The impact of starch on wheat falling number and the evolvement of starch structure in the developing endosperm of soft white winter wheat

A Thesis
Presented in Partial Fulfillment of the requirements for the
Degree of Master of Science
with a
Major in Food Science
in the
College of Graduate Studies
University of Idaho

by
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May 2019

AUTHORIZATION TO SUBMIT THESIS

This thesis of Yuezhen He, submitted for the degree of Master of Science with a Major in Food Science and titled "The impact of starch on wheat falling number and the evolvement of starch structure in the developing endosperm of soft white winter wheat," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

The United States Pacific Northwest in known to produce premium soft white wheat with consistent quality; however, the unexpected low falling number issue resulted in a $30 million and $140 million loss in 2014 and 2016, respectively. Wheat with a low falling number is considered poor-quality due to the elevation of α-amylase activity, which leads to a quick liquefaction of starch and decreases flour paste viscosity. The primary causes of low falling number are pre-harvest sprouting and late maturity α-amylase, both triggered by unusual weather pattern (e.g. pre-harvest rain and temperature shock). We hypothesize that weather impacts starch structural development and influences starch functionality (e.g., viscosity). Our previous study supports this hypothesis and reveals a starch developmental change in some low falling number wheat. To identify a solution for the low falling number issue, we conducted a comprehensive review (Chapter 1) regarding the impact of starch and its interaction with other molecules on wheat falling number, which lead to another hypothesis that plant growing conditions play an important role in influencing starch structure evolvement. However, it was difficult to directly identify how environmental stress triggers the starch structural changes because it is not known how starch structure evolves during grain development in soft white wheat. Thus, we conducted a study to close this knowledge gap, and we investigated the evolvement of starch structure in developing kernels (Chapter 2). We systematically measured starch structural characteristics, including starch content, starch granule size distribution, the ratio of amylose to amylopectin, the development in the structure of amylopectin, and starch gelatinization temperature and enthalpy change. We divided the development of starch structure into three stages: the initial stage (Day 7 to Day 10 after anthesis), rapid accumulation stage (Day 14 to Day 28 after anthesis), and the maturity stage (Day 35 to Day 42 after anthesis). During the rapid accumulation stage, starch quantity increased rapidly, starch granule size distribution became a bimodal distribution, and starch crystalline structure gradually became more organized. After the plant reached physiological maturity (Day 35 after anthesis), starch structure continued developing during the starch maturity stage. Our findings suggest that when environmental stress occurs during the rapid accumulation stage, it can critically change starch structure and impact starch functionality. Future work to identify the genetic controls of the development of starch structure during the

rapid accumulation stage will be helpful to develop a new wheat variety with a high resistance to weather changes and may help solve the low falling number issue.

ACKNOWLEDGEMENTS

I acknowledge the financial support from Idaho Wheat Commission (Boise, ID), Ardent Mills, LLC (Denver, CO), the College of Agriculture and Life Sciences and the Bi-State School of Food Science at the University of Idaho (Moscow, ID). I would like to express my thanks to my advisor Dr. Amy Lin for providing me an opportunity to complete my master's study under her guidance and for her support during my graduate studies at the University of Idaho. Dr. Lin's role as a mentor during my master's training encouraged me to become a better person and researcher. Special thanks to my graduate committee members Dr. Carolyn Bohach on teaching me writing and presenting skills, and thanks to Dr. Kurtis Schroeder for providing me research materials and for helping me broaden my knowledge in agronomy. I am grateful for the insightful discussions and advice given by Dr. Eric Bertoft (Finland) regarding the concept of the building-block backbone model of amylopectin structure and the characterization of amylopectin internal molecular chains. I also thank Dr. Ian Tetlow at the University of Guelph (Ontario, Canada) for the discussions regarding starch synthesis and synthesis related enzymes. I also thank Dr. Cathy Wilson at the Idaho Wheat Commission (Boise, ID) for the guidance and discussions related to the wheat productions and falling number issue, Dr. Valerie Jean Lynch-Holm for the training and assistance with the microscopic examinations, and Ms. Melissa Mann (Lincoln, NE), Ms. Samantha Burns (Moscow, ID), and the Writing Center at the University of Idaho for editing my progress reports, manuscripts, and thesis. I also thank Mr. Russell S. Day for helping me collect wheat heads. I appreciate the assistance from helpers Vanessa Hernderz, Charlene Home, Amir Rezamand, Chia-Wei Lin, Adam Rosemore, Jess King, Dipesh Basnet, Hailey Kessler, Hailey Wright, Scott Jones, Victoria Ghielmetti, Puspita Sari Hamijoyo, Natasha Textor, and Eric Sanchez. Last but not the least, I would like to thank the supports from all my lab mates: Dr. Rohollah (Ali) Sadeghi, Dr. Bin Du, Dr. Fang Fang, Yu-Lian Lin, Min-Hui (Sunny) Tsai, Chen Chen, Yijing Shao, Oscar Huang, Angela Johnson, and Duncan Robertson.

DEDICATION

I lovingly dedicate this thesis to my father Yonghong He, my mother Duanyun Yao and all my family for their unconditional support.

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CHAPTER 1 IMPACTS OF STARCH AND THE INTERACTIONS BETWEEN STARCH AND OTHER MACROMOLECULES ON WHEAT FALLING NUMBER

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1.1 ABSTRACT

Wheat with a low falling number (FN) has been particularly prevalent in recent years and has resulted in a loss of more than $140 million in a single year in the wheat industry in the Pacific Northwest of the United States. FN measurement is a standard method for the evaluation of grain α-amylase activity, and a low FN indicates a reduction in hot wholemeal paste viscosity due to sprouting damage. Recent studies show that a low FN may result from a developmental change of starch and adverse effects of non-α-amylase macromolecules on wheat. In this review, we describe the principles of FN measurement and the relationship between FN and α-amylase. We also discuss the isozymes, locations, and inhibitors of wheat α-amylase. The effects of various aspects of starch, which is the substrate of α-amylase, on wheat FN are also discussed, including starch structural characteristics (e.g., starch granule architecture), starch susceptibility to α-amylase, and the interaction between starch and non-starch macromolecules (e.g., lipids). Studies on the effects of planting environments (e.g., temperature) and agronomic practices (e.g., irrigation and fertilization) on both starch paste viscosity and FN are also reviewed. This paper highlights the importance of considering the impacts of starch and the interactions of starch and other macromolecules, including wheat α-amylase, on wheat FN, which is important for developing strategies to solve the low FN problem.

1.2 INTRODUCTION

The decrease in falling number (FN) has critically and directly impacted trading prices of wheat and has resulted in a significant economic loss to the wheat industry, especially wheat growers. FN measurement is a standard method for evaluating grain α-amylase activity (AACC International, 2010a). An FN between 300 and 450 seconds (s) or higher is desired,

and a value below 300 s implies sprouting damage and sprouting-related poor end-use quality. A low FN often leads to a discount in trading prices. For example, wheat grains were discounted 25 US cents per bushel for every 25 s below the cutoff (300 s) in the United States (Delwiche, Vinyard, & Bettege, 2015; *Personal Communication,* Idaho Wheat Commission, Boise, ID, United States, February 16, 2017). The recent outbreak of the low FN issue in the Pacific Northwest, including Idaho, Washington, and Oregon resulted in a loss of $140 million in a single year to the wheat industry (Campbell, 2016). The low FN issue is not unique to the United States; it has also been reported in Australia (Mares, 1993; Mares, Mrva, & Panozzo, 1994), the United Kingdom (Kettlewell, Sothern, & Koukkari, 1999), Japan (Yanagisawa, Nishimura, Amano, Torada, & Shibata, 2005), and China (Xiao, Zhang, Yan, & Lin, 2002).

The causes of low FN in wheat are conventionally classified into two criteria: pre-harvest sprouting (PHS) and late maturity α-amylase (LMA). PHS is usually caused by a continuous rain after grains reach physiological maturity and before they are harvested (Mares & Mrva, 2014). LMA, also known as prematurity α-amylase, is a high isoelectric point (pI) α-amylase synthesized during the middle to late stages of grain development and ripening. LMA is usually produced when there is a sudden change in wheat growing temperature, such as a decrease from a temperature higher than 25 °C to a temperature lower than 18 °C (or 12 °C at night) 20 – 30 days after anthesis (Mares & Mrva, 2014). Both PHS and LMA have elevated α-amylase activities, leading to a rapid liquefaction of starch pastes and consequently a low FN (Flintham, Adlam, Bassoi, Holdsworth, & Gale, 2002; Mrva, Cheong, Yu, Law, & Mares, 2009). In the recent breakout of the low FN issue, some low FN wheat (e.g., FN < 300 s) surprisingly lacked significant α-amylase activity, and the cause for the reduction in FN in that wheat is unknown (Steber, 2016b). In our previous research, a developmental change of starch was revealed in some low FN wheat with an increased proportion of B-type wheat starch, which has a relatively lower paste viscosity and a higher susceptibility to wheat α-amylase than A-type wheat starch (Shao, 2018). Starch, the substrate of α-amylase, is the major contributor of wholemeal paste viscosity. Removing starch from the wholemeal, which initially has a viscosity of 200 rapid visco units [RVU]), can decrease the viscosity to 10 RVU (Morris, King, & Rubenthaler, 1997). Other than starch (60% –70% w/dry wt), wheat wholemeal contains other macromolecules, which do not provide obvious viscosity but can

interact with starch and impact the flour paste viscosity and FN measurement. Those macromolecules include proteins (8% –18% w/dry wt), lipids (2% –3% w/dry wt), and non-starch polysaccharides (e.g., arabinoxylan and β-glucan, 2% –3%, w/dry wt) (Chung, Park, Ohm, & Howitt, 2009; Posner, 2000).

The characteristics of starch and other macromolecules are affected by wheat growing environments, such as temperature, fertilization, and water availability. Shi et al. (1994) reported that hot weather (e.g., 40 °C) is associated with a reduction in starch accumulation and the average size of starch granules. High temperature also causes an increase in starch lipids and amylose content. These changes lead to an increase in starch gelatinization temperature and a decrease in starch swelling capacity, and both negatively correlate with starch paste viscosity (Shi, Seib, & Bernardin, 1994). The effects of macromolecules on wholemeal paste properties is substantial, and macromolecular characteristics can be significantly impacted by wheat growing environments and agronomic practices.

1.3 FALLING NUMBER

1.3.1 FN measurement

Alpha-amylase hydrolyzes starch and promotes the liquefaction of starch pastes, thus disrupting bread dough structure during baking. Therefore, quantifying α-amylase activity is important to the wheat industry for maintaining consistent product quality. Several methods, including the FN test, have been developed to measure α-amylase activity in grains (Hagberg, 1960). Most of the methods are based on the Wohlgemuth principle, in which degraded starch binds less to iodine than native starch, and, thus produces a lighter color in the test (Hagberg, 1961a). The measurement results are expressed in “Sandstedt, Kneen, and Blish” (SKB) units (Hagberg, 1961a). The FN test, which differs from other common tests, is based on the change in hot wholemeal paste viscosity (Hagberg, 1960, 1961b). In this test, seven grams (total weight with 14% [w/w] moisture, equivalent to 6.02 g dry wt) of wheat wholemeal is mixed with 25 mL of water in a test tube (21 × 220 mm) and shaken vigorously 20 times. The test tube, with a stirrer fixed on top of the tube, is immersed in a boiling water bath for five seconds. The wholemeal suspension is further mixed by moving the stirrer up and down twice per second for 55 s. The stirrer is then released and dropped from the top to the bottom of the tube. The time in seconds, including the 60 s before the stirrer is released, for the stirrer to

reach the bottom of the tube is defined as FN (Hagberg, 1961b). Flours with high α-amylase activity are hydrolyzed rapidly during the measurement, resulting in a rapid liquefaction and, consequently a shorter falling time.

Although FN measurement is based on the change in paste viscosity, the falling of the stirrer does not represent the viscosity measured by other commonly used viscometers (e.g., Rapid Visco Analyzer). The speed of the stirrer passing through the paste at each time point is different in the FN measurement. This phenomenon is due to the variable shearing forces generated by the stirrer. The shearing forces come from two directions: the vertical and the horizontal direction of the falling stirrer while moving. Other visocometers, such as the Rapid Visco Analyzer (RVA) and Amylograph®, apply a constant shear rate to the paste so that the shearing force is in the same direction as the movement of the paddle (RVA and Amylograph® use paddles, instead of a stirrer), and the resistance of the paste to the shearing force is recorded as its viscosity (Deffenbaugh & Walker, 1989; Ross, Walker, Booth, Orth, & Wrigley, 1987). Thus, the change in hot wholemeal paste viscosity during the FN measurement is more variable than the measurements of other viscometers because of the additional shearing force generated by the stirrer.

FN measurement has a unique heating profile because, unlike other tests, it mimics the heating rate in baking bread dough and takes a similar amount of time to reach the starch gelatinization temperature of 55 °C to 65 °C. For FN measurement, it takes about 30 s, while the actual baking of a 70-g dough at 250 °C takes about 40 s to reach the starch gelatinization temperature (Perten, 1964). Unlike the FN test, most of the viscometric tests (e.g., RVA and Amylograph®) gradually heat starch suspension (~1.5 °C per min) and measure the peak (maximum) viscosity (AACC International, 2010b, 2010c). Alpha-amylase activity can also be directly measured by Wohlgemuth-based methods or other commercial analysis kits (e.g., Megazyme α-amylase assay Ceralpha Method kit and α-Amylase SD Assay Kit) (McCleary & Sheehan, 1987; McKie & McCleary, 2015). However, direct measurement of α-amylase activity is usually represented by the increase in hydrolytic products or the decrease in substrate concentration after reaching maximal hydrolysis, which does not normally occur during baking (Perten, 1964).

1.3.2 FN and wheat trades at the international market.

FN measurement is widely adopted by the wheat industry for detecting sprouting grains. It is also accepted by several international organizations for measurement of α-amylase activity in grains or flours. These organizations include the International Association for Cereal Chemistry (International Association for Cereal Science and Technology (ICC), Approved 1968; Last revised 1995), the AACC International (formerly American Association of Cereal Chemists) (AACC International, 2010a), and the International Standardization for Organization (ISO, 2009). The FN test is also used to evaluate the quality of grains in many countries (Delwiche et al., 2015), but each country has a different requirement for FN. In Canada, the required FN in red spring wheat for exporting is in the range of 380 s to 450 s (Canadian Wheat Commission, 2017; Hatcher, 2005). In Australia, the standard FN for Australian utility grade hard wheat and Australian premium white varieties is 200 s and 350 s, respectively (Grain Trade Australia, 2018). In the United Kingdom, the standard FN for soft wheat and hard wheat is 220 s and 250 s, respectively (Home Grown Cereals Authority, 2014).

In the United States, the FN test is not only used for exporting but is also adopted by inland ports and elevators. Federal and state laboratories perform the FN test according to Federal Grain Inspection Service Directive 9180.38 (Federal Grain Inspection Service Directive, 2013). Exporters usually use 300 s as the minimum requirement, and grain elevators usually reduce the price of grains with an FN lower than 250 s. Low FN grains are segregated into the feed market or blended into other grains. However, these practices do not prevent a price discount, and neither do they solve the financial crisis of wheat growers. As a result, some growers give up planting wheat the following planting season or reduce their hectarage, leading to a decrease in planted acres of wheat. For example, the wheat-planted areas in Idaho were decreased from 1.27 to 1.19 million hectares three years after the breakout of the low FN issue that occurred in 2013 (U.S. Department of Agriculture National Agricultural Statistics Service, 2016, 2017). Such a decrease in planted hectares may impact the competitiveness of Idaho in the global wheat market.

1.3.3 Correlation between FN and end-use quality

A low FN may indicate poor end-use quality because a low FN wheat has a quick liquefaction during baking. Wheat flour with an FN in the range of 200 s to 250 s can make good bread crumbs with ideal texture, color, and loaf volume (Perten, 1964). Bread crumbs become sticky when using a flour with an FN lower than 150 s because the starch in that flour is severely hydrolyzed by α-amylase and cannot form a continuous starch-protein matrix to provide a desired bread dough structure (Hug-Iten, Handschin, Conde-Petit, & Escher, 1999; Scanlon & Zghal, 2001). Although a low FN is associated with undesired texture, using a high FN flour does not guarantee excellent baking quality. For example, bread crumbs made by flour with a high FN (i.e., over 300 s) were found to have an undesirable dry texture (Perten, 1964).

A low FN is an indicator of high α-amylase activity, which, in conventional view, adversely affects end-use quality. However, having a small amount of α-amylase does not negatively impact baking quality. The addition of α-amylase (i.e., fungal α-amylase) is a popular practice in the industry in order to improve baking attributes (Rosell, Haros, Escrivá, & Benedito de Barber, 2001; Blaszczak, Sadowska, Rosell, & Fornal, 2004). Marti et al. (2017) also reported that adding a small amount of sprouting wheat (less than 2%), which provided α-amylase improved bread volume and softness because starch hydrolysates are beneficial to yeast fermentation which leads to enhanced bread baking quality (Marti, Cardone, Nicolodi, Quaglia, & Pagani, 2017). LMA-affected wheat, with an increase of two to three times the hydrolytic activity compared with unaffected wheat, did not have a significantly negative impact on bread quality; although that wheat has reduced FNs (e.g., 210 s to 240 s) (Newberry et al., 2018).

When the FN measurement was developed in the 1960s, bread quality was the primary concern. Currently, the FN test is also used to predict the quality of many other products such as sponge cakes and sugar snap cookies. For sponge cake, its volume is a critical quality requirement. A decrease in FN from 400 s to 150 s leads to a decrease in sponge cake volume by approximately 4%. When FN is further decreased to 100 s, the volume is decreased by about 15% (Finney, Natsuaki, Bolte, Mathewson, & Pomeranz, 1981; Kaldy & Rubenthaler, 1987). However, a recent study with a larger sample size of 55 soft white wheat samples, found that the correlation between FNs and sponge cake volume is low, with a coefficient of

determination (R^2) value below 0.42 (Kiszonas, Engle, Pierantoni, & Morris, 2018). This contradictory result indicates that using FN to evaluate wheat quality for making sponge cake or similar products needs further investigation. For sugar-snap cookies, cookie diameter is the major quality requirement but does not correlate with FNs (Kaldy & Rubenthaler, 1987; Kiszonas et al., 2018). The FN test is a rapid method for evaluating grain α-amylase activity and predicting wheat quality for making bread dough. However, the FN test has been inappropriately used to evaluate wheat quality for making various types of wheat products. Some low FN wheat is discounted or segregated to the feed market, but that wheat, in fact, is not poor-quality for making sugar-snap cookies and similar products. The industry is exploring new methods to properly evaluate grain quality and trading prices to avoid economic loss and enhance agricultural sustainability.

1.4 FALLING NUMBER AND ALPHA-AMYLASE

1.4.1 FN and α-amylase activity

FN and α-amylase activity have a linear relationship when FN is in the range of 200 s to 300 s (Souza, Costa, & Kratochvil, 2011). When FN is out of this range, it is curvilinearly correlated with α-amylase activity (Yu et al., 2015). To adjust the curvilinear correlation into a linear correlation, FN is converted to the "liquefaction number" (LN) using the equation below. The relationship between LN and α-amylase activity becomes linear with an R^2 value of 0.975 (Perten, 1964).

$$LN = \frac{6000}{FN - 50}$$

However, such a relationship has been challenged, and some low FN wheat do not have significant α-amylase activity (Steber, 2016b), indicating that α-amylase is not the only factor impacting FNs.

1.4.2 Alpha-amylase isozymes in low FN wheat

Wheat has two major groups of α-amylase isozymes with different characteristics: high pI (6.0 - 6.5) and low pI (4.5 - 4.8). Some α-amylases do not fit in this classification and have a pI value outside of both ranges (e.g., pI 10.0) (Daussant & Renard, 1987). Other common terms to describe wheat α-amylase are malt α-amylase and green α-amylase. Malt α-amylases

belong to the high pI group and are produced during the germination period by genes in *α-Amylase1 (α-Amy1)* (Gale & Ainsworth, 1984). Green α-amylases have low pI values and are produced during the endosperm development or germination periods by *α-Amylase2* (*α-Amy2)* (Cheng, Oldach, Mrva, & Mares, 2014). LMA (late maturity α-amylase) and PHS (pre-harvest sprouting), the two major causes of low FN in wheat, have different combinations of α-amylase isozymes. LMA is associated with the α-amylases produced by *α-Amy1* with a high pI, while PHS contains the α-amylases produced by both *α-Amy1* and *α-Amy2* with a mix of high and low pI α-amylases (Barrero et al., 2013; Mieog, Janeček, & Ral, 2017). A comparison of α-amylases in PHS and LMA is summarized in Table 1.

In addition to *α-Amy1* and *α-Amy2*, *α-Amylase3* (*α-Amy3*) and *α-Amylase4* (*α-Amy4*) are also highly expressed when PHS and LMA occur. Mieog et al. (2017) hypothesized that the isozyme produced by *α-Amy4* is involved in the LMA-affected wheat because it is the only isozyme co-expressed with the isozyme produced by *α-Amy1*. Mieog et al. (2017) also hypothesized that the four isozymes synergistically hydrolyze starch. They found that the isozymes produced by *α-Amy1* and *α-Amy4* are more efficient in hydrolyzing short glucan chains on the surface of the starch granule, while the isozymes produced by *α-Amy2* and *α-Amy3* hydrolyze long chains, such as amylopectin B chains, and allow complete starch degradation. The roles of the four isozymes, especially the isozymes produced by *α-Amy3* and *α-Amy4,* have yet to be confirmed, but it is an important topic because it affects starch hydrolysis in wheat.

1.4.3 Alpha-amylase distribution in low FN wheat.

The distribution of α-amylase in low FN wheat varies under different conditions. PHS is induced when grains are physiologically mature, and high humidity stimulates germination. Alpha-amylase in PHS wheat is initially synthesized in the scutellum area, which is close to the germ area (the proximal end of a grain; Figure 1.1-A). Later, the synthesis continues in the aleurone regions in the embryo area, which is activated by gibberellic acid (gibberellins), and the synthesis location gradually moves toward the brush areas (the distal end of a grain) (Gubler, Millar, & Jacobsen, 2005; Mares, 1984). With abundant starch in the endosperm, alpha-amylolysis in PHS wheat generates a fair quantity of oligomers from starch to support kernel germination, and the degradation process generates a porous appearance on starch

granules (Figure 1.2-A). In contrast, LMA is induced while grains are not physiologically mature (e.g., 25 to 30 days after anthesis), and grains, after they are mature, do not have sprouted appearance like PHS grains (Figure 1.2-B). Mrva, Wallwork, and Mares (2006) examined the location of dead aleurone cells associated with the synthesis of α-amylase and reported that the dead aleurone cells in LMA-affected wheat is scattered randomly throughout the aleurone layer in the grain (Figure 1.1-B). The α-amylase in LMA-affected wheat might still remain in the aleurone regions where α-amylase is synthesized; however, research has yet to corroborate this.

In the past, identifying the cause of low FN was not critical because the existence of low FN, not the cause, determined the trading prices. However, with a large amount of low FN wheat and massive economic losses, the industry is looking for solutions, including utilizing low FN wheat in foods, in order to maintain reasonable trading prices. Since wheat grains with PHS or LMA may have different biochemical changes in the kernels, they have different end-use qualities. Thus, identifying the cause of low FN in wheat has become an important matter. The confocal microscope techniques that Mares et al. (2006) developed, demonstrate programmed aleurone cell death associated with the synthesis of α-amylase. This method can differentiate LMA (scattered randomly throughout the aleurone layer) from germination (condensed dead cells close to wheat germ area; Figure 1.1). Mares et al. (1994) also developed an assay, in which a wheat grain is cut into two halves separating the proximal (embryo side) and distal side (brush side), and their α-amylase activity is measured. LMA-affected wheat has similar α-amylase activities in both the proximal and distal halves of the grain, and such distribution pattern is recognized as typical of the late maturity α-amylase syndrome. Mares et al. (1994) reported that such a pattern is contradictory to the distribution of α-amylase in other grains with elevated α-amylase activity, where the enzyme is located almost entirely in the proximal half of the grain. The morphology of starch granules is another useful indicator. Starch granules in LMA-affected wheat are not severely hydrolyzed by α-amylase and maintain an intact appearance, whereas those of PHS affected wheat have many pores on the surface (Figure 1.2-A). Both PHS and LMA do not affect all of the grains in the same field homogenously, and only a portion of grains have elevated α-amylase activity (Olaerts, De Bondt, & Courtin, 2017; Mares, Mrva, & Panozzo, 1994). It is important to examine a fair amount of grains (e.g., 20 grains) to identify the cause of low falling number.

