COLD SENSITIVITY IN SOUTHERN HIGHBUSH (*Vaccinium corymbosum* L. interspecific hybrid) FLORAL BUDS

by

LAUREN ELIZABETH REDPATH

(Under the Direction of Erick Smith)

ABSTRACT

Blueberries are an important commercial crop in Georgia, averaging $16,750 ha\(^{-1}\) yr\(^{-1}\) over the last 10 yrs. Hard winter freezes and spring frosts are detrimental to low chill blueberries [*V. corymbosum* (L.) and *V. darrowii* (Camp.) hybrid complex] which flower and crop earlier than many rabbiteye [*Vaccinium virgatum* (Aiton) syn. *ashei*] and northern highbush cultivars (*V. corybosum*). Southern highbush blueberry (SHB) floral buds differentiate the preceding summer, developing into fall. Blueberries acclimate in late fall, becoming less susceptible to cold temperatures and freezes. Longer photoperiods and warmer temperatures signal deacclimation with buds losing their hardiness as internal organs develop towards budbreak. Cold hardiness of blueberry floral buds can be measured in several different methods with varying degrees of success. Cold hardiness is an important topic when considering diverse production climates that can have temperatures that exceed chill accumulation thresholds, stimulating growth, and where frost protection is necessary for economical survivability.

INDEX WORDS: deacclimation, dormancy, northern highbush, rabbiteye, southern highbush, ice nucleation
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CHAPTER 1

BUD DEVELOPMENT AND THE IDENTIFICATION OF COLD INJURY IN SOUTHERN Highbush Blueberry (Vaccinium corymbosum L. interspecific hybrid) FLORAL BUDS

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Introduction

Blueberry (Vaccinium spp) is a perennial fruiting crop that produces floral buds on one-year old wood. The floral buds develop on new woody growth, remain dormant through late fall into early spring, and will flower when temperature and photoperiod requirements are met. However, blueberry can flower under short days (8 h) and warm temperatures (21 °C) even without chill accumulation (Arora et al., 2004; Banados and Strik, 2006; Bieniasz, 2012). Acclimation in blueberries coincides with the onset of dormancy and enables floral buds to withstand lower temperatures (Rowland, 2008; Wisniewski et al., 2003). During dormancy, floral buds withstand freezing temperatures and chill hours are accumulated. When the requisite environmental and physiological conditions are met, the buds are able to deacclimate and break dormancy (Retamales and Hancock, 2012).

As floral buds deacclimate and progress through fruit set, cold hardiness is reduced and the temperature at which freeze damage occurs, rises (Aslamarz et al., 2010; Miranda et al., 2005; Rieger, 1989; Salazar-Gutierrez et al., 2014; Spiers, 1978; Wolf and Cook, 1992). In deacclimated buds and fruit, the potential for crop loss increases as temperatures reach 0 °C or below (Miranda et al., 2005; Rieger, 1989). Freeze related crop loss is exemplified in a 2007 spring freeze, commonly known as the “Easter freeze” by fruit growers, which reduced southeastern blueberry production by 100% in many states including, Illinois, Minnesota, and Tennessee. The largest southeastern blueberry producing state, Georgia, lost 65% of its crop (Warmund et al., 2008), amounting to a $ 64.9 M loss (USDA, 2008). How blueberry floral buds develop, accumulate chill, and are affected by freeze will be reviewed.
**Bud Development**

Blueberry buds develop from the vegetative meristem on one-year-old wood in the summer and progress toward anthesis the following spring (Darnell et al., 1991). Blueberry inflorescent buds have multiple flowers per bud and develop at end of the shoot with vegetative buds below the floral buds (Retamales and Hancock, 2012). Blueberries grown in some climates with a long growing season may experience two flushes of growth, late spring and mid-summer, and will produce floral buds on both flushes. These flushes have distinctive effect on bloom timing, harvest, and fruit quality; fruit that develop on summer flush may be smaller in size in some varieties and growing regions (Retamales and Hancock, 2012).

The transition of vegetative meristem to reproductive (floral) meristem results from signals stimulated by plant growth hormones (Guimond et al., 1998; Lang, 1965). Within inflorescences such as blueberry, differentiation of each meristem occurs acropetally (Barton, 2010; Bieniasz, 2012), as such the terminal floral meristem differentiates first followed by the lateral floral meristems (Kovaleski et al., 2015). Blueberry floral bud induction is noted by a flattened dome with peripheral bract primordial separation (Kovaleski et al., 2015).

In northern highbush blueberry (NHB; *V. corymbosum*), the flower primordium differentiates in August, forming the meristematic center (Bieniasz, 2012). Sepals continue to develop into the autumn, along with anther and carpel primordia, which develop at the sepal base (Bieniasz, 2012). In southern highbush blueberries (SHB; *V. corymbosum* interspecific hybrids) ‘Emerald’ and ‘Jewel’, outer whorl sepals, petals, anthers, and pistils, form and expand into late fall (Kovaleski et al., 2015). Anther and ovule formation and development has been observed throughout winter in SHB ‘Sharpblue’ (Huang et al., 1997). Highlighting the contrast between NHB and SHB, filaments and anthers in NHB are the last developing organs before entering
dormancy (Bieniasz, 2012). Huang et al. (1997) observed morphological development throughout dormancy in SHB ‘Sharpblue’. Comparing NHB and SHB, Huang et al. (1997) and Bieniasz (2012) both observed mid-winter development of tapetum and microspore mother cells, which preceded pollen tetrad formation.

**Dormancy**

The role of dormancy in woody perennial plants has been further subdivided into paradormancy, endodormancy, and ecodormancy (Lang et al., 1987). Paradormancy is dormancy controlled by physiological characteristics of the plant, external to the dormant bud. Often associated with paradormancy is apical dominance and the imposition of dormancy on lateral buds (White et al., 1998). Endodormancy is controlled by floral buds (Lang et al., 1987). In many perennial plants, including blueberries, endodormancy requires chilling unit accumulation (Fadón et al., 2015). Dormancy ends when the chilling requirement is reached and the temperature and photoperiod are above critical thresholds. Conversely, if the temperature remains below the critical threshold, the plant will remain dormant, in a state of ecodormancy. Ecodormancy is dormancy controlled by environmental conditions unfavorable for plant growth, such as low temperatures (Campoy et al., 2011).

Chill accumulation is a factor of genotypic variation within blueberry, NHB accumulate between 800 and 1,000 chill hours, while varieties of SHB and rabbiteye blueberries [RE; V. virgatum (Aiton) syn. ashei] require < 550 and ~600 chill hours, respectively (Darnell et al., 1998; Ehlenfeldt et al., 2012; Retamales and Hancock, 2012; Spiers et al., 2006). Northern adapted species of blueberry such as V. constablaei (Gray) and V. angustifolium (Aiton) have
greater cold tolerance than NHB. Southern adapted blueberry species, e.g. *V. darrowii* (Camp.), *V. tenellum* (Aiton), and RE, are progressively more sensitive to cold (Ehlenfeldt et al., 2012; Rowland et al., 2005). Cold sensitive cultivars predominantly acclimate faster and reach their minimum floral bud freezing lethal temperature earlier than cold hardy genotypes (Ehlenfeldt et al., 2012; Rowland et al., 2008). Due to lower chill requirements, SHB are precocious compared to NHB (Ehlenfeldt et al., 2012; Rowland et al., 2005), predisposing them to spring frosts. Blueberry cold hardiness parameters have been previously based upon research done with NHB cultivars (Flinn and Ashworth, 1994a). However, minimal work has been reported in SHB. ‘Legacy’ has been characterized for cold hardiness in genotype comparison studies (Rowland et al., 2008; Rowland et al., 2005). The chill hour requirement of ‘Legacy’ is 500 h to 600 h, whereas many commercially grown SHB have chill requirements of 150 h to 300 h (Retamales and Hancock, 2012).

Blueberry will flower without receiving chill hours, which belies the hypothesis endodormancy in blueberry (Darnell et al., 1998). Hall and Ludwig (1961) treated blueberries with various photoperiods, discovering short day photoperiods have a more profound effect on flowering in blueberries. Further, floral induction decreased with increasing temperatures (Hall and Ludwig, 1961; Spann et al., 2004). In a study involving the photoperiod of NHB, ‘Duke’, ‘Elliot’, and ‘Bluecrop’ (chilling requirement of 800 h) (Retamales and Hancock, 2012) under long day greenhouse conditions, the plants would break bud without entering endodormancy (Banados and Strik, 2006). In another study, SHB ‘Gulfcoast’, RE ‘Tifblue’, and NHB ‘Bluecrop’, all flowered after receiving only half their chilling requirement when treated with 15 h long day conditions at 15 °C (Arora et al., 2004).
However, a lack of chill hour accumulation can extend bloom period causing a prolonged harvest (Egea et al., 2003; Erez, 2000; Luedeling et al., 2009). Adequate chill hour accumulation is necessary for buds of many woody plants, including many blueberry cultivars, to bloom with uniformity (Egea et al., 2003). Different chill models have been developed to best calculate chill hours for varying regions and crops (Luedeling and Brown, 2011). Common chill models include: the chill hour model, a simple model where chill hours are calculated below 7 °C (sometimes seen as hours between 7 °C and 0 °C) (Retamales et al., 2012; Spiers and Marshall, 2003; Spiers et al., 2006); the Utah model, which calculates chill hour accumulations as a full hour between certain temperatures, and a half hour at temperatures bordering that range, and subtracting chill hours if higher temperatures are reached (Anderson and Richardson, 1987; Richardson et al., 1974); and the dynamic model, a newer complex model to account for chill negation, often used in places with warmer climates (Zhang and Taylor, 2011).

**Ice Nucleation within the Bud**

Chill is important for blueberry production; however, sub-zero temperatures can damage plant tissue, open the plant to infection, and ultimately reduces yield. Pure water melts at 0 °C; however, water may freeze below its melting point. The condition where water remains liquid below 0 °C is at a state of equilibrium provided it is not subjected to disturbance or the solution is metastable. When a substance remains as a liquid below its melting point, the solution is considered supercooled (Ashworth, 1991; Burr et al., 2001; Wisniewski et al., 2004). Supercooling of water in a plant is critical to minimize damage during freeze events. Supercooled solutions energetically favor the transition to a solid, but need stimulus to form a crystal that will propagate freezing (Ashworth, 1991; Wisniewski et al., 2004).
Stimulus involved in ice nucleation could be homogeneous where a critical temperature is met and water molecules crystalize spontaneously (Burr et al., 2001; Quamme et al., 1982). Or water molecules align on a substrate in the solution, which catalyzes nucleation. This is referred to as heterogeneous ice nucleation, where nucleation is initiated at a point within the solution and propagates throughout the solution (Niedermeier et al., 2011). These phenomena are time, temperature, and solution dependent (Wisniewski et al., 2004).

Supercooling of a solution is dependent on the volume, presence of impurities, and temperature range. The singular hypothesis (Stansbury and Vali, 1965) suggests that the presence of impurities can act as catalyst for ice nucleation within the sample at a specific temperature and in relation to volume. This suggests that larger volume solutions have greater number of potential impurities and ice nucleation increases with decreasing temperatures. Singular hypothesis is dependent on fixed sample temperature and does not account for rate of cooling nor duration. However, the stochastic hypothesis suggests that ice nucleation is a random event dependent on time, volume, and composition, where a water droplet will have an equal chance of freezing at a given temperature and time. The probability of this event increases with decreasing temperatures (Niedermeier et al., 2011). Also, the rate of cooling will affect the amount of ice nucleation. Hence, larger sample size with slow cooling rates will increase the ice nucleation potential, thereby reducing supercooling and increasing the freezing threshold temperature (Ashworth, 1984; Ashworth, 1991; Ashworth et al., 1985).

However, neither proposed hypotheses are strongly suited to explain observations under experimental conditions. As discussed in Ashworth (1991), multiple experiments that were conducted to determine which hypothesis supports observations, there are anomalies in temperature, time, and volume that suggest both homo- and heterogeneous ice nucleation
hypotheses can be applied to the observations. Regardless, ice nucleation and subsequent freezing is affected by sample size, solute concentration, and compartmentation. Ashworth et al. (1984b) observed water freezes at lower temperatures when isolated within cavities connected to areas of bulk water via pores of 100nm or less. Ashworth (1984b) also observed that adding solutes to the solution depressed the freezing temperature within the cavities. In *Prunus* spp, xylem discontinuity was observed in dormant floral buds and bud tissue could be supercooled. When vascular differentiation resumed and xylem was continuous, supercooling was lost (Ashworth; 1984a). Xylem conductivity has a critical role in the propagation of ice and demonstrates that small volumes of water have the ability to depress freezing temperatures in plants.

**Plant Tissue Cold Injury**

Cold injury in buds is caused by the nucleation and propagation of ice within sensitive tissues (Pearce, 2001). Once ice is initiated, the pattern of ice propagation enables tolerance of extracellular ice and avoidance of intracellular ice formation via freezing point depression and supercooled water (Kishimoto et al., 2014; Workmaster et al., 1999). A supercooled solution maintains its liquid state below its natural freezing point through associative properties of dissolved solutes (George and Burke, 1984). In freeze-tolerant plants, ice propagation first occurs extracellularly (Levitt, 1980). Plants are more capable of recovering from extracellular ice formation; intracellular ice propagation frequently results in cold injury (Griffith, 1996; Mccully et al., 2004). The extracellular tissue creates an ice sink, drawing water from surrounding tissues and converting it to ice. This ice sink creates a cavity causing mechanical damage and cell collapse to surrounding tissues (Ashworth et al., 1989). Intracellularly, the solution supercools,
lowering the temperature at which internal cell membrane disruption occurs (McCully et al., 2004).

The cells avoid freeze damage through depressing the freezing point through supercooling the intracellular water content of floral organs until critical temperature is reached and ice is formed (Ashworth et al., 1988). Supercooling occurs below the freezing point of the subtending tissue, whereby the plant can avoid freezing injury in that tissue (Andrews and Proebsting, 1987; Burke et al., 1976; Kang et al., 1998; Mathers, 2004). Intracellular freezing depends on the rate of cooling (Marmiroli et al., 1986; Mazur, 1963; Steponkus et al., 1983). If a plant tissue cools quickly in a natural setting, equilibrium is unable to be maintained, and supercooling will eventually be overcome by intracellular freezing. Conversely, if chilled slowly, the tissue dehydrates to maintain equilibrium between intra- and extracellular aqueous vapor pressures, preventing intracellular freezing (Mazur, 1969). Nuclear Magnetic Resonance (NMR) data from Quamme et al. (1982) illustrated that as temperature decreases, so does the liquid water content of the intracellular tissues. The cell dehydrates as it contracts, slowly releasing water to the extracellular ice pool. The remaining intracellular liquid becomes concentrated, depressing its natural freezing point (Ishikawa and Sakai, 1981; Levitt, 1980).

Flinn and Ashworth (1994) observed acclimated buds to have less ice formation than deacclimated or non-acclimated floral buds. The absence of ice formation in acclimated blueberry buds indicated extraorgan freezing (Flinn and Ashworth, 1994b; Sakai, 1982). Extraorgan freezing hypothesizes that water from nearby tissues migrate toward the lower vapor pressure areas or cavities as a mechanism of frost survival for the reproductive organs. Flinn and Ashworth’s (1994) study found rachises, florets, and pedicels did not have sufficient cavities for
ice formation; however, in the bracts, bud scales, and floret scales, ice formation was observed in blueberry buds.

The extracellular propagation of ice results in a deficit of aqueous vapor pressure (Burke et al., 1976). Intracellularly, the cell water supercools, and vapor pressure exceeds that of the extracellular water (Mazur, 1969), creating a pressure gradient wherein water diffuses through the plasma membrane to freeze extracellularly (Quamme et al., 1982b). The movement of intracellular water to extracellular propagating ice avoids the formation of intracellular ice crystals (Sakai, 1960; Sakai and Otsuka, 1967), resulting in intracellular dehydration and the concentration of solutes (Mazur, 1969).

Ice propagation rate depends upon water potential gradient, cell wall and membrane permeability, and cell surface area (Ashworth, 1991; Flinn and Ashworth, 1994b). Segregation of ice propagation was observed in several studies (Ashworth et al., 1989; Ishikawa and Sakai, 1985; Quamme, 1978). Ice propagation is preferential in larger pore spaces, and is limited by smaller diameter pores such as microcapillaries, which in turn aid in supercooling cells (Ashworth et al., 1992; Ashworth and Abeles, 1984). Similar to the lowered freezing temperature of cavities compared to bulk freezing found by Ashworth (1984), Jones et al. (2000) found that freezing in large pores of Vitis vinifera stems precludes ice progression into lateral appendages such as buds in the same manner (Jones et al., 2000). If significant supercooling takes place, ice crystal growth penetrates this barrier affecting the less cold tolerant floral organs (Zachariassen and Kristiansen, 2000).

On a whole bud scale, Quamme and Gusta (1987) found that water preferentially moved out of the base of the peach floral buds to the pith and bud scales. The preferential diffusion out of the flower primordium was due to its significantly lower mean water potential; the vascular
tissue at the base of the bud, the bud scales, and the stem bark had higher mean water potentials. The stem xylem had a significantly higher mean water potential than all other floral parts; consequently increasing attached stem length in addition to accessory flower parts (scales, internode, and lateral buds) corresponded to a significantly higher ice nucleation temperature of peach flower bud (Quamme and Gusta, 1987). Without these parts, ice nucleation occurred at a significantly lower temperature in the flower primordium (Quamme and Gusta, 1987).

Kishimoto et al. (2014b) found a similar trend in blueberries in which the ice nucleation temperature of both leaves and flower buds were lower than stems. Infra-red thermography showed ice nucleation activity begins in apical and basal regions of blueberry stems, progressing toward the leaves and flower buds (Kishimoto et al., 2014). Further, bud location also had an effect on freezing injury in blueberries; apical buds were more susceptible to freezing injury, regardless of maturity stage (Biermann et al., 1979).

Though not well understood, lack of development of vascular tissue contributes towards the barrier between stems and overwintering dormant peach buds (Ashworth, 1984). Ashworth (1982) reported vascular differentiation and xylem continuity was not established between floral buds and connecting tissue until deacclimation in peach. In NHB, the vascular system between the florets and the parent plant was established in late fall with mature xylem extending into the ovaries; however, stamen and pistil xylem maturity remained undifferentiated until after deacclimation (Ashworth, 1984; Flinn and Ashworth, 1994b). Though lacking xylem maturity, ice crystallization and propagation was not associated with xylem continuity in blueberry. Light micrographs of acclimated blueberry buds illustrated ovarian xylem vessel continuity, scanning electron microscopy displayed an absence of ice cavitation in acclimated blueberry florets. Frosts
occurring after deacclimation and xylem continuity establishment facilitated ice propagation within reproductive tissues (Flinn and Ashworth, 1994b).

