Phenolic analysis of sample #284 taken on 13 Jun 2006 from clump #4 at the Crystal Creek site. Note the difference in the y axis scale.

There was a strong seasonal pattern to phenolic content with very low content and fewer types in the new expanding stems in June which increases rapidly into September then slowly increases into October or November. During the winter the content varies

some (Fig. 12). The June stems also had about 10 fewer peaks than those collected the rest of the year (Fig. 11).

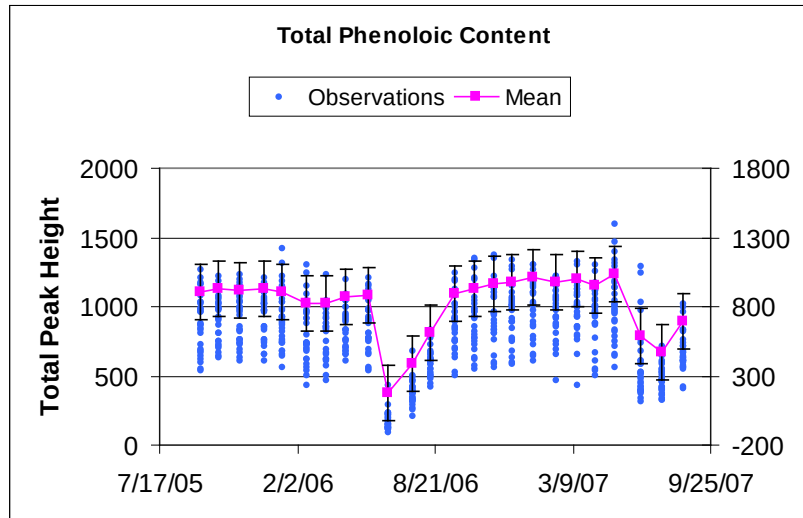


Figure 12. Phenolic content at each sample date (n=30)

There were more phenolic compounds in the short clumps than in the releasing clumps at Blacktail Cr. and just the opposite at Soda Butte Cr. They were equal at Crystal Cr. (Fig. 13.)

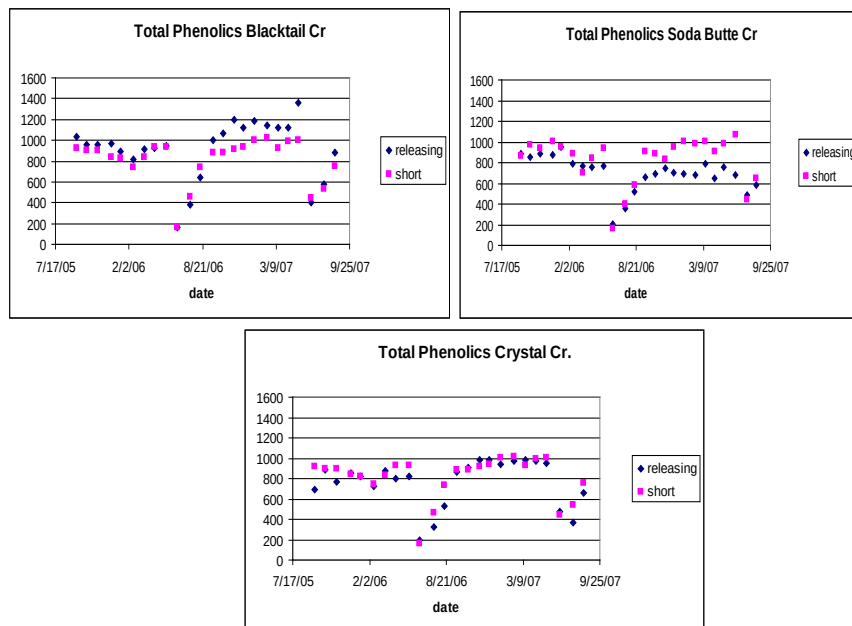


Figure 13: The average (n=5) of total phenolic compounds, by month, in short and releasing willows at the Blacktail, Crystal, and Soda Butte sites.