1.5 ALPHA-AMYLASE INHIBITORS IN WHEAT

An α-amylase inhibitor, silver nitrate, has been used to inhibit the hydrolytic activity of wheat α-amylase during FN measurement and other viscosity tests in order to diminish the impact of wheat α-amylase on the measurement of flour paste viscosity (Hutchinson, J.B., 1966; Batey, Curtin, & Moore, 1997). However, there are several endogenous α-amylase inhibitors in wheat, and their influence on the FN measurement has not yet been reported. The major α-amylase inhibitors in wheat are proteins, minerals, and phenolic compounds, and their existence is affected by the genetic and environmental background of wheat.

1.5.1 Proteinaceous inhibitors

Wheat has a unique protein known as α-amylase inhibitor, and its amount is 0.3% to 1.0% of the total protein content in wheat kernels. This proteinaceous inhibitor is located in the endosperm (Kneen & Sandstedt, 1946; Shainkin & Birk, 1970) and is involved in seed formation and starch deposition. It also plays a role in preventing the growth of bacteria, fungi, and insects (Kneen & Sandstedt, 1946; Takase, 1994). During the grain filling period, the quantity of α-amylase proteinaceous inhibitor continues to increase until grains reach full maturity (Pace, Parlamenti, Rab, Silano, & Vittozzi, 1978). Buonocore, Petrucci, and Silano (1977) reported that many wheat albumins contain some proteinaceous inhibitors that are phylogenetically related and coded by a small number of genes; thus, the content of proteinaceous inhibitors may vary among cultivars. The hydrolytic characteristic of the proteinaceous inhibitor in wheat has not been determined, but a similar α-amylase proteinaceous inhibitor in barley was found to decrease 81% of wheat α-amylase activity in an in vitro study (Weselake, Macgregor, & Hill, 1983). However, the impact of proteinaceous inhibitors on FN has not been reported.

1.5.2 Mineral inhibitors

Wheat contains various minerals, such as magnesium, phosphate, sulfur, and potassium (Hussain, Larsson, Kuktaite, & Johansson, 2010). Hussain et al. (2010) determined the mineral content in 321 genotypes of wheat and identified 27 different minerals. Copper and zinc are most abundant with 5.6 mg/kg and 41.6 mg/kg, respectively. Both copper and zinc can inhibit the activity of bacterial α-amylase, and 1 mM of copper and zinc can decrease

bacterial α-amylase activity by 74% and 27%, respectively (Cordeiro, Martins, & Luciano, 2002). These findings indicate that minerals in wheat can potentially interfere with α-amylolysis and, consequently, impact FNs.

1.5.3 Phenolic compound inhibitors

Wheat contains large amounts of phenolic compounds, which can reduce the activity of α-amylase and, consequently, affect paste viscosity. In general, wheat contains 7 to 12 g garlic acid equivalent (GAE)/kg of phenolic compounds (Žilić, 2016). These compounds are primarily located in the aleurone layer and the bran. Thus, removing wheat bran results in the loss of the majority of these compounds and a 66% reduction in antioxidant activity (Liyana-Pathirana & Shahidi, 2006). Brazier-Hicks et al. (2009) identified 70 phenolic compounds in wheat, and these phenolic compounds are classified into various groups according their structural characteristics. Among those groups, phenolic acids, flavonoids, and pranthocyanidins in wheat have been shown to affect α-amylase activities (Funke & Melzig, 2005; Gonçalves, Mateus, & De Freitas, 2011; Kim, Kwon, & SoN, 2000).

Phenolic acids inhibitors. The major phenolic acid in wheat is ferulic acid, which accounts for 75% to 93% of the total phenolic acids in wheat grains (Serpen, Gökmen, Karagöz, & Köksel, 2008). Ferulic acid can decrease α-amylase activity with a half maximal inhibitory concentration (IC50) of 5 mM (Funke & Melzig, 2005). Phenolic acids (i.e., ferulic acid) interfere with α-amylase activity through the non-competitive inhibition mechanism. They bind to α-amylase and change the conformation of α-amylase, thus reducing its hydrolytic activity (Funke & Melzig, 2005).

Flavonoids inhibitors. Flavonoids are the major pigments in wheat and generate the colors purple, blue, or red in wheat grains (Hosseinian, Li, & Beta, 2008). Žilić, Serpen, Akıllıoğlu, Janković, & Gökmen (2012) reported that bread wheat and durum contain 213 mg/kg to 259 mg/kg of flavonoids. Some flavonoids, such as cyanidin 3-glucoside and luteolin, have been shown to inhibit α-amylase (Kim et al., 2000; Wiese, Gaertner, Rawel, Winterhalter, & Kulling, 2009) through the non-competitive inhibition mechanism (Kim et al., 2000; Wiese et al., 2009). An in vitro study showed that luteolin (5mg/mL) decreases over 50% of α-amylase activity (Kim et al., 2000), which indicates a potential impact on FN.

Proanthocyanidin inhibitors. Fresh wheat bran contains 20 μg/g to 40 μg/g of proanthocyanidins, which are colorless phenolic compounds (McCallum & Walker, 1990). The main forms of proanthocyanidins in wheat are prodelphinidin B-3 and procyanidin B-3 (Dinelli et al., 2009). Procyanidin is a potent inhibitor of α-amylase, and its inhibition capability positively correlates with its degree of polymerization (Gonçalves et al., 2011). Though proanthocyanidins are colorless, some red wheat contain higher amounts of proanthocyanidins than white wheat because the complex formed by proanthocyanidins and ferric irons or copper is colored (McCallum & Walker, 1990; Miyamoto & Everson, 1958). Wheat colors do not have a direct relationship with α-amylase activity. However, colored wheat, such as red wheat, might have a higher quantity of both colored (i.e., flavonoids) and colorless (i.e., proanthocyanidins) compounds that affect α-amylolysis and impact FN.

1.6 THE SUBSTRATE OF ALPHA-AMYLASE: STARCH

1.6.1 Starch granular morphology and starch pasting properties.

Wheat kernels contain 63% to 72% (w/dry wt) of starch (Lineback & Rasper, 1988), and starch paste viscosity highly correlates with wheat flour paste viscosity with an R^2 value of 0.85 (Blazek & Copeland, 2008). Wheat starch, similar to triticale (a wheat-rye hybrid) and barley starch, consists of two types of starch granules: large (diameter > 10 μm) and disk-shaped A-type granules and small (diameter < 10 μm) and spherical B-type granules. The existence of C-type granules with a diameter less than 5 μm has also been reported. However, it has been speculated that C-type granules are developing granules and will become A- or B-type starch (Raeker, Gaines, Finney, & Donelson, 1998; Singh, Singh, Isono, & Noda, 2010). Ao and Jane (2007) reported that A-type granules have a lower gelatinization and pasting temperature, but have a higher peak, trough, final, and setback viscosity than B-type granules. When mixing A- and B-type granules in different ratios, the reconstituted starch with a high proportion of A-type granules has a relatively high viscosity (Ao & Jane, 2007).

A- and B-type granules are synthesized differently and have different chemical compositions. Starch consists of two α-glucans: amylose and amylopectin, and both are composed of linear α-glucans through α-(1→4) linkages and branched through α-(1→6) linkages. Amylose has long linear chains with few α-(1→6) linkages (0.3% - 0.5%), and amylopectin is a much larger molecule with more α-(1→6) linkages (4% - 5%). Amylose,

with longer linear glucans, can easily retrograde with other linear chains or form amylose-lipid complexes, in which both phenomena can negatively impact starch paste viscosity (Hizukuri, Abe, & Hanashiro, 1996). Amylopectin, with more branches, has a complicated molecular chain distribution, and thus, amylopectin molecular chains are further classified into A, B1, B2, B3, and B4 chains according to the molecular chain length and the relationship with other amylopectin chains (Hizukuri, Abe, & Hanashiro, 1996). Having a high quantity of long chains (i.e., B2, B3, and B4) positively correlates with starch paste viscosity (Jane et al.,1999). When comparing A- and B-type wheat starch granules, A-type granules have more amylose and long-B2 amylopectin chains than B-type granules. Also, A-type granules have a relatively low amount of lipid-amylose complex (Geera, Nelson, Souza, & Huber, 2006a) and therefore, less restriction in granule swelling, leading to a higher paste viscosity (Ao & Jane, 2007; Tester & Morrison, 1990). We hypothesize that starch granule size impacts FNs. Our previous study supports this hypothesis and reveals that some low FN wheat, without significant sprouting, have an increased proportion of B-type starch granules (Shao, 2018). Our findings suggest that in addition to α-amylase, starch developmental changes also affect FN.

1.6.2 Effects of Wx gene products on starch paste viscosity

Wild-type wheat (*Triticium aestivum* L.*;* common wheat; normal wheat) has three homologous waxy genes, *Wx-A1*, *Wx-B1*, and *Wx-D1*. Partial waxy mutants carry null alleles at one or two *Wx* loci, and waxy mutants have null alleles at all three loci (Chao et al., 1989; Graybosch, Guo, & Shelton, 2000; Nakamura, Yamamori, Hirano, & Hidaka, 1993). Thus, waxy mutants lack granule-bound starch synthases (GBSS) (EC 24.1.21), which are encoded by *Wx* genes (Graybosch et al., 2000; Nakamura et al., 1993). Common, partial waxy, and waxy wheat differ in amylose content, amylose-lipid complex content, amylopectin structure, paste viscosities, and FNs. Partial waxy and waxy mutants are amylose-reduced wheat and have little amylose-lipid complex, which affects paste viscosity (Garimella Purna, Shi, Guan, Wilson, & Graybosch, 2015; Geera et al., 2006a; Kim & Huber, 2010b; Tester & Morrison, 1990). Also, waxy mutants have a low ratio of amylose to amylopectin in weight, which has a higher molecular weight and larger molecular size (i.e., the *Z*-average radius of gyration) than the amylopectin of common wheat (Yoo & Jane, 2002). Thus, partial waxy and waxy mutants

have a high peak viscosity and low pasting and gelatinization temperature. However, partial waxy and waxy mutants, despite their high paste viscosity, have relatively low FNs (Graybosch et al., 2000). Starch in waxy wheat gelatinizes and reaches maximum viscosity rapidly. As shown in Figure 1.3, the viscosity of waxy wheat reaches the highest viscosity and begins to decrease at about 3.5 min, while common wheat reaches its highest viscosity at about 6 min (Garimella Purna et al., 2015; Graybosch et al., 2000). Rapid gelatinization of partial waxy and waxy wheat results in a more gelatinized starch, which is more susceptible to α-amylase than granule starch, at the initial stage of heating. In addition, rapid gelatinization allows more hydrolysis because α-amylase is still active at the temperature when waxy wheat is gelatinized. When starch in common wheat is fully gelatinized at 65 – 70 °C, α-amylase is less active due to the high temperature. Therefore, during FN measurement, waxy wheat experiences active hydrolysis of both granule starch and gelatinized starch, whereas the predominant reaction in common wheat starch is granular starch hydrolysis (Shao, 2018).

In addition to rapid gelatinization, waxy starch granules are usually hydrolyzed more rapidly than common starch (also described as normal starch, which refers to starch with a significant amount of amylose) granules because of its weak granular architecture. Waxy starch granules have more internal cavities and voids than normal starch and are more susceptible to α-amylase (Srichuwong & Jane, 2007). Some commercial wheat is partial waxy wheat, such as Alturas, Golden Spike, and UI Silver (Marshall et al., 2018), and using the FN test to evaluate its quality or determine its trading price is a disadvantage to that wheat.

1.6.3 Effects of damaged starch on starch paste viscosity

Damaged starch contains small starch fragments generated during milling or grinding that negatively impacts paste viscosity and affect the consistency of the FN test results. An increase in damaged starch content from 6.5% to 12.0% results in a decrease in starch peak viscosity by 500 Cp (Liu et al., 2014). Damaged starch is also more susceptible to enzymes (e.g., α-amylase) due to the reduction in particle size and increased water adsorption (Liu et al., 2014; Ma et al., 2016). The content of damaged starch in wheat flour is about 5% to 8% (Duyvejonck, Lagrain, Pareyt, Courtin, & Delcour, 2011), and hard wheat flour has higher damaged starch content than soft wheat flour (Roman-Gutierrez, Guilbert, & Cuq, 2002). Starch damage is associated with milling techniques (e.g., grinding intensity and time), and

grinders with different cutting mechanisms can generate different amounts of damaged starch (Ross & Kongraksawech, 2018). Ross et al. (2018) reported that the damaged starch content of winter wheat milled by eight millers was significantly different with the damaged starch content ranging from 2.15% to 5.55% (Gaines et al., 1987; Ross & Kongraksawech, 2018). For FN measurements, hammer (e.g., Perten Lab Mill 3100), cyclone (e.g., UDY Cyclone Sample Mill), and burr type grinders (e.g., Romer Mill Model 2, Bunn commercial coffee grinder) are recommended by the *AACCI* for preparation of wholemeal flour (AACC International, 1995). The difference in the content of damaged starch generated by different mills affects FNs, which further supports the fact that the impact of low FNs on trading prices needs to be considered.

1.6.4 Starch susceptibility to wheat alpha-amylase

The susceptibility of starch to α-amylase determines how fast starch is hydrolyzed and liquefied and consequently, impacts FNs. Starch in granular form is less susceptible to α-amylase than in gelatinized form. However, granule starch hydrolysis affects FN the most because α-amylase is less active at the starch gelatinization temperature (Shao, 2018). Granule starch hydrolysis is affected by granule size, granule shape, the presence of pores on the granule surface, existence of channels and cavities in granules, and its crystalline structure. Spherical and small B-type granules are hydrolyzed by α-amylase about 2 – 3 times faster than disk-shaped and large A-type granules (Kruger & Marchylo, 1985). This phenomenon is also seen in triticale and barley starch (MacGregor, 1979; Salomonsson, Heneen, Larssonraznikiewicz, Carlsson, & Karlsson, 1989). The high susceptibility of B-type granules to α-amylase is due to their large total surface area. B-type granules have a 1.4 – 2.0 m^2/g higher specific surface area than large starch granules (Kim & Huber, 2010b; MacGregor, 1979) and thus, are more susceptible to α-amylase.

Starch granules with pores, which appear on the surface of granules, and channels and cavities, which are found inside of granules, are hydrolyzed more rapidly as α-amylase can enter granules through these openings (Fannon, Hauber, & BeMiller, 1992). Thus, starch in sprouting wheat usually has a porous appearance. Some starch, such as disk-shaped A-type wheat starch, do not have visible pores but have weak spots where α-amylase can attack (Lin et al., 2006). The weak spots in disk-shaped A-type wheat starch are located in the groove

area, and A-type granules in the wheat with elevated α-amylase activity (i.e., sprouting wheat, α-amylase-over-expressed mutants) usually have significant damage at the groove (Fig. 4). The weak spots in B-type starch granules distribute randomly on their surface, and the partially degraded granules usually have dents randomly distributed on the granule surface. Internal channels and cavities allow α-amylase to travel and degrade starch from the granule surface to the hilum, where starch molecules are less organized and more susceptible to α-amylase (Fannon, Hauber, & BeMiller, 1992; Whan et al, 2014). Once α-amylase hydrolyzes starch all the way to the hilum, α-amylase can degrade starch very rapidly, and the degradation begins to move toward the granule surface. When starch granules are hydrolyzed at this level, they are often broken into small fragments and thus, have a larger total surface area. Pores and channels are naturally present in some starch because starch molecules are not homogeneously organized in starch granules (Lin et al., 2006). Pores and channels can be enlarged physically (e.g., dehydration) or enzymatically (e.g., enzyme hydrolysis) (Kim & Huber, 2008; Li, Vasanthana, Hoover, & Rossnagel, 2004), allowing further hydrolysis and a greater impact on FNs.

Both disk-shaped large A-type and spherical small B-type starch granules in commercial wheat have A-type polymorphic crystalline. Starch crystalline polymorphs are classified into A, B, and C types based on their X-ray diffraction patterns and are associated with starch molecular arrangement (Hizukuri, Abe, & Hanashiro, 1996). This classification is not based on granule size and shape and does not have a direct relationship with another classification used for wheat (or barley) starch. Starch polymorph types have a strong correlation with α-amylase hydrolysis efficiency (Jane et al., 2003). A-type-polymorph starch (e.g., wheat, barley) is highly susceptible to α-amylase. B-type-polymorph starch (e.g., potatoes) is relatively resistant to α-amylase. The susceptibility of C-type-polymorph starch (e.g., peas) to α-amylase is between that of A-type and B-type (Jane et al., 2003). A-type-polymorph starch generally has more pinholes and channels in starch granules than B-type-polymorph starch. Starch with A-type polymorphs also has shorter amylopectin chains, and the double helices of the branch chains are less ordered than those of starch with B-type polymorphs. The less ordered crystalline structure and the presence of pinholes in A-type-polymorph starch allow α-amylase to hydrolyze the granules more rapidly than B- and C-type-polymorph starch (Jane, 2006; Jane et al., 2003). In addition to the granule structure, amylose content also affects

starch polymorphism. Both normal and waxy maize starches are A-type polymorphs, but the high-amylose maize starch is a B-type polymorph. Barley starch also has a high amylose content and has a B-type or mixed B-type polymorph. The V-type polymorph, which is the typical polymorph of amylose-lipid complex, is also observed in the high-amylose barley starch (Regina et al., 2012). High-amylose wheat is new to the commercial market. Its polymorphic type is different from that of other commercial wheat and is another variable that can affect the FN measurement.

1.6.5 Environmental effects on starch characteristics

Wheat planting environments (i.e., temperature) and agronomic management (i.e., fertilization, irrigation) can impact FNs through their effect on starch quantity and quality. However, it is challenging to make recommendations on agronomic practices to improve the quality of starch as little is known in this area. Also, wheat cultivars and environmental factors vary in studies, making it difficult to compare or to advance the findings from the existing literature. For example, fertilizers can be applied before sowing or at the early stage of grain filling (Li et al., 2013; Ni et al., 2012). The quantity of fertilizer used in a study may not be appropriate for a different study due to the difference in wheat planting density, soil nutrients, and other environmental factors. Wheat with different genetic backgrounds also react to environmental factors differently, but the genetic information of research materials is not always provided in the literature. ~~In the past~~, growers usually have not had an advantage on trading price by providing wheat with a higher than expected quality. Thus, the focus was always maximizing the yield and reaching the desired protein content. However, the low FN crisis has motivated some wheat growers to seek solutions by adjusting their agronomic practices, such as adjusting fertilization guidelines in order to enhance starch content and quality.

Fertilization. Nitrogen, sulfur, and phosphorous are the three primary elements in wheat fertilization. In some regions, potassium is applied to enhance the growth of wheat (*Personal communication*). Research has shown that the application of these fertilizers impact starch and starch pasting properties beyond the effects on yields and protein content. However, such an impact on starch is not consistent in research, and it does not have a dose-response to applied fertilizers. Furthermore, most of the data show that the impact of fertilizers on starch depends

on cultivars, growing seasons, and other environmental factors (i.e., water availability, temperature).

Nitrogen fertilization. Nitrogen fertilization is aimed at maximizing the yield and increasing protein content with little intention on promoting starch functionality. Although protein and starch are the two primary components in flour, the ratio of protein to starch is not a strong indication of starch functionality (i.e., paste viscosity).Nitrogen fertilization might increase the synthesis of enzymes (i.e., starch branching enzyme, starch synthase), which are essential for starch disposition and development (Dupont & Altenbach, 2003; Rahman et al., 1995; Dong, Sang, Peng, Wang, & Yang, 2007; Li et al., 2018). Nitrogen fertilization also increases both starch content and the proportion of amylopectin in weight (Fu et al., 2008), leading to an increase in wholemeal flour paste viscosity (Zheng et al., 2012). However, the reports on the impact of nitrogen fertilization on starch quantity and quality are not consistent in the literature. Nowotna et al. (2007) reported that applying a high quantity of nitrogen fertilizer to triticale results in a high proportion of small starch granules, low amylose content, high starch gelatinization temperature, and low FNs. Li et al. (2013) had a similar observation, and their SEM images showed an increased proportion of small starch granules in nitrogen-fertilized winter wheat. However, Li et al. (2013) reported that nitrogen fertilization did not affect starch content, amylose amount, and peak viscosity but slightly increased final viscosity, setback viscosity, and onset gelatinization temperature.

The effect of nitrogen on starch quality varies among cultivars. In the studies of Fu et al. (2008) and Zheng et al. (2012), the cultivars selected in their studies, which had protein contents of 12% and 13%, respectively, did not have a dose response to nitrogen fertilizer. Spring wheat responds to nitrogen fertilizer by increasing its protein content, while winter wheat increases starch content and large (A-type) starch granules (Fredriksson, Salomonsson, Andersson, & Salomonsson, 1998). Both waxy and common wheat responded similarly to nitrogen fertilization by increasing starch content from ~60% to ~70% in a greenhouse study (Ni, Xu, Feng, & Wang, 2011).

Sulfur fertilization. Nitrogen fertilization is often accompanied with sulfur fertilization to increase yields because plants produce cysteine when the levels of its precursor *O*-acetylserine are adequate, and the concentration of *O*-acetylserine in plant cells is dependent on nitrogen (Hesse, Nikiforova, Gakiere, & Hoefgen, 2004). However, nitrogen-sulfur

fertilization is performed without the intention of promoting paste viscosity although applying a mixture of sulfur and nitrogen fertilizers do increase starch content, starch swelling power, and starch paste viscosity (Li et al., 2013; Shen, Zhu, Guo, Wang, & Ma, 2006), and spring and winter wheat have different responses to sulfer fertilization.

For spring wheat, sulfur fertilization alone does not have much effect on starch content but the addition of sulfur to nitrogen fertilizer has a positive effect on starch paste viscosity (Klikocka et al., 2016). For winter wheat, sulfur fertilization, after applying nitrogen-based fertilizers, causes an increase in starch content by about 7% (Tao et al., 2018). Even under heat-stress, which causes a decrease in starch content, sulfur fertilization increases starch content by 8% to 10% (Tao et al., 2018). It is not necessary to maximize sulfur fertilization in order to promote flour paste viscosity or FN because the effect of sulfur fertilization on starch paste viscosity is not a dose-response (Li et al., 2013; Shen et al., 2006). The proper amount of sulfur fertilizer for specific cultivars and soil conditions needs to be determined.

Phosphorus fertilization. Phosphorus profoundly affects the yield and wheat starch quality because it plays an important role in transferring and storing energy in plants. The balance between 3-phosphoglycerate and inorganic phosphorus can alter the activity of ADP-glucose pyrophosphorylase (AGPase), which in turn regulates starch synthesis (MacDonald & Strobel, 1970). Phosphorus fertilization can increase starch and amylopectin content and the proportion of extremely small starch granules in winter wheat; however, not all of the cultivars had such responses to phosphorus fertilization (Li et al., 2013; Ni et al., 2012).

Phosphorus fertilization does not always promote starch content or starch paste viscosity (not a dose-response), and the reports on this objective are inconsistent in research (Zheng et al., 2012; Zhang, Li, Fu, Li, & Li, 2018). Such inconsistant findings are due to the fact that phosphorus is involved in several biologic pathways, which affect starch differently. Phosphorus fertilization can increase starch content, the numbers and size of channels and cavities in starch granules, the susceptibility to starch degrading enzymes, starch degrading enzyme activity, and gene expression associated with starch synthesis (Zhang et al., 2018). Among those impacts, the promotion of starch synthesis activity and starch accumulation can increase starch paste viscosity. However, enhanced channelization, increased susceptibility to starch degrading enzymes, and increased activity of starch degrading enzymes have an adverse effect on starch paste viscosity (Garimella Purna et al., 2015; Li et al., 2004). In

addition, excess phosphorus fertilization can cause a shorter grain filling period and a higher respiration, leading to the loss of sugar and energy (Zhang et al., 2018). Thus, a positive outcome of phosphorus fertilization requires a balance among all the interactions, and therefore, the optimal amount of phosphorus fertilizer needs to be determined for specific cultivars and environments.

1.6.6 Water availability: Irrigation and water stress

Water availability has a significant impact on starch content and starch granule size and thus, starch paste viscosity. Well irrigated wheat, compared with rain-fed wheat, has an increased starch content and starch paste viscosity (Geera, Nelson, Souza, & Huber, 2006b). Without irrigation, the proportion of B-type granules is increased from 7% to 15% (volume percentage) (Dai, Li, Zhang, Yan, & Li, 2016; Dai, Yin, & Wang, 2009; Dai et al., 2008), leading to a decrease in paste viscosity. Another study conducted in a greenhouse showed that induced water stress at 5 – 9 days after anthesis led to an increase in the proportion of B-type granules and a decrease in granule size (Fábián, Jäger, Rakszegi, & Barnabás, 2011). The timing of irrigation is critical to starch granule size distribution. Irrigation during the anthesis stage is associated with an increase in B-type starch granules; conversely, irrigation during the grain filling period is associated with an increase in the proportion of A-type granules (Dai et al., 2016; Dai et al., 2009). The change in the proportion of A- and B-type granules is dependent on the timing of irrigation because the accumulation of A- and B-type granules starts at different periods, and water availability affects the synthesis of starch. Singh et al. (2008) reported that water stress at 15 days after anthesis is associated with reduced pasting temperature, reduced amylose content, increased peak and final viscosity, and increased setback viscosity; these effects may be the outcome of the negative impact of water stress on the synthesis of A-type granules. Response to irrigation is also cultivar dependent, and some cultivars are not affected at all (Singh et al., 2008). Generally, irrigation has a positive impact on starch paste viscosity and FNs.