Freezing patterns are tissue specific events. The concentration and distribution of water within floral organs can be visualized using nuclear magnetic resonance microimaging (NMRI). NMR microscopy detects the difference in proton relaxation times from acclimated and deacclimated buds, allowing analysis of the bud water state (Ishikawa et al., 2000). The density of these mobile protons displays non-frozen tissue within buds, thus when water is bound, proton mobility ceases creating a non-image indicating frozen water (Faust et al., 1991). Ishikawa et al. (2000) used $^1$H-NMR imaging to look at the mobile water content of _Rhododendron japonicum_ floral inflorescences. The authors reported decreased signal intensity in the bud scales and axis indicating either tissue dehydration or partial freezing. At the same time, there was an observed slight increase in the signal intensity of the florets preceding intracellular freezing (Ishikawa et al., 2009; Price et al., 1997). $^1$H-NMR also detected decreased floret size, in _Rhododendron japonicum_ and _Acer japonicum_ as the samples were cooled to lethal temperatures (Ishikawa et al., 1997a; Ishikawa et al., 1997b). Erez et al. (1998) determined availability of free water in plants was indicative of cold resistance as opposed to the previously theorized endodormancy conditions (Erez et al., 1998; Faust et al., 1991). This was determined by quantifying the availability of free water in peach buds (via magnetic resonance imaging, MRI) which had and had not acquired appropriate chilling accumulation under forcing conditions (Erez et al., 1998). Rowland et al. (1992) used NMR to visualize water restriction in dormant NHB cultivars ‘Duke’ and ‘Bluecrop’ during deacclimation. Proton mobility increased as the buds deacclimated (Rowland et al., 1992). Price et al. (1997) studied the freezing pattern of _Rhododendron japonicum_ florets, correlating NMR data to exothermic events produced by freezing events using
differential thermal analysis (DTA). Decreased intensity of mobile protons occurred at -7 °C, which corresponded to a DTA high temperature exotherm (HTE) peak at the same temperature. HTEs correspond to extracellular freezing events, (Burke et al., 1976; Quamme, 1974). The florets froze at lower temperatures compared to other floral organs (floral bud scales froze at -7 °C), their proton mobility ceasing between -14 °C and -21 °C. This was consistent with DTA exotherms between -17 °C and -24 °C (Price et al., 1997).

**Tissue Analysis of Cold Hardiness**

Cold hardiness is measured by exposing plant material to freezing temperatures and quantifying resulting tissue damage (Calkins and Swanson, 1990). Different methods of quantifying cold damaged tissues include measuring plant viability after a freezing treatment, electrolyte leakage of disrupted membranes, chlorophyll content, and thermal analysis (Bigras and Colombo, 2001). Often, more than one method is used for confirmation of plant cold hardiness as these methods do not always produce similar results as in the case of blueberries (Flinn and Ashworth, 1994a).

*Freezing Tolerance Tests (FT)* are methods where plant tissue is placed in a temperature-controlled chamber. Either whole or excised plant tissues are evaluated at or over a range of temperatures. After exposure to the treatment, plant material is removed, stabilized for a period of time at a temperature above freezing, and evaluated for physiological changes due to injury. Evaluation of plant material susceptibility to freeze is analyzed through tissue dissection or forcing vegetative and floral buds (Burr et al., 2001). Forcing consists of placing plant tissue in favorable conditions to promote growth and after a period of time the buds can be assessed for mortality (Campoy et al., 2011). Bud mortality is expressed in relation to the lethal temperature
Electrolyte leakage tests cell membrane disruption, where increased electrolyte conductivity is indicative of cold injury. Similarly, electrical impedance spectroscopy measures extracellular resistance. Burr et al. (2001) reported that in tandem with increasing cold damage in plant tissue, intracellular electrolyte leakage and extracellular conductivity both increased in conifers. Similarly, in olives there was a good correlation between data obtained through electrolyte leakage and impedance spectroscopy \((r^2 = 0.98)\), as well as between electrolyte leakage and fractal analysis \((r^2 = 0.95)\) (Azzarello et al., 2008).

Differential Thermal Analysis (DTA). DTA measures the internal change in temperature of the sample through thermocouples as the chamber and tissue temperature progressively cools. DTA is used to observe the freezing of supercooled water, which identifies low temperature exotherms (LTE) (Quamme et al., 1995). Profile peaks of an exothermic event are observed as either extracellular water freezing, indicated by an initial peak (HTE), or injury to internal tissues as indicated by later peaks (LTE) (Sakai, 1979).

Previous studies in fruit crops report exothermic peaks representing both HTE and LTE events in peaches \((P. persica)\) (Andrews et al., 1983; Miranda et al., 2005; Quamme, 1974), apple \((Malus domestica)\) (Quamme et al., 1972a), and blueberry (Quamme et al., 1972b). In apple stem tissue, HTEs were observed as a discontinuous spread of ice, such as from the bark to the xylem (Quamme et al., 1972a). Whereas, blueberry stems were not associated with an exothermic event relating bark to xylem injury (Quamme et al., 1972b). In bud tissue, the two exothermic events represent of cooling events of bud scales and bud primordia. The HTE is associated with a freezing event within the bud scales and the LTE is associated with an
intracellular event within the bud primordia (Wisniewski and Fuller, 1999). Bud scales freeze first and act as ice sinks for nucleating activity producing an HTE (Ishikawa et al., 2000). Removal of bud scales and transitional leaves produced only one exotherm in both peach and persimmon (*Diospyros kaki*) (Kang et al., 1997). In persimmon, the exotherm had an LTE of -20.7 °C, which was colder than the single exothermic event of the bud with scales at -14.3 °C (Kang et al., 1997). Kang’s (1997) results were similar to Quamme’s (1987) findings where the flower primordia had a lower lethal temperature when bud scale tissue was removed. Peach primordia had a single exothermic event higher than the LTE (-14.1 °C) yet lower than the HTE (-8.4 °C) of the bud as a whole, at -11 °C (Kang et al., 1997). Kang et al. (1997, 1998) determined that the $LT_{50}$ value obtained from the freezing temperatures of persimmon floral buds correlated well to the singular exothermic event represented by DTA of floral buds (Kang et al., 1997; Kang et al., 1998).

Warmund et al. (1990) established that the exothermic peaks of floral buds of multiple blackberry and raspberry cultivars corresponded to cold injury in plant tissue freezing tests (Warmund and George, 1990). In further studies on *Ribes* species, Warmund et al. (1991) found floral buds of black and red currants to produce multiple LTEs, suggesting freeze injury to multiple floral buds within the raceme. The number of LTEs were not representative to the number of floral buds, suggesting simultaneous freeze events of buds within the raceme (Warmund et al., 1991). In another study on blackberries, Warmund et al. (1992) also found that LTEs produced from DTA did not corresponded to a bud’s developmental stage (Warmund et al., 1992).

Supercooling of floral buds has been observed in many deciduous fruiting crops using DTA: peach, apricot (*P. armeniaca*), sweet and sour cherry (*P. avium* and *cerasus*), persimmon,
and blueberry (Ashworth, 1984; Kang et al., 1997; Quamme et al., 1982a; Viti et al., 2013; Wittenbach and Bukovac, 1972). Quamme (1974) associated freezing events of supercooled water of several *Prunus* species (apricot, plum, cherry, and peach) to freezing injury in their respective flower buds (Quamme, 1974). In the deacclimation of peach and sweet cherry from deep supercooling to bud break, the range of the DTA associated peaks in floral buds widened. As buds developed closer to budbreak, fewer exotherms were produced, occurring at increasingly warmer temperatures. Parallels were established between increasing DTA exotherms and freezing injury at increasing ice nucleation temperatures as deacclimation progressed (Andrews et al., 1983).

In NHB, DTA measurements did not reflect the $LT_{50}$ values obtained from plant tissue freezing methods (Flinn and Ashworth, 1994a). The DTA results had consistently higher exotherms compared to the $LT_{50}$ floral bud temperatures from FT (Flinn and Ashworth, 1994a). DTA exotherms for SHB from acclimation to deacclimation have not been characterized. SHB typically thrive in environments with milder winters in comparison to SHB cultivars. In a study comparing SHB ‘Legacy’ to three RE and three NHB cultivars, ‘Legacy’ had a higher $LT_{50}$ and lost hardiness sooner than the three NHB (Ehlenfeldt et al., 2012). This suggests warmer critical freezing temperatures could be anticipated in low chill cultivars of blueberry, leading to a difference in exotherms. This is exemplified in studies involving regionally different pecans and sour cherries (Mathers, 2004; Volk et al., 2009).

*Microscopy.* Sites of ice formation can be determined through microscopic techniques. Fixing tissue in formalin-acetic-acid-alcohol (FAA) is the first step in a commonly used embedding and sectioning process. Paraffin-embedding the sample hardens the tissue, allowing for microtome sectioning. Subsequent microscopy distinguishes dehydrated and cold damaged
tissues, structures, and bud development (Ashworth, 1982; Olson and Steeves, 1983). An alternative to paraffin embedding is cryosectioning. This technique involves a high-pressure freezing method in liquid nitrogen and sectioning using a cryostat. Cryosectioning morphologically preserves samples and can produce thinner sections than most microtomes (Chen and Zhao, 2005; McCully et al., 2010).

Additional methods which forgo microsectioning include using a scanning electron microscopy and confocal microscopy. In scanning electron microscopy, morphological development and cold injury, such as the pattern of ice cavity formation, can be viewed (Ashworth et al., 1989; Engin and Uenal, 2007). Confocal microscopy is able to delineate live cells from dead cells using fluorescein diacetate and propidium iodide stains (Jones et al., 2016; Truernit and Haseloff, 2008).

**Cold Mitigation**

Cold tolerance is a plants’ ability to acclimate to low winter temperatures, survive, and thrive the following season (Rowland et al., 2008). For deciduous plants, as photoperiod shortens and average daily temperature decreases, plants begin to acclimate. The plants will deacclimate as photoperiod lengthens and average daily temperature increases. As blueberries lose cold hardiness, floral bud organ injury due to freezing temperature occurs at increasing temperatures (Ehlenfeldt et al., 2015). This impacts the producer’s decision to mitigate economic loss through a form of frost protection.

Frost protection can be achieved to varying degrees with air movement, heaters, and sprinkler irrigation. Wind machines and orchard heaters were developed for areas where water
may not be available for agricultural irrigation during freeze events. However, most blueberry production regions have access to sufficient water for overhead irrigation. Applying water through overhead irrigation during a freeze event distributes water over the plant. The resulting water application freezes and encapsulates the plant tissue in ice. As water freezes, a small amount of heat is released, called the latent heat of fusion, the heat energy is absorbed by the plant and protects it from freeze damage (Lyrene and Williamson, 1997; Perry, 1998). Wind and decreasing temperature counteracts the latent heat of ice formation. Irrigation must be applied at a rate where ice formation is greater than water evaporation and heat loss; as a general rule if temperature is below -5 °C and wind speed above 16 km/h frost protection may not be effective (Snyder and Melo-Abreu, 2005).

Frosts occur as advective or radiative; understanding the type of frost determines mitigation. During an advective frost, cold air blows into an area and replaces the warmer air that was present before the weather change. It is associated with moderate to strong winds, no temperature inversion, and low humidity. Temperatures will drop below freezing and stay for an extended period. Advection frosts are difficult to protect against. Orchard heaters will be ineffective because the wind will remove the heat from the orchard and with overhead irrigation bubbles will form in the ice reducing conductivity of latent heat of freezing water.

A radiative frost occurs when the sky is clear, there is little or no wind, and is often associated with a temperature inversion (where the temperature increases with elevation above the ground) (Snyder and Melo-Abreu, 2005). Radiation frosts occur because of heat loss in the form of radiant energy. Temperatures at the orchard floor fall quickly, dew/frost point is met and frost occurs (Perry, 1998). With radiative frost, wind machines, orchard heaters, and overhead irrigation have demonstrated a reduction in freeze damage; however, when conditions are below
-5 °C and wind speed above 16 km/h there may be no economic return from frost mitigation (Pitts, 1989; Perry, 1998).

**Summary**

Blueberries are a commercially important crop that can be severely impacted by freezing temperatures. Similar to most deciduous perennial fruiting crops, blueberry forms floral buds in the previous season on that season’s woody growth and overwinters to bloom after a period of chill accumulation. Hardiness can be determined through various methods, not all of which are suitable for blueberry floral bud lethal freezing temperature determination. Freezing tolerance tests of tissue subjected to temperature and time increments, have proven to be reliable and inexpensive. DTA has been demonstrated not to reflect hardiness temperatures seen in situ or in freezing tolerance testing. The use of NMR and techniques of microscopy have shown where the proliferation of ice and the subsequent damage was caused in floral bud tissue after freezing. For blueberry, much of the cold hardiness work has been performed on cultivars that require a significant amount of chill. For low chill blueberries, photoperiod may be of more importance than chill accumulation, which may involve different critical freezing temperatures. Studies into the freezing tolerance of low chill blueberries could have impact blueberry on the management and frost protection.
CHAPTER 2

LETHAL TEMPERATURE ANALYSIS OF SOUTHERN HIGHBUSH BLUEBERRY

(Vaccinium corymbosum L. interspecific hybrid) FLORAL BUDS

\[1\] Redpath, L.E., Smith, E., Malladi, A., and Chavez, D. To be submitted to *Hortscience*. 
Abstract

Blueberry production in southeastern U.S. utilizes low chill blueberries, which are susceptible to freeze damage. Tolerance to freezing temperatures of floral buds in northern highbush blueberry [NHB; *Vaccinium corymbosum* (L.)] has been previously described; however, southern highbush blueberry (SHB; *V. corymbosum* interspecific hybrids) cold tolerance has not been well characterized. The objective of the study was to determine SHB’s dormant floral bud sensitivity to freeze by evaluating excised floral buds (EFB) and floral buds attached to a stem segment (AFB) using differential thermal analysis (DTA) and freeze tolerance analysis (FT) with pre-treatments of storage at 4.0 °C (FT4; 2015-2016 and 2016-2017) or -2.0 °C (FT-2; 2016-2017) overnight. Floral buds of ‘Emerald’ and ‘Farthing’ were sampled till budswell during two seasons (2015-2016 and 2016-2017) from a commercial farm in Lakeland, GA. Samples were pretreated then subjected to either DTA (-2.0 to -27.0 °C gradient program, decreasing at 4.0 °C h⁻¹) or FT in a programmable freezer (FT4 and FT-2; -3.0 to -21.0 °C gradient, removing bud tissue at -3.0 °C increments). The FT4 and FT-2 samples were stored for a week at 4.0 °C and evaluated for floral bud tissue damage. To evaluate floral bud damage, a lethal temperature threshold of 50% floral bud damage (LT₅₀) was used as a critical loss threshold. In 2015-2016, ‘Emerald’s lowest LT₅₀ was on 25 Jan at -18.0 °C in FT-2 treated AFB and ‘Farthing’s lowest LT₅₀ was on 8 Feb at -19.0 °C for AFB with FT-2 analysis. In 2016-2017, ‘Emerald’ and ‘Farthing’ had the lowest LT₅₀ on 5 Jan with FT-2 treated AFB at -19.0 °C and -20.2 °C, respectively. ‘Emerald’ and ‘Farthing’ FT-2 EFB LT₅₀ were 46-53% higher in temperature with a difference of 10.2 °C and 9.5 °C, respectively, compared to AFB FT-2 on 5 Jan. Neither cultivar’s EFB FT4 or FT-2 showed hardiness below -13.0 °C throughout the season in both years. In 2016-2017, comparisons made between FT-2 and FT4 AFB showed FT-2 was tolerant
to colder temperatures in both cultivars. DTA exotherms were not consistently detected and detected exotherms did not reflect FT analysis, suggesting DTA is not an appropriate method to estimate blueberry floral bud cold hardiness. Individual bud weight and length were measured and showed increases in weight and length both seasons. In conclusion, SHB evaluated in this study had greater sensitivity to cold than previously described NHB and SHB ‘Legacy’.
Introduction

Georgia consistently ranks in the top five blueberry producing states in the U.S., bringing in an average of over $6,750 ha\(^{-1}\)yr\(^{-1}\) over the last 10 yrs (USDA, 2016). In the same period, harvested acreage nearly tripled going from 2,428 ha in 2005 to 6,960 ha in 2015 (USDA 2016). In 2015, Georgia harvested an average yield of 5,021 kg·ha\(^{-1}\). In addition, the increase in hectares harvested contributed toward doubling Georgia’s blueberry production revenue since 2009 (USDA, 2016). Hard winter freezes and spring frosts are detrimental for Georgia’s blueberry production, reducing crop load by 50% or more as seen in many southeastern states between the years 2006 and 2007 (Warmund et al., 2008).

Dormancy is necessary for blueberry production. Floral buds harden against cold temperatures when entering dormancy during acclimation. The release from dormancy and resumption of morphological progression to budbreak occurs during deacclimation and it is noted by the loss of cold hardiness (Aurora et al., 2004; Bieniasz, 2012; Junttila et al., 1983). As they exit dormancy and progress to budswell, the buds become more susceptible to frost (Ehlenfeldt et al., 2012; Rowland et al., 2005). After dehardening, subsequent freezing temperatures can be detrimental to survivability of buds (Flinn and Ashworth, 1990; Flinn and Ashworth, 1994a; Warmund et al., 2008; Warmund et al., 1992).

Presently, dormancy is described in three specific physiological states: paradormancy, ecodormancy, and endodormancy. Paradormancy is dormancy established by the plant; however, a remote location within the plant regulates dormancy in a tissue. An example of this is lateral bud dormancy due to auxin flow to the terminal bud (White et al., 1998). Ecodormancy is dormancy controlled by environmental factors such as temperature, moisture, and short day
photoperiods present during winter (Lang et al., 1987). Endodormancy is dormancy controlled by the dormant tissue.