The dominant peak at retention time 7.8 minutes (Fig. 14), total phenolic content (Fig 15), and residual phenolic content (Total minus retention time 7.8, Fig 16) were subjected to a paired-sample t-test to assess differences in treatment within site as well as treatment differences between sites (Table 1).

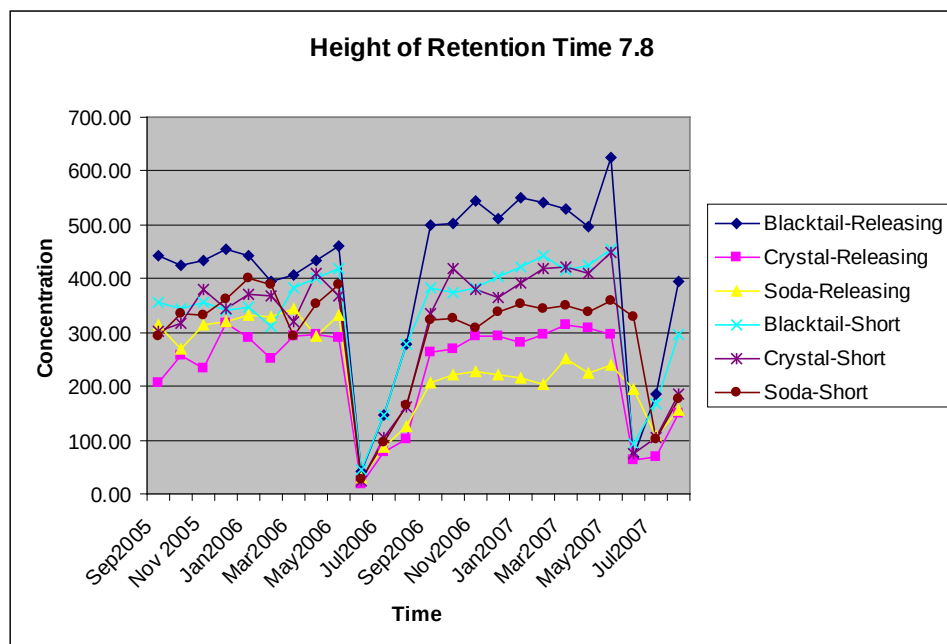


Figure 14: The concentration of an unknown, abundant phenolic compound in willow tissue having a retention time of 7.8 minutes. Values are the monthly average derived from 5 samples of either releasing or short willows from 3 different sites over a 2-year (September 2005 through August 2007) period.