1.6.7 Temperature

The optimal growth temperature of wheat is between 17 °C and 23 °C (Porter & Gawith, 1999). Wheat grown in areas with high temperature (e.g., 37/28 °C day/night) has been shown

to have reduced starch accumulation, an increased proportion of A-type starch, and decreased size of both A- and B-type granules (Al-Khatib & Paulsen, 1984; Hurkman & Wood, 2011; Kolderup, 1975; Nicolas, Gleadow, & Dalling, 1984; Shi et al., 1994; Sofield, Evans, Cook, & Wardlaw, 1977; Spiertz, 1977). High planting temperature is also associated with an increase in amylose content (Hurkman & Wood, 2011; Shi et al., 1994), but this phenomeon is cultivar dependent (Matsuki, Yasui, Kohyama, & Sasaki, 2003). The effect of planting temperature on amylopectin structure is not completely clear. Shi et al. (1994) reported that high temperature (40 °C) during the grain filling period results in more short-chain amylopectin (DP 10-16) and fewer medium chains (DP 17-21). However, Matsuki et al. (2003) reported that high temperature is associated with more short (DP 6-12) and medium chains (DP 13-34); this effect is similar to the impact of high temperature on maize and rice.

Planting temperature can also affect starch structural characteristics because starch synthesis enzymes are sensitive to temperature variations. Temperatures above 25 °C adversely affect enzyme reaction rates (i.e., soluble starch synthase), and a prolonged period of exposure to high temperature (above 20 °C in wheat) causes permanent loss of enzyme activity. Thus, a high planting temperature leads to a low starch yield (Keeling, Banisadr, Barone, Wasserman, & Singletary, 1994). The sowing time, at which the temperature may vary, can also impact starch structural characteristics and paste viscosity because the activities of starch synthesis enzymes are different at different temperatures (Sharma, Tiwari, Gupta, Rane, & Singh, 2018).

1.7 BEYOND THE ENZYME AND SUBSTRATE: INTERACTIONS OF STARCH AND NON-ALPHA-AMYLASE MACROMOLECULES

1.7.1 Proteins

Protein alone does not contribute considerably to flour paste viscosity. However, proteins can affect flour paste viscosity through their interactions with starch, and such interactions are related to both storage proteins (i.e., gluten) and starch granule associated proteins. Wheat contains 10% - 12% (w/dry wt) storage proteins, which can form a gluten matrix through the interchain disulfide bonds between glutenin and gliadin (Shewry & Halford, 2002; Shewry, Tatham, Barro, Barcelo, & Lazzeri, 1995). The gluten matrix becomes a physical barrier and limits the accessibility of starch to water, which is a critical

need for starch gelatinization. Consequently, the existence of storage proteins results in a delay of starch gelatinization and the time it takes to reach the highest paste viscosity (Jekle, Muhlberger, & Beeker, 2016; Chen, Deng, Wu, Tian, & Xie, 2010). Chen et al. (2010) also reported that mixing gluten with starch decreased the peak viscosity of the reconstituted flour. Wheat starch granules contain about 0.2 % (w/w) protein, which is referred to as starch granule associated protein, and it can be located in the interior or on the surface of starch granules (Rahman et al., 1995). The existence of starch granule associated proteins negatively correlates to starch paste viscosity. Li et al. (2016) removed starch granule associated protein, using an alkaline solution that greatly increased the peak, setback, and final viscosity of the starch paste and decreased pasting temperature and peak time during the RVA measurement. Removing those starch granule associated proteins facilitates amylose leaching from starch granules and promotes starch granule swelling, which leads to a higher pasting viscosity (Li et al., 2016).

1.7.2 Non-starch polysaccharides

Wheat flour contains 2% to 3% (w/dry wt) non-starch polysaccharides, which can affect the leaching of starch molecules from starch granules during gelatinization; thus, non-starch polysaccharides can have an effect on starch granule swelling and starch paste viscosity (Sasaki, Yasui, & Matsuki, 2000; Shi & Bemiller, 2002). Arabinoxylan is the major non-starch polysaccharide in wheat flour, and one gram of dry water-soluble arabinoxylan can hold ten grams of water (Kweon, Slade, Levine, & Gannon, 2014). Adding rye arabinoxylan to wheat flour shortens the pasting time (Harasztos et al., 2016), and adding a high concentration (3% w/w) of non-starch wheat polysaccharides to wheat starch decreases starch peak viscosity and increases breakdown (Sasaki et al., 2000). Wheat arabinoxylan structural characteristics, including molecular weight, the ratio of xylose to arabinose, and side-chain substitution and distribution, vary among cultivars (Izydorczyk & Biliaderis, 1993; Saulnier, Sado, Branlard, Charmet, & Guillon, 2007) and are affected by the planting temperature. The arabinoxylan in winter wheat can prevent ice formation in cold weather, and a decrease in the ratio of xylose to arabinose increases solubility (Kindel, Liao, Liske, & Olien, 1989). The effect of arabinoxylan on FN has not been reported. In our previous report, it has been observed that the quantity of water-soluble arabinoxylan increased in some low FN wheat

(Shao, 2018). More studies are needed to elucidate the role of arabinoxylan in wholemeal paste viscosity.

1.7.3 Lipids

The starchy endosperm of wheat contains 2% to 3% crude lipids (Hargin, Morrison, & Fulcher, 1980). Approximately 40% of the lipids are associated with wheat starch (Chung et al., 2009), and wheat lipids affect starch swelling during gelatinization (Tester & Morrison, 1990). Starch lipids consist of phospho- and galactolipids, which are part of the lipid layer derived from the remnant of degraded amyloplast after starch is synthesized (Hargin et al., 1980). Lipids on the surface of starch granules can form an amylose-lipid complex, which reduces water absorption and amylose leaching during gelatinization (Gerits, Pareyt, & Delcour, 2015). Removing lipids with methanol or enzymes (i.e., lipase and phospholipase) significantly increases wheat flour paste viscosity and starch swelling capacity (Gerits et al., 2015; Tester & Morrison, 1990). In contrast, adding extra fatty acids decreases flour (i.e., corn flour) paste viscosity (Démé , Peuvrel-Disdier, & Vergnes, 2015). Starch lipid content is associated with the size of starch granules (Geera et al., 2006a; Kim & Huber, 2010a). A- and B-type granules are synthesized differently and have different amount of lipids. Small B-type wheat starch granules have a relatively high amount of starch lipids, and it negatively correlates to FN. Also, B-type granules have a relatively larger surface area, which generates more lipid-layers from degraded amyloplast (Bechtel & Wilson, 2003; Kim & Huber, 2010b).

1.8 CONCLUSION

The economic loss caused by the reduction in wheat FN is critical to both wheat growers and the wheat industry. FN measurement was developed to assist the industry in detecting sprouting damage in order to ensure product quality. The method was designed to show the reduction in starch paste viscosity due to increased α-amylase activity. However, the paste viscosity of starch can be affected by many factors such as starch structural characteristics, the interactions between starch and other macromolecules, and the relationship of starch with starch degrading enzymes. Starch paste viscosity is also impacted by wheat cultivars, growing environments, and agronomic managements. Starch paste viscosity affects not only FN but also wheat end-use quality, regardless of the types of products produced. The low FN issue

needs to be solved, and research should focus on end-use quality, instead of the falling number, in order to increase agricultural sustainability and the profitability of growers

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1.10 TABLES AND FIGURES

Table 1.1 Categorization of α-amylases in PHS and LMA affected wheat

Categories	Pre-harvest sprouting (PHS)	Late maturity α-amylase (LMA)
Isozymes[1]	α−Amylase 1 & α−Amylase 2 [(a)]	α−Amylase 1 & α−Amylase 4 [2; (b, c, d)]
Isoelectric point	High & low pI [(e, f)]	High pI [(b, c, g, h)]
Enzyme generating stage[3]	Malt & green [(a, i)]	–
Synthesis location[4]	Close to the scutellum [(h)]	Scattered in grain [(h)]

Note. [1] The names of isozymes are associated with the *α-Amylase* (*Amy;* i.e., *α-Amylase1, α-Amylase2, α-Amylase4*) genes controlled the production of those isozymes. The common abbreviations used in the literatures are AMY and TaAMY. [2] LMA is characterized by abnormally elevated level of AMY1 in the aleurone layer during grain development through harvest, however, Mieog et al. (2017) reported the potential involvement of AMY4 in the LMA phenotype. [3] Current literature does not describe α-amylase in LMA-affected wheat as either malt or green α-amylase. [4]See Figure 1.1.

[(a)] Gale & Ainsworth, 1984; [(b)] Mares & Mrva, 2008; [(c)] Newberry et al., 2018; [(d)] Mieog et al., 2017); [(e)] Kruger & Marchylo, 1985; [(f)] Verity, Hac, & Skerritt, 1999; [(g)] Mares & Mrva, 2014; [(h)] Mrva, Wallwork, & Mares, 2006; [(i)] Olered & Jönsson, 1970

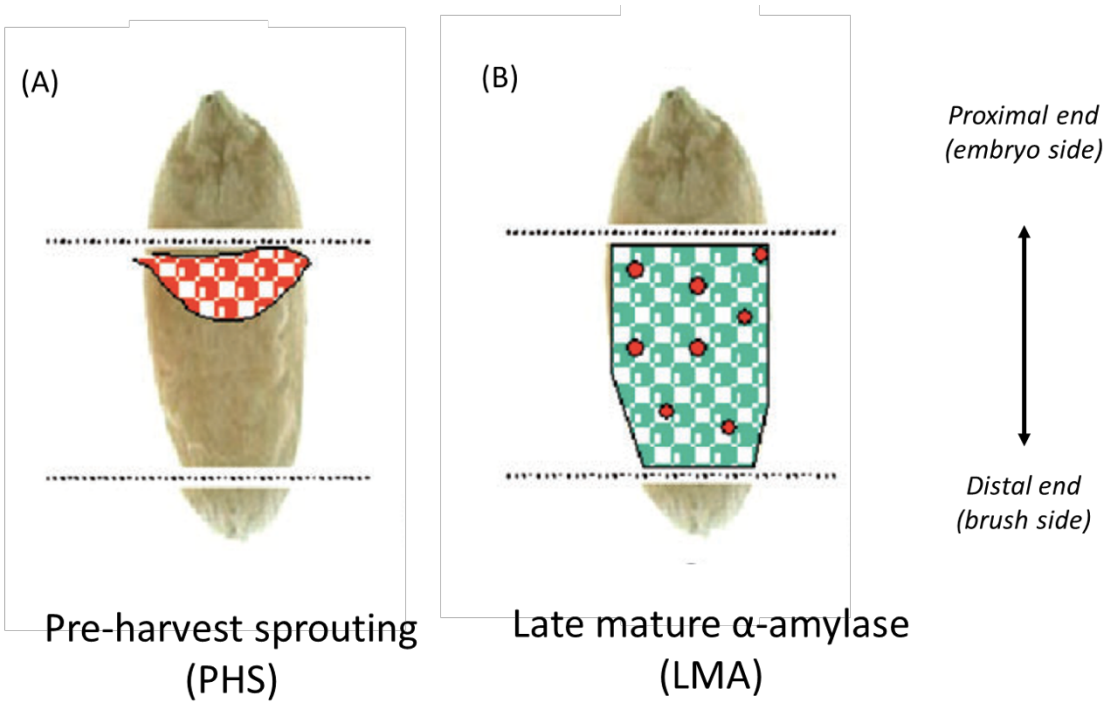

Figure 1.1 Distribution of dead aleurone cells in pre-harvest sprouting (A) and late-maturity α-amylase affected (B) wheat kernels. Red and green dots represent dead and live aleurone cells, respectively. The double-headed arrows demonstrated the descriptive ends and sides of wheat kernels. Reprinted with minor editing from "α-Amylase and programmed cell death in aleurone of ripening wheat grains," by K. Mrva, M. Wallwork, and D. J. Mares, 2006, Journal of Experimental Botany, 57(4), p. 884. Copyright 2006 by the authors. Reprinted with permission.

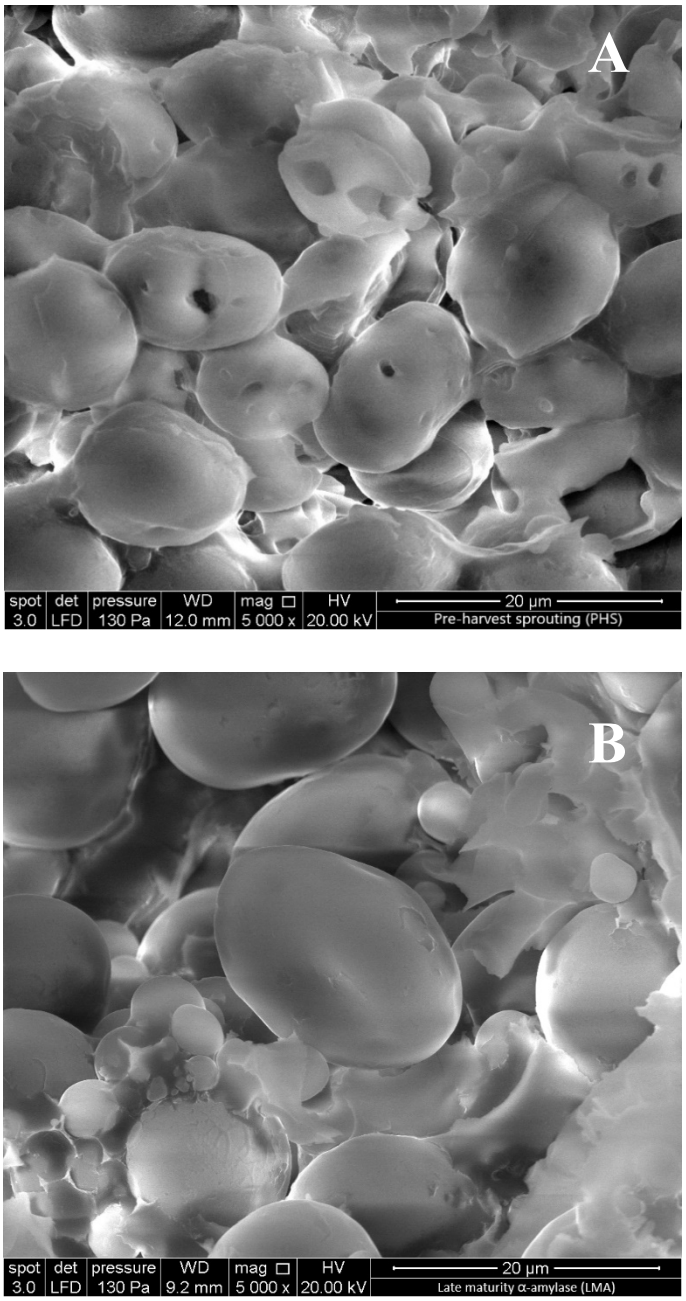

Figure 1.2 SEM images of starch granules of pre-harvest sprouting wheat (A) and late-maturity α-amylase affected (B) wheat. The holes shown on the granule surface in Figure (A) are generated by starch degrading enzymes (i.e., α-amylase). Both images are the soft white winter wheat SY Ovation grown in the Pacific Northwest in the Unites States.

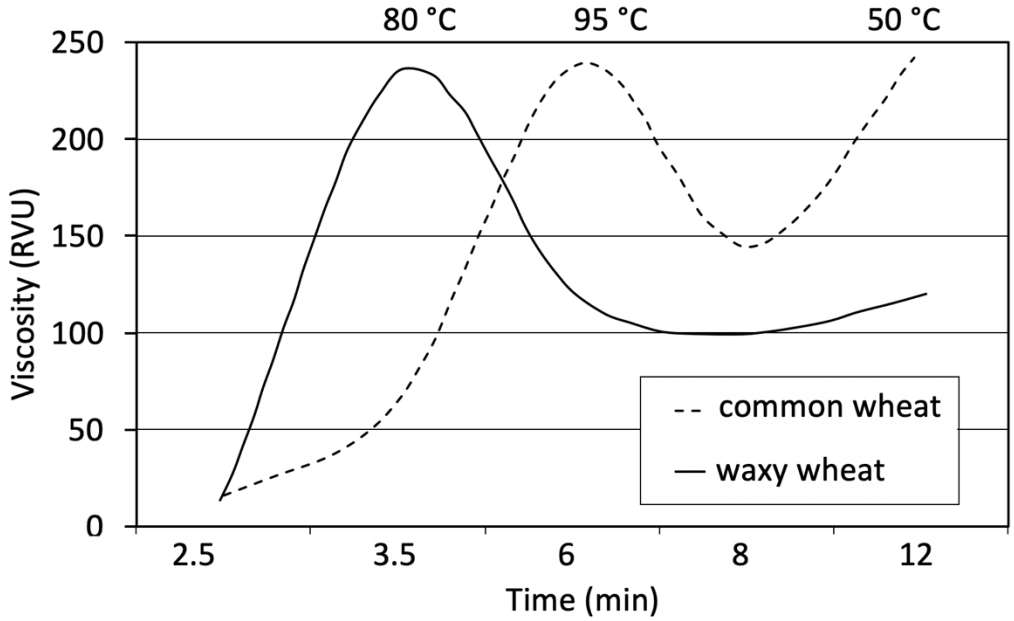

Figure 1.3 Paste viscosity of waxy (solid line) and common wheat (dashed line) measured by an RVA. Reprinted from "Aberrant falling numbers of waxy wheats independent of α-amylase activity," R. A. Graybosch, G. Guo, and D. R. Shelton, 200, Cereal Chemistry, 77(1), p. 2. Reprinted with permission.

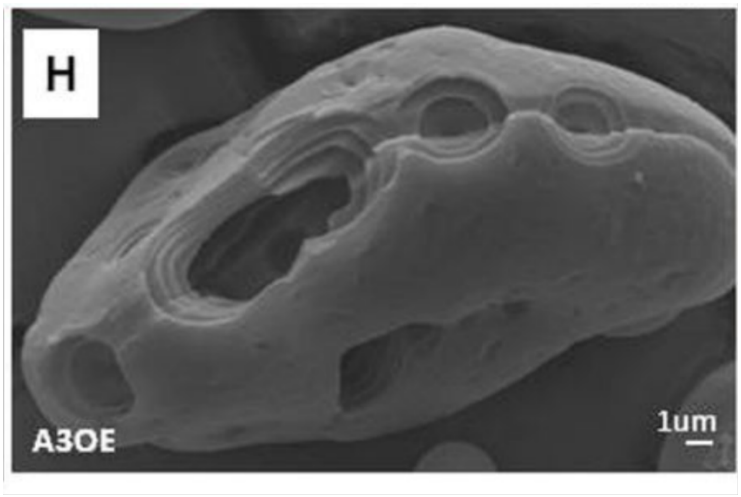

Figure 1.4 SEM images of wheat starch granules in overexpressed α-amylase wheat. Reprinted from "Engineering α-amylase levels in wheat grain suggests a highly sophisticated level of carbohydrate regulation during development," A. Whan, A. S. Dielen, J. Mieog, A.F. Bowerman, H. M., Robinson, K. Byrne, …, and J.P. Ral, 2014, Journal of Experimental Botany, 65(18), p. 5452. Reprinted with permission.

CHAPTER 2 EVOLVEMENT OF STARCH STRUCTURE IN THE DEVELOPING ENDOSPERM OF SOFT WHITE WINTER WHEAT

2.1 ABSTRACT

Low falling number (FN) issue resulted a loss of $140 million in 2016 in the wheat industry in Pacific Northwest (PNW). The reduction of FN indicates an elevation of α-amylase activity, which hydrolyzes starch and decreases the flour paste viscosity. From the previous study, our group revealed a developmental change starch granule in some low FN wheat. We hypothesized the starch synthesis can be influenced by some environmental factors (e.g. temperature) and consequently change the starch characteristics (e.g. granular size, amylose content), which further impact the FN and end-use quality. However, there was a knowledge gap of lacking information about starch developmental change of soft white wheat. The objective of this study was to investigate the starch synthesis during the wheat development period in order to understand the impact of environmental factors on starch property changes. Starch was isolated from two soft white wheats, SY Ovation and UI Sparrow, harvested throughout the grain development period, and further fractionated into large and small starch granules to examine structural and properties. Our findings revealed the soft white wheat on Day 7 and Day 14 initiated of large A-type starch granule and small B-type granule, respectively. Day 14 to Day 28 was the most important period for starch synthesis because starch content increased rapidly and reached to the plateau. Although the wheat reached physiological maturity on Day 35, the amylose content and thermal properties (i.e. enthalpy change and temperature range) further adjusted until Day 42. The different critical development stages of soft white wheat can be used to identify the influenced starch properties when environmental stress happened at a particular stage. Future research and breeding program can be benefit by the information through genetic control on developing varieties with a high resistance to the environmental stresses and found the potential solution on the low falling number issue.

2.2 INTRODUCTION

Wheat is an important crop in the Pacific Northwest in the United States. The six wheat classes include hard red winter, hard red spring, hard white, durum, soft white, and soft red winter (McFall & Fowler, 2009); the production of soft white wheat is critical to the agricultural economy in the Northwest. Three states: Idaho, Washington, and Oregon, generate 92% of the production of soft white wheat in the United States (U.S. Wheat Associates, 2017) and over 90% of production is exported outside of the United States to countries such as Philippines, Japan, Korea, Indonesia, Yemen, and Thailand (U.S. Department of Agriculture National Economic Research Service, 2019; U.S. Department of Agriculture Federal Grain Inspection Service, 2019). Growers' profits are primarily determined by the yield, but falling number, a measurement of grain α-amylase activity, can directly impact the grain price or cause a purchase rejection. The falling number test was developed in 1961 in order to identify invisible sprouting damage (Hagberg, 1960, 1961; Perten, 1964). The grain with low falling number, which is measured in seconds, was given a reduced price. For example, in 2016, wheat in the United States was devalued $0.25 for every 25 seconds the falling number decreased below 300 seconds (Delwiche, Vinyard, & Bettge, 2015; Personal communication, Idaho Wheat Commission, Boise, ID, United States). Low falling number wheat was segregated into the feed market or blended with other wheat. The Northwest is known to consistently provide premium, quality wheat. However, the unexpected reduction of falling number, regardless of wheat varieties or planted areas, caused losses of $100 million and $140 million in 2014 and 2016, respectively (Campbell, 2016; Weaver, 2018; Personal communication, Idaho Wheat Commission, Boise, ID, United States). The low falling number crisis directly affects growers' profits, disturbs the market supply capacity, causes a decrease of production acres, and negatively impacts the competitiveness in the global market.

The falling number test demonstrates how quickly starch is liquefied by elevated α-amylase activity. Thus, a wheat sample with starch that liquefies quickly, has a lower falling number. In this measurement, wheat wholemeal flour was mixed with water in a falling number test tube, and a stirrer was inserted into the tube. Then, the flour-water mixture was heated in boiling water to gelatinize starch, which generates a viscous flour paste. Then, the stirrer was dropped from the top to the bottom of the tube, and the time (seconds) the stirrer

took to reach the bottom is defined as falling number (AACC International, 2010). A falling number below 300 seconds indicates an elevated α-amylase activity and poor end-use quality. This assumption has been challenged by researchers, but the falling number measurement is still a standard method implemented in the United States and other countries.