SHB and NHB blueberries are thought to be endodormant during the winter (Fadón et al., 2015; Lang et al., 1987). Release from endodormancy is determinant upon satisfied physiological factors. Chilling is a requirement for endodormancy release in many woody plants (Crabbe and Barnola, 1996), including blueberry (Rowland et al., 1999; Spiers and Marshall, 2003). Once sufficient chill hours have been accumulated, flower buds can break dormancy (Wilkie et al., 2008). Chill hour accumulation determines suited regional adaptability for blueberries; NHB requires greater chill hours than SHB, consequently, NHB are grown in colder climates (Retamales and Hancock, 2012). SHB germplasm contains V. darrowii (Camp.), an evergreen blueberry species, that does not have a dormant period; as such SHB has a lower chill requirement and a shorter dormancy period than NHB (Lyrene, 2008a; Lyrene, 2005; Retamales and Hancock, 2012).

Temperature and moisture are important factors in dormancy. Bittenbender and Howell (1975) found that an increase in either temperature or moisture content decreased bud hardness of the NHB ‘Jersey’, albeit through different mechanisms (Bittenbender and Howell, 1975). Photoperiod is also an important factor in budbreak. NHB cultivars ‘Duke’, ‘Elliot’, and ‘Bluecrop’ grew continuously and broke bud without entering endodormancy when treated to long day photoperiod conditions (Banados and Strik, 2006; Hall and Ludwig, 1961). These experiments showed that environmental conditions resulting in ecdormancy have a greater effect on budbreak than chill hour requirement of endodormancy in blueberry. Nevertheless, the lack of adequate chill can cause variation in development. Banados and Strik (2006) reported NHB kept in long day photoperiod conditions did not initiate flower buds, where short day
photoperiod treated plants initiated flowers after two weeks. Treatments of varying chill hours in ‘Perlette’ grapes found that increasing chill duration decreased days until budbreak upon transferred to 22 °C; further, increased chill also increased budbreak percentage and uniformity (Dokoozlian, 1999). No accumulated chill hours in ‘Perlette’ resulted in less than 40% budbreak occurring over a longer period of time compared to vines receiving 50 or greater chill units when moved to 22 °C. Increasing chill hour accumulation, increased bud break uniformity in ‘Perlette’ grapes (Dokoozlian, 1999).

Recently, the necessity of chilling requirement in SHB has come into question. In an experiment involving three SHB cultivars ‘Sharpblue’, ‘Wannabe’, and ‘Gulf Coast’, continuous nitrogen application allowed the plants to forgo dormancy without deleterious effects. Lacking cold temperatures, the plants did not harden (Darnell et al., 1998). Spann et al. (2003) determined that flower buds in SHB cultivars ‘Misty’ and ‘Sharpblue’ did not break when kept at 21 °C/18 °C day/night temperatures and treated to conditions of short day photoperiod or short day photoperiod with an interrupted night; long day photoperiod treated plants achieved bloom. When transferred to a long day photoperiod, short day photoperiods as well as short day photoperiods with interrupted night plants began to bloom after two weeks (Spann et al., 2003).

SHB genetic contributor, V. darrowii, was able to develop flower buds on old canes in all conditions after 8 weeks of photoperiod treatment; SHB ‘Sharpblue’ and ‘Misty’ did not develop flower buds on short day-interrupted night or long day conditions (Spann et al., 2003). Flower bud initiation was significantly greater under short day conditions in V. darrowii, ‘Sharpblue’, and ‘Misty’, than either short day-interrupted night or long day after 8 weeks. (Spann et al., 2003).
The lethal temperature of plants can be measured in a number of ways including differential thermal analysis (DTA), freeze tolerance testing (FT), and electrolyte leakage (Burr et al., 2001). In DTA, exothermic events are measured in a thermos electric module, and a DTA profile is recorded as temperature decreases over time. A DTA profile displays peaks at which exothermic events occur, often having both high temperature and low temperature exothermic events (HTE and LTE, respectively). An HTE in general represents the extracellular water freezing, such as supercooling in the bud scales, whereas an LTE represents an intracellular supercooling event within the floral organs (Wisniewski and Fuller, 1999). In NHB, DTA has been difficult to quantify, producing a single exothermic event observed at -8 °C, which was significantly warmer than the FT (Flinn and Ashworth, 1994a). Predominantly used for blueberry is FT, which is performed using either a glycol bath or a freeze chamber (Ehlenfeldt et al., 2009; Ehlenfeldt et al., 2012; Ehlenfeldt et al., 2015; Rowland et al., 2008; Rowland et al., 2005). Electrolyte leakage is used to measure electrical conductivity of blueberry stems; however, blueberry floral bud electrolyte leakage has not been characterized. The greatest increase in electrical conductivity occurred between −8 °C to −16 °C in blueberry stems of different germplasm; the conductivity of all species continued to increase until -24 °C (Liu Wan Ping et al., 2012). In rabbiteye [RE; Vaccinium virgatum (Aiton) syn. ashei] cultivars, electrolyte leakage of blueberry branches increased with decreasing temperature until -20 °C. Floral bud FT of the same plants determined the lethal temperature at 50% floral bud damage (LT₅₀) was between -13.3 °C to -25 °C (Kim et al., 2014).

NHB cold tolerance has been studied from acclimation to deacclimation (Ehlenfeldt et al., 2012; Ehlenfeldt et al., 2015; Flinn and Ashworth, 1994a; Rowland et al., 2005; Rowland et al., 2013). Flinn and Ashworth (1994) researched the floral bud cold hardiness of NHB
‘Berkeley’, with two different methods, DTA and FT, using a segment of stem with the floral bud attached (AFB), as well as excised floral buds (EFB) (Flinn and Ashworth, 1994a). They reported that ‘Berkeley’ was hardy from -34.5 °C to -20 °C before budswell using FT in AFB tissue (Flinn and Ashworth, 1994a); EFB tissue had higher hardiness temperatures of -22.5 °C to -8.1 °C. The NHB cultivars ‘Bluecrop’, ‘Little Giant’, and ‘Northsky’ were analyzed for lethal temperatures from late September to early April; average minimum lethal temperature was -27.4 °C (7 Feb), -28 °C (29 Nov – 7 Feb), and -28 °C (7 Nov – 6 Mar), respectively (Ehlenfeldt et al., 2012). Concomitantly, SHB ‘Legacy’ had a minimum lethal temperature of -24.1 °C in early winter and progressively lost cold tolerance toward budswell (Ehlenfeldt et al., 2012).

NHB characteristically have higher chill requirements than SHB (Retamales and Hancock, 2012); however, characterization of lower chill SHB has not been researched extensively. ‘Legacy’ has a chill requirement of 500 to 600 chill hours (Krewer and NeSmith, 2006; Lyrene, 2004); whereas producers in southern Georgia are growing cultivars with much lower chill requirements (200-400 chill hours) and potentially higher lethal temperatures toward budswell. Certain low chill SHB cultivars have been evaluated for midwinter hardiness, including ‘Millennia’ (-13.2 °C), ‘Sapphire’ (-14.7 °C), ‘Sharpblue’ (-15.8 °C), ‘Misty’ (-17.2 °C), ‘Biloxi’ (-17.2 °C), ‘Star’ (-18.7 °C), ‘Windsor’ (-19.5 °C), ‘Echota’ (-24.7 °C), and ‘Reveille’ (-25.1 °C) (Ehlenfeldt et al., 2009).

Previous studies of floral bud lethal temperature of SHB cultivars have been evaluated at a single time point. The objective of the study was to determine SHB, ‘Emerald’ and ‘Farthing’, dormant floral bud sensitivity to freeze by evaluating excised floral buds (EFB) and floral buds attached to a stem segment (AFB) using FT and DTA with pre-treatments of storage at 4.0 °C (FT4) or -2.0 °C (FT-2) overnight, though the winters of 2015-2016 and 2016-2017.
Methods and Materials

Plant material. One year old fruiting shoots of ‘Emerald’ and ‘Farthing’ within a contiguous row per cultivar were harvested at Mathis Farms, a commercial blueberry farm in Lakeland, GA. Material was collected on 2 Nov, 16 Nov, 7 Dec, 21 Dec, 12 Jan, 25 Jan, 1 Feb, 8 Feb, and 15 Feb of the 2015-2016 winter. In 2016-2017 winter samples were collected on 29 Nov, 13 Dec, 5 Jan, 17 Jan, 24 Jan, and 31 Jan. Shoots were randomly collected from a minimum of 30 plants. Samples were stored in moist paper towels within a sealable plastic bag and sent to the University of Georgia Griffin Campus, Griffin, GA, where they were held at 4 °C until preparation and analyses.

In preparation, leaves were removed from all shoots. Shoots were selected at random and their buds excised per cultivar. Remaining shoots were trimmed to 5 cm segments containing floral buds. Sixteen shoots per cultivar were cut to 12 cm to be used in bloom forcing. Bloom forcing was preformed from 25 Jan – 15 Feb of 2016, and 24 Jan of 2017.

DTA. Twelve buds were excised, wrapped in a wetted tissue (Kimwipe®, Irving, TX), and held overnight (12 h) at 4 °C. Following pretreatment of 4 °C, the buds were placed on thermos electric modules (TEM) for DTA. From 25 Jan to 15 Feb, an additional pre-treatment was supplemented, adding a second DTA set held at an overnight temperature of -2°C. From 25 Jan to 15 Feb, four attached floral buds, represented as a single bud on a 5 cm shoot, were also analyzed for exothermic events. Attached buds were treated to both overnight temperatures of -2°C and 4 °C. The shoots were taped down such that the bud scales of the attached bud were firmly pressed against the thermocouple plate. Reference shoots (4) of the same size that did not contain buds were also placed on thermocouples. All thermocouple plates were covered with aluminum foil shells to prevent movement. The ESPEC EY-101 (Tabai Espec Corp., Osaka,
Japan) chamber was then cooled at rate of 4°C∙h⁻¹. A 700x Control Module CR7 (Campbell Scientific Inc., Logan, UT) data logger recorded the electric output from the exothermic events of the tissues in the ExceLINX (Keithley Instruments, Cleveland, OH) program. Exothermic events were displayed as peaks in the DTA profile.

**FT.** To determine the temperature at which floral bud damage is observed, eight sets of six EFB were randomly selected from the remaining buds. Each set of buds was placed on a tissue (Kimwipe®, Irving, TX), folded, and moistened with D.I. water. Each sample set was placed in individual freezer bags and sealed. This preparation was also done for 5 cm stem cuttings containing AFB. For each FT run, 48 excised and 5 cm stems segments were prepared for each cultivar. One sample set containing six replicates of each tissue type, EFB and AFB, was placed in temperature-designated bags of 4 °C, -3 °C, -6 °C, -9 °C, -12 °C, -15 °C, -18 °C, and -21 °C.

Prepared samples sets were treated to an overnight temperature of 4 °C from 16 Nov to 12 Jan 2015-2016. From 25 Jan to 15 Feb of the 2015-2016 winter, the overnight treatment was altered to -2 °C using the ESPEC EY-101 (Tabai Espec Corp., Osaka, Japan) freeze chamber to induce ice nucleation activity and avoid excessive supercooling (Anisko and Lindstrom, 1996). During the 2016-2017 winter, the experiment was evaluated with pre-treatments of both 4 °C and -2 °C.

Bags were organized and placed on a rack in the ESPEC EY-101 (Tabai Espec Corp., Osaka, Japan) freeze chamber. Removal rates occurred every -3 °C, from -3 °C until -21 °C. A control bag of EFB and AFB was held at constant 4 °C for the duration of the experiment (approximately 8 days). Three temperature probes were placed in different freezer bags, simulating the tissue temperature inside the sample bags. These probes were interspersed...
between the sampling bags in the chamber. The temperatures recorded from these three probes were averaged to estimate the sample temperature and removal time. The temperature program cooled the ESPEC EY-101 (Tabai Espec Corp., Osaka, Japan) at 4°C·h⁻¹. As the chamber cooled, the bags were removed at their indicated temperatures. Removal was quickly done to minimize the influence of outside temperature on temperature fluctuation of the freeze chamber. Following program completion of samples held at -2 °C overnight, samples treated to 4 °C overnight were transferred and run sequentially during the 2016-2017 winter.

Following the FT, samples were stored in a refrigerator at 4°C for a week to allow for physiological changes associated with freeze damage to set in (e.g. browning). Evaluations involved dissecting buds with a razor to evaluate for damage. Each bud was evaluated under a light microscope for coloration changes specific to tissue damage. Similarly, inflorescence mortality was determined per individual floral bud. Critical bud freezing temperature was determined by the lethal temperature representing 50% bud necrosis (LT₅₀) on a logistic scale (Proebsting, 1961).

*Bud Water Content.* Fresh bud weight and length were taken using Mettler Toledo ML203E scale (Mettler Toledo, Columbus, OH) and Traceable® calipers (VWR International, Radnor, PA). Subsets of ten buds from each cultivar were measured on each sampling date. After fresh measurements were taken, samples were placed in an oven (Thermo Electron Corporation, Beverly, MA) at 60 °C. After drying for 48 h, sample weight and length was measured.

*Statistical Analysis.* FT temperature inflection points were determined using a logistic curve in JMP Pro version 13 (SAS, Cary, NC). All other analyses were evaluated with SAS’s 9.4 Proc GLM (SAS Institute Inc., Cary, NC, U.S.) and means were separated at \( P<0.05 \) level using Tukey’s honest significant difference (HSD) test.
Results

DTA. The relationship between detected DTA exotherms for either pretreatments, -2 °C or 4 °C, and either FT-2 or FT4 LT\textsubscript{50} were significantly different in both ‘Emerald’ and ‘Farthing’ (\textit{P} < 0.05) (data not shown). FT-2 and FT4 produced lower freezing events than DTA for either pretreatment; the relationship was significantly different in both tissue types of ‘Farthing’ and ‘Emerald’ (data not shown). The exothermic events seemed to indicate bulk water freezing.

\textit{FT}. The relationship between tissue types, AFB and EFB, was also significantly different in FT4 of both cultivars for most sampling dates in the winter of 2015-2016. The AFB FT LT\textsubscript{50} was significantly lower than EFB from 21 Dec to 8 Feb in ‘Emerald’ by 26-96%. The AFB FT4 LT\textsubscript{50} was significantly lower than EFB for ‘Farthing’ on 21 Dec and FT-2 data from 25 Jan to 8 Feb by 59-95%; on 12 Jan where there was no significant difference, though AFB had a lower LT\textsubscript{50}. AFB FT-2 LT\textsubscript{50} reached an average minimum temperature of -17.0 °C for ‘Emerald’ on 25 Jan and -18.6 °C for ‘Farthing’ on 8 Feb. EFB minimum LT\textsubscript{50} was -13.0 °C for FT4 for ‘Emerald’ on 12 Jan and was -12.1 °C for FT-2 ‘Farthing’ on 15 Feb (Table 2.1).

Comparing tissue types of either pre-treatment temperature of ‘Emerald’ in winter 2016-2017, AFB LT\textsubscript{50}s were significantly lower by 30-116% for FT-2 and 27-41% lower for FT4 compared to respective EFB results from 29 Nov to 17 Jan. FT-2 EFB were significantly higher than FT-2 AFB for every sampling date, except 24 Jan, where the difference was not significant. FT4 EFB were significantly higher than the AFB counterparts from 29 Nov until 17 Jan by 27-41% (Table 2.2).

There was no significant difference between the FT-2 and FT4 LT\textsubscript{50} in ‘Emerald’ EFB in 2016-2017. FT-2 AFB LT\textsubscript{50} were consistently lower than FT4 AFB LT\textsubscript{50} for every collection
date, ranging 12-40%. The difference was significant for all collection dates except 17 Jan. FT-2 was not consistently lower than FT4 in ‘Emerald’ EFB (Table 2.2). The lowest AFB LT50 of ‘Emerald’ occurred on the same sampling date in both pre-treatments temperatures: 5 Jan; the FT4 LT50 was -14.5 °C, while the FT-2 LT50 was -19.0 °C (Table 2.2).

From 13 Dec to 24 Jan ‘Farthing’ FT-2 and FT4 AFB achieved lower LT50s in 2016-2017 than respective ‘Emerald’ buds (Table 2.2). Similar to ‘Emerald’, ‘Farthing’ tissue type data showed AFB to have lower LT50s than EFB. The FT-2 LT50 of EFB was significantly higher than FT-2 AFB for every sampling date by 37-87% (Table 2.2). The LT50 of FT4 EFB was between 10-73% higher compared to FT4 AFB for every sampling date; the difference was significant from 13 Dec to 17 Jan.

Concerning the effect of temperature pre-treatment in ‘Farthing’ EFB, there was no significant difference between the LT50 of FT-2 and FT4 for any collection date during the 2016-2017 winter. The LT50 of FT-2 AFB was lower than FT4 AFB for every collection date ranging from 6-53%; this difference was significant for all dates except 17 Jan and 31 Jan. On 5 Jan, ‘Farthing’ AFB reached the minimum LT50, coinciding with ‘Emerald’ minimum LT50; FT-2 ‘Farthing’ AFB reached -20.2 °C, and FT4 AFB reached -17.0 °C (Table 2.2).

‘Emerald’ AFB on 21 Dec, 12 Jan, and 25 Jan of winter 2015-2016, were more cold tolerant than at 7 Dec, with LT50s 27%, 28%, and 34% lower than 7 Dec. There was a significant increase of 21% in AFB LT50 on 1 Feb, followed by a 16% decrease on 8 Feb. AFB LT50 significantly increased 37% from 08 Feb to 15 Feb (Fig. 2.1A).

During the 2016-2017 winter, the LT50 of FT4 AFB from 29 Nov to 17 Jan were between 36-48% lower than the LT50 of AFB collected on 24 Jan in ‘Emerald’. FT-2 AFB had
significantly higher LT_{50}s on 24 Jan and Jan 31 from hardier mid-winter buds collected on 13 Dec and 5 Jan by 24-40% (Fig. 2.2A).

The LT_{50} of ‘Farthing’ AFB showed a steadier rate of hardening. AFB LT_{50} from 16 Nov to 7 Dec 2015, with no significant difference until 21 Dec where it decreased by 20%. ‘Farthing’ AFB lethal temperature increased on 12 Jan by 15%, which was not significantly different from 21 Dec. ‘Farthing’ AFB LT_{50} continued to significantly decrease from 12 Jan to 25 Jan by 30% in 2016. On 15 Feb, AFB LT_{50} increased 12%; though it was not significantly different from the LT_{50}s from 21 Dec to 8 Feb in 2015-2016 (Fig 2.1B).