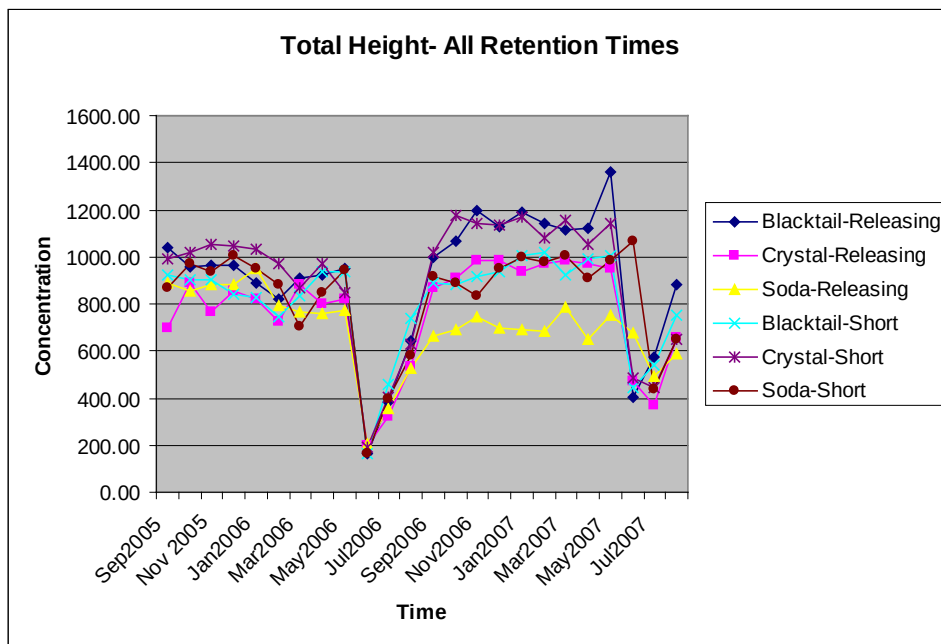


Figure 15: The concentration of all phenolic compounds present in willow tissue across all retention times. Values are the monthly average derived from 5 samples of either releasing or short willows from 3 different sites over a 2-year (September 2005 through August 2007) period.

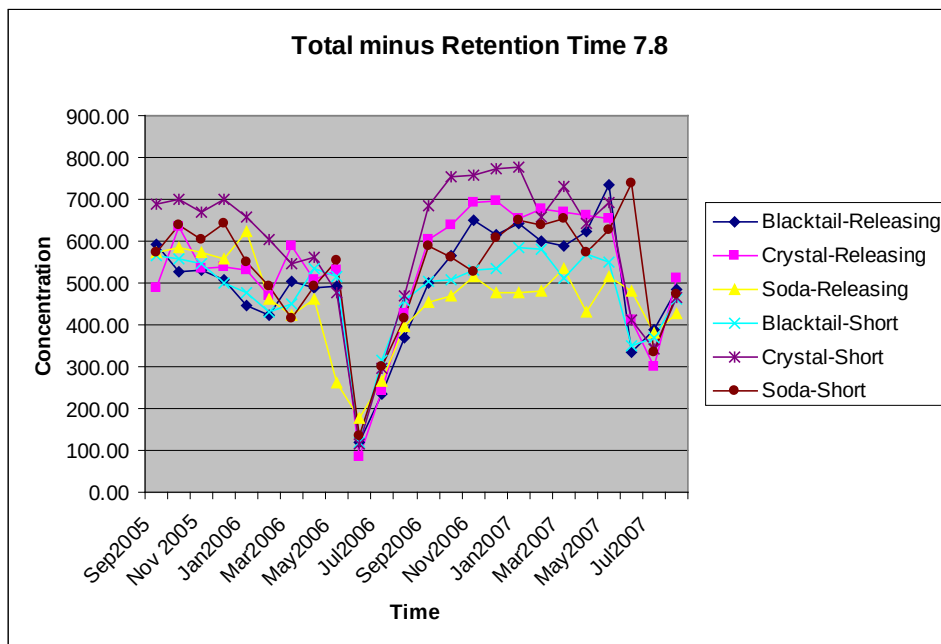


Figure 16: Concentration of “remaining” phenolics when subtracting the concentration of an unknown, abundant compound at retention time 7.8 minutes from the total concentration of all phenolic compounds. Values are the monthly average derived from 5 samples of either releasing or short willows from 3 different sites over a 2-year (September 2005 through August 2007) period.

Table 1: Results of paired-sample t-tests for willows of different height status at 3 different sites. All comparisons are significant unless noted as "non-significant (NS)". For sites, Blktl = Blacktail Creek; Crystl = Crystal Creek; and Soda = Soda Butte Creek; For treatments, R or Rel = releasing willows; S or Shrt = short willows