Research has shown that the elevation of α-amylase activity (or quantity) is not the only cause for the decrease of FN (He, Lin, Chen, Tsai, & Lin, 2019). Starch, the substrate of α-amylase, provides the viscosity of wholemeal flour paste, and its relationship (e.g., susceptibility to the enzyme) with α-amylase has a direct influence on FN and the end-use quality (Finney, Natsuaki, Bolte, Mathewson, & Pomeranz, 1981; Kaldy & Rubenthaler, 1987; Perten, 1964). Some low FN wheat indeed has a lack of α-amylase activity (Steber, 2016b), and some low FN wheat has a developmental change of starch in that it has an increased proportion of small B-type wheat starch granules (Shao, Tsai, He, Chen, Wilson, & Lin, 2019). Starch, in some plants, such as wheat, barley, and rye, consists of two types of starch granules: large, disk-shaped A-type granules (diameter > 10 μm), and small, spherical B-type starch granules (Peng, Gao, Abdel-Aal, Hucl, & Chibbar, 1999). A- and B-type wheat starch granules are synthesized differently and differ in their structure and physical and chemical properties (Ao & Jane, 2007; Kruger & Marchylo, 1985). An increase of B-type granules leads to a lower paste viscosity and a higher susceptibility to α-amylase; therefore, B-type wheat starch liquefies quicker than A-type wheat starch. Consequently, such a developmental change of starch negatively impacts the FN (Shao et al., 2019). We hypothesized the developmental change of starch in the low falling number wheat was triggered by environmental stresses (e.g., temperature), which influence starch synthesis resulting in the change of starch functionality (e.g., paste viscosity), FN, and wheat end-use quality. However, it is challenging to prove that wheat starch synthesis in low falling number wheat is affected by the environment because of a of lack of information regarding the evolvement of starch structure during grain development. In this study, our objective is to fill that gap and advance current knowledge by revealing how starch structure develops during the grain filling period in soft white wheat in order to understand how starch structure and functionality can be manipulated by the environment at particular times during grain development.

2.3 MATERIALS AND METHODS

2.3.1 Materials

Two popular soft white winter wheats (*Triticum aestivum* L.), SY Ovation and UI Sparrow, were selected for this study. Both wheat varieties were developed in the Pacific Northwest in the United States and are known for their good falling numbers, which are usually above 300 seconds. However, the SY Ovation wheat had a low falling number in over 80 percent of the planting locations when the low falling number issue broke out in the region in 2016. UI Sparrow was also affected but only about 40 percent of the locations had a low falling number (Steber, 2016a; Schroeder et al, 2017, unpublished data). Both wheats were planted in October of 2016 and harvested in July of 2017 in Tammany, Idaho. We began to sample wheat kernels four days after 90% of the wheat in our field reached the flowering (anthesis) stage, which occurs when 50% of spikelets within the same wheat head are flowering (Waduge, Xu, Bertoft, & Seetharaman, 2013; Kalinga, Bertoft, Tetlow, Liu, Yada, & Seetharaman, 2014). The images of a wheat head and spikelet are shown in Figure 2.1 and 2.2. Both wheat varieties physiologically matured on Day 35 after anthesis when they lost the green pigment on the wheat head (glumes and grains), flag leaves, and strand (Mares, 1989), and the wheat on Day 42 was identified as ripened and ready to harvest. The entire research strip plot was harvested on Day 49 (Schroeder and White, 2018).

We handpicked wheat heads on Day 4, Day 7, Day 10, Day 14, Day 21, Day 28, Day 35, and Day 42 after anthesis, and samples were placed in a cooler filled with dry ice before they were shipped to the laboratory. Then, all of the wheat heads were washed gently with deionized tap water to remove soil and debris. We collected the kernels located at the middle section of the wheat heads, which excluded the top five and bottom five spikelets (Figure 2.1). This process was performed on ice to diminish the interference of biochemical activities (e.g. enzyme hydrolytic activity) with our analyses. Collected wheat kernels were immediately transferred and stored in liquid nitrogen for further examination.

The sieves with 50-µm, 75-µm, and 150-µm pore sizes were purchased from VWR International (Radnor, PA), and the sieve with a 10-µm pore size was purchased from Advantech Manufacturing, Inc. (New Berlin, WI). Syringe filters with various sizes of membranes, made of polytetrafluoroethylene (PTFE), were purchased from GE Healthcare (Chicago, IL) and Thermal Scientific Inc. (Rockwood, TN). The examinations and analyses

described below were conducted in a laboratory with a humidity level of 10 ± 2 % and at a room temperature of 20 °C to 22 °C. This study was conducted in a location with a pressure of 29.68 Hg and an elevation of 2709 feet (825 m) above sea level where the temperature of boiling water was 97.09 °C.

2.3.2 Isolating starch from developing wheat kernels

Starch was isolated following Shinde, Nelson, and Huber (2003) with modifications. Thirty-five grams of frozen kernels were ground in a juice blender (Osterizer 6650; Sunbeam Products, Inc., Boca Raton, FL) for one minute. The wholemeal flour slurry was suspended in 0.02 M hydrochloric acid (350 mL, prepared by diluting the commercial hydrochloric acid solution [36.5% to 38%, v/v], Macron, Avantor Performance Materials LLC, Center Valley, PA) and stirred for 10 min, followed by the addition of sodium bisulfite (0.1750 g, ≥ 97%, J.T. Baker, Avantor Performance Materials LLC, Center Valley, PA) and thimerosal (0.0035 g, ≥ 97%, Sigma Aldrich, St. Louis, MO). The pH was adjusted to 7.6 by adding 0.05 M Tris-(hydroxymethyl) aminomethane buffer (Trizma® base, ≥ 99.8%, Sigma-Aldrich, Inc. St. Louis, MO). Then, the protein in the wholemeal flour was hydrolyzed by protease, which was prepared by combining 0.1750 g protease (XIV type, Sigma-Aldrich, St. Louis, MO) and 0.02 M hydrochloric acid solution (10 mL; described above). The protein was hydrolyzed at 21°C for 1 h with continuous horizontal shaking (about 120 rpm) in a water bath shaker (American Optical Scientific Instrument Division, Buffalo, NY). This condition was determined according to our preliminary study (data not shown). The slurry was filtered through a sieve with a 150-µm pore size to remove bran fragments, and the first filtrate was collected. The retentate (i.e. wheat bran or large kernel pieces) on the sieve were mixed with deionized tap water (4 °C) and stirred for 30 s, passed through a sieve with a 150-µm pore size, and collected as the second filtrate. The first and second filtrate was collected and combined together and then centrifuged at 5,500 ×*g* for 20 min at 4 °C. The pellet was collected and fully suspended in an 80% (w/v) cesium chloride solution (50 mL, ≥ 99%, Fisher Scientific, Fair Lawn, NJ) and then centrifuged at 14,000 ×*g* for 20 min at 4 °C. After centrifugation, the brown layer on the top of the white starch pellet was scraped by a spatula, transferred into another container, suspended in an 80% (w/v) cesium chloride solution (25 mL), and then

centrifuged at 14,000 ×*g* for 20 min at 4 °C. This procedure was repeated twice to fully recover the starch from the brown layer. The combined starch was suspended with 200-mL deionized water and then recovered by centrifugation at 9,500 ×*g* for 20 min at 4 °C; this washing procedure was repeated at least four times until cesium chloride was completely removed. An aliquot (about 200 to 400 µL) of silver nitrate solution (0.1 M, ≥ 99%, Sigma Aldrich, St. Louis, MO) was added into the supernatant to validate that the supernatant was free of chloride ions. The purified starch was suspended homogenously with 100-mL deionized tap water and passed through a sieve with a 75-µm pore size to remove the trace amounts of insoluble fibers. Starch was recovered after it was filtered through a glass microfiber filter with a retention size of 1.2 µm (Whatman®, GE Healthcare Life Sciences, Maidstone, United Kingdom) and air-dried in a hood for 48 h.

2.3.3 Measuring the falling number of wholemeal flour

Kernels collected on Day 42 were milled using a Udy cyclone sample mill (Udy Corporation, Fort Collins, CO) consisting of a screen with a pore size of 0.5 mm. The falling number of the wholemeal flour was measured following the AACCI method (AACC International, 2010) using a falling number machine (Perten Falling Number® 1000, Perten Instrument, Inc., Springfield, IL). Deionized tap water (25.0 mL at the room temperature) was added to 7.0 g wholemeal flour (14% moisture) in a falling number tube, then homogenously mixed using a shaker for 3 s (Shakematic 1095, Perten Instrument, Inc., Springfield, IL). A stirrer was inserted into the falling number tube, and the falling number tube was placed in boiling water for 5 s and stirred vertically for 55 s with the stirrer. Then (after a total of 60 s), the stirrer was released by the machine, and the time for the stirrer to drop to the bottom of the tube was recorded as the falling number.

2.3.4 Quantifying the moisture content of the developing kernels

Wheat kernels, which were stored in liquid nitrogen, were weighed (0.5 – 0.6 g total wt) into 2-mL microtubes, which were placed in a basket filled with dry ice. Then, the kernels were placed in liquid nitrogen again, followed by freeze drying in a Flexi-Dry MP Manifold Lyophilizer (FTS Systems, Inc., Stone Ridge, NY) for 48 h. We measured the loss of kernel

weight after freeze drying. Then, the freeze-dried kernels were ground using a mortar and pestle, passed through a sieve with a 0.5-mm pore size, and dried (0.1 g) in an air force oven (Lab-Line Instruments, Inc, Melrose Park, IL) controlled at 105 °C for 12 h. The moisture content was calculated based on the total weight lost during the freeze- and oven-drying. Each sample was measured in triplicate.

2.3.5 Quantifying starch amount in developing kernels

The starch content was quantified using the Megazyme total starch kit (Megazyme u.c., Wicklow, Ireland). In brief, the wholemeal flour was mixed with 2 M potassium hydroxide (≥ 87.2%, Fisher Scientific, Hampton, NH) for 20 min in an ice bath, and hydrolyzed by α-amylase and amyloglucosidase into glucose at 50 °C for 30 min. Glucose was quantified by conducting a glucose-oxidase-plus-peroxidase-based reaction, and then, the glucose quantity was used to calculate the starch amount.

2.3.6 Separating large and small starch granules

Wheat starch granules were separated following the method developed by Dhital, Shrestha, and Gidley (2010) with modifications. Starch granules (5 g dry wt) were suspended in a 250-mL graduated cylinder filled with 250 mL deionized tap water. The graduated cylinder (4 cm × 30 cm) was sealed with parafilm and inverted five times to make a homogenous suspension, which took about 30 s to 45 s. The suspension was set at room temperature for 58.8 min; then, a pipette, connected with a vacuum pump, was used to collect the top 10-cm solution. Next, the graduated cylinder was refilled with deionized tap water to the 250-mL mark and repeated the procedure until the top 10 cm of the solution did not contain any starch granules, which was validated by examining the solution with a polarizing light microscope (Nikon Eclipse E600, Nikon Instruments, Melville, NY). The collected top suspension, containing small starch granules, was filtered through a sieve with a 10-μm pore size, and recovered by centrifugation at 9,500 ×*g* at 4 °C for 20 min. The remaining suspension in the graduated cylinder, containing large starch granules, was recovered by centrifugation under the same conditions as described above. The starch pellet was dried at room temperature in a fume hood for 48 h.

2.3.7 Analyzing starch thermal properties

A differential scanning calorimeter (DSC; model Diamond, Perkin-Elmer Co., Norwalk, CT) was used to evaluate starch thermal properties. Starch (3.0 mg, dry wt) was weighed into stainless-steel DSC pans and moistened with 6 μL of deionized tap water. The pan was hermetically sealed and equilibrated at room temperature for one hour prior to examination. Data were collected at a heating rate of 10 ºC/min over a temperature range of 25 ºC to 130 ºC. The temperature and enthalpy calibration for the instrument were performed with pure indium, which has a melting point of 156.6°C and heat of fusion of 28.45 J/g.

2.3.8 Examining the particle size distribution of starch granules

Starch (0.030 g, dry wt) was suspended with 40 μL of 10% (v/v) Tween® 80 solution in a 2-mL microtube (Difco Laboratories Inc., Detroit, MI) and followed by adding 1.5 mL deionized tap water to the starch-Tween solution mixture. The suspension was thoroughly mixed by a vortex at a high speed for one minute. An aliquot (10 μL) of the suspension was then transferred to a 600-mL beaker containing 400 mL deionized tap water and examined with a particle size analyzer (AccuSizer 780, Particle Sizing Systems, Santa Barbara, CA) with continuous stirring. Each sample was examined six times, and the suspension was thoroughly mixed between each measurement.

2.3.9 Examining the morphology of developing wheat kernels and starch granules

Wheat kernels were cut across the center of the grain (Figure 2.3) using a double-edged razor blade and then covered by 500-μL fixative solution at 4 °C for 24 h. The fixation solution consisted of 2% (v/v) paraformaldehyde, 2% (v/v) glutaraldehyde, and 0.1 M sodium phosphate buffer at pH 7.2. Both paraformaldehyde and glutaraldehyde were diluted from stock solutions (purity ≥ 95.5%) purchased from Electron Microscope Science (Hatfield, PA), and the sodium phosphate buffer was prepared by dissolving sodium phosphate dibasic and sodium phosphate monobasic (purity ≥ 98%, J.T. Baker, Avantor Performance Materials LLC, Center Valley, PA) in deionized tap water. Next, we added deionized tap water (1 mL) and washed the fixative solution three times. After removing the water from the last washing, an aliquot (300 μL) of 1% (v/v) osmium tetroxide (diluted from 4% [v/v] osmium tetroxide aqueous solution with purity ≥ 99.9%, Structure Probe, Inc., West Chester, PA) was added

onto the top of the fixed kernels, and the samples were kept at 4 °C. After 12 h, wheat kernels were dehydrated by adding 500 µL of 30% (v/v) ethanol two times with a gradual increase of the concentration to 50%, 70%, 80%, 90%, 95%, and 100% (v/v) of ethanol. The ethanol-dehydrated kernels were further dried in a Samdri-PVT-3B critical point dryer (Tousimis Research Co., Rockville, MD). Dried kernels were placed on aluminum scanning electron microscope (SEM) stubs with an adhesive carbon tape and examined using a Quanta 200F SEM (Field Electron and Ion Company, Hillsboro, OR) at a low vacuum condition with an accelerating voltage of 20 kV.

2.3.10 Examining the chain-length distribution of starch molecules

Gelatinizing starch Wheat starch (80 mg, dry wt) was suspended in 800 µL deionized tap water; then, 7.2 mL dimethyl sulfoxide (DMSO, 99.97%, Fisher Scientific, Hampton, NH) was added into the suspension. The starch-DMSO mixture was heated in boiling water with gentle stirring at 50 rpm for 1 h. The mixture was transferred onto another stirring plate without heating and stirred gently for another 12 h. Four times volume (32 mL) of 100% (v/v) ethanol were added drop by drop and set at room temperature for 30 min. The ethanol-starch suspension was centrifuged at 5,000 ×*g* for 20 min at room temperature, and then, the supernatant was discarded. The pellet was re-suspended in 0.8-mL deionized tap water, and then 7.2-mL DMSO was added to the suspension. The starch-DMSO mixture was heated in boiling water again for another 20 min with gentle stirring at 50 rpm. Then, the gelatinized starch solution was cooled down to room temperature for further analysis (Next section: debranching starch molecules).

Debranching starch molecules Gelatinized starch (6 mL, 10 mg/mL, Section: gelatinizing starch) was precipitated from the starch-DMSO solution by adding 24 mL ethanol (100% [v/v]), then set at room temperature for 30 min, and centrifuged at 4,000 ×*g* for 20 min. The starch pellet was re-dissolved in 4.86 mL deionized tap water and heated in a boiling water bath for 20 min with gentle stirring. Then, the mixture was cooled to approximately 40 ℃. An aliquot (810 µL) of starch solution was transferred into a 2-mL microtube and mixed with 90 µL of 1 M sodium acetate buffer (pH 5.0; prepared using acetic acid, 100%, Fisher Scientific, Hampton, NH; and sodium acetate, ≥ 99.0%, J.T. Baker, Avantor Performance Materials LLC, Center Valley, PA). Enzyme Solution I and Enzyme Solution II, which

contain varying amounts of pullulanase (from *Klebsiella planticola,* about 30 U/mg of pullulan at 40 °C with pH 5.0, Megazyme u.c, Wicklow, Ireland) and isoamylase (from *Pseudomonas* sp., 240 U/mg of oyster glycogen at 40 °C with pH 4.0, Megazyme u.c., Wicklow, Ireland) were prepared. Enzyme Solution I was a mixture of 1 μL pullulanase, 1 μL isoamylase, and 93 μL 0.1 M sodium acetate buffer (pH 5.0); Enzyme Solution II was a mixture of 1 μL pullulanase, 1 μL isoamylase, and 3 μL 0.1 M sodium acetate buffer (pH 5.0). An aliquot of Enzyme Solution I (50 μL) was added to the starch solution, and debranching was performed in a 40 ℃ water bath with horizontal shaking at 80 rpm. After 1 h, the rest of Enzyme Solution I (45 μL) was added to the mixture, and debranching continued for another hour before adding 5 μL Enzyme Solution II. Then, debranching continued for another 5 h. The enzymes were added multiple times in order to maintain high hydrolytic activity during the debranching reaction (total seven hours). We avoided using a high quantity of enzymes in this study due to concerns of interference with the chromatography; in addition, we wanted to avoid creating undesired side reactions (e.g., transferase reaction) (Lin, Chang, Chou, & Lu, 2011). Enzyme activities were inactivated by heating the mixture in a boiling water bath for 10 min. After cooling to room temperature, an aliquot (200 μL) of debranched starch solution was transferred to a new 2-mL microtube and diluted with 800 μL 0.1 M sodium nitrate with 0.02% (w/v) sodium azide, which was the same as the mobile phase of the chromatography, for obtaining optimum intensity of the RI signal. Then, an aliquot of 30 μL of 0.1 M barium acetate solution (> 99%, Acros Organics, Thermo Scientific, Belgium) was added, and the solution was gently mixed for one minute to remove the ammonium sulfate, which can interfere with the chromatography by generating a peak with the same elution volume as starch molecular peaks (Lin et al, 2011). The barium sulfate precipitates were removed by centrifuging at 4000 ×*g* for 5 min, and the supernatant was filtered through a 2.7-μm pore size syringe filter.

Analyzing starch molecule chain-length distribution The debranched starch molecules were injected onto a High-Performance Size Exclusion Chromatography (HPSEC) system equipped with Waters 1525 Binary HPLC pump (Waters Co., Milford, MA), a Rheodyne® injector Model 7725i with 200 μL sample loop (IDEX Corporation, Rohnert Park, CA), and a Waters 2410 refractive index (RI) detector (25 °C, Waters Co., Milford, MA). A guard column OHpak SB-G 6B (50 mm × 6 mm, Shodex, Showa Denko KK, Japan) and a series of

two size exclusion columns, OHpak SB-804 HQ (300 mm × 8 mm) and SB-802 HQ (300 mm × 8 mm, Shodex, Showa Denko KK, Japan), were connected and placed in a column oven (Model 5CH, Waters Co., Milford, MA) set at 50 °C. Columns were eluted with 0.1 M sodium nitrate with 0.02% (w/v) sodium azide at 0.4 mL/min (Lin et al., 2011). The calibration curve of retention volume and molecular weight was made with a series of pullulan standards (Shodex Standard P-82, JM Science Inc., Grand Island, NY) with molecular weights of 212000, 112000, 22800, and 5900 Da, and maltohelptaose (> 90%, TCI Chemical Trading Co., Ltd., Tokyo, Japan) and maltotetraose (> 90%, Megazyme, Wicklow, Ireland) with molecular weights of 1153 and 667 Da. The chromatogram was recorded and processed using Waters® Breeze™ Software Version 3.30 (Waters Co., Milford, MA).

2.3.11 Data analysis

Results were presented in the format of mean (*M*) and standard deviation (*SD*). The significant difference of samples collected at different days was analyzed by one-way analysis of variance (ANOVA) and Tukey's test at a significance level $\alpha = .05$. A student independent *t* test was also used to compare the difference between the starch granules of SY Ovation and UI Sparrow at the same growing stage (significant level $\alpha = .05$). Statistical software, SPSS® (Version 25, IBM Cooperation, Armonk, NY), and was used for analyzing the data.

2.4 RESULT

2.4.1 Temperature and precipitation during the wheat growing period

The two soft white winter wheat varieties were planted on October 3, 2016 in Tammany, Idaho, and had different growth rates in the same field. A fully mature heading is defined as when the wheat head emerges above flag leaf ligule as shown in Figure 2.4. SY Ovation wheat fully headed on May 27, 2017, flowered on June 1, 2017 (defined as zero days after anthesis; Day 0), reached physiological maturity on July 6, 2017 (Day 35), and was harvested on July 13, 2017 (Day 42); UI Sparrow wheat fully headed on June 2, 2017, flowered on June 4, 2017 (Day 0), reached physiological maturity on July 9, 2017 (Day 42), and was harvested on July 16, 2017 (Day 49). A weather station was installed in the field to record the temperature and precipitation (Figure 2.5). During the grain development period (from late May to the mid of July), the highest temperature in a day varied from 16 °C to 38 °C, and the

lowest temperature in a day ranged from 4 °C to 19 °C. The temperatures in our study were slightly higher than the historical average of temperatures (since 1882; National Weather Service, 2017) during the grain development period of soft white winter wheat, which the highest temperature in a day varied from 21 °C to 34 °C and the lowest temperature in a day ranged from 9 °C to 16 °C. The period from Day 20 to Day 30 is a sensitive time in which temperature shocks can trigger late-maturity α-amylase and lead to a reduction of falling number (Mrva and Mares, 2001; Mares and Mrva, 2014). Temperature fluctuations over 10 °C within a short time period (e.g., two days) at about anthesis to Day 6 that observed in the Pacific Northwest area in 2014 has also been speculated as another trigger of low falling number (Shao et al., 2019). During our study, temperature conditions were considered average for the season, and we did not observe a heat or cold shock or an extreme temperature fluctuation.

Pre-harvest rain is the other primary cause of low falling number, which was not observed in 2017. According to the National Weather Service (2017), the greatest amount of precipitation primarily occurs during the kernel development period (May and June in this study), and the precipitation averages about 31.15 mm in late May to June and 0.25 mm in July. In our study, the precipitation was 26.64 mm from May to June and 0 mm in July, and there were only nine rainy days during the grain development period. Daily precipitation ranged from 0.25 mm to 8.32 mm at the initial stage of the grain development period; after Day 20, there was no detectable precipitation.

2.4.2 The morphology of the developing wheat kernels

To examine the developing endosperm and starch, wheat kernels were cross-sectioned (demonstrated in Figure 2.3) and examined using an SEM. On Day 7, the wheat kernels of SY Ovation were very small (length 2.3 mm × width 1.6 mm) with a thick aleurone layer (0.25 mm) that surrounded the C-shaped endosperm (1.75 mm × 0.25 mm) (Figure 2.6A). The kernel size increased quickly in the first three weeks: 2.6 mm × 1.8 mm (Day 10), 3.2 mm × 2.4 mm (Day 14), 3.4 mm × 2.5 mm (Day 21), 3.6 mm × 2.9 mm (Day 28), and 4.0 mm × 3.0 mm (Day 35) (Figure 2.6, B-F). After reaching grain maturity on Day 35, the wheat kernels began to dehydrate and ripen, and the size of the wheat kernels decreased to 3.5 mm × 2.7 mm on Day 42 (Figure 2.6G). The aleurone layers became thinner during grain development:

0.3 mm (Day 7 and Day 10), 0.2 mm (Day 14), and became less than 0.1 mm after Day 21. The size of the endosperm increased quickly in the first three weeks and continued increasing until harvest: 1.6 mm × 0.4 mm (Day 7), 2.2 mm × 0.7 mm (Day 10), 2.5 mm × 1.5 mm (Day 14), 2.9 mm × 2.3 mm (Day 21), 3.6 mm × 2.9 mm (Day 28), 4.0 mm × 3.0 mm (Day 35), and 3.5 mm × 2.7 mm (Day 42). On Day 28, we observed that the endosperm had occupied the entire space of the kernels.

The development of the UI Sparrow kernels was very similar to that of SY Ovation. On Day 7, the wheat kernels of UI Sparrow were also very small (2.3 mm × 1.4 mm); the kernels increased quickly in the first three weeks and continued increasing until Day 35: 2.9 mm × 2.0 mm (Day 10), 3.1 mm × 2.0 mm (Day 14), and 3.1 mm × 2.0 mm (Day 21), 3.2 mm × 2.7 mm (Day 28), 3.6 mm × 2.8 mm (Day 35); and then slightly decreased to 3.5 mm × 2.6 mm on Day 42 (Figure 2.7, B-G). On Day 7, a thick aleurone layer (0.4 mm) that surrounded the C-shaped endosperm (1.4 mm × 0.3 mm) (Figure 2.7A). The aleurone layers became thinner during kernel development: 0.4 mm (Day 7), 0.3 mm (Day 10), 0.2 mm (Day 14), and became less than 0.1 mm after Day 21. The size of the endosperm increased in the first three weeks and continued increasing until Day 35: 1.4 mm × 0.3 mm (Day 7), 1.9 mm × 0.9 mm (Day 10), 2.7 mm × 1.5 mm (Day 14), 2.8 mm × 1.6 mm (Day 21), 3.2 mm ×2.7 mm (Day 28), 3.6 mm × 2.8 mm (Day 35); and then decreased to 3.5 mm × 2.6 mm (Day 42). As with SY Ovation, we observed that the endosperm occupied the entire space of the kernels on Day 28. The wheat kernels of UI Sparrow were approximately 0.1 mm to 0.4 mm smaller than the wheat kernels of SY Ovation from Day 21 to Day 42.