‘Farthing’ AFB LT_{50} displayed a parabolic curve in both pre-treatments in winter 2016-2017. In FT4 AFB, 29 Nov, 24 Jan, and 31 Jan had significantly different LT_{50}s than mid-winter buds, ranging from 33% to 67% higher than buds on 5 Jan and 17 Jan in winter 2016-2017. In 2016-2017 FT-2 ‘Farthing’ AFB, only 5 Jan was significantly lower than 29 Nov, 24 Jan, and Jan 31 by 29%, 37%, and 51%, respectively (Fig. 2.2B).

*Bud Water Content.* In 2015-2016 fresh weight, dry weight, and change in bud moisture content of ‘Emerald’ significantly increased on 15 Feb from 8 Feb by 84%, 54% and 9%, respectively (Table 2.3). In ‘Farthing’ fresh and dry weight were not significantly different when comparing 15 Feb data from 12 Jan or 1 Feb (Table 2.3) data during the 2015-2016 winter. Bud moisture content of ‘Farthing’ was not significantly different across dates. The bud moisture content increased on 15 Feb in ‘Emerald’ and coincided with an increase in AFB LT_{50} (Fig. 2.1A & Table 2.3).

In 2016-2017, bud fresh weight in both ‘Emerald’ and ‘Farthing’ increased on 24 Jan from 17 Jan by 75% and 71%, respectively (Table 2.4). Bud fresh weight also increased from 24
Jan to Jan 31 by 46% and 52% in ‘Emerald’ and ‘Farthing’ respectively (Table 2.4); the difference was significant between both dates in ‘Farthing’. The dry weight of ‘Emerald’ and ‘Farthing’ increased on Jan 31 by 53% and 46%, respectively. In ‘Farthing’ there was no significant difference among the bud moisture content between the collection dates; ‘Emerald’ bud moisture content was significantly greater on 24 Jan and Jan 31 from 29 Nov - 5 Jan by 19-27% (Table 2.4).

In 2015-2016, fresh bud length is greatest on 15 Feb (526 CH) in both ‘Emerald’ and ‘Farthing’ increasing by 23% and 22% respectively from 8 Feb; however, it is not significantly different from mid-winter bud length in ‘Farthing’ (Table 2.5). Bud fresh length and dry length measurements increased for both cultivars as they approached budswell (Table 2.5) in 2016, though the difference was only significant in ‘Emerald’, which increased 34% in fresh bud length and 25% in dry bud length (Table 2.5). Increase in bud fresh length was significant in 2017 for ‘Emerald’ and ‘Farthing’ from 17 Jan to 31 Jan by 46% and 34%, respectively (Table 2.6). Dry length was also significantly higher by 26% in ‘Emerald’ and 22% in ‘Farthing’ from 24 Jan to 31 Jan in 2017 (Table 2.6).

**Discussion**

*DTA.* Critical bud freezing temperature has been established via differential thermal analysis (DTA) in a variety of fruit crops, such as peach (Ballard et al., 1999; Pedryc et al., 1999; Quamme, 1974), persimmons (Kang et al., 1998), and grapes (Clark et al., 1996; Ferguson et al., 2011; Gao et al., 2014; Quamme, 1986). DTA measures the exothermic signature wherein latent heat is released as supercooled water freezes within the bud (Burr et al., 2001). The lethal
temperature at which the tissue freezes corresponds to tissue cold damage (Quamme et al., 1982b). The detected DTA exothermic events in SHB produced lethal temperatures significantly higher than FT. These higher temperatures may be the result of bud scale high temperature exotherms or bulk water freezing. Similar findings have been reported by Flinn and Ashworth (1994), in NHB buds cooled at a rate of 2 °C·h⁻¹ (Flinn and Ashworth, 1994a). Further, relationships between exotherms and bud stage or bud moisture content were not found in SHB (data not shown), similar to findings by Warmund in Ribes and Rubus (Warmund et al., 1991; 1992).

*FT.* In the results of our study, AFB had significantly lower LT₅₀s than EFB in both years of the study 2015-2016 to 2016-2017 (Table 2.1 and 2.2). Flinn and Ashworth (1994a) similarly found that attached buds had a lower lethal temperature than excised buds. Our results are further substantiated by DTA results of V. vinifera by Quamme (1986) which found that excised buds had a significantly higher LT₅₀ than attached buds.

In 2016-2017, FT-2 AFB had lower LT₅₀s than either FT-2 or FT4 in both ‘Emerald’ and ‘Farthing’. Further in 2016-2017, FT4 AFB had lower LT₅₀s than either FT-2 or FT4 in both ‘Emerald’ and ‘Farthing’, for the majority of the collection dates. In 2016-2017, there were no significant differences between the pre-treatments within EFB in either cultivar; however, differences were significant within AFB, in which FT-2 had lower LT₅₀s than FT4, for both cultivars (Table 2.2).

In 2015-2016, ‘Emerald’ had lower LT₅₀s than ‘Farthing’ when treated to FT4; however, when treated to FT-2 from 25 Jan to 15 Feb, ‘Farthing’ had lower LT₅₀s (Table 2.1). From 13 Dec to 24 Jan in 2016-2017, ‘Farthing’ AFB had lower LT₅₀s than respective ‘Emerald’ buds in either FT-2 or FT4 (Table 2.2).
The SHB ‘Emerald’ and ‘Farthing’ have approximately 250 and 350 recommended chill hour accumulation, respectively (Retamales and Hancock, 2012), which is much lower in comparison to either previously studied NHB cultivars (800+ recommended chill hours) or SHB ‘Legacy’ (500 - 600 recommended chill hours) (Krewer and Nesmith, 2006; Retamales and Hancock, 2012). In Ehlenfeldt’s (2012) research, ‘Bluecrop’, ‘Little Giant’, and ‘Northsky’ had consistently lower LT\(_{50}\)s than ‘Legacy’ from 17 Oct to 10 Apr in both years of the study. Also reported in Ehlenfeldt’s (2012) study, ‘Legacy’ preceded the NHB cultivars in development and budbreak. Thus, treated to the same conditions, lower-chill cultivars are less hardy and break bud earlier than high-chill cultivars. Similarly, ‘Emerald’, which has a lower chill requirement than ‘Farthing’, preceded ‘Farthing’ in loss of hardiness in 2015-2016 (Fig. 2.1 and Table 2.1); however, no difference was evident in 2016-2017 (Fig 2.2 and Table 2.2).

In addition to chill hours, environmental conditions such as temperature are important in budbreak (Hall and Ludwig, 1961; Spann et al., 2004; Arora et al., 2004). Georgia is a subtropical climate compared to New Jersey, leading to earlier deacclimation and bud break of low-chill cultivars. While known that SHB are less hardy and break bud earlier than NHB (Ehlenfeldt et al., 2012), SHB hardiness has been previously uncharacterized for southern regions. In our study, ‘Emerald’ and ‘Farthing’ broke bud mid-February in 2016 and end of January in 2017. Earlier budbreak in southern regions puts the buds at greater risk for late spring freezes. Knowledge of SHB deacclimation is essential in understanding SHB hardiness and integral for frost management programs.

In a mid-winter study of SHB lethal temperature, Ehlenfeldt (2009) found a broad range of temperatures, from ‘Millennia’, which had a 2-year average of -13.2 °C, to ‘Echota’ and ‘Reveille’, which had 2-year averages temperature of -24.7°C and -25.1 °C, respectively.
‘Echota’ and ‘Millennia’ both contain 25% *V. corymbosum* in their genotype. ‘Millennia’ also contains *V. darrowii*, which could confer traits of chill susceptibility and a respectively higher LT$_{50}$; conversely, ‘Echota’ does not have either *V. darrowii* or *V. angustifolium* (Aiton) in its genotype, and has a lower mid-winter LT$_{50}$ (Ehlenfeldt et al., 2009). In comparison, ‘Emerald’ has greater than 25% *V. darrowii* (Lyrene, 2008b); ‘Farthing’ was bred from ‘Windsor’ and FL96-27, both SHB cultivars (Lyrene, 2008c). In Ehlenfeldt’s (2009) study, ‘Windsor’ reached a mid-winter lethal temperature average of -19.5 °C. In our study ‘Farthing’ reached a similar minimum LT$_{50}$ of -18.6 °C on 8 Feb in 2016, and -20.2 °C on 5 Jan in 2017 (Table 2.1 & 2.2). Ehlenfeldt (2009) measured a specific time point, where our study has a collection of timepoints from which maximum bud cold hardiness can be selected. ‘Misty’, a grandparent of ‘Emerald’, was also included in Ehlenfeldt’s (2009) study. ‘Misty’ had a LT$_{50}$ of -17.2 °C (Ehlenfeldt et al., 2009). In 2016, ‘Emerald’ produced a comparative LT$_{50}$ of -17.0 °C on 25 Jan in 2016 (Table 2.1); in 2017, ‘Emerald’ minimum LT$_{50}$ occurred lower at an earlier date, -19.0 °C on 5 Jan (Table 2.2). Mid-winter LT$_{50}$s in Ehlenfeldt’s study (2012) were taken on 25 Jan and 1 Feb in 2006 and 18 Jan and 24 Jan in 2007. Further differences can be attributed to regional climate, Ehlenfeldt’s study (2009) took place in New Jersey which has a greater chill hour accumulation than Georgia. Our study expands the knowledge of SHB winter hardiness. This study further extrapolates upon acclimation and deacclimation information which can be useful for future studies determining the impact of weather on SHB hardiness.

Cold sensitive genotypes reach their minimum floral bud lethal temperature earlier compared to cold hardy genotypes (Ehlenfeldt et al., 2012; Rowland et al., 2008). Rowland et al. (2008) and Ehlenfeldt et al. (2012) preformed FT using a glycol bath to determine the LT$_{50}$ of different blueberry genotypes through dormancy. A single SHB, ‘Legacy’, was used as a
comparison for multiple NHB and RE cultivars. ‘Legacy’ had higher lethal temperatures than all the NHB included in the study in addition to two of the three RE cultivars. Compared to the data reported in our study on either ‘Emerald’ (minimum LT$_{50S}$ of 17.0 °C and 19.0 °C in 2015-2016 and 2016-2017, respectively) or ‘Farthing’ (minimum LT$_{50S}$ of 18.6 °C and 20.2 °C in 2015-2016 and 2016-2017, respectively) from 2015-2017, ‘Legacy’ had a lower minimum lethal temperature (-24.1 °C). The mid-winter hardiness of ‘Legacy’ quantified by in the study by Rowland et al. (2008) reported a higher temperature than Ehlenfeldt et al. (2012) at -16 °C, higher than either ‘Emerald’ or ‘Farthing’ in our study. Rowland et al. (2008) measured at a single time point, which could have been earlier or later than peak hardiness temperature of the bud.

Subsequent LT$_{50}$ measurements after ‘Legacy’s’ minimum LT$_{50}$ produced consecutively increasing LT$_{50S}$, where NHB ‘Little Giant’ and ‘Northsky’ maintained their minimum LT$_{50}$ for 10 and 18 weeks respectively (Ehlenfeldt et al., 2012). In concert with losing hardiness, ‘Legacy’ broke bud earlier than the NHB cultivars (Ehlenfeldt et al., 2012; Rowland et al., 2008; Rowland et al., 2005). The SHB in our study were low chill cultivars; correspondingly, they had a shorter period of winter hardiness compared to the NHB in both the Rowland et al. (2008) and Ehlenfeldt et al. studies (2012). Similar to results of ‘Legacy’ in Ehlenfeldt et al. (2012), both ‘Emerald’ and ‘Farthing’ reached a point of maximum hardiness and proceeded to lose bud hardiness. This trend is noticeable in 2016-2017 winter (Fig. 2.2A & B); in winter 2015-2016 both cultivars reached a maximum bud hardiness whereupon their LT$_{50}$ began to increase, though subsequent LT$_{50S}$ fluctuated in ‘Emerald’ and were not significantly different in ‘Farthing’ (Fig. 2.1A & B).
In addition to ‘Legacy’ this singular point of maximum bud hardiness followed by loss of hardiness was also seen in ‘Tifblue’, ‘US 1043’, ‘US 1056’, and ‘Bluecrop’. ‘US 1056’ and ‘Bluecrop’ maximum bud hardiness occurred weeks later than ‘Tifblue’ and ‘US1043’ (Ehlenfeldt et al., 2012). ‘Bluecrop’ is a NHB cultivar with 94% V. corymbosum. Interestingly ‘US 1056’ and ‘US 1043’ are both RE blueberries and have the same species germplasm composition, though ‘US 1056’ reaches a lower minimum lethal temperature than ‘US 1043’.

Ehlenfeldt (2012) found that the maximum bud hardiness occurred 20 Dec in the three cold sensitive cultivars, ‘US 1043’, ‘Tifblue’, and ‘Legacy’. In our study ‘Emerald’ and ‘Farthing’ differed reaching a maximum cold hardiness in late Jan to early Feb in 2016, and in 2017 the LT50 coincided on 5 Jan in both cultivars.

As they lost hardiness, ‘Emerald’ and ‘Farthing’ LT50 rose to -10.4 °C (‘Emerald’) and 16.4 °C (‘Farthing’) by 15 Feb in 2016 and was between -11.5 °C and 13.6 °C by 31 Jan in 2017. The LT50 of ‘Legacy’ on 10 Apr was -11.4 °C, comparable to our SHB cultivars (Ehlenfeldt et al., 2012). Ehlenfeldt et al. (2012) ceased collection on 10 Apr; ‘Tifblue’, ‘Legacy’, and ‘US 1043’ were between advanced stages of bud swell and budbreak, comparable bud stages to ‘Farthing’ and ‘Emerald’; ‘Bluecrop’ and ‘US 1056’ reached bud swell, while ‘Little Giant’ and ‘Northsky’ were earlier in development. In contrast of the studied SHB cultivars in our research, ‘Legacy’ had a longer period between reaching the minimum LT50 to budbreak (16 weeks from 20 Dec – 10 Apr) compared to ‘Emerald’ and ‘Farthing’ (1-4 weeks).

**Bud Water Content.** Increase in bud water content is associated with deacclimation of buds and the increase in cold sensitivity. Biermann (1979) established that under field conditions artificially dehydrated attached buds were able to survive lower temperatures than hydrated buds (Biermann et al., 1979). In our study ‘Emerald’ in 2016 and both ‘Emerald’ and ‘Farthing’ in
2017 increased in bud weight, indicating budswell. Bud moisture content significantly increased in ‘Emerald’ from 8 Feb to 15 Feb in 2016 and from 17 Jan to 24 Jan in 2017. Loss of hardiness reported by FT analysis, showing an increase in LT$_{50}$ in buds at budswell compared to mid-winter tight buds. In 2016, ‘Emerald’ AFB were significantly less hardy on 15 Feb from 8 Feb (Fig. 2.1A & B) and on 24 Jan from 17 Jan in 2017 (Fig. 2.2A), corresponding to a significant increase in average bud moisture content in ‘Emerald’ (Table 2.3 and Table 2.4).

**Conclusion**

AFB were hardier than EFB for both cultivars. FT-2 was significantly lower than FT4 in AFB of both cultivars. Exotherms established by the DTA profile were significantly warmer than FT. SHB lethal temperature decreases from late fall, reaches a maximum bud hardiness, and subsequently increases until budbreak. Loss of hardiness coincided with increase in bud moisture content in ‘Emerald’ as well as the increase in bud fresh weight of both cultivars in 2017.
CHAPTER 3

OBSERVATIONS OF BUD DEVELOPMENT AND COLD INJURY IN SOUTHERN HIGHBUSH BLUEBERRY (*Vaccinium corymbosum* L. interspecific hybrid) FLORAL BUDS

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Abstract

A microscopic evaluation was designed to identify dormant floral bud growth and freeze damage in southern highbush blueberries [SHB; *Vaccinium corymbosum* (L.) interspecific hybrids]. The objectives were to identify developmental progression of SHB floral buds through winter dormancy and to observe their response to freezing temperatures by using microscopy techniques of paraffin embedding and confocal microscopy. Shoots with floral buds attached were collected every 3 to 4 weeks (Nov – Feb) from SHB ‘Emerald’ and ‘Farthing’ at a farm in Lakeland, GA. Samples collected were either preserved in fixative, formaldehyde-acetic acid-ethyl alcohol (FAA) and stored at -2.0 °C overnight and freeze tolerance tested (FT; -3.0 to -21.0 °C gradient, removing bud tissue at -3.0 °C increments). Samples treated to freezing temperatures were stored at 4.0 °C for a week and either fixed in FAA, prepared for confocal microscopy, or evaluated for percent damaged floral buds. All fixed buds were dehydrated with ethanol and embedded with paraffin. Paraffin embedded buds were sectioned at 10 μm via rotary microtome. Sections were stained with 0.01% toluidine blue and viewed under a light microscope. Specimens viewed under a confocal microscope were non-fixed samples. Buds were untreated or subjected to FT and stained with Fluorescein diacetate (FDA; green fluorescence, live cells) or propidium iodide (PI; red fluorescence, necrotic cells). Ovule development in untreated samples increased in size through the sample period by 296% and 615% for both ‘Emerald’ and ‘Farthing’, respectively. Untreated styles and anthers elongated throughout the sampling period by 153% and 632% for ‘Emerald’ and ‘Farthing’, respectively. Microspore mother cells were observed in meiosis forming haploid microspores, and by mid-Feb, tapetum cells had formed into complete pollen tetrads. ‘Emerald’ bud development progressed ahead of ‘Farthing’. Mid-winter sampling coincided with formation of pollen tetrads and enlargement of styles, anthers, and ovules.
Browning was observed in paraffin embedded toluidine stained samples, which were similar to damage observed in FT. This suggests that the browning may be associated to necrotic tissue. In toluidine stained samples, browning was observed to increase with decreasing temperatures until -18.0 °C when all reproductive organs displayed browning. Under confocal microscopy, green florescence was observed at -12.0 °C and -15.0 °C in style, anther, and ovule tissue for both cultivars. Ovules had green florescence as low as -18.0 °C; however, by -21.0 °C no floral tissue fluoresced green. Confocal and paraffin embedded samples displayed red fluoresced cells or browning, respectively, in the outer whorls followed by styles, placenta, anthers, and then ovules. In conclusion, microscopic images revealed growth throughout dormancy for both SHB evaluated. Observations of necrotic tissue from FT and confocal microscopy were associated with browning observed in toluidine stained samples that underwent FT.
**Introduction**

Blueberry buds develop in the summer of the preceding year on that season’s woody growth. Within a single floral bud, 5-12 flowers develop (Litwinczuk et al., 2005; Smolarz, 2006). Southern highbush blueberry [SHB; *Vaccinium corymbosum* (L.) interspecific hybrids] floral buds mature throughout the winter, progressing toward anthesis the following spring (Darnell et al., 1991). Northern highbush blueberry [NHB; *V. corymbosum* (L.)] floral buds mature until acclimation whereupon floral organ development enters stasis as the buds harden; bud development resumes at the end of January (Bieniasz, 2012; Gough et al., 1978). Petals and sepals are the first of the floral organs to form, which develop by late autumn (Bieniasz, 2012; Kovaleski et al., 2015). Huang et al. (1997) saw differences in SHB from NHB developmental progression, where anther and ovary development continued throughout winter in SHB ‘Sharpblue’. Progressing towards budbreak, differentiation of pollen tetrads and ovule development take place (Huang et al., 1997). Styles and anthers elongate preceding budbreak (Spiers, 1978).