<u>Site/Treatment</u>	<u>Peak Ht @ 7.8 min.</u>	<u>Total Phenolic Content</u>	<u>Residual (total - peak 7.8)</u>
Blktl-R vs Blktl-S	Rel > Shrt	Rel > Shrt	Rel > Shrt
Crystl-R vs Crystl-S	Shrt > Rel	Shrt > Rel	Shrt > Rel
Soda-R vs Soda-S	Shrt > Rel	Shrt > Rel	Shrt > Rel
Blktl-R vs Crystl-R	Blktl > Crystl	Blktl > Crystl	Crystl > Blktl
Blktl-R vs Soda-R	Blktl > Soda	Blktl > Soda	NS
Crystl-R vs Soda-R	NS	NS	Crystl > Soda
Blktl-S vs Crystl-S	Blktl > Crystl	Crystl > Blktl	Crystl > Blktl
Blktl-S vs Soda-S	Blktl > Soda	NS	Soda > Blktl
Crystl-S vs Soda-S	NS	Crystl > Soda	Crystl > Soda

Releasing willows had significantly greater ($p < .05$) peak heights/concentrations at retention time 7.8 minutes, as well as total phenolic content, than short willows at Blacktail. The concentrations were reversed at Crystal and Soda Butte, where short willows had significantly greater peak heights at retention time 7.8 minutes, total, and residual (total minus 7.8-minute retention time) phenolic content. Releasing willows at Blacktail had significantly greater peak heights at 7.8 minutes retention time and total phenolic content than either Crystal or Soda Butte. Releasing willows at Crystal, however, had greater residual phenolic content than either Blacktail or Soda Butte. Conversely, short willows at Blacktail only had greater peak heights at retention time 7.8 than short willows at Crystal or Soda Butte. The short willows at Crystal had significantly greater total and residual phenolic content than at either Blacktail or Soda Butte, but Soda Butte short willows had significantly greater residual phenolic concentration than short willows at Blacktail. In addition, the second year of the study experienced significantly greater total, residual, and peak height in retention time 7.8 minutes in releasing and short willows at both the Blacktail and Crystal sites. No yearly differences were observed in the releasing or short willows at Soda Butte (Figures 14, 15, and 16).

Objective 2-: Identify some of the phenolic compounds involved in the ungulate/willow interactions

Identification of compounds in the willow bulk extract using compound standards were analyzed with an Agilent LC-MSD system. Standards obtained were salicortin and tremulacin (from Dr. Richard Lindroth at University of Wisconsin) and salacin (Sigma-

Aldrich, St. Louis, MO). Comparisons of peaks representing compounds in the willow extract were made using both UV spectrum (nm 280) and mass spectrum (for both scan and sim modes). By comparing the retention times of the standard compounds with retention times for compounds in the willow extract, the compound at retention time 15.503 minutes was identified (Figure 1). Identification was verified by the UV spectrum and LC/MS spectrum for salicortin (Figure 2). Further verification was noted by the LC-MS spectra based on the retention time match for the salicortin standard vs. the compound with the same retention time in the willow sample (Figure 3), as well as the mass spectral data for these same compounds (Figure 4).

The other phenolic glycoside standard, tremulacin, was not found in the willow extract based on UV spectral data and the LC-MS spectral data. Using HPLC retention times and UV spectral data from the willow extract and the salacin standard, a minor peak with a 7.215 retention time was identified as salacin. Finally, LC-MS analysis revealed the molecular weights of four unidentified compounds (peak 9.018 retention time, M.W. 357; peak 9.546, M.W. 285; peak 10.103, M.W. 331; retention time of 19.078, M.W. 427). Fractionation of these compounds from the willow extract and subjecting the fractions to further UV and LC-MS spectral analysis may lead to their identification.