2.4.3 The morphology of developing starch granules

The starch granules of the wheat endosperm were visible on Day 7 in both SY Ovation and UI Sparrow; they were very small, with a diameter of 2 mm to 4 mm, respectively, and were spherical in shape (Figure 2.8a and Figure 2.9a). From Day 7 to Day 10, the granule size increased to 5 μm for SY Ovation (Figure 2.8b) and 8 μm for UI Sparrow (Figure 2.9b) and were relatively uniform in size. On Day 14, both large and small starch granules were observed on the endosperm. The large granules were disk shaped while the small starch granules were spherical in shape. Throughout the starch development, the size of the starch granules continuously increased. For SY Ovation, the SEM revealed that the size of large

starch granules continuously increased: 14 µm (Day 14), 22 µm (Day 21), 23 µm (Day 28), 27 µm (Day 35), and then decreased to 25 µm (Day 42). A similar observation of the change in the size of the granules was also found in small starch granules. The granules initially increased: 2.5 µm (Day 14), 5.5 µm (Day 21), 7 µm (Day 28 and Day 35), and then they decreased to 6 µm (Day 42). UI Sparrow had a similar pattern with an increase in the diameter of the granules from Day 14 to Day 28 and then the granules maintained a similar size from Day 35 until Day 42. The large starch granule sizes were 20 µm (Day 14), 22 µm (Day 21), 24 µm (Day 28), 25 µm (Day 35), and 27 µm (Day 42). The small starch granule sizes were 2 µm (Day 14), 3 µm (Day 21), 5 µm (Day 28), 6 µm (Day 35), and 7 µm (Day 42).

2.4.4 The falling number of mature wheat

The falling number of SY Ovation and UI Sparrow was 345 s and 337 s, respectively, which meets the industrial expectation of above 300 s (Figure 2.10). There was no significant difference in the falling numbers of the two varieties (p = .567 at significant level α =. 05).

2.4.5 Moisture content of developing wheat kernels

The moisture content of SY Ovation kernels did not have a significant change in the first two weeks: 72.62% (Day 7), 73.59% (Day 10), and 72.66% (Day 14; p = .097), and then, the moisture content quickly decreased to 64.65% (Day 21), 51.25% (Day 28), and 42.24% (Day 35) (p < .001, Table 1, Figure 2.11). After Day 35, the moisture content decreased dramatically to 8.67% on Day 42. We observed a similar change in the moisture content of the UI Sparrow wheat kernels. The moisture content of UI Sparrow wheat kernels did not have a significant change between Day 7 (73.54%) and Day 10 (72.81%) (p = .190), and then, it decreased to 71.39% (Day 14), 61.00% (Day 21), 48.13% (Day 28), and 37.11% (Day 35; p < .001). After Day 35, the moisture content reduced dramatically to 9.05% on Day 42 (Table 2.1).

2.4.6 Starch content of developing wheat kernels

During the grain development period, starch is accumulated in the endosperm and starch content continued increasing until harvest. According to the starch accumulating rate, we categorize starch synthesis into three stages: the initial stage (Day 7 to Day 10), the rapid

growing stage (Day 14 to Day 28), and the maturity stage (Day 35 to Day 42) (Table 2.1, Figure 2.12). For SY Ovation, the starch content was 20.90% on Day 7 and 19.29% on Day 10, and starch accumulation between Day 7 and Day 10 had no significant increases (p = .207). The rapid accumulation stage began on Day 14 and the starch content increased significantly on each day samples were collected: 21.67% (Day 14), 49.80% (Day 21), and 60.48% (Day 28). When the starch synthesis reached the maturity stage, the starch content did not have a significant change and was 62.85 % on Day 35 and 60.67% on Day 42. UI Sparrow wheat had a similar starch content development pattern as SY Ovation. At the initial stage of starch synthesis, the starch content on Day 7 (20.57%) and Day 10 (19.08%) was similar. At the rapid growing stage, starch content increased significantly on each sample collecting day: 26.19% (Day 14), 54.70% (Day 21), and 61.43% (Day 28) (p < .001). The starch content reached a plateau on Day 28 and there was no significant change at the maturity stage: 63.84 % (Day 35) and 62.00% (Day 42). SY Ovation and UI Sparrow wheat did not have significant differences in the starch content at the initial stage Day 7 (p = .551) and Day 10 (p = .336) (Figure 2.12), but UI Sparrow wheat accumulated starch much faster than SY Ovation from Day 10 to Day 14 and maintained starch content, then both wheats reached the same starch content (~60 %) on Day 28 and maintained the similar amount of starch content on Day 35 (p = .737) and Day 42 (p = .056) until harvest (Day 49).

2.4.7 Starch granule size distribution of developing wheat kernels

Wheat starch, similar to barley and rye, has two distinct starch granules: A- and B-type granules. In mature or commercial wheat, A-type starch has a diameter larger than 10 μm with a disk shape, and B-type starch is smaller than 10 μm with a spherical shape. A- and B-type granules are synthesized differently and differ in their structure and properties. To examine the development of granule size, starch granule sizes were detected by a particle size analyzer, and granules with a diameter above 10 μm are defined as large granules and the rest (diameter < 10 μm) are small granules. The quantity of granules with a particular diameter is represented as volume percentage, which refers to the volume comprised of starch granules of a particular diameter (e.g., > 10 μm or < 10 μm) compared with the total volume which is comprised of all of the detected particles in the percentage (Figure 2.13 and 2.14; Table 2.1). At the initial stage of starch synthesis (Day 7 and Day 10), SY Ovation wheat had a normal

distribution of starch granule size with a peak volume percentage at a diameter of 5 μm, and about 94.71% of starch granules had a diameter smaller than 10 μm (Figure 2.13, Table 2.1). On Day 10, we observed the main peak at a diameter of 5 μm and a shoulder at a diameter of 8 μm. The bimodal distribution became obvious on Day 14; first peak (peak at 5 μm), second peak (peak at 14 μm) and the groove between the two peaks were observed at 7 μm. On Day 21, the second peak at about 14 μm shown on Day 14 became the primary peak, and the peak diameter had increased to 21 μm; the volume percentage of the two peaks peaked at a diameter of 5 μm and 21 μm with a volume percentage of 6.48% and 93.52%. From Day 28 to Day 42, the distributions were similar to each other in that the primary peak was peaked at 5 μm, the other peak was peaked at 21 μm, and the groove between the peaks was approximately 10 μm. The volume percentage of the primary peak, which indicated a small starch population, had increased to 21.59% on Day 28, followed by a slight decrease to 15.75% on Day 35 and another decrease to 10.31% on Day 42.

Similar to SY Ovation, UI Sparrow wheat had a normal distribution of starch granule size on Day 7, and the peak had a shoulder on Day 10 (Figure 2.14, Table 2.1). The peak distribution became very broad, and the shoulder on Day 10 gradually shifted to the right and became an obvious peak on Day 14; the first peak reached 5 μm, the second peak reached 13 μm, and the groove was observed at 7 μm. The second peak on Day 14, which represented the population of large starch granules, became the primary peak on Day 21, which had a volume percentage of 94.07% and peaked at 22 μm; the peak of small starch granules had a volume percentage of 6.53 % and peaked at 5 μm, and the groove reached 10 μm. On Day 28, the peak of small starch granules greatly increased to the volume percentage of 22.90 %. The starch granule size distribution, including the peak diameters of both peaks and grooves, was identical to Day 21, and remained the same until Day 42. The only change was the volume percentage of small starch granules decreased on Day 35 (11.72%) and Day 42 (12.15%).

2.4.8 The amylose and amylopectin content of starch in developing wheat kernels

The amylose and amylopectin content of starch at different growing days were analyzed by HPSEC-RI, and the area under the curve was used to represent the percentage of amylose and amylopectin. The starch molecules were eluted into four fractions. Fraction I (10.4 – 11.5 mL) and Fraction II (11.5 – 14.0 mL) were comprised of the debranched amylose

molecules with a molecular weight larger than 2×10^4 Da; and Fraction I and Fraction II were similar to the fractions reported by (Bertoft, Piyachomkwan, Chatakanonda, & Sriroth, 2008), which was defined as long amylose chains and short amylose chains, respectively. According to Bertoft et al., the fraction of long amylose chains represents linear amylose molecules and the fraction of short amylose chains represents branched amylose molecules. Fraction III (14.0 – 15.0 mL) was comprised of long amylopectin chains with a molecular weight in the range of 2×10^4 Da to 4×10^3 Da, and Fraction IV (15.0 – 16.8 mL) was comprised of short amylopectin chains with a molecular weight in the range of 4×10^3 Da to 1×10^3 Da. The chain length of amylopectin was represented as the degree of polymerization (DP), and Fraction III had a chain length in the range of 27 -120 while Fraction IV was DP 6 - 27.

The large starch granules of SY Ovation wheat had similar amylose content on Day 7 (10.67%) and Day 10 (11.42%; $p = .2$) (Table 2.2). There was a significant increase ($p < .001$) of the amylose content of large SY Ovation starch granules: 15.78% (Day 14), 19.30% (Day 21), 22.53% (Day 28), 23.60% (Day 35), and 25.44% (Day 42). The development of small starch granules was different from large starch granules in SY Ovation wheat. The amylose content of small SY Ovation starch granules significantly increased from 13.92% (Day 14), to 17.90% (Day 21). Then, there was an insignificant increase on Day 28, and amylose content reached 19.29% ($p = .07$). The amylose content of small SY Ovation starch further increased to 23.79% on Day 35 and 26.29% on Day 42. The amylose content of large UI Sparrow starch granules had a similar development to the large SY Ovation starch granules, which gradually increased during the grain development period:10.08% (Day 7), 12.10% (Day 10), 16.44% (Day 14), 19.81% (Day 21), 23.46% (Day 28), to 24.86% (Day 35; Table 2.2). After Day 35, the amylose content of large starch granules remained at 24.86% (Day 35) and 25.13% (Day 42) with no significant difference ($p = .903$). The amylose content of small UI Sparrow starch granules had a slightly different development from the small SY Ovation starch, which had a continuous and significant increase on each sample collecting day during the entire grain development period: 12.21% (Day 14), 16.31% (Day 21), 21.15% (Day 28), 25.46% (Day 35), and 26.96% (Day 42 , $p < .001$). Starch molecules are composed of both amylose and amylopectin; therefore, the increase of amylose content represents the decrease of amylopectin content.

Amylopectin is highly branched molecules, and the evolvement of chain-length distribution is associated with the formation of crystalline structure. During the grain development period, we observed minor changes in the content of long amylopectin chains (Fraction III) of the large SY Ovation starch (Table 2.3): it increased from 14.63% (Day 7) and 13.53% (Day 10) to 15.63% (Day 14), and 16.22% (Day 21). Then, the long amylopectin chain content decreased to 14.33% (Day 28), to 13.48% (Day 35), and finally to 14.42% (Day 42). The percentages of were based on the total starch molecules (sum of amylose and amylopectin). The change of short amylopectin chain content of large SY Ovation starch was more extensive than long amylopectin chain: there was no significant difference between the starch content of 74.70% (Day 7) and 75.05% (Day 10). After Day 10, there was a significant decrease ($p < .001$) of the starch content from 68.59% (Day 14), to 64.49% (Day 21), to 63.14% (Day 28), to 62.92% (Day 35), and finally to 60.15% (Day 42). For the small SY Ovation starch granules, the change of both long amylopectin chain and short amylopectin chain were similar to their change in the large SY Ovation wheat. The content of long amylopectin chain decreased by the following percentages: 15.22% (Day 14), 14.95% (Day 21), 14.09% (Day 28), 12.61% (Day 35), to 12.47% (Day 42), and the content of short amylopectin chain decreased by the following percentages: 70.86% (Day 14), 67.15% (Day 21), 66.62% (Day 28), 63.60% (Day 35), to 61.23% (Day 42). The percentage decreases of short amylopectin chain (about 9%) higher than the decreases of the long amylopectin chain (about 3%).

The change of long amylopectin chain and short amylopectin chain in UI Sparrow starch was similar to the changes in SY Ovation starch. The long amylopectin chain in the large UI Sparrow was increased from 14.83% (Day 7) and 13.45 % (Day 10) to 16.07% (Day 14) and 15.52% (Day 21), followed by decreased to 13.96 % (Day 28), 13.22% (Day 35), and 14.28% (Day 42).; and the short amylopectin chain in the large UI Sparrow starch was decreased from 75.09% (Day 7) and 74.46% (Day 10) to 67.49% (Day 14), 64.67% (Day 21), 62.58% (Day 28), 61.92% (Day 35), and 60.58% (Day 42). In the small UI Sparrow starch, the long amylopectin chain content was decreased from 15.10% (Day 14) and 15.26% (Day 21) to13.29% (Day 28), 12.87% (Day 35), and 11.98% (Day 42)., and the short amylopectin chain content was decreased 72.69% (Day 14), 68.43% (Day 21), 65.57% (Day 28), 61.67% (Day 35), and 61.06% (Day 42).

2.4.9 Thermal properties of starch in the developing kernels

The thermal properties of starch granules, including onset gelatinization temperature (To), peak gelatinization temperature (Tp), conclusion gelatinization temperature (Tc), gelatinization temperature range (the difference between onset and conclusion temperature, $\Delta T = Tc - To$), and the enthalpy change (ΔH), were examined by using a DSC and the data were tabulated in Table 2.4. The large SY Ovation starch granules had onset temperatures of 55.57 ℃ (Day 7), 54.08 ℃ (Day 10), 53.57 ℃ (Day 14), 57.33 ℃ (Day 21), 58.11 ℃ (Day 28), 57.80 ℃ (Day 35), and 58.00 ℃ (Day 42). During the grain development period, there was a minor decrease on the onset gelatinization temperature from 55.57 ℃ (Day 7) to 54.08 ℃ (Day 10; $p < .001$) and then remained around 53.57 ℃ (Day 14; $p = .683$ at significance level $\alpha = .05$). Later, the onset gelatinization temperature increased from 53.57 ℃ (Day 14) to 57.33 ℃ (Day 21, $p < .001$), and remained relatively similar at 58.11 ℃ (Day 28), 57.80 ℃ (Day 35) and 58.00 ℃ (Day 42, $p = .297$ at significant level $\alpha = .05$). There were no significant changes in the conclusion gelatinization temperatures, and thus, the gelatinization temperature range had a similar trend as the onset temperature. The gelatinization temperature range first decreased from 16.96 ℃ (Day 7) to 13.80 ℃ (Day 10), and then remained relatively similar: 9.70 ℃ (Day 14), 11.07 ℃ (Day 21), and 9.54 ℃ (Day 28; $p = .197$ at significant level $\alpha = .05$). The gelatinized temperature range further significantly decreased to 7.63 ℃ (Day 35) and then to 8.84 ℃ (Day 42, $p < .001$). The enthalpy change for SY Ovation large starch granules first maintained similar values at 8.45 J/g (Day 7), 7.50 J/g (Day 10), and 8.42 J/g (Day 14). Then the enthalpy change increased to 10.47 J/g (Day 21; $p < .001$). The enthalpy change further increased to 12.02 J/g (Day 28) and then to 11.04 J/g (Day 35) and finally, was reduced to 9.65 J/g (Day 42).

The changes in the thermal properties of large and small starch granules were different than SY Ovation. The onset gelatinization temperature first increased from 53.06 ℃ (Day 14) to 57.46 ℃ (Day 21) and remained around 58.16 ℃ (Day 28). Then, to further decreased to 55.71 ℃ (Day 35) and stayed at 55.17 ℃ on Day 42. A significant increase of the conclusion gelatinization temperature was found in small starch granules. It first increased from 65.55 ℃ (Day 14) to 72.02 ℃ (Day 21), and then gradually decreased to 69.99 ℃ (Day 28), to 68.11 ℃ (Day 35), and finally to 68.20 ℃ (Day 42). The gelatinization temperature range for the small starch granules was no difference from 12.49 ℃ (Day 14) to 14.56 ℃ (Day 21, *p*

=.052 at significance level α = .05). Then, it significantly decreased to 11.84 ℃ (Day 28) and remained relatively stable around 12.40 ℃ (Day 35) and 13.03 ℃ (Day 42; p =.281 at significance level α = .05). There was limited variation in the enthalpy change; it varied from 9.11 J/g (Day 14) to 10.98 J/g (Day 21 and Day 35).

The large starch granules from UI Sparrow showed a different trend than SY Ovation. The onset gelatinization temperature significantly increased two times. It first increased from 51.04 ℃ (Day 7), to 50.71 ℃ (Day 10), then to 54.08 ℃ (Day 14; $p < .001$), and the second time it increased from 54.57 ℃ (Day 14) to 58.58 ℃ (Day 21, $p < .001$), and then, there was no significant difference at 57.99 ℃ (Day 28), 58.40 ℃ (Day 35), and 57.51 ℃ (Day 42, p =.297 at significant level α = .05). There were no obvious changes found in the conclusion gelatinization temperatures of the UI Sparrow large granules. The gelatinization temperature range of SY Ovation large starch granules followed a similar trend as the onset gelatinization temperatures. It first decreased from 14.25 ℃ (Day 7) to 13.30 ℃ (Day 10), and then maintained a value around 12.21 ℃ (Day 14). It further decreased to 9.47 ℃ (Day 21) and remained at 9.54 ℃ (Day 28). The number ($p < .001$) decreased to 7.07 ℃ (Day 35) and then to 8.27 ℃ (Day 42). The enthalpy change for SY Ovation large starch granules maintained a value around 7.75 J/g (Day 7), 7.49 J/g (Day 10), and 7.22 J/g (Day 14; p =.937 at significant level α = .05). The enthalpy change was then:11.86 J/g (Day 21; $p < .001$), 11.70 J/g (Day 28), 11.33 J/g (Day 35), and 10.36 J/g (Day 42; p = .193 at significance level α = .05).

UI Sparrow small starch granules had a different thermal property trend than the large starch granules of UI Sparrow but were similar to the small granules of SY Ovation. The onset gelatinization temperature first increased from 51.54 ℃ (Day 14) to 58.47 ℃ (Day 21), and then gradually decreased: 56.68 ℃ (Day 28), 56.21 ℃ (Day 35), and 54.61 ℃ (Day 42). A significant increase of the conclusion temperature was found in the small starch granules. The conclusion temperature first increased from 70.89 ℃ (Day 14) to 72.64 ℃ (Day 21), and then gradually decreased from 69.37 ℃ (Day 28), to 66.10 ℃ (Day 35), and finally, to 66.73 ℃ (Day 42). The temperature range for the small starch granules continuously and significantly decreased:19.35 ℃ (Day 14), 14.17 ℃ (Day 21), 12.70 ℃ (Day 28), and 9.89 ℃ (Day 35; $p < .001$). Then, the temperature range slightly increased back to 12.12 ℃ on Day 42. Similar to the enthalpy change in SY Ovation small starch granule, there was no obvious

trends in the UI Sparrow small starch granules: the enthalpy change value varied from 9.27 J/g (Day 42) to 10.94 J/g (Day 28).

2.5 DISCUSSION

2.5.1 Initial stage of starch synthesis: Day 7 to Day 10 after anthesis

The purpose of this study is to investigate how starch structure evolves during the grain development period in order to understand how starch structure and functionality can be manipulated by the environment at particular times during grain development. We focus on starch structural properties were starch content, granule size distribution, amylose and amylopectin content, and thermal properties, which all critical to the starch pasting viscosity and falling number (He et al., 2019).

In the initiation stage, the two selected soft white wheat varieties, starch granules in wheat endosperm cells were observed at Day 7 for the first time during the grain development period. Starch granule size was small with a diameter about 5 μm and spherical shape. Until Day 10, starch granule size did not have a significantly change, and we did not observe the bimodal distribution of starch granule size until Day 14 after anthesis. Our observation is similar to that of Evers (1971) and Kalinga et al. (2014) who investigated a Finland spring wheat and an Canada east hard red spring, respectively. Both of these studies found that the endosperm cell developed at about four days after anthesis, and the endosperm starch granules were observed at about seven to ten days after anthesis (Evers, 1971; Kalinga et al., 2014). Parker (1985) hypothesized that starch granules observed at the initial stage (the first week after anthesis) were young A-type granules, which are large, with a diameter above 10 μm when reaching maturity. Although the granule size did not have many changes during the first ten days, amylose content increased from 10.67 % (SY Ovation) and 10.08% (UI Sparrow) on Day 7 to 11.42% (SY Ovation) and 12.10% (UI Sparrow) on Day 10. The active genetic expression of the enzyme starch synthase I, which is known for being responsible for the elongation of starch molecular chains, at the initial stage of grain development might contribute to the increase of amylose content in the first week after anthesis (Peng, Hucl, & Chibbar, 2001). Granule-bound starch synthase is also very active at this stage that directly promotes the synthesis of amylose (Zhao, Dai, Jiang, & Cao, 2008).

2.5.2 Rapid accumulation stages of starch synthesis: Day 14 to Day 28 after anthesis

Starch granules accumulated rapidly during Day 14 to Day 28, in which starch amount increased from 20% (Day 14) to 60% (Day 28) in both soft white wheat varieties. Our observation agrees with the literature that starch accumulation begins at Day 9 and lasts until Day 33 after anthesis in U.S. hard red spring and Chinese winter wheat (Altenbach, DuPont, Kothari, Chan, Johnson, & Lieu, 2003; Dai, Yin, & Wang, 2009; Yang, Zhang, Wang, Xu, & Zhu, 2004). Research has shown that the endosperm cell in wheat kernels stop devising to multiple cells but continue enlarging the size after Day 14 and the pericarp tissue begins to degenerate, which allows the endosperm to gradually increase its volume and mass (Figure 2.6 and 2.7); Bechtel, Abecassis, Shewry, & Evers, 2009; Bechtel, Gaines, & Pomeranz, 1982; Briarty, Hughes, & Evers, 1979; Evers, 1971).

During the rapid accumulation period of starch synthesis, we observed the bimodal distribution of starch granule size on Day 14 that indicates the initiation of B-type starch granules (diameter < 10 μm at mature) and continuously increase of the size of A-type starch granules (diameter >10 μm at mature) (Figure 2.8c and 2.9c) (Kalinga et al., 2014; Parker, 1985).

The quantity of granules with a particular diameter is represented as volume percentage, which refers to the volume comprised of starch granules of a particular diameter (e.g., > 10 μm or < 10 μm) compared with the total volume which is comprised of all of the detected particles in the percentage. The data of our starch granule size showed that the volume percentage of small starch granule gradually decreased from approximately 92 - 94% (Day 7) to 6% (Day 21) and then increased quickly to 22% (Day 28) followed by a decrease again. The detected small particles can be B-type granules or young A-type granules. Parker (1985) and Bechtel, Zayas, Kaleikau, and Pomeranz (1990) reported that the primary starch granules at the initial stage of grain development is young A-type starch; thus, we hypothesize the decrease of the volume percentage of small particles during Day 7 to Day 14 is a result of the size development of young A-type starch granules. Although B-type granules are initiated on Day 14, the total quantity of B-type granules is relatively lower compared with A-type granules (about 10% of total starch content in mature grain). From Day 21 to Day 28, the increase of the volume percentage of small particles is due to increase in the number of the B-type starch granules. Bechtel et al. (1990) reported the initiation of C-type starch granules

(diameter < 5 μm at mature) on Day 21 that would lead to an increase of the volume percentage of small particles (Bechtel et al., 1990). However, it is speculated that C-type starch granules are simply developing granules that will later grow into A-type or B-type starch granules (Raeker, Gaines, Finney, & Donelson, 1998; Singh, Singh, Isono, & Noda, 2010). In the current study, we did not separate granules with a diameter smaller than 5 μm but focused on the development of A- and B-type granules.

We separated large and small starch granules based on the difference of their gravities. It would be reasonable to suggest that large granules are A-type starch and small granules are B-type starch in the mature grains. However, at the initial stage of starch synthesis, the small-granule fraction (92-94% of the total starch content) is a mix of young A- or fully developed B-type starch granules. In the rapid accumulation stage of starch synthesis, the primary large granules are A-type starch, which has a distinct disk shape under a SEM; the small granules would be still a mix of young A-type granule and well-developed B-type granule.