Budswell, associated with style and anther elongation, occurs in SHB before NHB (Ehlenfeldt et al., 2012). Cold hardiness and bud stage development of NHB ‘Bluecrop’, ‘Little Giant’, and ‘Northsky’, and SHB ‘Legacy’, was observed from New Jersey plantings from acclimation to deacclimation (Ehlenfeldt et al., 2012). In Ehlenfeldt’s (2012) study, ‘Legacy’ lost cold hardiness and reached budswell before weeks NHB cultivars.

Colder temperatures induce freezing point depression within buds (Kishimoto et al., 2014). Freezing of the extracellular solution increases the water potential gradient out of the cell, concentrating the intracellular solutes and depressing the freezing point (Flinn and Ashworth, 1994a; Franks, 1982). Supercooling occurs when the solution decreases below its freezing point.
Intracellular ice nucleation is catalyzed when a critical temperature is reached (Ashworth et al., 1988).

Ice nucleates first in susceptible tissues, propagating to other floral organs with decreasing temperatures (Levitt, 1980; McCully et al., 2004; Warmund et al., 1992). The pattern of ice nucleation, extent of damage due to cold, discoloration, and membrane disruption are hypothesized to be visible through microscopy. The objectives of this study are to delineate blueberry developmental progression, from acclimation to budbreak, in two SHB cultivars, ‘Emerald’ and ‘Farthing’. Further, this study aims to characterize tissue susceptibility and patterns of ice nucleation in freeze treated buds.

**Methods and Materials**

*Plant material.* Shoots with floral buds were collected from ‘Emerald’ and ‘Farthing’ plants at a commercial blueberry farm in Lakeland, GA (Mathis Farms). Material was collected on 2 Nov, 16 Nov, 7 Dec, 21 Dec, 12 Jan, 25 Jan, 1 Feb, 8 Feb, and 15 Feb of 2015-2016 winter. During 2016-2017, buds were collected on 29 Nov and 17 Jan. Buds were collected in field and placed in vials containing formaldehyde-acetic acid-ethyl alcohol (FAA), a mixture of 50% ethanol, 5% glacial acetic acid, 10% formaldehyde and 35% water.

*FT.* Separate samples were stored in moist paper towels within a plastic bag and sent to the University of Griffin Campus, Griffin, GA, where they were held at 4 °C for up to 2 hours until leaf tissue removal from shoots. Shoots were trimmed to 5 cm. Replicates of 6 stem-attached floral buds (AFB) per sample set were grouped and placed in moistened tissue in freezer bags and sealed.
In both 2015-2016 and 2016-2017 winter, eight sample sets with six replicates were used. Sample sets were placed in temperature-designated bags of 4 °C, -3 °C, -6 °C, -9 °C, -12 °C, -15 °C, -18 °C, and -21 °C. The freezer bag containing the control sample subset was stored at 4 °C for the duration of the experiment. The remaining seven sample sets were organized and stored in an environmental control chamber overnight at -2 °C. Following, the environmental control chamber, ESPEC EY-101 (Tabai Espec Corp., Osaka, Japan), containing the samples applied a temperature program which decreased 4°C·h⁻¹ at 0% relative humidity. Chamber temperature was constantly decreasing at 4 °C·h⁻¹, samples were removed once the target temperature was reached (approximately every 45 min). Removal occurred every -3 °C, from -3 °C until -21 °C. Three temperature probes were placed in different freezer bags, simulating the tissue temperature inside the sample bags. These probes were interspersed between the sampling bags in the chamber. The temperatures recorded from these three sensors were averaged to estimate the sample temperature and removal time. Removal was performed quickly to minimize the influence of outside temperature on temperature fluctuation of the freeze chamber.

Following cold temperature treatment, samples were stored at 4 °C for a week. Buds were either excised and placed in FAA or dissected for bud mortality evaluation.

**Paraffin Embedding.** Developmental progression of floral organs in ‘Emerald’ and ‘Farthing’ was observed through paraffin embedding throughout the winter of 2015-2016.

The method for paraffin embedding tissues was modified for longer dehydration and filtration times to account for the woody tissue of blueberry buds (Ruzin, 1999). Samples were removed from FAA and dehydrated. Buds were placed in 10 mL of an initial concentration of 50% ethanol, 25% tert-butyl alcohol (TBA), and 25% water. Vials were placed on a Lab-Line® Orbit Environ-Shaker (Lab-line Instruments, Inc., Melrose Park, IL) for 2 h at 30 °C. In the
second step of the cycle, the solution was poured off and replaced by a solution of 25% ethanol, 50% TBA, and 25% water. Samples were placed on a shaker for 2 h and kept at 30 °C. In the third step of the cycle, the solution was poured off and replaced by a solution of 10% ethanol, 75% TBA, and 15% water. Vials were placed on a shaker for 2 h and kept at 30 °C. In the final two steps (steps 4 and 5) of the cycle, the solution was poured off and replaced by a 100% solution of TBA for 2 h each. Vials were placed in an oven (Thermo Electron Corporation, Beverly, MA) at 28 °C.

Following dehydration, the buds were infiltrated with Paraplast® (McCormick Scientific LLC, St. Louis, MO) embedding media. The final solution of TBA was poured off, and replaced with 75% TBA and 25% melted Paraplast®. The solution was kept at 60 °C for 12 h. Vial caps were loosened to promote alcohol evaporation. In the following step, the solution was poured off and replaced with 50% TBA and 50% paraffin, which infiltrated at 60 °C for 12 h. In the third step, the solution was poured and replaced with a new solution of 25% TBA and 75% paraffin, which infiltrated at 60 °C for 12 h. The fourth and fifth steps consisted of 100% paraffin concentrations, infiltrating at 60 °C for 12 h. The last step involved discarding the paraffin and infiltrating another 100% concentration of paraffin for 2 h at 60 °C. Buds and paraffin were then poured into cubical plastic wells and filled with paraffin.

The paraffin embedded tissue was sliced at 10 µm on a Reichert Histostat rotary microtome (Reichert Technologies, Depew, NY). Sections were floated in a water bath at 45 °C. Sections were placed on slides on a hot plate at 55 °C for 2 h, adhering paraffin sections to slides.

Next, slides were placed in a staining cuvette containing 100% xylene for 3 minutes, dissolving the paraffin. This was followed by placement in cuvettes of xylene/ethanol (50-50
solution), ethanol 100%, ethanol/water (50-50), 0.01% toluidine blue and boric acid solution, and deionized water for 3 minutes each. Permount mounting medium (Fisher Scientific, Hampton, NH) was added to dried slides and secured with a cover slip.

Developmental progression of embedded buds was evaluated in four floral buds which had all floral organs visible. Floral buds were selected from three excised buds at 1 month intervals. Floral organ growth was evaluated from field fixed samples. Tissue-specific cold damage of buds subjected to freezing temperatures were imaged from 1 Feb collected samples in 2016. In winter 2016-2017, freeze treated samples were evaluated on 13 Dec and 17 Jan. An Olympus BX51 (Olympus, Tokyo, Japan) epi-fluorescence microscope was used to view the tissue with an Olympus DP70 (Olympus, Tokyo, Japan) camera.

Confocal microscopy. Confocal microscopy was used to determine live and dead cells within freeze treated floral buds in winter on 17 Jan 2017. Specimens viewed under a confocal microscope were fresh, non-fixed samples. Fluorescein diacetate (FDA) is a fluorescent green stain that stains living cells, necessitating fresh samples. Samples were treated to FT. After incubation at 4 °C for a week, six buds were visually evaluated for browning and floral bud death to determine the lethal temperature at which 50% floral bud cold injury occurred (LT_{50}) (Skinner and Garland-Campbell, 2008). LT_{50} was determined by floral bud necrosis due to cold injury in freeze treated samples of both cultivars. Bud cold hardiness was determined by the inflection temperature at 50% bud necrosis on a logistic scale (Proebsting, 1961). Remaining fresh samples around the LT_{50}, as well as a control and negative control, were cut using Personna® double edged razor blades (Personna, Verona, VA) and treated to FDA and propidium iodide (PI) stain (Jones et al., 2016; Krasnow et al., 2008; Truernit and Haseloff, 2008).
Fluorescein diacetate (FDA) working concentration is typically 0.1 µg/mL – 10 µg/mL (Krasnow et al., 2008). Blueberry buds consist of woody tissue; thus, a higher concentration of 10 µg/mL was used to increase permeation within cell layers. PI was prepared at 50 µg/mL. Multiple sections per floral bud were hand cut with a Personna® double edged razor blades (Personna, Verona, VA). Cuttings were immersed in FDA for 25 minutes, allowing the tissue to take up the stain, before moved to the PI solution for another 25 minutes. Cuttings were placed upon a slide with deionized water and a cover glass slip.

A z-stack of the bud was created with a Zeiss LSM 510 (Zeiss, Oberkochen, Germany). Images were max projected via Zen 2.1 (black) (Zeiss, Oberkochen, Germany).

The LT$_{50}$ of confocal microscopy buds was determined by the averaged inflection point of a logistic curve of three floral buds (Proebsting, 1961). Floral buds with any red fluorescing organs were determined dead.

Statistical Analysis. FT temperature inflection points were determined using a logistic curve in JMP Pro version 13 (SAS, Cary, NC). ImageJ (NIH, Bethesda, MD) was used to determine length and area via pixel to µm ratio conversion in microscopy images of ovaries, ovules, style, and placenta of four distinct floral buds per cultivar and collection date. One-way ANOVA, and Tukey HSD ($\alpha = 0.05$) significance test were performed in JMP Pro version 13 (SAS, Cary, NC).

Results

Paraffin embedding. Winter developmental progression in both ‘Emerald’ (Fig 3.1) and ‘Farthing’ (Fig 3.2) were observed in 2015-2016, visible changes are evident from 2 Nov to 8
Feb. Developmental changes are further quantified by floral organ growth seen in Table 3.1 and 3.2.

With 5 accumulated chill hours on 2 Nov, ‘Emerald’ bud morphology displayed both male and female reproductive organs, including anthers, filaments, style, ovaries, and undefined ovules (Fig. 3.1A). The stylar canal has clear definition in the floral bud. Vascular tissue became more defined on subsequent collection dates where extension into the style and filament is evident (Fig. 3.1B-D). On 7 Dec, anther filaments appeared larger. From 2 Nov to 7 Dec, the ovary, ovule, and placental area all increased by 42% to 45%; the style length increased 15%. From 7 Dec to 11 Jan, the ovule increased size by 43%, and the style and anthers elongated by 42% (Fig. 3.1C). Pollen grain differentiation is visible on buds with greater than 100% stylar elongation from Dec to Jan. The increase in ovule size corresponded to greater ovule definition and decreased placental size from Dec to Jan (Fig 3.1C). Ovaries lost uniform rounded appearance, ovarian cavity depressions were noticed in Jan and Feb. At bud swell on 8 Feb, ‘Emerald’ buds had further stylar elongation increasing by 19% (Fig. 3.1D). Again, anthers increased proportional to the style (Fig. 3.1D). Ovules increased in size by 23% from Jan to Feb. Ovary, ovule, and placenta significantly increased from Nov to Feb in ‘Emerald’, increasing by 163%, 296% and 212%, respectively (Table 3.1). The style increased between each date, nearly tripling in length from Nov to Feb; however, the difference was not significant. Pollen tetrad formation was complete in the majority of floral buds by 8 Feb (Fig. 3.1D).

Morphological development from acclimation to budbreak of ‘Farthing’ progressed similar to ‘Emerald’. Between 2 Nov and 7 Dec, ‘Farthing’ floral buds displayed ovule differentiation and growth, increasing 49% (Fig. 3.2A & B). Further, anthers and style elongated from 2 Nov to 7 Dec, the style increasing 50% (Table 3.2). The ovaries and placenta also
increased in size. Between Dec to Jan, ‘Farthing’ buds displayed significant increases in style length, ovules, and ovaries, all increasing greater than 50% (Table 3.2). Continued style and anther elongation is observed on 8 Feb. Pollen tetrads are visible in the anthers. Ovarian depressions observed in ‘Emerald’ in Jan and Feb, are visible in 8 Feb ‘Farthing’ sections (Fig. 3.2D). The ovary to placenta mass ratio increases from Nov to Feb in ‘Farthing’, indicative of the diminishing placenta.

‘Emerald’ anthers from 7 Dec and ‘Farthing’ on 21 Dec were not mature and did not have pollen formation (Fig. 3.3A and 3.4A). Tapetum cells surrounding the pollen sac and microspore mother cells are visible in anther sections on 21 Dec in ‘Emerald’ and 11 Jan in ‘Farthing’ (Fig. 3.3B and 3.4B). As of 1 Feb, microspore mother cells are dispersed in elongated anthers in both cultivars (Fig. 3.3C and 3.4C), ‘Emerald’ has some tetrad formation (data not shown). Mature pollen tetrads formation is visible in ‘Emerald’ and ‘Farthing’ on 8 Feb (Fig. 3.3D and 3.4D).

**Confocal microscopy.** Fluorescein diacetate (FDA) displays living cells with a green fluorescence, stimulated by enzymatic activity. Propidium Iodide (PI) highlights dead cells in red fluorescence.

‘Emerald’ samples taken on 17 Jan, 2017 and treated to FT showed live anthers, stylar canals, ovules, and vascular tissue at -12 °C (Fig. 3.5B & G). There was no difference in organ fluorescence between control buds at 4 °C (Fig. 3.5A & F) and -12 °C freeze treated buds (Fig. 3.5B & G). Buds treated to -15 °C did not have differences in organ fluorescence from -12 °C (Fig. 3.5C & H). At -18 °C ovules and vascular tissue fluoresced green (Fig. 3.5D & I). Less vascular fluorescence is observed at -18 °C in comparison to -15 °C and -12 °C treated buds. At -21 °C, no floral bud organs fluoresced green in ‘Emerald’ on 17 Jan (Fig. 3.5E & J).
On 17 Jan 2017, ‘Farthing’ buds had much lower lethal temperatures (Table 3.3). Buds had green fluorescing anthers, style, ovules, and vascular tissue from 4 °C to -18 °C (Fig. 3.7A-D & E-I). Green fluorescence displayed at -18 °C (Fig. 3.7B & G) was not visually different from control buds at 4 °C (Fig. 3.7A & F). Similar to ‘Emerald’ and FT LT50 (Table 3.3), at -21 °C no green fluorescence was visible within ‘Farthing’ floral buds on 17 Jan (Fig. 3.7E & J).

Bud mortality in ‘Emerald’ was above 50% in FT from 4 °C to -12 °C until -15 °C, whereat bud mortality was 60%. Bud mortality was 44% and 100% at -18 °C and -21 °C, respectively in ‘Emerald’. ‘Farthing’ bud mortality was below 50% until -21 °C, whereat it was 89% (Table 3.3).

Discussion

Paraffin embedding. In this study we observed blueberry bud differentiation throughout the winter in SHB, which contrasts previous findings in NHB (Bieniasz, 2012). Our observations were similar to work on SHB, Sharpblue, floral buds (Huang et al., 1997). All flower reproductive organs continued to increase in definition and size from acclimation in Nov until budswell in Feb. Huang et al. (1997) observed tapetal cell, sporogenous cell, placenta, and ovule development in Dec. Tapetal development and sporogenous cells are seen from 21 Dec in ‘Emerald’ (Fig. 3.3B) and from 11 Jan in ‘Farthing’ (Fig. 3.4B). Whereas placental and ovule development are noticed earlier in ‘Emerald’ (Fig. 3.1) and ‘Farthing’ on 2 Nov (Fig. 3.2), increasing in size on 7 Dec (Table 3.1 & 3.2). In ‘Sharpblue’, as well as ‘Emerald’ and ‘Farthing’, floral buds increased in size and anthers reached earlier meiotic stages in Jan. Pollen
tetrads were visible in ‘Sharpblue’ in Jan, whereas the majority of ‘Emerald’ and ‘Farthing’
floral buds did not have mature pollen tetrads until 8 Feb.

Toluidine blue stains nucleic acids blue (Obrien et al., 1964). Toluidine blue is also a
differential stain which stains lignin in cell walls purple (Sakai, 1973). Killing freezes damage
cells, decreasing nucleic acid concentrations (Gusta and Weiser, 1972). Floral bud tissue
browning due to cold injury parallels Daniell et al. (1969) who similarly observed stress related
brown tissue staining with toluidine blue. Elodea (Elodea canadensis Michx) leaf cells treated to
52 °C and stained with toluidine blue showed brownish blue stained chloroplasts in the
cytoplasm, associated with necrosis (Daniell et al., 1969). Chloroplasts contain nucleic acids;
when damaged, the absence of blue staining is evident. Tissue browning was also observed
surrounding damaged lenticels in mango fruit (Nguyen, 2015). Nguyen (2015) observed lenticel
tissue browning in both unstained and toluidine blue stained images. Nguyen (2015) observed
healthy cells walls stained purple with toluidine blue. Lenticel damage observed by Nguyen
(2015) was caused by water turgidity strain in the cell walls, this stress displayed enzymatic
browning evident on the fruit exterior. In comparison, damaged lenticel toluidine blue staining
showed discoloration surrounding the brown lenticel, indicating phenolic accumulation (Nguyen,
2015; Vermerris and Nicholson, 2006). Phenolic compounds are released from damaged
vacuoles and are oxidized by the enzyme, polyphenol oxidase, causing tissue browning
(Holderbaum et al., 2010; Vaughn and Duke, 1984). Another study which explores toluidine blue
staining and phenolic compounds in damaged tissue looked at the zebra chip disease in potato
tubers through autofluorscence and toluidine blue staining (Miles et al., 2010). Plants affected by
the zebra chip disease display symptoms of vascular browning (Miles et al., 2010). Toluidine
blue stained tubers displayed healthy blue colored vascular tissue, contrasted with diseased
vascular tissue appearing as red-brown (Miles et al., 2010). Autofluorogenic phenolic compounds, which are associated with the hypersensitive response, are visible at infected areas due to rapid cell death (Miles et al., 2010). Miles et al. (2010) found no fluorogenic compounds in the healthy vascular tissue, while the diseased tissue which exhibited red-brown toluidine blue stain, fluoresced.