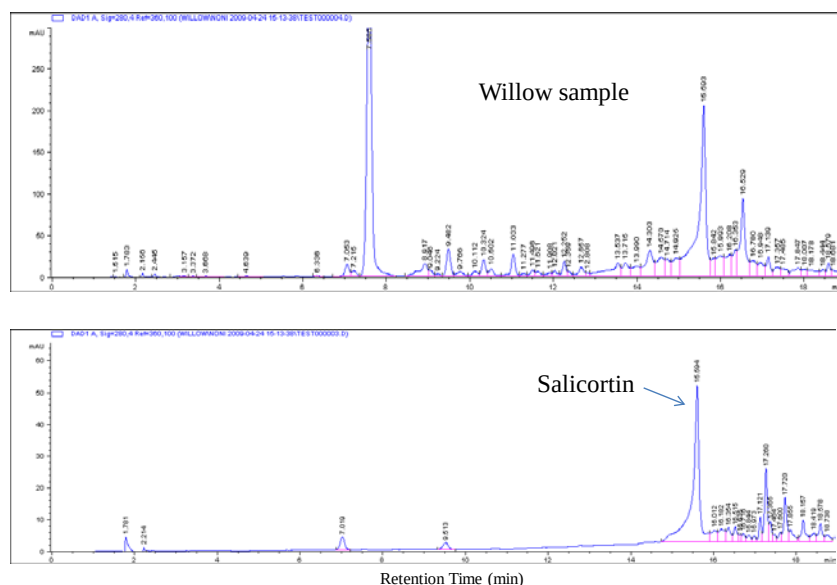


Figure 1: UV chromatogram showing the identification of the phenolic glycoside salicortin in willow samples. Identification of peak number 7.215 (upper diagram) as salacin was obtained using HPLC-UV spectral analysis.

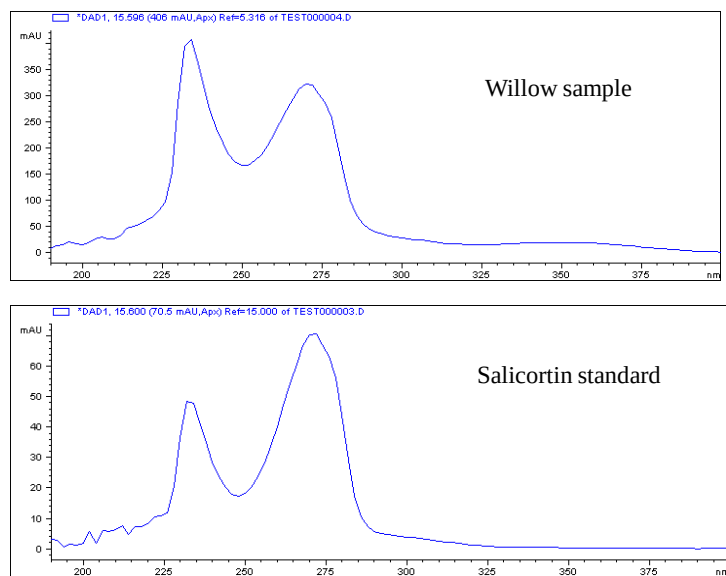


Figure 2: Identification of the phenolic glycoside salicortin in willow samples using UV spectrum (mm 280).

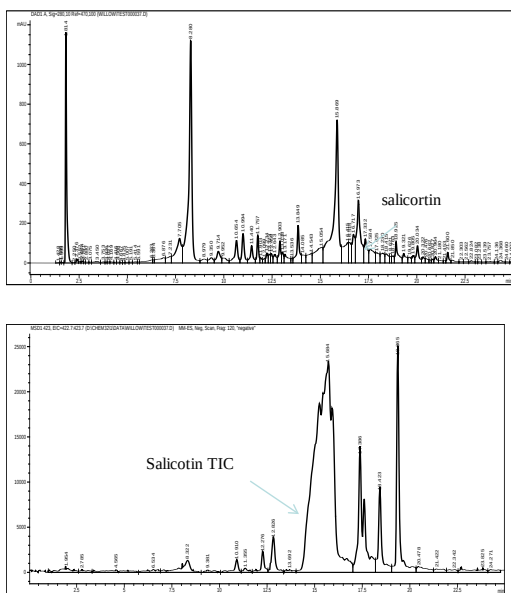


Figure 3: LC-MS analysis showing the results of UV and mass spectrometry analysis for salicortin in the willow sample