We quantified the amylose content of both large and small starch granules and observed a continuous increase of amylose content in both fractions from Day 14 to Day 28. Morrison and Gadan (1987) reported the similar findings that the amylose content of un-fractioned starch (comprised both large and small starch granules) of two UK winter wheat varieties continued increasing from Day 14 to Day 28, and Yang et al. (2004) explained that such phenomena is due to the active granule-bound starch synthase that promotes the synthesis of amylose. During the period from Day 14 to Day 28, small starch granules contained about 2% to 4% less amylose content than the large starch granules (Table 2.1); however, when reaching the maturity (Day 42), small starch granules had approximately 1% higher amylose content than large starch granules. Our findings do not agree with Ao and Jane (2007) and Kim and Huber (2010) that the small B-type starch has less amylose content than large A-type starch in matured hard winter wheat and matured soft white spring wheat (variety Jubilee). In Ao and Jane's study (2007), the amylose content of A- and B-granules of a hard winter wheat (variety Wesley) are approximate 35% and 27%, respectively. In Kim and Huber's study (2010), the amylose content of A- and By-granules of a soft white spring wheat (variety Jubilee) are approximately 26% and 21%, respectively. The amylose content of our A- and B-granules in matured soft white winter wheat are approximate 26% and 25%, respectively. We assumed the difference between large and small granules are associated with

granule bound starch synthase. The enzyme variations could be due to the fact that the initiation of B-type starch granules occurred approximately one week later than the large A-type starch granules and this delayed synthesis caused a lower amylose content in the B-type starch granules.

We revealed a continuous increase of onset gelatinization temperature (To) and enthalpy change (ΔH) of the large-granule fraction during this rapid accumulation stage of starch synthesis, and there was a significant difference among the samples collected from each day. The onset gelatinization temperature represents the needed temperature to initiate the dissociation of highly organized starch granule architecture; thus, an increase of onset gelatinization temperature indicates an enhancement of the organization of starch molecules. It can be caused by the formation of a helical structure due to the development of amylose in which having more amylose or longer amylose chain length promotes the formation of a helical structure (Matveev et al., 2001). The evolvement of amylopectin structure is another contributor to the increase of onset gelatinization temperature. During the starch synthesis process, amylopectin chains better align with each other to form double helices due to a decrease in the number of building blocks per cluster or an increase in the inter-block chain length (Vamadevan, Bertoft, & Seetharaman, 2013) Similar amylopectin structure change was found in the long chain amylopectin (DP 27-120) content from Day 14 to Day 28 for both large and small starch granule was about 3% to 4% higher than the other starch synthesis stages (Table 2.3). Kalinga et al (2014) also found amylopectin had longest internal chain length from Day 14 to Day 28. Therefore, the increase content and chain length of the long chain amylopectin indicates the change of the alignment of amylopectin molecular chains, which are in the form of double helices in the crystalline lamellae. Such a change directly impacts the onset genitalization temperature. An advanced study to examine of the change of the chain-length distribution of amylopectin molecules and the activity of debranching enzymes will elucidate an increase of onset gelatinization temperature.

Another novel finding during the rapid accumulation stage of starch synthesis is the continuous and significant increase of gelatinization onset temperature (To) and enthalpy change (ΔH). The onset gelatinization temperature refers to the temperature starch start to dissolve or melt, and the enthalpy change presents the needed energy to completely dissolve or melt the highly organized crystalline structure of starch granules. An alteration of the onset

gelatinization temperature and enthalpy change indicates a variation in the quality of the starch crystalline structure (Cooke & Gidley, 1992), which is greatly affected by the characteristics of amylopectin molecules (Vamadevan et al., 2013). Well-developed crystalline is structured with more double helices due to the longer chain length of the external chains of amylopectin; such well packed crystalline (e.g. A-type crystalline) needs more energy to uncoil molecule chains and dissolve (melt) the crystalline structure and have a higher enthalpy change (Cooke & Gidley, 1992; Vamadevan et al., 2013). Our data obtained from DSC showed a significant and continuous increase of enthalpy change and an increase of onset gelatinization temperature in both large and small starch granules with some variations: the onset gelatinization temperature increased 5 °C from Day 14 to Day 28 and the enthalpy change increased about 2 - 4 J/g from Day 14 and Day 28. The observations were further supported by Waduge, Xu, and Seetharaman (2010), who found crystalline structure of both large and small starch granules were the mixture of A-type and B-type crystalline before Day 14, and then adjusted to a well-packed A-type crystalline on Day 28. Due to the packing differences, A-type crystallites melt at higher temperature compared to B-type crystalline (Vamadevan et al., 2013). Therefore, the onset gelatinization temperature and enthalpy change increased from Day 14 to Day 28. An advanced study to examine the evolvement of fine amylopectin structure will expose the mechanism of the alteration of enthalpy change during this period.

2.5.3 Maturity stage of starch synthesis: Day 35 to Day 42 after anthesis

After Day 28, starch content in wheat kernels reached the highest quantity during the granule development period and the quantity remained relatively unchanged from Day 35 to Day 42 after anthesis. Wheat kernels were fully developed by Day 35. After this time, the wheat kernels reached their greatest weight and diameter and began to dehydrate and ripen (Altenbach et al., 2003; Bechtel et al., 2009; Dupont & Altenbach, 2003; Evers, 1970). Both wheat varieties physiologically matured on Day 35 after anthesis when they lost the green pigment on the wheat head (glumes and grains), flag leaves, and grain ~~strand~~ (Mares, 1989), and the wheat from the Day 42 was identified as ripened and ready to harvest. The research strip plot was harvest by combine at about Day 45 after anthesis (Schroeder and White, 2018).

Although the wheat reached physiological maturity on Day 35 after anthesis and starch quantity peaked on Day 28, we found that the starch structure continued to evolve even after this period. For example, the starch granule size distribution on Day 35 and Day 42 was different from Day 28 in both SY Ovation and UI Sparrow wheat kernels. The volume percentage of small starch granules of SY Ovation was 21.59% on Day 28, it first decreased to 15.75% (Day 35) and further decreased to 10.31% (Day 42). The similar decrease of the small starch granule volume percentage was also found in UI Sparrow: it decreased from 22.90% (Day 28) to 11.72% (Day 35), but then remained no significant change at 12.15% on Day 42 after anthesis. The volume percentage of small starch granules in the present study was about 5% to 15% lower than the reported values (Geera, Nelson, Souza, & Huber, 2006b; Raeker et al., 1998; Stoddard, 1999). The reasons could be the varieties difference as described in Stoddard (1999) that 130 examined bread wheat lines from Australia showed 17% difference in the volume percentage of small starch granule (from 23% to 50%).

In addition to the change of starch granule distribution in SY Ovation and UI Sparrow, after wheat reached physiological maturity, the amylose content of large starch granules continued increasing another 1% between Day 28 and Day 35 and reached 24% (SY Ovation) and 25% (UI Sparrow) on Day 35. The continuous increase of amylose content was particularly significant in small granules, in which it increased another 4% between Day 28 and Day 35 and kept increasing by 2% (SY Ovation) and 3% (UI Sparrow) the following week (Table 2.1). We hypothesize the significant development of amylose content in small granules is due to the lack of starch branching enzymes in small granules. Ahmed et al. (2015) reported that large A-type starch granules contain starch synthase (starch synthase I and IIa), starch branching enzyme I, and phosphorylase; however, small B-type granules only hold starch synthase (I and IIa) (Ahmed, Tetlow, Ahmed, Morell, & Emes, 2015). The role of starch synthase is to generate linear glycan, which granule-bound starch synthase use it as a substrate and enhance amylose content (Pfister & Zeeman, 2016; van de Wal, D'Hulst, Vincken, Buléon, Visser, & Ball, 1998). The function of the starch branching enzyme is to cleave an α-1,4-glucan chain, transfer this cleaved portion to the carbon 6 position of a glucose unit on the same or another glucan chain, and then, form a branch chain that linked by α-1,6 glyosidic linkages (Pfister & Zeeman, 2016). Starch synthase is active at about Day 10 to Day 15 after anthesis (Peng et al., 2001), which differs from the starch branching enzyme

that is present and active at Day 18 to Day 32 after anthesis in wheat kernels (Morell, Blennow, Kosar-Hashemi, & Samuel, 1997; Shewry et al., 2009). It is reasonable to speculate that the lack of branching enzymes in the B-type starch granules in the late stage of grain development, promotes the continuous development of amylose.

We also observed a minor alteration of enthalpy change in both large and small starch granules from Day 35 and Day 42, which indicates an adjustment of the amylopectin crystalline structure by glycan trimming through the starch debranching enzymes (Kalinga et al., 2014; Vamadevan et al., 2013).

2.5.4 The alteration of starch synthesis and its influence on falling number

Starch synthesis is influenced by the growing environment, such as growing temperature, irrigation, and fertilization (He et al., 2019). Use growing temperature as an example, when wheat growing in a high temperature condition (e.g. 37/28 ℃ day/night), it has been shown to reduce the starch accumulation period, increase the proportion of A-type granule, and decrease the granular size of both A- and B-type starch granules (Al-Khatib & Paulsen, 1984; Hurkman & Wood, 2011; Shi, Seib, & Bernardin, 1994). The reason is due to the high growing temperature (above 25 ℃) adversely influence the reaction rate of starch synthesis enzymes, like starch synthase, and long period exposure to the high temperature cause permanent enzyme activity loss (Keeling, Banisadr, Barone, Wasserman, & Singletary, 1994). Therefore, the high temperature exposure could reduce the starch yield and properties, and further influence the wheat wholemeal flour functionality (i.e. pasting viscosity) and falling number.

The reduction of falling number is also triggered by environmental stress. The two primary causes of low falling number in wheat are pre-harvest sprouting and late mature α-amylase. Sprouting is promoted by pre-harvest rains, and late mature α-amylase is believed to be triggered by cold temperature shock or heat temperature shock during the period of Day 20 to Day 30 after anthesis (Mares & Mrva, 2014). Researchers in the Northwest of the United States also observed that temperature fluctuation might be another cause of late mature α-amylase related to low falling number (Steber, 2016). Starch is the substrate of α-amylase, and its interaction with α-amylase, which is determined by starch structure, has a direct impact on the performance in the falling number test. Starch also directly contributes to the

viscosity of the flour paste and has a critical role in influencing the end-use quality of the flour, particularly in soft wheat, which has a relatively low quantity of protein compared with hard wheat and durum. The quality of popular products made using soft wheat (e.g., crackers, Asian sponge cake, etc.) rely on the quality and quantity of starch in that wheat. However, the influence of environment stress on starch synthesis associated with falling number in soft wheat has not been reported. Our research revealed that starch accumulated quickly from Day 14 to Day 28 after anthesis in both varieties of soft white wheat. Our findings indicate that the environmental stress, which occurred from Day 14 to Day 28 after anthesis, directly affected the quantity of starch. Our hypothesis is supported by a study led by Al-Khaib and Paulsen (1984) of hard red winter wheat, which revealed that a heat shock, i.e., 37 °C during the day and 28 ℃ at night, from Day 14 to Day 28 after anthesis is associated with the reduction of starch content in wheat. More researches are needed to on starch characteristics on different temperature condition during the rapid accumulation stage to advance the understanding on soft white wheat.

Our research also identified another critical period during which an alteration of starch synthesis can greatly impact flour paste viscosity. Our data showed that large A-type starch granules were initiated on Day 7 after anthesis and small B-type starch granules were initiated on Day 14 to Day 28 in soft white wheat. The size of both large and small granules reached a constant granule size distribution after Day 28 after anthesis. A- and B-type starch granules are synthesized differently in that different synthesis enzymes are involved in generating A- and B-type granules. Thus, the difference between A- and B-starch granules are not as simple as their size and shape but also involves their structure and properties. Our previous research has showed that having a higher proportion of B-type granules can lead to a reduction of flour paste viscosity and consequently, negatively affects falling number (Shao et al., 2019). The primary reasons of such a reduction of viscosity are due to the lower starch pasting viscosity and higher susceptibility of B-type starch granule to wheat α-amylase compared with A-type wheat starch (Ao & Jane, 2007; Geera, Nelson, Souza, & Huber, 2006a; Kim & Huber, 2010) (Naguleswaran, Li, Vasanthan, Bressler, & Hoover, 2012). Thus, the promotion of generation of B-type starch granules during starch synthesis would negatively affect falling number; on the other hand, an increase in the production of A-type starch granules would benefit flour paste viscosity and falling number. The period, from Day 7 to Day 28 after anthesis, is critical

to the ratio of A- and B-type starch granules, and the environmental stress that occurs in this period can alter the ratio and change starch pasting property and the starch susceptibility to wheat α-amylase, and consequently, impact the falling number. Our hypothesis was supported by research reported by Fábián et al. (2011) who found that water stress occurring on Day 5 to Day 9 after anthesis increased the proportion of the B-type starch granules and decreased the size of both A- and B-type starch granules (Fábián et al, 2011).

Our research revealed that amylose content of B-type starch granule continued increasing during the entire grain development period in both SY Ovation and UI Sparrow. The role of amylose in generating paste viscosity is that amylose restricts the swelling of starch granules and negatively affects starch paste viscosity. Our previous study revealed that some low falling number wheat has an increased amylose content along with an increased in the proportion of B-type starch granules (Shao et al., 2019). Current literature has shown high planting temperature (37 °C to 40 °C) is associated with an increase in wheat amylose content (Hurkman & Wood, 2011; Shi et al., 1994); however, this phenomenon may be variety specific (Matsuki, Yasui, Kohyama, & Sasaki, 2003). Therefore, more research is needed to investigate potential environmental influences on starch thermal properties and amylose content.

2.6 CONCLUSION

Understanding and solving the low falling number issue are critical to the profitability and sustainability of the wheat industry. We hypothesized was environmental changes trigger starch developmental changes, impact on its properties and functionalities (e.g. viscosity), and further affect falling number. The present study focused on the starch developmental change in soft white wheat during the wheat development period as the first step to support the hypothesis.

The developmental changes of starch properties that related to the pasting properties and falling number test were identified at each growing stage. Day 7 and Day 14 were the initiation of the large A-type and small B-type starch granule. Day 14 to Day 28, starch content rapidly accumulated, the large and small starch granule proportion changed, the amylose and amylopectin content, and thermal property changes both quality and quantity. Wheat kernels reached to its physiological maturity on Day 35. After that, although the starch

content and granule size distribution did not have significant changes, the starch crystalline structure was further adjusted to became more organized alignment until the harvest Day 42. Our findings suggest the environmental stress, which occurs at these stages, can critically impact starch functionality, such as starch paste viscosity, due to the impact on starch structure evolvement. For the advance research in the future, such as applying cold shock in a controlled environment, the knowledge generated in this study will be helpful to predict and interpret the impact on wheat falling number.

Our current study revealed the evolvement of starch structure and such information can be used to assist the research and breeding program on developing a new variety with a higher resistance to the developmental changes, and this can be a potential solution to managing low falling numbers.

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2.8 TABLES AND FIGURES

Table 2.1 The starch content, moisture content, and the volume percentage of the large and small starch granules of SY Ovation and UI Sparrow during grain development period (n = 3)

Varieties	Day after anthesis	Moisture content (%)	Starch content* (%)	Volume %	
				Small granule (diameter < 10 μm)	Large granule (diameter > 10 μm)
SY Ovation	7	74.62 ± 0.47 [a]	20.90 ± 0.39 [a]	94.71 ± 1.41 [a]	4.74 ± 0.81 [a]
	10	73.59 ± 0.56 [a]	19.29 ± 0.49 [a]	79.34 ± 1.26 [b]	20.66 ± 1.31 [b]
	14	72.66 ± 0.16 [a]	21.67 ± 0.57 [a]	42.71 ± 2.19 [c]	57.76 ± 1.91 [c]
	21	64.65 ± 0.97 [b]	49.80 ± 0.88 [b]	6.48 ± 0.67 [d]	93.52 ± 0.67 [d]
	28	51.25 ± 0.53 [c]	60.48 ± 0.98 [c]	21.59 ± 1.27 [e]	78.41 ± 1.27 [e]
	35	42.24 ± 0.43 [d]	62.85 ± 1.00 [c]	15.75 ± 1.18 [f]	84.25 ± 1.18 [f]
	42	8.67 ± 0.07 [e]	60.67 ± 0.19 [c]	10.31 ± 0.85 [f]	90.05 ± 1.12 [f]
UI Sparrow	7	73.54 ± 0.30 [a]	20.57 ± 0.81 [a]	92.47 ± 1.12 [a]	7.53 ± 1.12 [a]
	10	72.81 ± 0.12 [a]	19.08 ± 0.47 [a]	75.90 ± 1.09 [b]	24.10 ± 1.09 [b]
	14	71.39 ± 0.21 [b]	26.19 ± 0.76 [b]	32.93 ± 1.05 [c]	67.07 ± 1.05 [c]
	21	61.00 ± 0.47 [c]	54.70 ± 0.85 [c]	6.53 ± 1.09 [d]	94.07 ± 0.99 [d]
	28	48.13 ± 0.06 [d]	61.43 ± 0.93 [d]	22.90 ± 1.90 [e]	77.10 ± 1.90 [e]
	35	37.11 ± 0.42 [e]	63.84 ± 0.90 [d]	11.72 ± 1.15 [f]	88.28 ± 1.15 [f]
	42	9.05 ± 0.08 [f]	62.00 ± 1.10 [d]	12.15 ± 0.90 [f]	87.85 ± 0.90 [f]

Note: All results were presented in *M* and *SD*. The difference among samples collected on different days with the same variety (either SY Ovation or UI Sparrow) in each analysis was compared. The values followed by different superscripts are significantly different (one-way ANOVA test and Tukey's test, $p < .001$ at significance level $\alpha = .05$).

* Starch content was calculated based on dry weight basis

Table 2.2 The amylose and amylopectin content of SY Ovation and UI Sparrow starch during the grain development period (n=3)

	Day after anthesis	SY Ovation		UI Sparrow	
		Amylose (%)	Amylopectin (%)	Amylose (%)	Amylopectin (%)
Large granules	7	10.67±0.14 [a]	89.33±0.14 [a]	10.08±0.30 [a]	89.91±0.29 [a]
	10	11.42±0.51 [a]	88.58±0.51 [a]	12.10±0.10 [b]	87.90±0.10 [b]
	14	15.78±0.13 [b]	84.22±0.14 [b]	16.44±0.24 [c]	83.69±0.20 [c]
	21	19.30±0.26 [c]	80.70±0.27 [c]	19.81±0.20 [d]	80.19±0.20 [d]
	28	22.53±0.24 [d]	77.31±0.08 [d]	23.46±0.37 [e]	76.77±0.25 [e]
	35	23.60±0.20 [e]	76.41±0.20 [e]	24.86±0.23 [f]	75.14±0.23 [f]
	42	25.44±0.33 [f]	74.71±0.33 [f]	25.13±0.14 [f]	74.92±0.14 [f]
Small granules	14	13.92±0.04 [a]	86.10±0.01 [a]	12.21±0.21 [a]	87.67±0.17 [a]
	21	17.90±0.80 [b]	82.10±0.81 [b]	16.31±0.37 [b]	83.69±0.38 [b]
	28	19.29±0.60 [b]	80.73±0.74 [b]	21.15±0.17 [c]	78.78±0.16 [c]
	35	23.79±0.04 [c]	76.21±0.04 [c]	25.46±0.27 [d]	74.54±0.28 [d]
	42	26.29±0.08 [d]	73.76±0.00 [d]	26.96±0.11 [e]	73.05±0.13 [e]

Note: All results were presented in *M* and *SD*. The difference in the amylose or amylopectin content in large and small granules of samples of the same variety collected on different days were statistically analyzed. The values followed by a different superscript are significantly different (one-way ANOVA and Tukey's test, $p < .05$ at significance level $\alpha = .05$).

Table 2.3 The long amylopectin chain (Fraction III) and short amylopectin chain (Fraction IV) content of SY Ovation and UI Sparrow starch during the grain development period (n=3)

	Day after anthesis	SY Ovation		UI Sparrow	
		Fraction III (%)	Fraction IV (%)	Fraction III (%)	Fraction IV (%)
Large granules	7	14.63 ± 0.16 [a]	74.70 ± 0.04 [a]	14.83 ± 0.15 [a]	75.09 ± 0.15 [a]
	10	13.53 ± 0.10 [b]	75.05 ± 0.43 [a]	13.45 ± 0.20 [b]	74.46 ± 0.30 [a]
	14	15.63 ± 0.12 [c]	68.59 ± 0.25 [b]	16.07 ± 0.10 [c]	67.49 ± 0.22 [b]
	21	16.22 ± 0.17 [d]	64.49 ± 0.13 [c]	15.52 ± 0.11 [d]	64.67 ± 0.12 [c]
	28	14.33 ± 0.11 [a]	63.14 ± 0.13 [d]	13.96 ± 0.16e	62.58 ± 0.23 [d]
	35	13.48 ± 0.16 [b]	62.92 ± 0.07 [d]	13.22 ± 0.13 [b]	61.92 ± 0.10 [d]
	42	14.42 ± 0.13 [a]	60.15 ± 0.22 [e]	14.28 ± 0.13 [e]	60.58 ± 0.15 [e]
Small granules	14	15.22 ± 0.05 [a]	70.86 ± 0.02 [a]	15.10 ± 0.14 [a]	72.69 ± 0.08 [a]
	21	14.95 ± 0.23 [a]	67.15 ± 0.58 [b]	15.26 ± 0.19 [a]	68.43 ± 0.20 [b]
	28	14.09 ± 0.42 [b]	66.62 ± 0.20 [b]	13.29 ± 0.14 [b]	65.57 ± 0.12 [c]
	35	12.61 ± 0.03 [c]	63.60 ± 0.07 [c]	12.87 ± 0.13 [b]	61.67 ± 0.20 [d]
	42	12.47 ± 0.06 [c]	61.23 ± 0.08 [d]	11.98 ± 0.04 [c]	61.06 ± 0.07 [e]

Note: All results were presented in *M* and *SD*. Fraction III (long chain amylopectin) and Fraction IV (short chain amylopectin) content was calculated based on the peak area percentage of Fraction III and Fraction IV from the HPSEC chromatogram (Figure 2.16-2.19). The one-way ANOVA and Tukey's test were conducted to determine the significant difference of the contents of long- or short- amylopectin chains of starch granules with the same size and variety on different days after anthesis. The values followed by a different superscript are significantly different (, $p < .05$ at significance level $\alpha = .05$).