Confocal microscopy. On 17 Jan at 4 °C to -12 °C, both confocal microscopy and FT of ‘Emerald’ indicated survivability of floral buds with LT50 of less than 10% and green fluorescence. Confocal microscopy of ‘Emerald’ FT buds with a removal temperature of -15 °C displayed red fluorescing style and anthers, preformed similarly to FT of AFB indicating 60% bud necrosis (Table 3.3 and Fig. 3.5). At -18 °C, confocal microscopy had green fluorescing ovules while the anthers, placenta, ovary, and style fluoresced red; FT had slightly lower mortality rate of 44%. At -21 °C, FT and confocal microscopy indicated 100% necrosis. The LT50 of ‘Emerald’ confocal buds was -17.8 °C (data not shown), lower than the FT LT50 at -15.5 °C on 17 Jan.

Confocal microscopy of ‘Farthing’ had similar floral bud necrosis as seen in FT (Table 3.3 and Fig. 3.7). Confocal microscopy displayed green fluorescent floral organs at -18 °C, which fluoresced red at -21 °C (Fig. 3.7). ‘Farthing’ AFB FT buds had a 37% mortality rate for buds treated to -18 °C, which increased to 89% for buds treated to -21 °C (Table 3.3). The LT50 of ‘Farthing’ confocal buds was -18.7 °C (data not shown), which paralleled the FT LT50 at -18.8 °C on 17 Jan.

Paraffin embedded ‘Emerald’ buds showed a slow gradation of damage from -15 °C (Fig. 3.6), where the acropetal region of the style and ovaries were affected by light browning (Fig. 3.6D). Similarly, confocal microscopy showed little cold injury at -15 °C (Fig. 3.5C & H). FT
‘Emerald’ bud browning increased in treatment temperatures of -18 °C and -21 °C (Fig. 3.6D & E), corresponding to dead tissues in confocal microscopy at the same temperatures (Fig. 3.5D, E, I, & J). ‘Farthing’ toluidine blue stained sections did not have significant browning until -21 °C (Fig. 3.8). Similarly, in confocal microscopy images of FT ‘Farthing’ floral buds, green fluorescence was observed in anthers, ovules, and style until -21 °C (Fig. 3.7). Both toluidine blue staining and confocal imaging corresponded to AFB FT LT<sub>50</sub> of ‘Farthing’ on 17 Jan, which had 63% bud survival at -18 °C, and 11% bud survival at -21 °C (Table 3.3).

**Conclusion**

SHB developed throughout winter; continual development in style anther, ovaries, and ovules of both cultivars was observed. Significant differences in ovary and ovule size are seen from November to February in both ‘Emerald’ and ‘Farthing’. Anther development began with tapetum and microspore mother cell formation in mid-December in ‘Emerald’, pollen tetrad formation was complete by early to late February in both cultivars.

Through confocal microscopy visualization of tissue specific damage at freezing temperatures could be observed; toluidine blue browning was observed at similar FT temperature treatments. Bud necrosis evaluated by LT<sub>50</sub> approximated discoloration associated with damage visualized in both methods of tissue staining. Floral organs appear to be affected by cold damage first in their outer whorls, and are followed by style, ovaries, anthers, and ovules.
CHAPTER 4

COLD INJURY IN RELATION TO SEASON AFTER LATE NITROGEN FERTILIZER APPLICATIONS SOUTHERN HIGHBUSH BLUEBERRY (Vaccinium corymbosum L. interspecific hybrid) FLORAL BUDS¹

¹Redpath, L.E., Smith, E., Malladi, A., and Chavez, D. To be submitted to American Pomological Society.
Abstract

Late season applications of nitrogen fertilizer can affect plant development and cold hardiness. Blueberries (*Vaccinium* spp.) grown in the southeastern U.S. are susceptible to spring freezes. Southern highbush blueberries (SHB; *V. corymbosum* interspecific hybrids) are early flowering and are economically the most important blueberry to southeastern U.S. growers. The objectives were to identify the effects of late fertilizer applications on SHB floral bud cold hardiness, nutrient uptake, and flower timing. To determine the timing effect of late season fertilizer applications on cold hardiness, nutrient composition, and spring growth, a completely randomized study using two fertilizers, NPK 10-10-10 (Super Rainbow) and NPK 46-0-0 (urea) was evaluated. There were 4 treatments with protocols 1) Aug; final fertilizer applied on Aug 23 with 10-10-10 at 28g per pot. 2) Sept; Final fertilizer applied on Sept 15 with 10-10-10 applied on Aug 23 at 20 g per pot and Sept 15 at 10 g per pot. 3) Oct; Final fertilizer applied on Oct 15 with 10-10-10 applied on Aug 23 at 10 g per pot, Sept 15 at 10 g per pot and on Oct 15 at 10g per pot. 4) urea; Final fertilizer applied on Sept 15 with urea applied on Aug 23 at 3 g per pot and Sept 15 at 3 g per pot. The treatments were applied to SHB ‘Emerald’ and ‘Star’ grown in pots with pine bark media. To assess the effect of fertilization and timing, dormant plant tissue was divided into root and shoots and analyzed for macro and micro nutrients. Floral bud tissue was collected, prepared as floral buds attached to stem segments (5 cm), stored at -2.0 °C overnight and freeze tolerance tested (FT; -3.0 to -21.0 °C gradient, removing bud tissue at -3.0 °C increments). Floral bud growth stages were evaluated on 16 shoots per cultivar and treatment from Jan 24 to Mar 14 at 3-4 day intervals. FT was not significantly different for ‘Star’ throughout treatments or sample dates. ‘Emerald’ on Jan 17 was hardiest with protocol 3 at -12.0 °C and was 44% harder than protocol 1. On Feb 8, the hardiest floral buds of ‘Emerald’ were
protocol 2 at -13.5 °C and were 29% hardier than protocol 1. Levels of N between roots and shoots was similar for both cultivars except for ‘Emerald’, protocol 1 had 42% more N in the shoots. For 10-10-10, N accumulation in roots and shoots increased with later fertilization, except ‘Emerald’ where the shoots were 1.5% N with protocol 1, which was 25% and 11% greater than protocols 2 and 3 in the shoots, respectively. All fertilizer treatments in ‘Emerald’ lost hardiness and broke bud earlier than ‘Star’. However, no significant difference was seen between treatments of bloom progression for ‘Emerald’. Protocol 3 advanced bloom progression in ‘Star’; however, none of the treatments increased the sensitivity of floral buds to cold. In conclusion, neither protocols 3 or 4 negatively affected ‘Star’s cold tolerance. ‘Emerald’ appeared to be more sensitive to cold with protocol 1. Urea did not negatively affect nutrient levels in shoot or roots. This work demonstrates that urea and mid-October fertilization did not decrease floral bud cold hardiness. Further, the effect on bloom timing was cultivar dependent.
Introduction

Blueberries (*Vaccinium* spp.) are grown in a wide range of climatic regions. Lowbush blueberries [LB; *V. angustifolium* (Aiton)] are grown commercially in plant hardiness zones 4b (-31.7 to -28.9 °C) to 6a (-23.3 to 20.6 °C) in Maine. Northern highbush blueberries (NHB; *V. corymbosum*) grown commercially in Michigan are in plant hardiness zones 6a (-23.3 to -20.6 °C) to 6b (-20.6 to -17.8 °C), these zones are buffered climatically by Lake Michigan creating a lake-effect snow buffer. Southern highbush [SHB; *V. corymbosum* (L.) interspecific hybrids] are commercially grown in plant hardiness zones 8b (-9.4 to -6.7 °C) to 9a (-6.7 to -3.9 °C) in Georgia. Each species and cultivar of blueberry has chill requirements that are met within each plant hardiness zone; however, each blueberry can be sensitive to cold damage if temperatures drop rapidly while still actively growing. Because of plant sensitivity to cold, recommendations for termination for fertilization, especially nitrogen, are suggested to be four to six weeks before the first frost (Krewer and Nesmith, 2006). This will allow the plants to harden-off to minimize freeze injury.

Blueberry production in Georgia is in a sub-tropical climate, where blueberries are actively growing into late fall. Fertilization timing has been suggested to terminate from late August to early October to accommodate a 4 to 6 week interval before the first freezing event (Krewer and Nesmith, 2006). However, some growers have observed mid-winter necrotic floral bud tissue in SHB ‘Star’ (E. Smith personal communication) and fertilization timing was suggested as the cause, though insufficient research is available.

Orchardists recognize that fertilization is necessary to maintain plant health and increase yield, especially nitrogen. In Washington State, ‘d’Anjou’ pear [*Pyrus communis* (L.)] trees were fertilized with ammonium-nitrate (NH$_4$NO$_3$) at two rates (0.15 kg N tree$^{-1}$ or 0.45 kg n tree$^{-1}$)
with two timings (late summer or late winter) over multiple years (Raese, 1997). The lower rate of N had lower vigor and growth than the late winter high rate application; however, yield was only affected by late winter low N rate application. Cold tolerance testing was conducted using electrolyte leakage of 1 to 2 year old shoots, which demonstrated that low rates of N were significantly hardier to early fall freezes; however, by late fall and into winter the cold tolerance was similar between the treatments (Raese, 1997).

In a fertilization rate trial on ‘Golden Delicious’ apple (*Malus x domestica* Borkh.), trees were hardier to cold temperatures at rates below 151 g N tree\(^{-1}\) during fall and winter (Raese et al., 2007). Trees that received greater amounts of nitrogen showed higher rates of respiration (CO\(_2\) concentration) than the rates below 151 g N tree\(^{-1}\) during late fall and winter, which indicates that carbohydrate reserves were being depleted (Raese et al., 2007). Sucrose and sorbitol are important reserves in dormant apple and freeze tolerant electrolyte leakage tests show cold hardiness is related to high levels of sugars (Raese et al., 2007).

Cold tolerance testing on greenhouse grown bilberries [*V. myrtillus* (L.)] with nitrogen applications of 6.5 mmol m\(^{-2}\) ammonium nitrate with calcium hydroxide (NH\(_4\)NO\(_3\) + Ca(OH)\(_2\)) and trace gas fumigation (CO\(_2\) and O\(_3\)) showed N applications had increased cold hardiness, whereas the fumigation did not affect cold hardiness (Taulavuori et al., 1997). In a similar study, N was applied at 200 kg N ha\(^{-1}\)yr\(^{-1}\) to lingonberry [*V. vitis-idaea* (L.)] resulting in accelerated frost hardening (Taulavuori et al., 2001). These works suggest that acceptance of the hypothesis that N fertilization decreases plant cold hardiness may be misplaced.

Peach (*Prunus persica*), western red cedar (*Thuja plicata*), Douglas-fir (*Pseudotsuga menziesii*), Aleppo pine (*Pinus halepensis*), and Norway spruce (*Picea abies*) did not show sensitivity to cold from late season applications of N (Edgerton and Harris, 1950; Hawkins et al.,
1995; Hellergren, 1981; Puertolas et al., 2005; Wiemken et al., 1996). However, newly rooted ‘Bloodgood’ Japanese maple (*Acer palmatum*) were sensitive to freezing injury when N was applied and the cuttings had not produced shoot growth when compared to N applied plants with shoot growth (Stimart et al., 1985). The mechanism of cold tolerance is complex, where in some species sensitivity is enhanced by applications of N in the late fall. However, as the winter progresses sensitivity is similar regardless of N application rate or timing. Generalizing that late season applications of N tend to make a plant sensitive to frost or cold appears misplaced and cold hardiness in relation to N applications tend to be species, cultivar, and physiological state dependent.

Blueberries thrive in acidic soils (pH 4.0-5.5) and require ammoniacal sources of nitrogen (Hanson, 2006). Plant growth and yield increases with increasing nitrogen in blueberry plants (Bryla and Machado, 2011; Finn and Warmund, 1997; Hanson and Retamales, 1992). Nitrogen fertilizer can be applied granularly or in a liquid solution with no significant impact to production (Williamson and Miller, 2009) and increasing the rate of nitrogen fertilizer in SHB ‘Misty’ and ‘Star’ improved production (Williamson and Miller, 2009). In South Georgia, ‘Star’ floral buds have been observed to have necrotic reproductive tissue in the spring, which may have been impacted by fertilization into mid-October. This suggests frost damage to tissue that has not hardened or acclimated. The objectives of this study were to determine if N fertilization timing effects on floral bud tissue sensitivity to freezing, nutrient uptake, and bloom progression in the spring.
Methods and Materials

Plant material and Fertilization. One gallon potted ‘Star’ and ‘Emerald’ plants were donated by Cornelius Farms, Manor, GA in April 2016. The plants had been fertilized via overhead fertigation at a rate of 50 ppm N in the irrigation water, N was applied each irrigation. The plants were transported from Manor, GA to the University of Georgia’s Alapaha Blueberry Research Station near Alapaha, GA (31°20’43.9”N, 83°14’27.1”W). The pots were placed on a gravel pad and all floral buds, flowers, and fruit were removed. Irrigation was automated and set for 30 min in the morning (0500) every other day with adjustments relevant to rain. By July 2016, many of the pots were root bound and all plants were transplanted into 3 gallon pots. During transplanting, the roots were loosened before being placed into the 3 gal pots and were planted no more than 2.5 cm below the original soil line in the 1 gal pots. The media used from Cornelius Farms was milled pine bark (0.6 to 9.5 mm particle size) and the transplant pine bark were chips at 1 to 3 cm in size.

A completely randomized study using two fertilizers, 10-10-10 (Super Rainbow, Agrium, Denver, CO) and 46-0-0 (Urea, Brownlee Farm Center, Tifton, GA), were applied to SHB ‘Emerald’ and ‘Star’. There were 4 treatments with protocols 1) final fertilizer applied on Aug 23 with 10-10-10 at 28g per pot. 2) Final fertilizer applied on Sept 15 with 10-10-10 applied on Aug 23 at 20 g per pot and Sept 15 at 10 g per pot. 3) Final fertilizer applied on Oct 15 with 10-10-10 applied on Aug 23 at 10 g per pot, Sept 15 at 10 g per pot and on Oct 15 at 10g per pot. 4) Final fertilizer applied on Sept 15 with urea applied on Aug 23 at 3 g per pot and Sept 15 at 3 g per pot. The urea treatment was included to determine the effect of fertilizing without the other micro and macro nutrients on the cold hardiness of floral buds. A rate of 2.8 g N was applied to the respective protocol on May 5, Jun 6, July 5, and after transplant on July 22. Fertilizer
A recommendation of 28 g 10-10-10 every 4-6 weeks is standard commercial practice for first year blueberry in southeastern U.S. (Krewer and Nesmith, 2006). In November, the plants were transferred to the University of Georgia’s Griffin Campus for further analyses.

**Nutrient Analysis.** Four plants per cultivar and treatment were removed from pots and cleaned of soil media before separating roots from shoot material. Root material was considered soil line and below. Roots and shoots were separated and placed in their respective labeled paper bag and set in a Grieve 13-261-27A forced air drying oven (The Grieve Corporation, Round Lake, IL) at 60 °C for 72 h or consistent weight. The samples were analyzed for N, P, K, Mg, Ca, S, B, Zn, Mn, Fe, and Cu (Waters Agricultural Laboratories, Inc., Camilla, GA), where the dried tissue was ground to pass a 20-mesh screen, the samples were reduced to ash in a muffle furnace, acid digested, and measured by inductive coupled plasma spectrophotometer (ICP) coupled to a Digiblock 3000 (SCP Science, Baie D’Urfé, Quebec, Canada). Nitrogen was determined through combustion of plant tissue using a LECO FP-428 N analyzer (LECO, ST. Joseph, MI, U.S.).

**FT.** Fresh samples were collected from random plants within each treatment on three dates during the winter of 2016-2017: 7 Dec (143 accumulated chill units, CH), 17 Jan (341 CH), 8 Feb (395 CH). Leaves were removed from all collected shoots. Shoots were trimmed to 5 cm. Sample sets of 16 buds per treatment and cultivar were placed into sealable freezer bags, wrapped in moistened paper towel, and stored at -2 °C overnight. The next day all prepared sample sets were analyzed for FT with a temperature program that decreased at a rate of 4°C·h⁻¹ in an ESPEC EY-101 (Tabai Espec Corp., Osaka, Japan) chamber. Removal rates occurred at -3.0 °C, -6.0 °C, -9.0 °C, -12.0 °C, -15.0 °C, -18.0 °C, and -21.0 °C. A temperature probe was placed in the same type of sample bag at three positions within the chamber next to the samples.
When the average temperature was met at each set point the corresponding samples were removed and stored at 4.0 °C for a week. After the storage period, the floral buds were bisected and viewed under a dissecting scope for percentage bud necrosis. The lethal temperature at 50% (LT$_{50}$) floral bud necrosis was determined on a logistic scale (Proebsting, 1961).

*Bloom observation.* From the remaining plants, sixteen randomly selected branches from each treatment (four branches per plant, four plants per treatment) were tagged and labeled for bloom observation. Buds were counted and individually checked twice weekly for bloom progression.

Bloom progression was recorded according to the MSU blueberry growth stages table: stage 1 = tight/dormant bud; stage 2 = bud swell with visible green tip; stage 3 = budbreak signified by separation of bud scales; stage 4 = tight cluster, individual flower apices are visible; stage 5 = early pink bud, individual floral apices appear pink and separated; stage 6 = late pink bud, flowers have elongated but corollas are still closed; stage 7 = early bloom, less than 50% of the corollas have opened; stage 8 = full bloom, greater than 50% of the corollas have opened; stage 9 = petal fall, greater than 50% of the corolla tubes have fallen off the flower; stage 10 = early green fruit, small pea size green fruit have expanded; stage 11 = late green fruit, expansion slows and fruit appear light green; stage 12 = fruit coloring, oldest fruit in the cluster change color to blue/pink (Michigan State University, 2016).