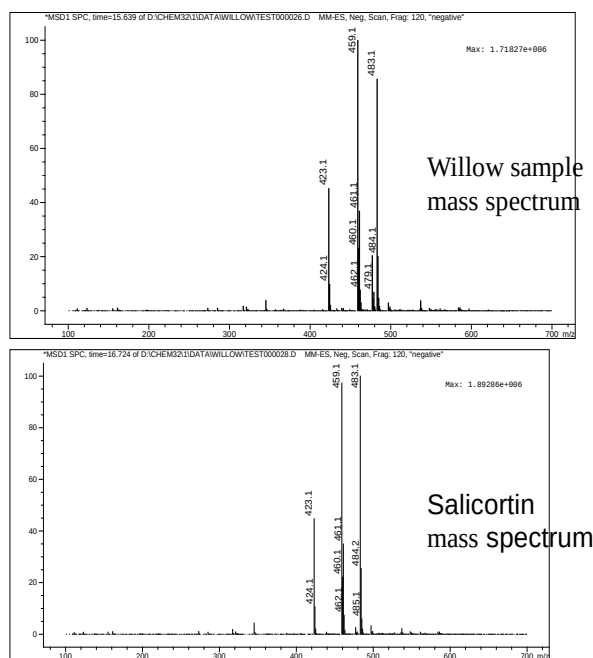


Figure 4: Mass Spectra showing the spectral data between salicortin standard and salicortin in the willow sample.

Objective 3- : Determine if phenolics are produced during the willow dormant period.

There are both positive and negative changes occurring through out the winter. A general decrease in February is followed by an increase. This differs from a peak in March reported by Thieme (1965). Phenolic content varied during the winter but the variation was not completely synchronous between the clumps (Figure 1).

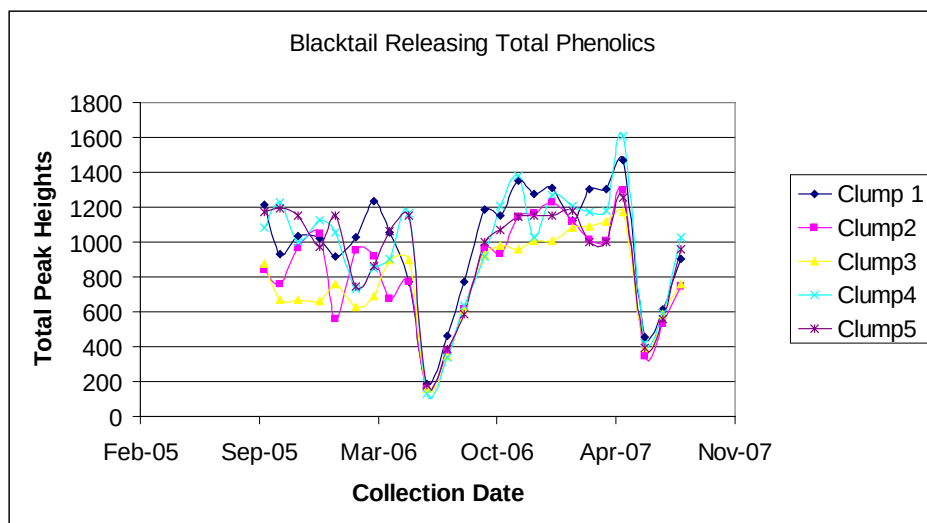


Figure 1. Phenolic content of the releasing clumps at the Blacktail site

Objective 4- : Determine if changes in phenolic concentrations are directly related to carbohydrate levels

A regression analysis between total sugars or total starch and heights of the phenolic peaks showed no significant relationship between phenolics and carbohydrates. It could be that phenolics, being only one of several metabolic products, do not have a noticeable relationship to the carbohydrate pool size. Starch content did drop to near zero beginning with the onset of freezing minimum temperatures in October and November. Starch content increased rapidly concurrently with phenolic content during the summer (Figure 1).