Table 2.4 Gelatinization temperature and enthalpy change of SY Ovation and UI Sparrow starch during the grain development period

Varieties	Granule type	Day after anthesis	To (°C)	Tp (°C)	Tc (°C)	ΔT (Tc-To)	ΔH (J/g)
SY Ovation	Large granules	7	55.57 ± 0.50 ^{b}	64.05 ± 0.48 ^{d}	72.53 ± 0.60 ^{c}	16.96 ± 0.10 ^{d}	8.45 ± 0.28 ^{ab}
		10	54.08 ± 0.21 ^{a}	60.43 ± 0.59 ^{b}	67.88 ± 1.70 ^{ab}	13.80 ± 1.49 ^{c}	7.50 ± 0.40 ^{a}
		14	53.57 ± 0.34 ^{a}	58.25 ± 0.35 ^{a}	64.34 ± 0.25 ^{ab}	9.70 ± 1.41 ^{bc}	8.42 ± 0.40 ^{ab}
		21	57.33 ± 0.54 ^{c}	61.18 ± 0.01 ^{bc}	68.40 ± 1.71 ^{b}	11.07 ± 1.17 ^{bc}	10.47 ± 0.61 ^{c}
		28	58.11 ± 0.01 ^{c}	61.84 ± 0.00 ^{c}	67.65 ± 0.66 ^{ab}	9.54 ± 0.68 ^{ab}	12.02 ± 0.28 ^{d}
		35	57.80 ± 0.04 ^{c}	60.84 ± 0.23 ^{bc}	65.43 ± 0.02 ^{ab}	7.63 ± 0.06 ^{a}	11.04 ± 0.25 ^{cd}
		42	58.00 ± 0.01 ^{c}	62.04 ± 0.22 ^{c}	66.84 ± 0.57 ^{ab}	8.84 ± 0.55 ^{ab}	9.65 ± 0.35 ^{bc}
	Small granules	14	53.06 ± 0.02 ^{a}	58.91 ± 0.33 ^{a}	65.55 ± 0.50 ^{a}	12.49 ± 0.52 ^{ab}	9.11 ± 0.17 ^{a}
		21	57.46 ± 0.89 ^{cd}	66.13 ± 0.11 ^{d}	72.02 ± 0.13 ^{d}	14.56 ± 1.03 ^{b}	10.98 ± 0.41 ^{b}
		28	58.16 ± 0.64 ^{d}	64.44 ± 0.36 ^{c}	69.99 ± 0.55 ^{c}	11.84 ± 0.09 ^{a}	9.49 ± 0.07 ^{a}
		35	55.71 ± 0.20 ^{cb}	61.09 ± 0.36 ^{b}	68.11 ± 0.16 ^{b}	12.40 ± 0.04 ^{a}	10.98 ± 0.29 ^{b}
		42	55.17 ± 0.07 ^{b}	60.67 ± 0.00 ^{b}	68.20 ± 0.06 ^{b}	13.03 ± 0.13 ^{ab}	9.91 ± 0.33 ^{a}
UI Sparrow	Large granules	7	51.04 ± 0.30 ^{a}	58.68 ± 0.28 ^{b}	65.29 ± 0.21 ^{b}	14.25 ± 0.08 ^{d}	7.75 ± 0.24 ^{a}
		10	50.71 ± 0.34 ^{a}	57.72 ± 0.13 ^{a}	64.01 ± 0.19 ^{a}	13.30 ± 0.53 ^{cd}	7.49 ± 0.67 ^{a}
		14	54.57 ± 0.54 ^{b}	60.07 ± 0.11 ^{c}	66.78 ± 0.25 ^{c}	12.21 ± 0.78 ^{c}	7.22 ± 0.84 ^{a}
		21	58.58 ± 0.02 ^{c}	62.08 ± 0.12 ^{e}	68.05 ± 0.22 ^{e}	9.47 ± 0.20 ^{b}	11.86 ± 0.38 ^{b}
		28	57.99 ± 0.39 ^{c}	61.32 ± 0.00 ^{d}	66.60 ± 0.04 ^{d}	8.61 ± 0.35 ^{ab}	11.70 ± 0.56 ^{b}
		35	58.40 ± 0.13 ^{c}	60.98 ± 0.24 ^{d}	65.47 ± 0.42 ^{d}	7.07 ± 0.29 ^{a}	11.33 ± 0.36 ^{b}
		42	57.51 ± 0.10 ^{c}	61.03 ± 0.23 ^{d}	65.78 ± 0.23 ^{d}	8.27 ± 0.13 ^{ab}	10.36 ± 0.43 ^{b}
	Small granules	14	51.54 ± 0.11 ^{a}	60.26 ± 0.10 ^{a}	70.89 ± 0.10 ^{bc}	19.35 ± 0.01 ^{a}	9.89 ± 0.56 ^{ab}
		21	58.47 ± 0.69 ^{d}	66.21 ± 0.63 ^{c}	72.64 ± 0.63 ^{c}	14.17 ± 0.06 ^{b}	10.49 ± 0.17 ^{bc}
		28	56.68 ± 0.25 ^{c}	63.01 ± 0.89 ^{b}	69.37 ± 0.89 ^{b}	12.70 ± 0.64 ^{c}	10.94 ± 0.11 ^{bc}
		35	56.21 ± 0.07 ^{cb}	60.91 ± 0.29 ^{a}	66.10 ± 0.29 ^{a}	9.89 ± 0.22 ^{d}	11.30 ± 0.11 ^{c}
		42	54.61 ± 0.62 ^{b}	60.17 ± 0.78 ^{a}	66.73 ± 0.78 ^{a}	12.12 ± 0.16 ^{c}	9.27 ± 0.06 ^{a}

Note: All results were presented in *M* and *SD*. The differences among the various parameters on different days after anthesis within the same variety (either SY Ovation or UI Sparrow) in each parameter was compared. The values followed by a different superscript are significantly different (one-way ANOVA and Tukey's test, $p < .05$ at significance level $\alpha = .05$)

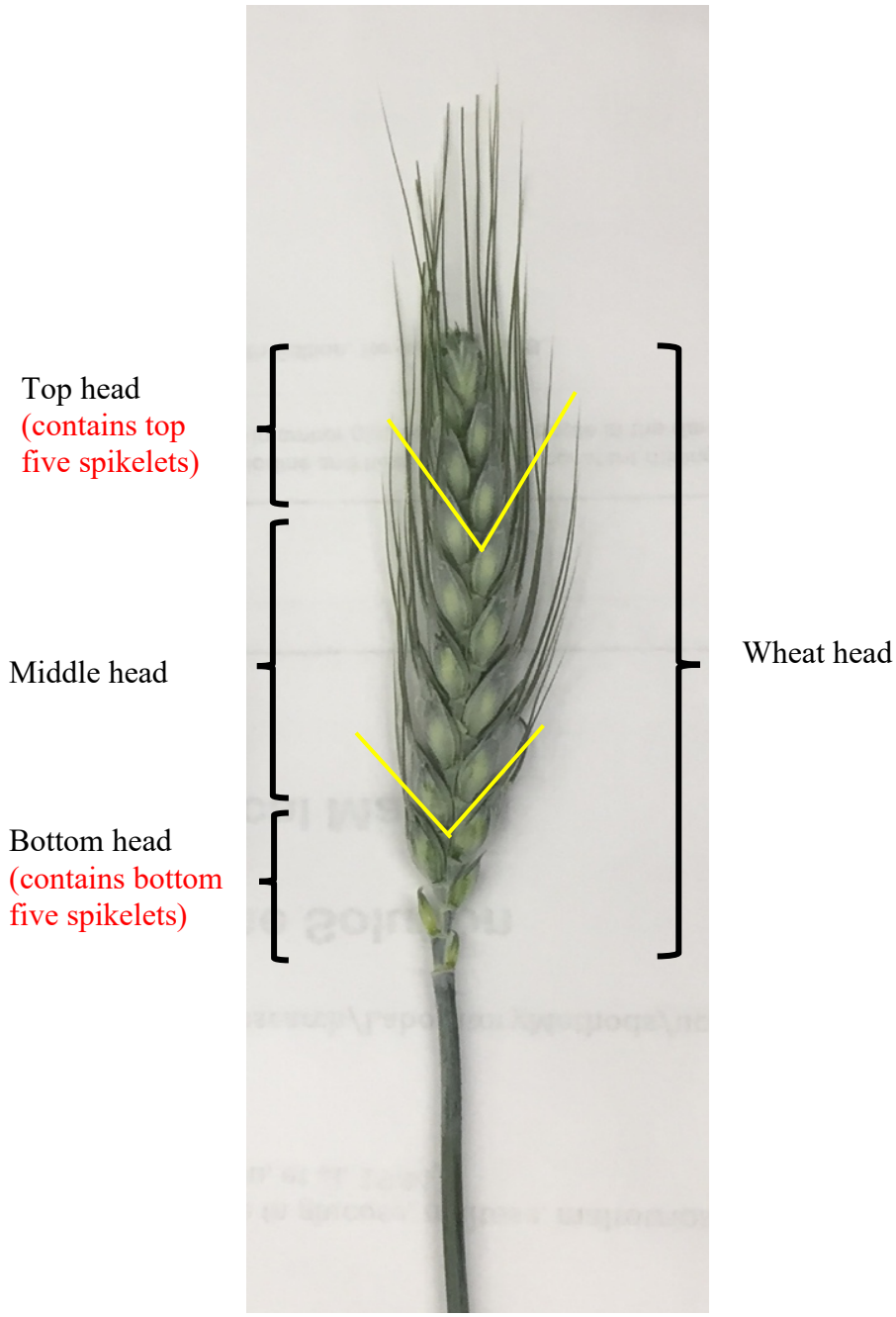

Figure 2.1 Sample preparation for determining and cutting the middle portion of the wheat heads (demonstration sample: Day 4, SY Ovation grown in Tammany ID, collected from the field on June 5, 2017)

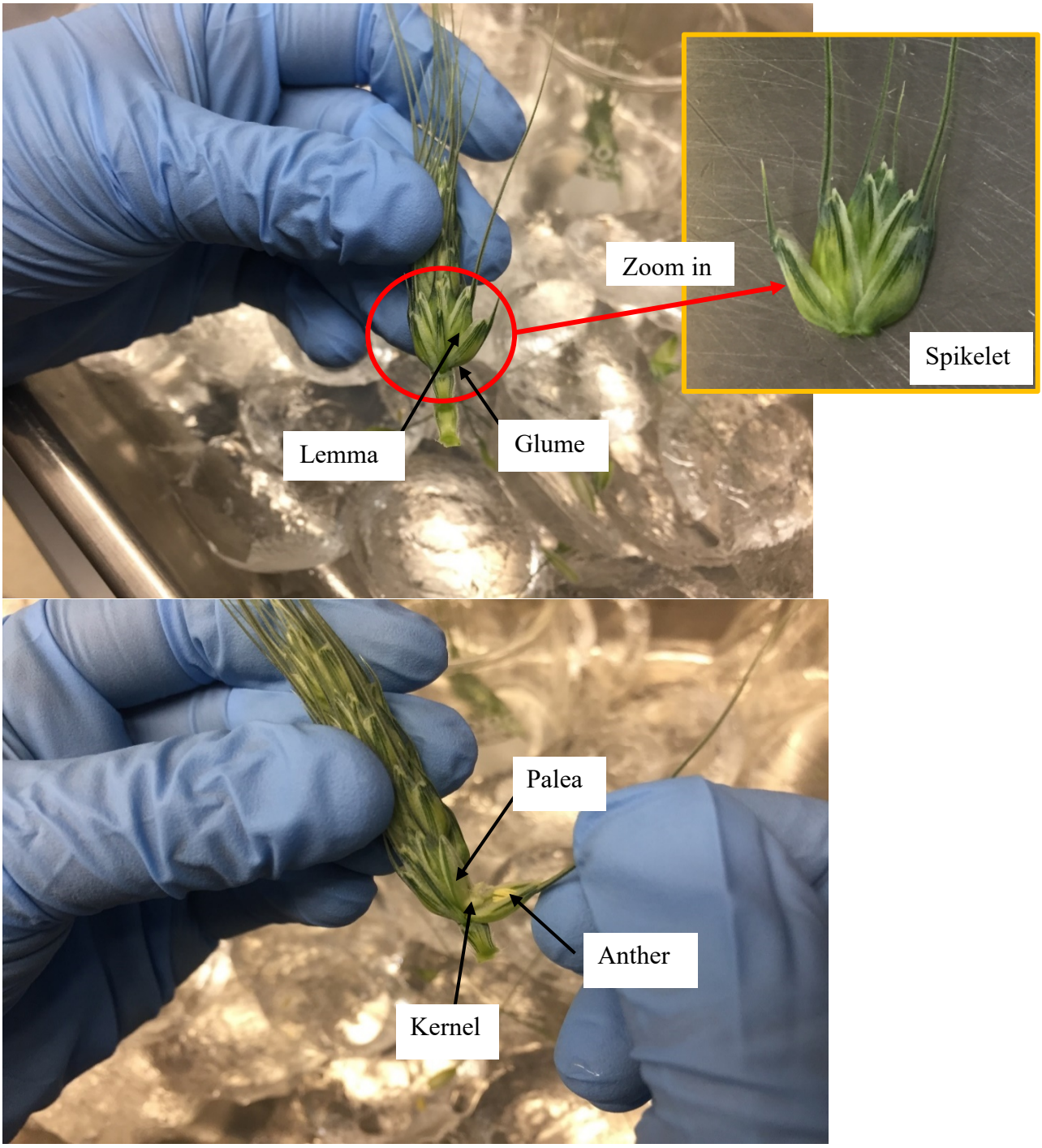

Figure 2.2 Plant anatomy of wheat spikelet on wheat head (demonstration sample: Day 4, SY Ovation grown in Tammany ID, collected from the field on June 5, 2017)

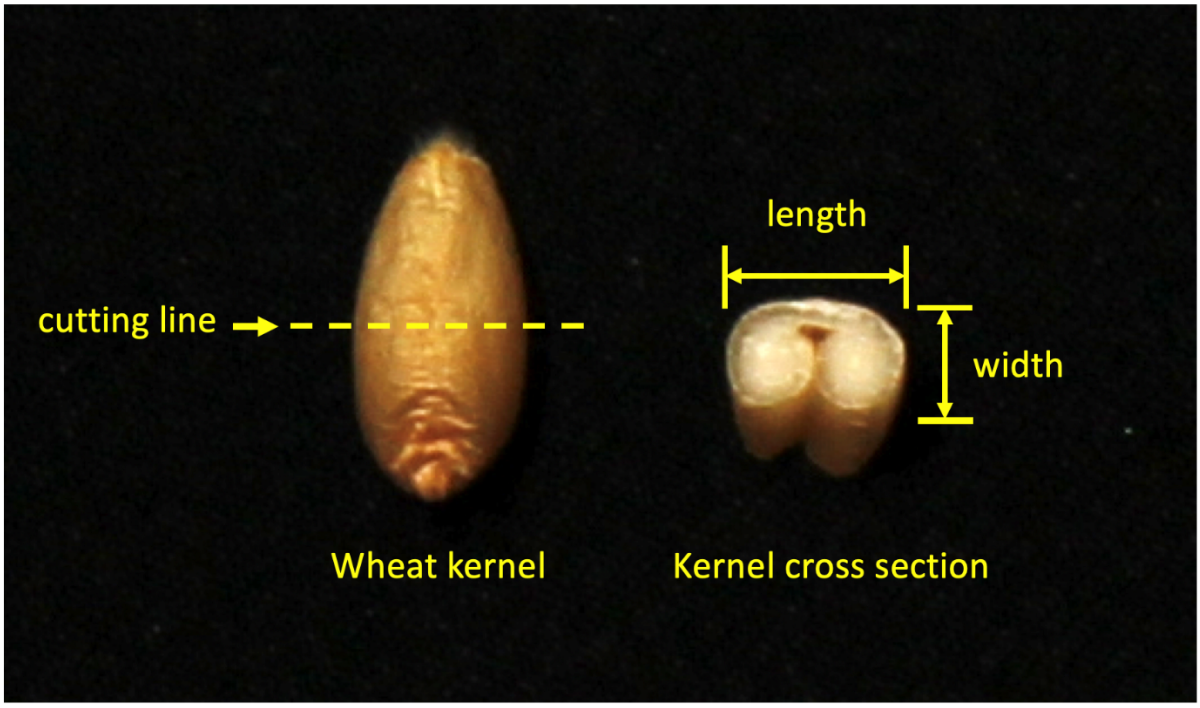

Figure 2.3 Microscope sample preparation. The cross section of a wheat kernel and the dimension terms.

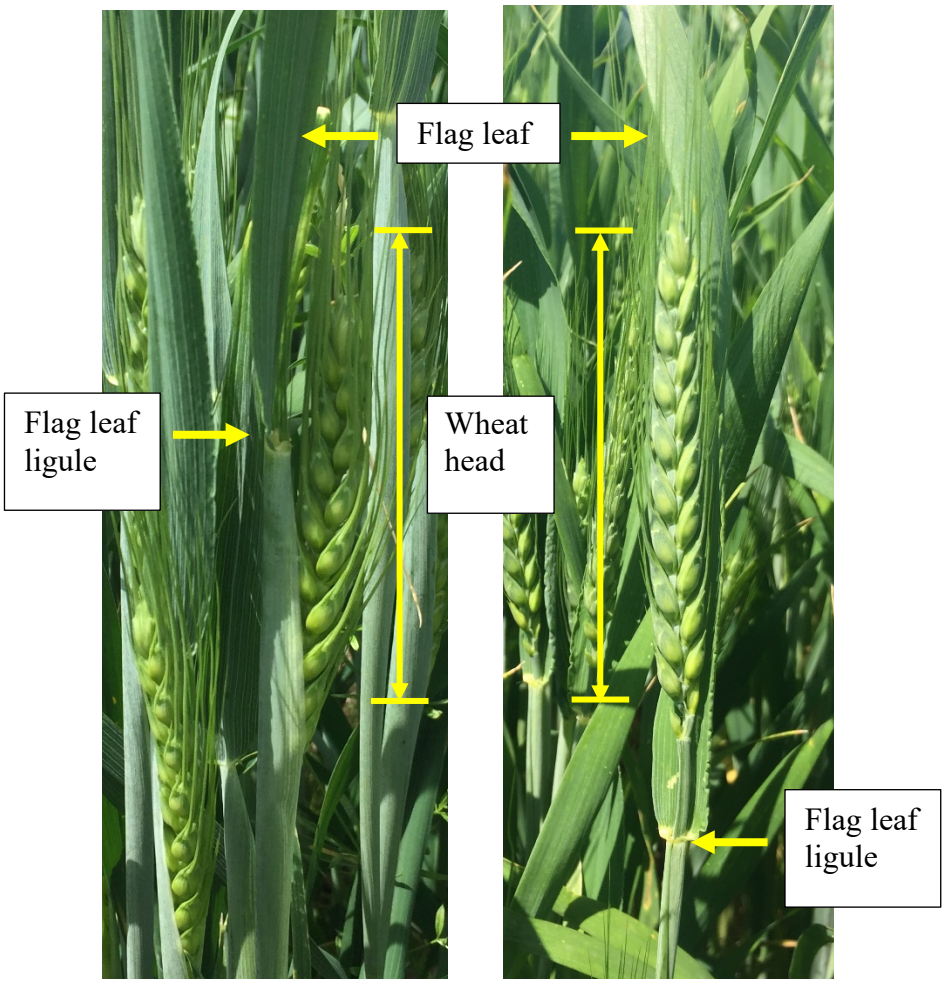

Figure 2.4 Heading in progress (left; May 31, 2017) and fully-grown wheat head (right; June 2, 2017). The demonstration sample is UI Sparrow wheat grown in Tammany, Idaho. The image on the left shows about four spikelets on the top of the wheat head emerging above the flag leaf ligule; the image on the right shows the wheat head completely emerged above the flag leaf ligule.

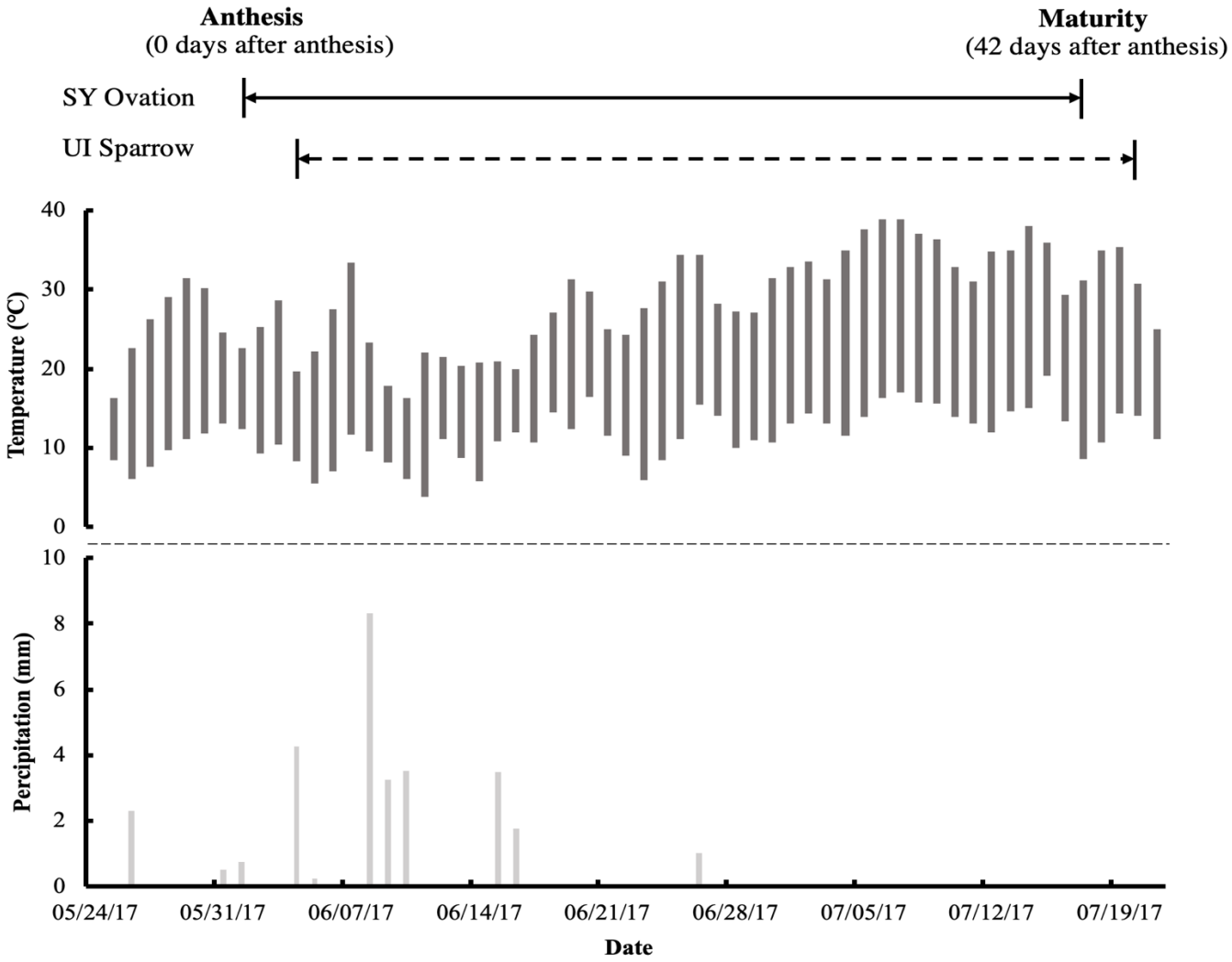

Figure 2.5 Temperature (top) and precipitation (bottom) in the field during the wheat growing period (May to July in 2017). The two figures share the bottom x-axis. The top lines show the growing period of SY Ovation (solid line) and UI Sparrow (dash line).

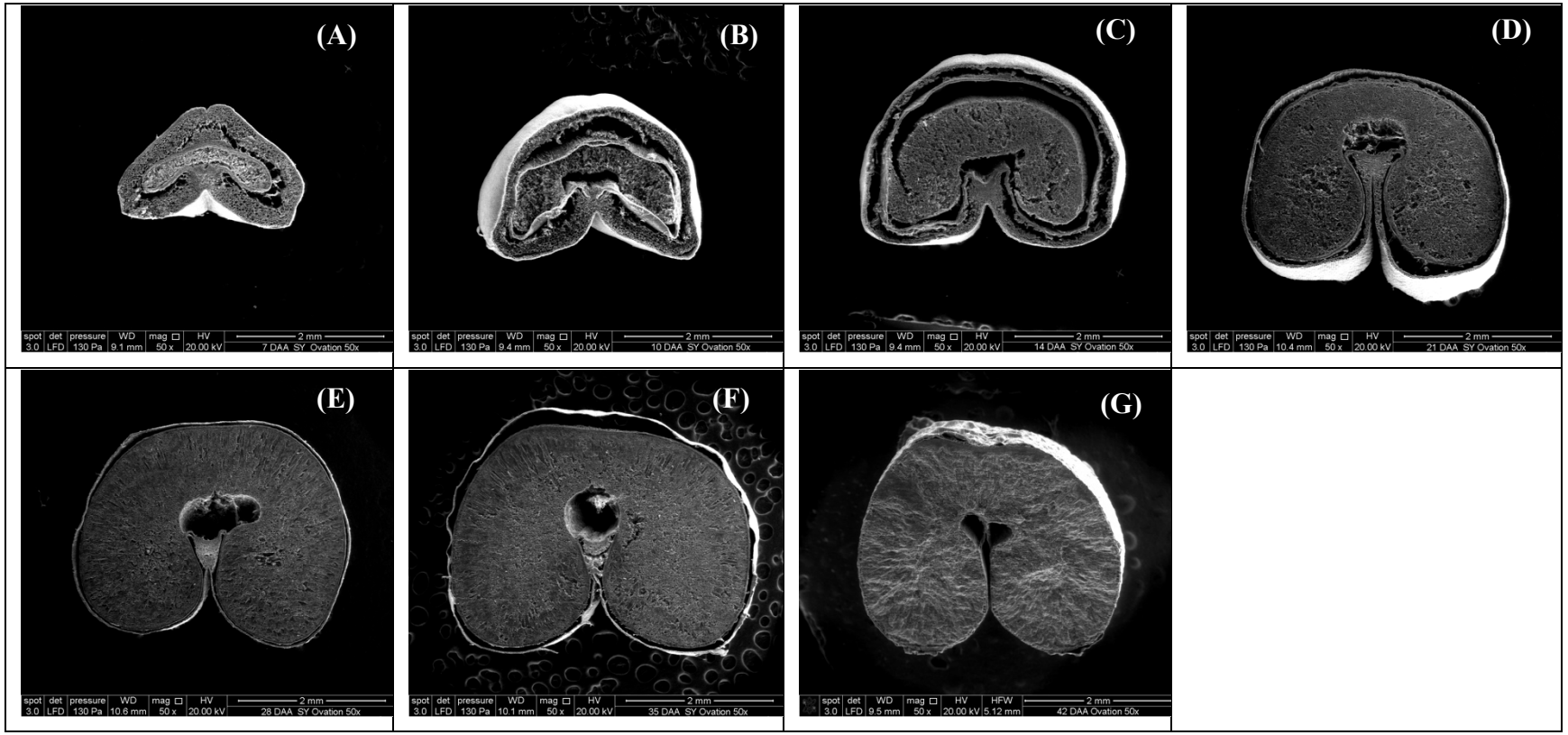

Figure 2.6 The cross section of SY Ovation wheat kernels on Day 7 (A), Day 10 (B), Day 14 (C), Day 21 (D), Day 28 (E), Day 35 (F), and Day 42 (G) after anthesis. The cross section was cut from the middle of the kernel as shown in Figure 2.3.