At the end of 7 weeks, the weighted average bloom stage per shoot was determined by calculating the summation of all buds at a designated bud stage and dividing by the total number of buds. Weighted averages of shoots were compared between plants.
Statistical Analysis. TA temperature inflection points were determined using a logistic curve in JMP Pro version 13 (SAS, Cary, NC). PROC GLM, SAS 9.4 (SAS, Cary, NC), determined mean nutrient content, LT_{50}, root and shoot weight, and bloom progression. Tukey HSD (α = 0.05) was determined by SAS 9.4 (SAS, Cary, NC).

Results

Nutrient analysis. Micro and Macro nutrients Mg, Ca and S of the roots and shoots for both ‘Star’ and ‘Emerald’ were similar when comparing treatments within roots or shoots. Some variability was noted in N, P, and K in the roots and shoots of both cultivars. ‘Emerald’ shoots had significantly more N in treatment 1 (Aug) than treatment 2 (urea) by 33.2%. ‘Emerald’ root treatment 3 (Oct) had significantly more N than 1 (Aug), 2 (Sept), and 4 (urea) by 43.4%, 29.4%, and 28.3%, respectively (Table 4.1). ‘Emerald’ root treatment 3 (Oct) had significantly more P than 1 (Aug) and 2 (Sept) by 60.0% and 43.3%, respectively. ‘Emerald’ shoot treatment 1 (Aug) had significantly more K than 2 (Sept) by 71.4%. ‘Emerald’ root P and shoot K were not significantly different (Table 4.1).

‘Star’ shoots had significantly more N in treatment 4 (urea) than treatment 1 (Aug) by 16.9%. ‘Star’ root treatment 4 (urea) had significantly more N than 1 (Aug) and 2 (Sept) by 20.0% and 17.0%, respectively (Table 4.1). ‘Star’ root treatment 3 (Oct) had significantly more P than 1 (Aug) by 26.7%. ‘Star’ shoot treatment 4 (urea) had significantly more P than 1 (Aug) by 20.0%. ‘Star’s root and shoot K were not significantly different (Table 4.1). Root and shoot weights were not significantly different between treatments in either cultivar.
FT. ‘Emerald’ samples taken from all treatments on 7 Dec had lower lethal temperatures than 17 Jan and 8 Feb. Significance between treatments was only observed on 17 Jan and 8 Feb in ‘Emerald’. On 17 Jan, Aug treated plants had a significantly higher LT$_{50}$ than Oct or Urea treatments by 50.5% and 31.7% respectively. On 8 Feb, Sept treated plants had a significantly lower LT$_{50}$ than all other treatments by 20.3-29.4%. (Table 4.2)

In ‘Star’, there was no significant difference observed between the treatments on any date; however, on 7 Dec, Sept treated plants had a mean LT$_{50}$ (–19.1 °C) 18.5-21.4% lower than the other treatments. Further, on 17 Jan, Aug and Oct treated plants had an LT$_{50}$ 27-28% and 22.3-23.4% lower, respectively, than Sept or Urea treatments. (Table 4.2)

Bloom observation. There was no significant difference seen between treatments in ‘Emerald’ (data not shown). All treatments reached stage 9 by the 7th week of study (Fig. 4.1). Differences were seen between treatments in ‘Star’. Throughout the observation period, Oct treated plants were at significantly later stages in developmental progression than Sept and Aug treated plants. By the 7th week of study, Aug treated plants reached bud stages 5 to 6, tight cluster/early pink bud; Sept treated plants reached stage 7, late pink bud; Urea and Oct treated plants reached stage 8, early bloom. (Fig. 4.1)

Discussion

Nutrient uptake and partitioning was not consistently affected by the treatments. The treatments displayed significant variation within cultivar; however, the effects were not similar between cultivars. In comparisons of N, P, and K in both cultivars, deficiencies were not observed. In addition, because dry weights, bloom timing, and cold hardiness were not affected negatively by
any late fertilization treatments, it can be suggested that late timing of fertilizer or use of urea is not a cultural practice that is injurious to dormant floral buds in ‘Emerald’ and ‘Farthing’. Though, bloom progression for ‘Star’ was advanced by late (treatment 1; Oct) fertilization in comparison to treatment 1 (Aug), which places the flowers at a susceptible stage for freeze damage.

Root and shoot material was taken in mid-January; by mid to late January, buds of all ‘Emerald’ treatments progressed beyond stage 2, while the majority of ‘Star’ buds had not yet reached stage 2. During leaf senescence, important nutrients are degraded by enzymes and mobilized for storage in woody parts of plants such as trunk and root tissue (Taiz and Zeiger, 2010). As budbreak approaches, these nutrients are remobilized and reallocated to actively growing regions of the plant. ‘Emerald’ plants lost their hardiness earlier than ‘Star’, corresponding to the earlier bloom development and possible reallocation of nutrients.

Raese et al. (2007) determined that moderate rates of N (0.45kg N tree\(^{-1}\)) applied in late winter increased the mid-winter cold tolerance compared to low rates of N (0.15kg N tree\(^{-1}\)) or late summer fertilizer applications (Raese, 1997). Conversely results of late winter applications of N (0, 0.15, 0.45, 0.91kg N tree\(^{-1}\)) in ‘Golden Delicious’ apple trees found low rates of N (151 g N tree\(^{-1}\) and below) significantly more hardy than higher rates of N in both the fall and winter (Raese et al., 2007). Later applications of neither N fertilizer nor urea had a significant difference on the mid-winter LT\(_{50}\) of either ‘Emerald’ or ‘Star’. Cold hardiness was affected by ending fertilization in Aug for ‘Emerald’ at the Jan 17 sampling was -8.0 °C compared to the -12.0 °C of treatment 3 (Oct). However, by Feb 8 the LT\(_{50}\)s were not significantly different. ‘Emerald’ flowered ahead of ‘Star’ and has low chill hour requirements, which suggests the floral bud may be physiologically active causing greater variability in hardiness per bud sample.
Apple trees that received greater applications of nitrogen displayed significantly greater shoot extension in the spring (Raese et al., 2007). Comparable results were observed in ‘Star’ nutrient analysis and bloom progression: late fertilizer applications resulted in increased N in the roots from Aug to Oct, bloom progression in Oct fertilized plants attained advanced bud stages earlier compared to Aug or Sept treatments.

Conclusion

Nitrogen and phosphorous was increased by the fertilizer treatments in roots of both cultivars. Fertilizer treatments did not affect cold sensitivity of ‘Star’. Oct treated ‘Star’ plants reached later developmental stages earlier than Aug or Sept treated plants. ‘Emerald’ lost hardiness and broke bud earlier than ‘Star’. ‘Emerald’ showed loss of cold hardiness by stopping fertilization in August mid-winter. For both ‘Emerald’ and ‘Star’ late season fertilization did not increase floral bud susceptibility to freezing temperatures.
CHAPTER 5

CONCLUSIONS

Blueberry floral buds were evaluated through methods of DTA and FT to determine low temperature lethal thresholds. Blueberry floral buds attached to stem or excised from the stem did not display low temperature exotherms after pre-treatment to -2 °C during DTA, indicating that DTA is not a suitable method for approximating floral bud cold hardiness in SHB. Attached floral buds were hardier than excised floral buds in both years of study. Tissue pre-treated at -2 °C had a lower LT50 than tissue with 4 °C pre-treatment. Floral bud sensitivity to freezing temperatures followed a parabolic curve where hardiness increased into mid-winter and began to lose sensitivity toward budbreak.

Throughout dormancy both ‘Emerald’ and ‘Farthing’ showed continued growth through microscopic methods. Styles and anthers elongated throughout the sampling period by 153% and 632% for ‘Emerald’ and ‘Farthing’, respectively. Microspore mother cells were observed in meiosis forming haploid microspores, and by mid-Feb, tapetum cells had formed into complete pollen tetrads. Browning was observed in paraffin embedded toluidine stained samples, which were similar to damage observed in FT. In toluidine stain samples, browning was observed to increase with decreasing temperatures until -18.0 °C when all reproductive organs displayed browning. Under confocal microscopy, green florescence was observed at -12.0 °C and -15.0 °C in style, anther, and ovule tissue for both cultivars. Ovules had green florescence as low as -18.0 °C; however, by -21.0 °C no floral tissue fluoresced green. Confocal and paraffin embedded
samples displayed red fluoresced cells or browning, respectively, in the outer whorls followed by ovules, styles, and then anthers. In conclusion, microscopic images revealed growth throughout dormancy for both SHB evaluated. Observations of necrotic tissue from FT and confocal microscopy were associated with browning observed in toluidine stained samples that underwent freeze tolerance testing.

Late fall nitrogen applications to ‘Emerald’ and ‘Star’ did not increase the floral bud sensitivity to cold. Variability in N-P-K was noted between the treatments, though the levels of nutrients did not appear to effect blooming for ‘Emerald’. ‘Star’ did display advanced flowering with late N applications. The use of a complete fertilizer or urea both appear sufficient and not detrimental to floral bud survival through the dormant period.
LITERATURE CITED


Proebsting, E. 1961. Cold hardiness of elberta peach fruit buds as influenced by nitrogen level and cover crops (77).


Table 2.1. Critical bud freezing temperatures at LT<sub>50</sub> for SHB varieties, ‘Emerald’ and ‘Farthing’, excised (EFB) and attached floral buds (AFB) using freeze tolerance testing (FT). Samples were collected from 16 Nov 2015 to 15 Feb 2016. Samples collected from 16 Nov to 12 Jan were pretreated to 4 °C (FT4) and samples collected from 25 Jan to 15 Feb used -2 °C pretreatments (FT-2).

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<td>12 Jan</td>
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Means followed by a different letter within a column and within a cultivar are significantly different at \( P < 0.05 \) according to Tukey HSD. Comparisons were done within a variety.
Table 2.2. Critical bud freezing temperatures at LT<sub>50</sub> for SHB varieties, ‘Emerald’ and ‘Farthing’, excised (EFB) and attached floral buds (AFB) using freeze tolerance testing (FT). Samples were collected from 29 Nov 2016 to 31 Jan 2017.

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<td></td>
<td>29 Nov</td>
<td>13 Dec</td>
<td>5 Jan</td>
<td>17 Jan</td>
<td>24 Jan</td>
<td>31 Jan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emerald</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-2 °C EFB</td>
<td>-10.5 a&lt;sup&gt;z&lt;/sup&gt;</td>
<td>-13.0 b</td>
<td>-8.8 a</td>
<td>-10.5 a</td>
<td>-10.5 ab</td>
<td>-9.9 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-2 °C AFB</td>
<td>-17.3 c</td>
<td>-18.1 c</td>
<td>-19.0 c</td>
<td>-15.5 b</td>
<td>-13.7 b</td>
<td>-13.6 c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 °C EFB</td>
<td>-10.5 a</td>
<td>-9.9 a</td>
<td>-10.3 a</td>
<td>-10.5 a</td>
<td>-11.2 ab</td>
<td>-11.0 ab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 °C AFB</td>
<td>-13.3 b</td>
<td>-14.0 b</td>
<td>-14.5 b</td>
<td>-13.8 b</td>
<td>-9.8 a</td>
<td>-11.5 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farthing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-2 °C EFB</td>
<td>-8.1 a</td>
<td>-10.5 a</td>
<td>-10.8 a</td>
<td>-10.5 a</td>
<td>-10.6 a</td>
<td>-9.3 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-2 °C AFB</td>
<td>-15.6 b</td>
<td>-18.4 c</td>
<td>-20.2 c</td>
<td>-18.8 b</td>
<td>-14.7 b</td>
<td>-13.4 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 °C EFB</td>
<td>-8.1 a</td>
<td>-10.5 a</td>
<td>-10.5 a</td>
<td>-9.7 a</td>
<td>-10.5 a</td>
<td>-10.5 ab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 °C AFB</td>
<td>-10.2 a</td>
<td>-14.1 b</td>
<td>-17.0 b</td>
<td>-16.8 b</td>
<td>-11.5 a</td>
<td>-12.6 b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>z</sup> Means followed by a different letter within a column and within a cultivar are significantly different at P < 0.05 according to Tukey HSD. Comparisons were done within a variety.
Table 2.3. Mean fresh weight, oven-dried weight, and change in weight of cultivars, ‘Emerald’ and ‘Farthing’ throughout winter 2015-2016.

<table>
<thead>
<tr>
<th>Date</th>
<th>Fresh Weight (mg)</th>
<th>Dry Weight (mg)</th>
<th>Change in Weight (%)</th>
<th>Fresh Weight (mg)</th>
<th>Dry Weight (mg)</th>
<th>Change in Weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 Nov</td>
<td>29.8 b^z</td>
<td>10.8 b</td>
<td>63.7 cd</td>
<td>26.6 c</td>
<td>10.0 e</td>
<td>62.2 a</td>
</tr>
<tr>
<td>7 Dec</td>
<td>34.2 b</td>
<td>13.5 b</td>
<td>60.8 d</td>
<td>28.1 c</td>
<td>10.5 de</td>
<td>62.3 a</td>
</tr>
<tr>
<td>21 Dec</td>
<td>43.3 b</td>
<td>15.8 b</td>
<td>63.6 cd</td>
<td>36.4 bc</td>
<td>12.6 cde</td>
<td>65.0 a</td>
</tr>
<tr>
<td>12 Jan</td>
<td>40.3 b</td>
<td>14.2 b</td>
<td>64.4 bc</td>
<td>46.5 ab</td>
<td>16.1 abc</td>
<td>64.8 a</td>
</tr>
<tr>
<td>25 Jan</td>
<td>39.3 b</td>
<td>13.2 b</td>
<td>66.0 bc</td>
<td>41.9 bc</td>
<td>14.5 bcde</td>
<td>64.8 a</td>
</tr>
<tr>
<td>1 Feb</td>
<td>47.5 b</td>
<td>15.2 b</td>
<td>67.5 b</td>
<td>50.18 ab</td>
<td>17.5 ab</td>
<td>64.4 a</td>
</tr>
<tr>
<td>8 Feb</td>
<td>44.8 b</td>
<td>14.4 b</td>
<td>66.2 bc</td>
<td>41.77 bc</td>
<td>15.3 bcd</td>
<td>61.9 b</td>
</tr>
<tr>
<td>15 Feb</td>
<td>82.4 a</td>
<td>22.2 a</td>
<td>72.0 a</td>
<td>60.86 a</td>
<td>20.3 a</td>
<td>65.8 a</td>
</tr>
</tbody>
</table>

R^2       0.43        0.32        0.48        0.34        0.36        0.19

P         <0.0001     <0.0001     <0.0001     <0.0001     <0.0001     0.0098

^z Means followed by a different letter within a column are significantly different at P < 0.05 according to Tukey HSD.
Table 2.4. Mean fresh weight, oven-dried weight, and change in weight of cultivars, ‘Emerald’ and ‘Farthing’ throughout winter 2016-2017.

<table>
<thead>
<tr>
<th>Date</th>
<th>Emerald Fresh Weight (mg)</th>
<th>Emerald Dry Weight (mg)</th>
<th>Emerald Change in Weight (%)</th>
<th>Farthing Fresh Weight (mg)</th>
<th>Farthing Dry Weight (mg)</th>
<th>Farthing Change in Weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29 Nov</td>
<td>18.8 c^z</td>
<td>8.4 b</td>
<td>56.1 c</td>
<td>13.7 c</td>
<td>5.4 d</td>
<td>59.0 bc</td>
</tr>
<tr>
<td>13 Dec</td>
<td>29.6 bc</td>
<td>12.3 b</td>
<td>57.5 c</td>
<td>19.5 c</td>
<td>8.6 cd</td>
<td>56.3 c</td>
</tr>
<tr>
<td>5 Jan</td>
<td>27.0 c</td>
<td>11.5 b</td>
<td>57.8 c</td>
<td>31.0 c</td>
<td>12.5 bc</td>
<td>59.5 abc</td>
</tr>
<tr>
<td>17 Jan</td>
<td>34.6 bc</td>
<td>13.4 b</td>
<td>61.0 bc</td>
<td>32.3 c</td>
<td>12.6 bc</td>
<td>61.4 abc</td>
</tr>
<tr>
<td>24 Jan</td>
<td>60.6 ab</td>
<td>18.0 ab</td>
<td>71.0 a</td>
<td>55.1 b</td>
<td>18.0 b</td>
<td>66.2 ab</td>
</tr>
<tr>
<td>31 Jan</td>
<td>88.7 a</td>
<td>27.5 a</td>
<td>68.6 ab</td>
<td>84.0 a</td>
<td>26.2 a</td>
<td>68.3 a</td>
</tr>
</tbody>
</table>

R^2 0.59 0.47 0.58 0.70 0.68 0.29

P <0.0001 <0.0001 <0.0001 <0.0001 <0.0001 0.002

^z Means followed by a different letter within a column are significantly different at P < 0.05 according to Tukey HSD.
Table 2.5. Mean fresh length, oven-dried length, and change in length of cultivars, ‘Emerald’ and ‘Farthing’ throughout winter 2015-2016.

<table>
<thead>
<tr>
<th>Date</th>
<th>Emerald</th>
<th></th>
<th></th>
<th>Farthing</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh Length (mm)</td>
<td>Dry Length (mm)</td>
<td>Change in Length (%)</td>
<td>Fresh Length (mm)</td>
<td>Dry Length (mm)</td>
<td>Change in Length (%)</td>
</tr>
<tr>
<td>16 Nov</td>
<td>4.6 b²</td>
<td>3.7 b</td>
<td>19.9 ab</td>
<td>5.7 c</td>
<td>4.8 b</td>
<td>15.9 bc</td>
</tr>
<tr>
<td>7 Dec</td>
<td>4.8 b</td>
<td>4.2 b</td>
<td>13.7 b</td>
<td>5.7 c</td>
<td>4.8 b</td>
<td>14.0 c</td>
</tr>
<tr>
<td>21 Dec</td>
<td>5.2 b</td>
<td>4.2 b</td>
<td>21.0 ab</td>
<td>6.2 bc</td>
<td>4.8 b</td>
<td>21.8 a</td>
</tr>
<tr>
<td>12 Jan</td>
<td>4.9 b</td>
<td>4.0 b</td>
<td>18.9 ab</td>
<td>6.8 ab</td>
<td>5.1 b</td>
<td>24.8 a</td>
</tr>
<tr>
<td>25 Jan</td>
<td>4.9 b</td>
<td>3.9 b</td>
<td>20.7 a</td>
<td>6.5 abc</td>
<td>5.1 b</td>
<td>22.3 a</td>
</tr>
<tr>
<td>1 Feb</td>
<td>5.1 b</td>
<td>4.0 b</td>
<td>21.9 a</td>
<td>6.8 ab</td>
<td>5.5 ab</td>
<td>19.9 ab</td>
</tr>
<tr>
<td>8 Feb</td>
<td>5.0 b</td>
<td>4.0 b</td>
<td>20.2 ab</td>
<td>6.5 bc</td>
<td>4.9 b</td>
<td>23.6 a</td>
</tr>
<tr>
<td>15 Feb</td>
<td>6.7 a</td>
<td>5.0 a</td>
<td>23.6 a</td>
<td>7.6 a</td>
<td>6.0 a</td>
<td>21.1 a</td>
</tr>
</tbody>
</table>

R²  0.37  0.28  0.15  0.28  0.24  0.31

P  0.0005 <0.0001 0.0036 <0.0001 <0.0001 <0.0001

²Means followed by a different letter within a column are significantly different at P < 0.05 according to Tukey HSD.
Table 2.6. Mean fresh length, oven-dried length, and change in length of cultivars, ‘Emerald’ and ‘Farthing’ throughout winter 2016-2017.