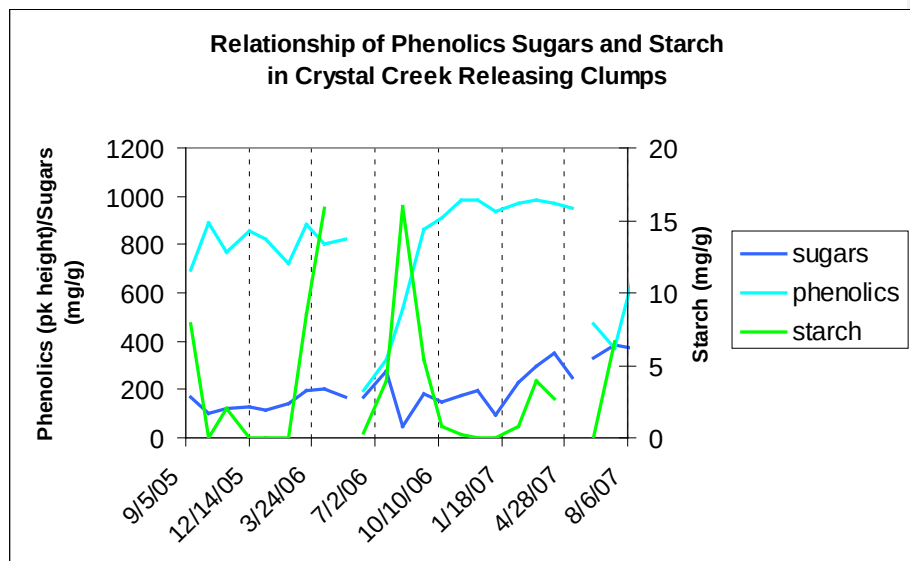


Figure 1. Seasonal changes in the relationship of phenolics, sugars and starch in the releasing clumps at Crystal Creek

Objective 5-: Identify the relationship between date of bud break and number of days with minimum temperature above 0°C

Bud break occurred in mid May both summers but in May of 2006 Soda Butte Creek had only five days with minimum above 0°C and Crystal Creek and Blacktail Creek had only 8 and no consecutive days occurred before the middle of the month. In May of 2007, Soda Butte Creek had seven days, Crystal Creek had five days and Blacktail Creek had two. Perhaps the willows in our study area are adapted to a cooler temperature threshold or something else is triggering the beginning of growth. It could also be that our temperature sensors are responding differently because they are not in an instrument shelter.

Objective 6-: Determine the timing of the end of willow growth period related to day-length

Growth had stopped by the time the August samples were collected indicating that day-length was the most probable cause. Leaves did not begin to turn color until well into September and early October. Photosynthates from leaves could therefore accumulate in stems for about six or seven weeks after growth ceases.

We did not find a good connection between climatic conditions and phenolic content or between phenolic content and willow height. Other factors are probably affecting current annual growth.

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Notes made on collection dates etc.

Aug 06 Soda Butte insect activity

Short

#5 defoliated

#4 defoliated

#3 75% defoliated

#2 85% defoliated

#1 85% defoliated

Releasing

#5 30% defoliated

#4 defoliated, 5% browsed

#3 defoliated, 15% browsed

#2 defoliated, 2% browsed

#1 not eaten

9 May 07 Willow samples

Soda Butte - Buds to early bud break

Crystal - early leaf, early anthesis

Blacktail == same as Crystal

15 Aug 07

Growth stopped

Short

SB 2 heavy Sawfly

SB1 light sawfly

Sb3 heavy sawfly

SB4 defoliated

Releasing

1 almost defoliated

2 mostly defoliated

3 most defoliated

4 mostly defoliated, little cag

5 Totally defoliated