<table>
<thead>
<tr>
<th>Date</th>
<th>Fresh Length (mm)</th>
<th>Dry Length (mm)</th>
<th>Change in Length (%)</th>
<th>Fresh Length (mm)</th>
<th>Dry Length (mm)</th>
<th>Change in Length (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29 Nov</td>
<td>4.2 b</td>
<td>3.5 b</td>
<td>16.4 a</td>
<td>4.5 d</td>
<td>3.7 c</td>
<td>17.3 bc</td>
</tr>
<tr>
<td>13 Dec</td>
<td>4.5 b</td>
<td>3.8 b</td>
<td>14.6 a</td>
<td>5.0 cd</td>
<td>4.4 c</td>
<td>13.1 c</td>
</tr>
<tr>
<td>5 Jan</td>
<td>4.4 b</td>
<td>4.0 b</td>
<td>10.2 a</td>
<td>5.5 cd</td>
<td>4.6 bc</td>
<td>16.7 bc</td>
</tr>
<tr>
<td>17 Jan</td>
<td>4.8 b</td>
<td>4.1 b</td>
<td>14.9 a</td>
<td>5.9 bc</td>
<td>4.1 c</td>
<td>29.8 a</td>
</tr>
<tr>
<td>24 Jan</td>
<td>5.4 ab</td>
<td>4.6 b</td>
<td>15.6 a</td>
<td>6.9 ab</td>
<td>5.4 b</td>
<td>22.2 ab</td>
</tr>
<tr>
<td>31 Jan</td>
<td>7.0 a</td>
<td>5.8 a</td>
<td>16.7 a</td>
<td>7.9 a</td>
<td>6.6 a</td>
<td>16.7 bc</td>
</tr>
</tbody>
</table>

R²  0.49  0.52  0.10  0.66  0.66  0.45  
P  <0.0001  <0.0001  0.3842  <0.0001  <0.0001  <0.0001

Means followed by a different letter within a column are significantly different at P < 0.05 according to Tukey HSD.
Table 3.1. ‘Emerald’ floral bud development in the ovary, ovule, placenta, and style. Cross-sectional area was determined of the ovary, ovule, and placenta, and style length were measured by ImageJ. Progression is monitored monthly with collections take on 2 Nov, 7 Dec, 11 Jan, and 8 Feb during winter of 2015-2016.

<table>
<thead>
<tr>
<th>Date</th>
<th>Ovary (µm)</th>
<th>Ovule (µm)</th>
<th>Placenta (µm)</th>
<th>Style (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Nov</td>
<td>1601.55 b</td>
<td>62.963 c</td>
<td>497.24 b</td>
<td>374.77 a</td>
</tr>
<tr>
<td>7 Dec</td>
<td>2795.81 ab</td>
<td>108.04 bc</td>
<td>902.84 ab</td>
<td>443.75 a</td>
</tr>
<tr>
<td>11 Jan</td>
<td>2685.01 ab</td>
<td>190.502 ab</td>
<td>773.14 ab</td>
<td>766.4 a</td>
</tr>
<tr>
<td>8 Feb</td>
<td>4217.12 a</td>
<td>249.574 a</td>
<td>1554.87 a</td>
<td>950.12 a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>R²</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.27</td>
<td>0.414</td>
<td>0.272</td>
<td>0.455</td>
</tr>
</tbody>
</table>

Means followed by a different letter within a row are significantly different at \( P < 0.05 \) according to Tukey HSD.
Table 3.2. ‘Farthing’ floral bud development in the ovary, ovule, placenta, and style. Cross-sectional area was determined of the ovary, ovule, and placenta, and style length were measured by ImageJ. Progression is monitored monthly with collections take on 2 Nov, 7 Dec, 11 Jan, and 8 Feb during winter of 2015-2016.

<table>
<thead>
<tr>
<th>Date</th>
<th>Ovary (µm)</th>
<th>Ovule (µm)</th>
<th>Placenta (µm)</th>
<th>Style (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Nov</td>
<td>526.1136</td>
<td>b²</td>
<td>31.063 b</td>
<td>290.01 b</td>
</tr>
<tr>
<td>7 Dec</td>
<td>1326.363 b</td>
<td>62.056 b</td>
<td>564.033 ab</td>
<td>315 bc</td>
</tr>
<tr>
<td>11 Jan</td>
<td>3151.72 a</td>
<td>159.562 a</td>
<td>830.635 a</td>
<td>716.8 ab</td>
</tr>
<tr>
<td>8 Feb</td>
<td>3564.769 a</td>
<td>222.118 a</td>
<td>803.147 a</td>
<td>1183.08 a</td>
</tr>
</tbody>
</table>

R² 0.63          0.655        0.378        0.76
P <0.0001        <0.0001     0.0045       0.0005

*Z* Means followed by a different letter within a row are significantly different at *P* < 0.05 according to Tukey HSD.
Table 3.3. Percentage of attached floral bud mortality in tissue treated to freeze tolerance testing (FT). Bud survival is evaluated per removal temperature on 17 Jan 2017 for ‘Emerald’ and ‘Farthing’.

<table>
<thead>
<tr>
<th>Removal Temperature</th>
<th>Emerald</th>
<th>Farthing</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 °C</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>-3 °C</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>-6 °C</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>-9 °C</td>
<td>0%</td>
<td>12%</td>
</tr>
<tr>
<td>-12 °C</td>
<td>8%</td>
<td>0%</td>
</tr>
<tr>
<td>-15 °C</td>
<td>60%</td>
<td>0%</td>
</tr>
<tr>
<td>-18 °C</td>
<td>44%</td>
<td>37%</td>
</tr>
<tr>
<td>-21 °C</td>
<td>100%</td>
<td>89%</td>
</tr>
</tbody>
</table>
Table 4.1. Percentage of N, P, K, for shoot and root material of ‘Emerald’ and ‘Star’ treated to various late fall applications of fertilizer\(^2\) 10-10-10 or 46-0-0 (urea) and analyzed of 2016-2017 season.

<table>
<thead>
<tr>
<th></th>
<th>%N</th>
<th>%P</th>
<th>%K</th>
<th>%N</th>
<th>%P</th>
<th>%K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoots</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (Aug)(^\text{z})</td>
<td>1.53</td>
<td>a(^y)</td>
<td>0.12</td>
<td>a</td>
<td>0.79</td>
<td>a</td>
</tr>
<tr>
<td>2 (Sept)</td>
<td>1.15</td>
<td>ab</td>
<td>0.08</td>
<td>a</td>
<td>0.23</td>
<td>b</td>
</tr>
<tr>
<td>3 (Oct)</td>
<td>1.36</td>
<td>ab</td>
<td>0.10</td>
<td>a</td>
<td>0.29</td>
<td>ab</td>
</tr>
<tr>
<td>4 (urea)</td>
<td>1.02</td>
<td>b</td>
<td>0.10</td>
<td>a</td>
<td>0.31</td>
<td>ab</td>
</tr>
<tr>
<td>P</td>
<td>0.0139</td>
<td></td>
<td>0.3226</td>
<td></td>
<td>0.0272</td>
<td></td>
</tr>
<tr>
<td>MSD</td>
<td>0.4068</td>
<td></td>
<td>0.0497</td>
<td></td>
<td>0.5196</td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (Aug)</td>
<td>0.89</td>
<td>b</td>
<td>0.07</td>
<td>b</td>
<td>0.18</td>
<td>a</td>
</tr>
<tr>
<td>2 (Sept)</td>
<td>1.10</td>
<td>b</td>
<td>0.10</td>
<td>b</td>
<td>0.19</td>
<td>a</td>
</tr>
<tr>
<td>3 (Oct)</td>
<td>1.56</td>
<td>a</td>
<td>0.17</td>
<td>a</td>
<td>0.26</td>
<td>a</td>
</tr>
<tr>
<td>4 (urea)</td>
<td>1.12</td>
<td>b</td>
<td>0.12</td>
<td>ab</td>
<td>0.20</td>
<td>a</td>
</tr>
<tr>
<td>P</td>
<td>0.0032</td>
<td></td>
<td>0.001</td>
<td></td>
<td>0.1315</td>
<td></td>
</tr>
<tr>
<td>MSD</td>
<td>0.4188</td>
<td></td>
<td>0.054</td>
<td></td>
<td>0.0938</td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\) Aug; final fertilizer applied on Aug 23 with 10-10-10 at 28g per pot 2) Sept. final fertilizer applied on Sept 15 with 10-10-10 applied on Aug 23 at 20 g per pot and Sept 15 at 10 g per pot. 3) Oct; Final fertilizer applied on Oct 15 with 10-10-10 applied on Aug 23 at 10 g per pot, Sept 15 at 10 g per pot and on Oct 15 at 10g per pot 4) urea; final fertilizer applied on Sept 15 with urea applied on Aug 23 at 3 g per pot and Sept 15 at 3 g per pot.

\(^{y}\)Means followed by a different letter within a column within roots and shoots are significantly different at \(P < 0.05\) per Tukey HSD.
Table 4.2. Measurement of lethal temperatures where 50% damage to floral bud tissue is recorded as LT$_{50}$ to ‘Emerald’ and ‘Star’ that fertilization was terminated in Aug., Sept., or Oct$^2$. TA was taken at three representative dates, 7 Dec (143 accumulated chill units, CU), 17 Jan (341 CU), and 8 Feb (395 CU).

<table>
<thead>
<tr>
<th></th>
<th>7 Dec 2016</th>
<th>17 Jan 2017</th>
<th>8 Feb 2017</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emerald</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Aug</td>
<td>-17.4 a$^y$</td>
<td>-8.0 a</td>
<td>-9.9 a</td>
</tr>
<tr>
<td>Sept</td>
<td>-15.4 a</td>
<td>-8.9 ab</td>
<td>-13.4 b</td>
</tr>
<tr>
<td>Oct</td>
<td>-17.5 a</td>
<td>-12.0 c</td>
<td>-10.7 a</td>
</tr>
<tr>
<td>Urea</td>
<td>-17.3 a</td>
<td>-10.5 bc</td>
<td>-9.5 a</td>
</tr>
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<td></td>
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<tr>
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<td>-16.6 a</td>
<td>-11.3 a</td>
</tr>
<tr>
<td>2 (Sept)</td>
<td>-19.1 a</td>
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<td>-13.8 a</td>
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<td>3 (Oct)</td>
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<td>-15.6 a</td>
<td>-10.7 a</td>
</tr>
<tr>
<td>4 (urea)</td>
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<td>-12.1 a</td>
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$^2$1) Aug; final fertilizer applied on Aug 23 with 10-10-10 at 28g per pot 2) Sept. final fertilizer applied on Sept 15 with 10-10-10 applied on Aug 23 at 20 g per pot and Sept 15 at 10 g per pot. 3) Oct; Final fertilizer applied on Oct 15 with 10-10-10 applied on Aug 23 at 10 g per pot, Sept 15 at 10 g per pot and on Oct 15 at 10g per pot 4) urea; final fertilizer applied on Sept 15 with urea applied on Aug 23 at 3 g per pot and Sept 15 at 3 g per pot.

$^y$Means followed by a different letter within a column are significantly different at $P < 0.05$ per Tukey HSD. Comparisons are done within a variety.
Figure 1.1. Dissected blueberry floral buds pre- and post-freezing treatment. (A) Untreated (4 °C) ‘Farthing’ floral bud with 0% bud necrosis (excised from an experimental sample of 5 cm stem section stem) and (B) treated (-12 °C) ‘Emerald’ floral bud with 80% bud necrosis.
**Figure 2.1.** Average FT LT$_{50}$ of attached ‘Emerald’ (A) and ‘Farthing’ (B) buds from autumn acclimation to budswell in 2015-2016. Means followed by a different letter within a line are significantly different at $P < 0.05$ according to Tukey HSD.
Figure 2.2. Average FT LT50 of attached ‘Emerald’ (A) and ‘Farthing’ (B) buds from autumn acclimation to budswell in 2016-2017. Means followed by a different letter within a line are significantly different at $P < 0.05$ according to Tukey HSD.
Figure 3.1. Morphological progression of singular ‘Emerald’ floral bud within inflorescence as seen on 2 Nov (A), 7 Dec (B), 11 Jan (C), and 8 Feb (D). Buds were paraffin embedded and toluidine blue stained images showing developmental progression of petals (pt), sepals (sp), anthers (a), style (s), filament (f), placenta (pl), ovary (o), and ovules (ov).
Figure 3.2. Morphological progression of singular ‘Farthing’ floral bud within inflorescence as seen on 2 Nov (A), 7 Dec (B), 11 Jan (C), and 8 Feb (D). Buds were paraffin embedded and toluidine blue stained images showing developmental progression of petals (pt), sepals (sp), anthers (a), style (s), filament (f), placenta (pl), ovary (o), and ovules (ov).
Figure 3.3. Anther development and pollen maturation of ‘Emerald’. Development progresses from immature anthers (a) seen on 7 Dec (A) to tapetum cells (tc) surrounding microspore mother cells (mm) by 21 Dec (B), reduction of tapetum cells and dissemination of microspore mother cells are seen on 01 Feb due to anther elongation (C), and on 8 Feb mature pollen tetrads (p) are seen (D). Buds were paraffin embedded and toluidine blue stained.
Figure 3.4. Anther development and pollen maturation of ‘Farthing’. Development progresses from immature anthers (a) seen on 21 Dec (A) to tapetum cells (tc) surrounding microspore mother cells (mm) by 11 Jan (B), reduction of tapetum cells and dissemination of microspore mother cells are seen on 01 Feb due to anther elongation (C), and on 8 Feb pollen tetrads (p) near maturation (D). Buds were paraffin embedded and toluidine blue stained.
Figure 3.5. Confocal microscopy images of cold treated ‘Emerald’ floral buds collected on 17 Jan 2017, held overnight at -2 °C, and subjected to varying freezing temperature treatments. Buds were subjected to different thermal treatments and differentially stained with Fluorescein diacetate (FDA), seen as green fluorescence, and propidium iodide (PI), seen as red fluorescence. FDA stains live cells, while PI stains nucleic acids visible only in damaged cells. Temperature treatments included 4 °C (A and F), -12 °C (B and G), -15 °C (C and H), -18 °C (D and I), and -21 °C (E and J). Decreased green fluorescence is noticed with decreasing temperatures suggesting increased tissue damage. Images showing tissue susceptibility of petals (pt), anthers (a), style (s), filament (f), placenta (pl), ovary (o), and ovules (ov) within a floral bud.
Figure 3.6. Paraffin embedding and toluidine blue stained images of ‘Emerald’ collected on 17 Jan 2017 and treated to -2 °C overnight. Buds were subjected to a temperature program decreasing 4 °C·h⁻¹ with removal rates every 3 °C from -3 °C to -21 °C. Above images approximate the lethal temperature at which floral buds suffered 50% cold injury and include buds with removal rates of 4 °C (A), -12 °C (B), -15 °C (C), -18 °C (D), and -21 °C (E). Images showing tissue susceptibility of petals (pt), sepals (sp), anthers (a), style (s), filament (f), placenta (pl), ovary (o), and ovules (ov) within a floral bud.
Figure 3.7. Confocal microscopy images of cold treated ‘Farthing’ floral buds collected on 17 Jan 2017, held overnight at -2 °C, and subjected to varying freezing temperature treatments. Buds were subjected to different thermal treatments and differentially stained with Fluorescein diacetate (FDA), seen as green fluorescence, and propidium iodide (PI), seen as red fluorescence. FDA stains live cells, while PI stains nucleic acids visible only in damaged cells. Temperature treatments included 4 °C (A and F), -12 °C (B and G), -15 °C (C and H), -18 °C (D and I), and -21 °C (E and J). Decreased green fluorescence is noticed with decreasing temperatures suggesting increased tissue damage. Images showing tissue susceptibility of petals (pt), anthers (a), style (s), filament (f), placenta (pl), ovary (o), and ovules (ov) within a floral bud.
Figure 3.8. Paraffin embedding and toluidine blue stained images of ‘Farthing’ collected on 17 Jan 2017 and treated to -2 °C overnight. Buds were then run on a temperature program decreasing 4 °C/h with removal rates every 3 °C from -3 °C to -21 °C. Above images approximate the lethal temperature at which floral buds suffered 50% cold injury and include buds with removal rates of 4 °C (A), -15 °C (B), -18 °C (C), and -21 °C (D). Images showing tissue susceptibility of petals (pt), sepals (sp), anthers (a), style (s), filament (f), placenta (pl), ovary (o), and ovules (ov) within a floral bud.
Figure 4.1. Bud development of ‘Star’ is followed from 24 Jan to 14 Mar in treatments of late season N application ending in August, October, and September, in addition to a urea treatment. Bloom progression was recorded according to the MSU blueberry growth stages table: stage 1= tight/dormant bud; stage 2= bud swell with visible green tip; stage 3= budbreak signified by separation of bud scales; stage 4 = tight cluster, individual flower apices are visible; stage 5= early pink bud, individual floral apices appear pink and separated; stage 6 = late pink bud, flowers have elongated but corollas are still closed; stage 7 = early bloom, less than 50% of the corollas have opened; stage 8 = full bloom, greater than 50% of the corollas have opened; stage 9 = petal fall, greater than 50% of the corolla tubes have fallen off the flower; stage 10 = early green fruit, small pea size green fruit have expanded; stage 11 = late green fruit, expansion slows and fruit appear light green; stage 12 = fruit coloring, oldest fruit in the cluster change color to blue/pink.
(Michigan State University, 2016).