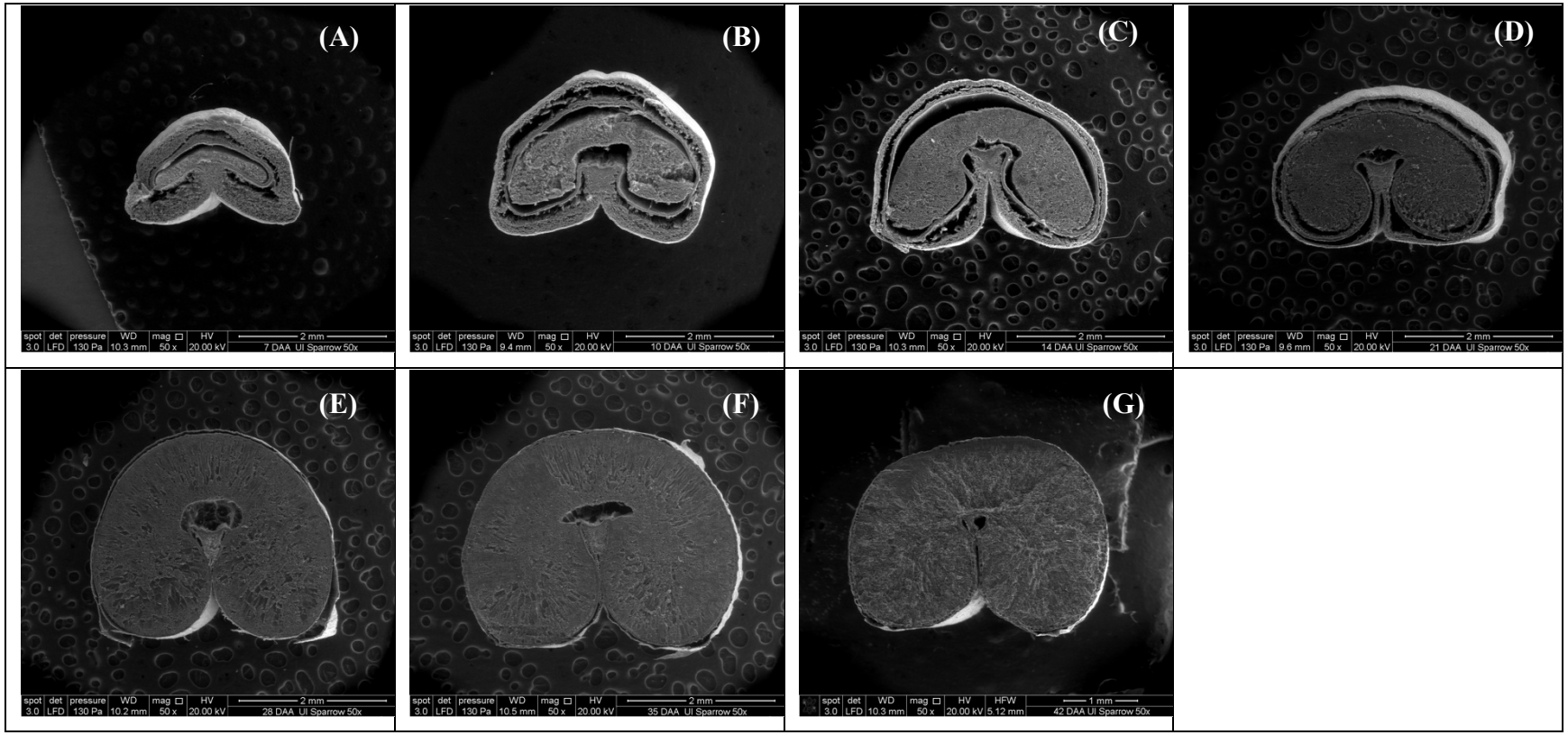

Figure 2.7 The cross section of UI Sparrow wheat kernels on Day 7 (A), Day 10 (B), Day 14 (C), Day 21 (D), Day 28 (E), Day 35 (F), and Day 42 (G) after anthesis. The cross section was cut from the middle of the kernel as shown in Figure 2.3.

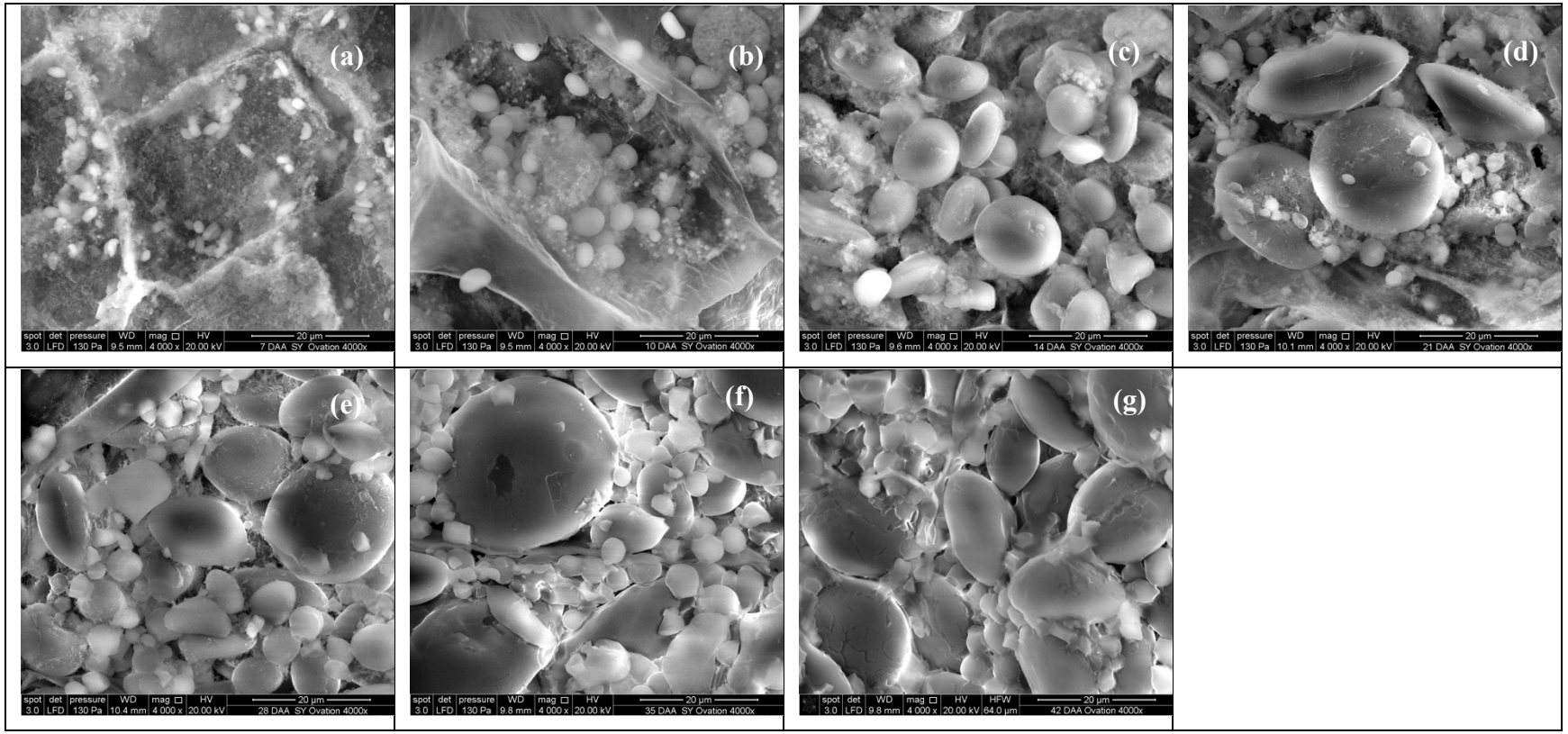

Figure 2.8 Starch in the endosperm of SY Ovation wheat on Day 7 (a), Day 10 (b), Day 14 (c), Day 21 (d), Day 28 (e), Day 35 (f), and Day 42 (g) after anthesis.

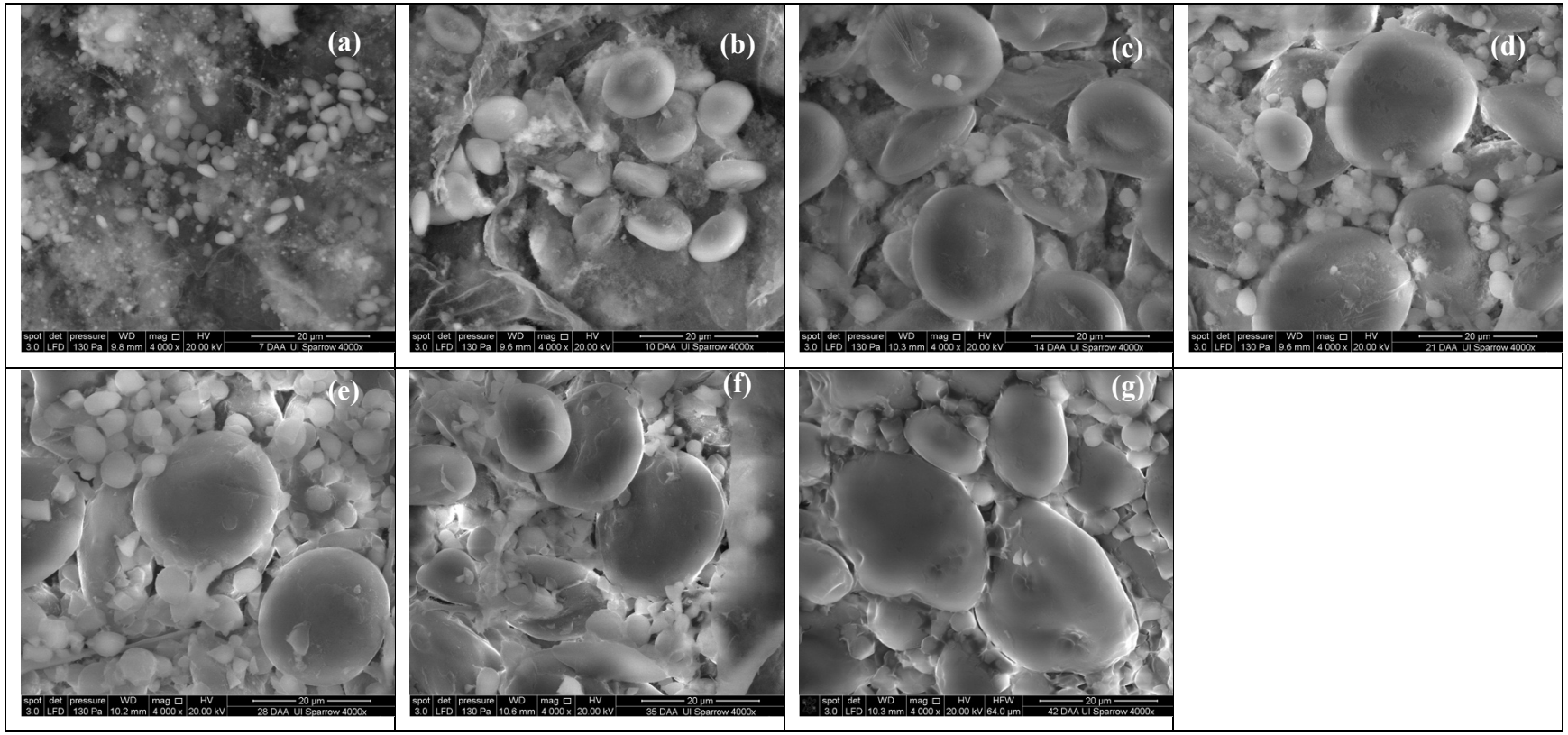

Figure 2.9 Starch in the endosperm of UI Sparrow wheat on Day 7 (a), Day 10 (b), Day 14 (c), Day 21 (d), Day 28 (e), Day 35 (f), and Day 42 (g) after anthesis.

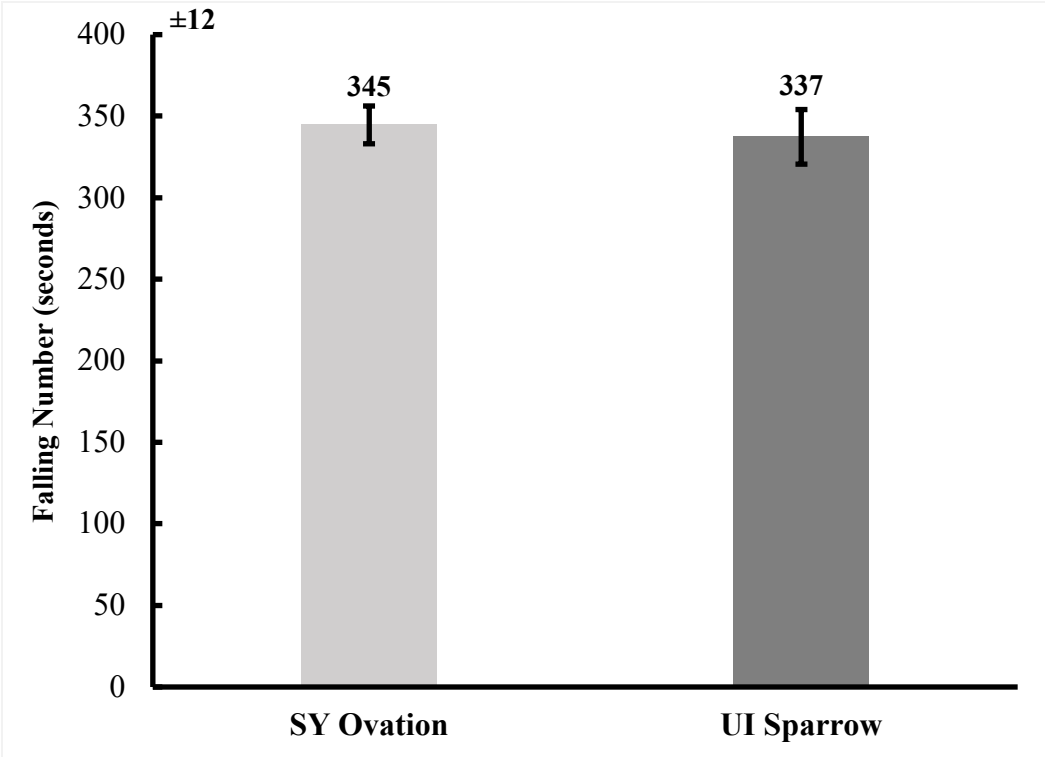

Figure 2.10 Falling numbers of the SY Ovation and UI Sparrow wholemeal flour (n=3). The values were presented in *M* and *SD*. No significant difference between the falling number of the two wheat varieties (Student t-test, $p = .567$ at significance level $\alpha = 0.05$)

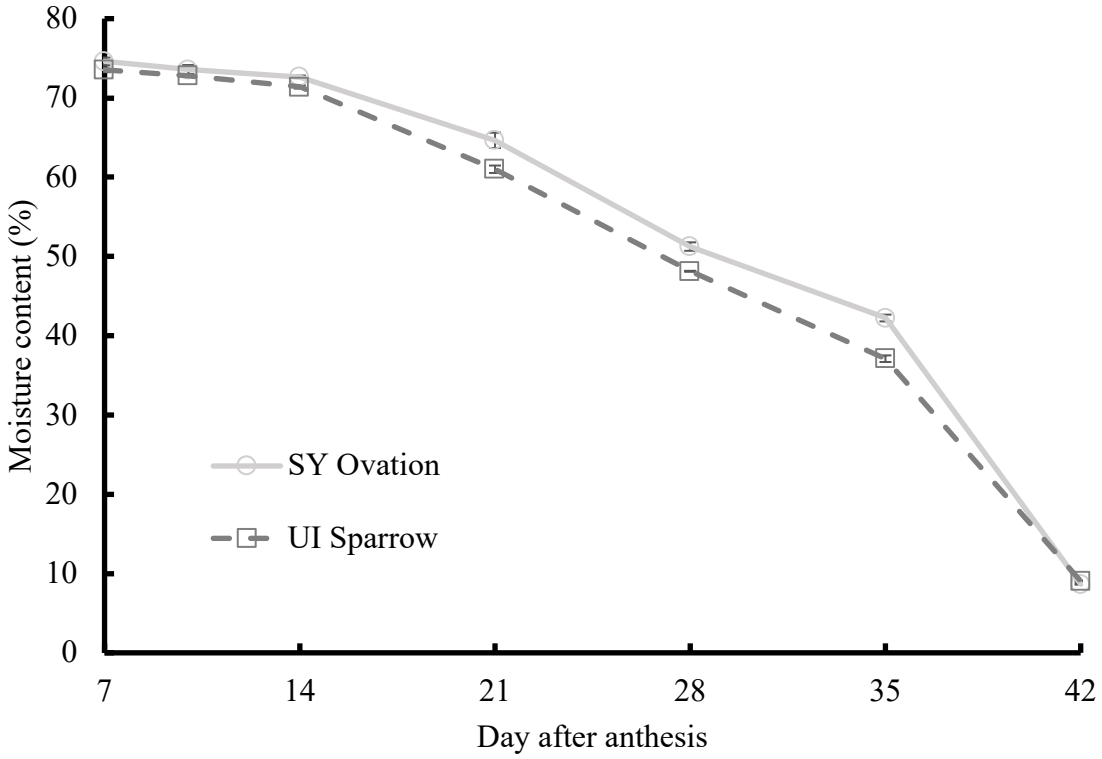

Figure 2.11 The change of moisture content (%) in SY Ovation (dashed line and UI Sparrow (solid line) kernels during the grain development period.

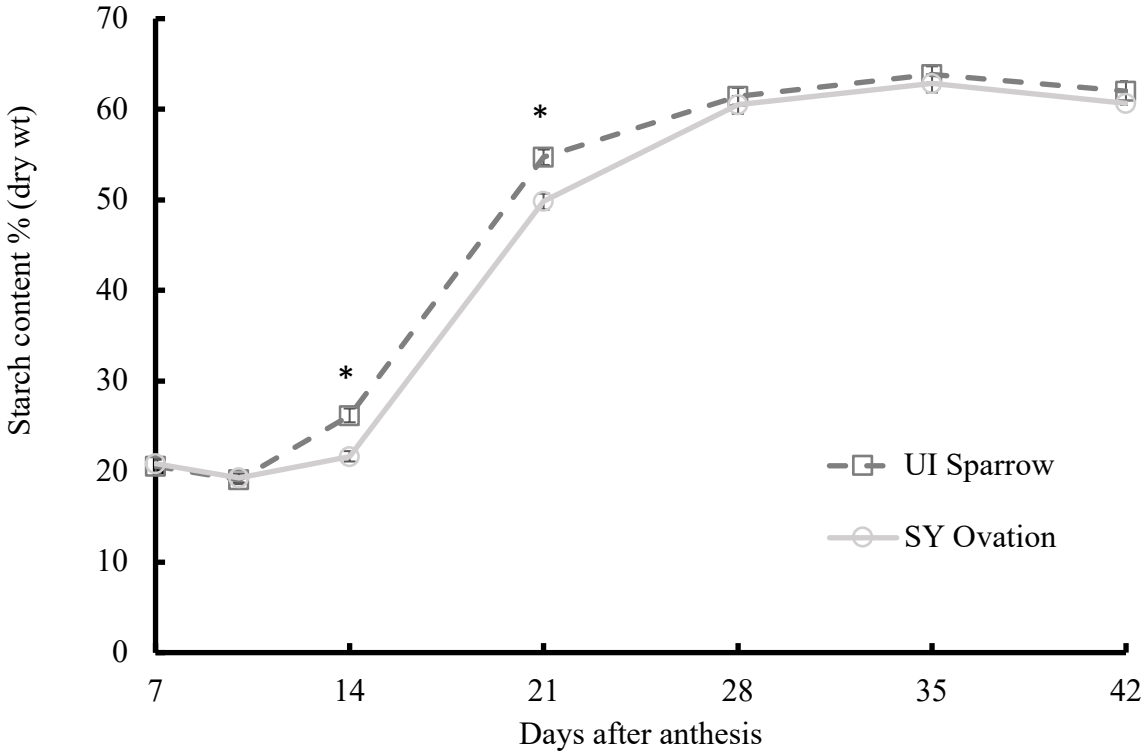

Figure 2.12 The change of starch content (%, on dry weight basis) during the grain development period. The asterisk,*, indicates the significant difference at $\alpha = .05$ level between the UI Sparrow and SY Ovation (Independent t-test, Day 14 after anthesis $p = .002$, Day 21 after anthesis $p < .001$)

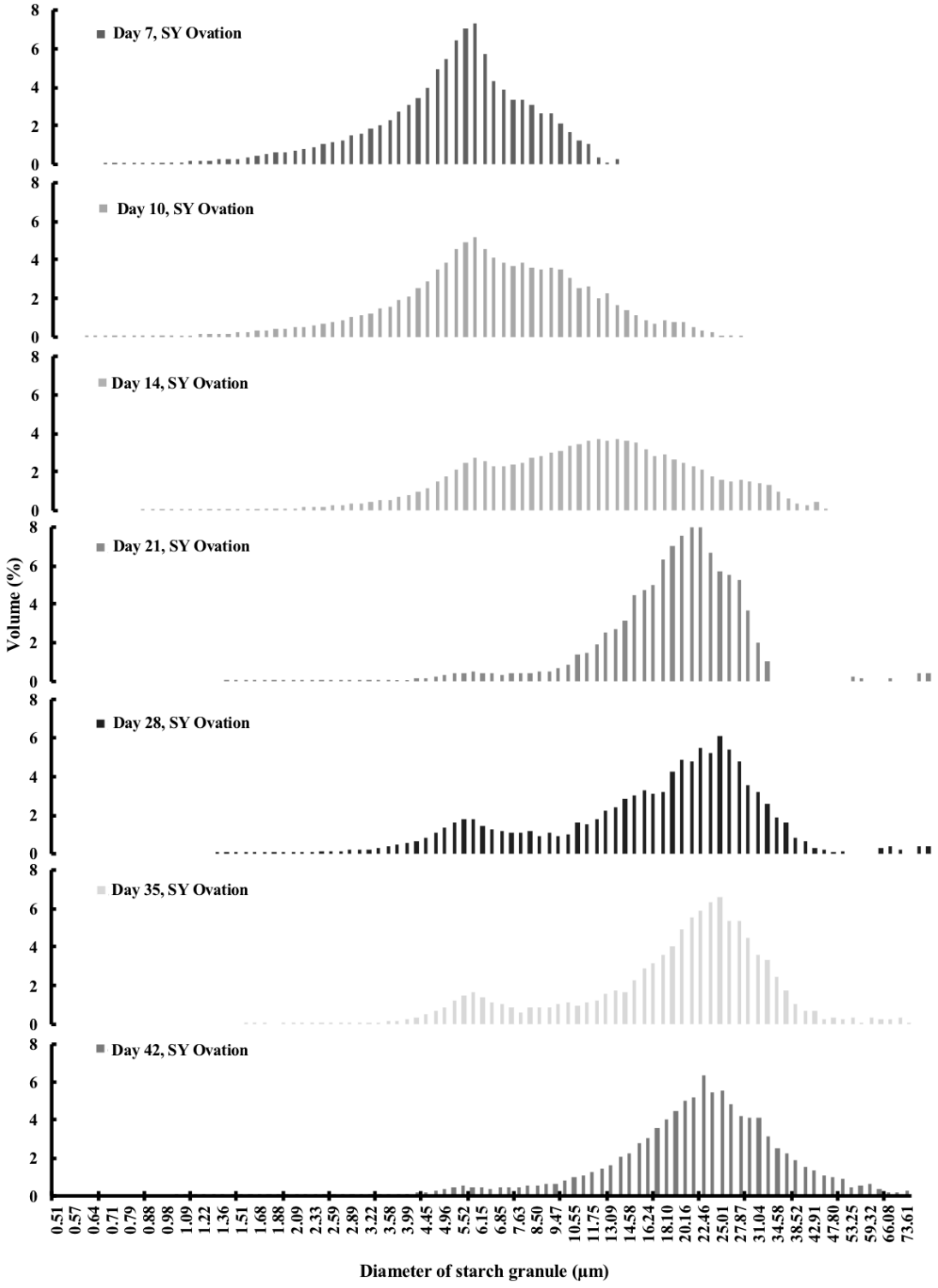

Figure 2.13 The change of granule size distribution of SY Ovation starch during the grain development period

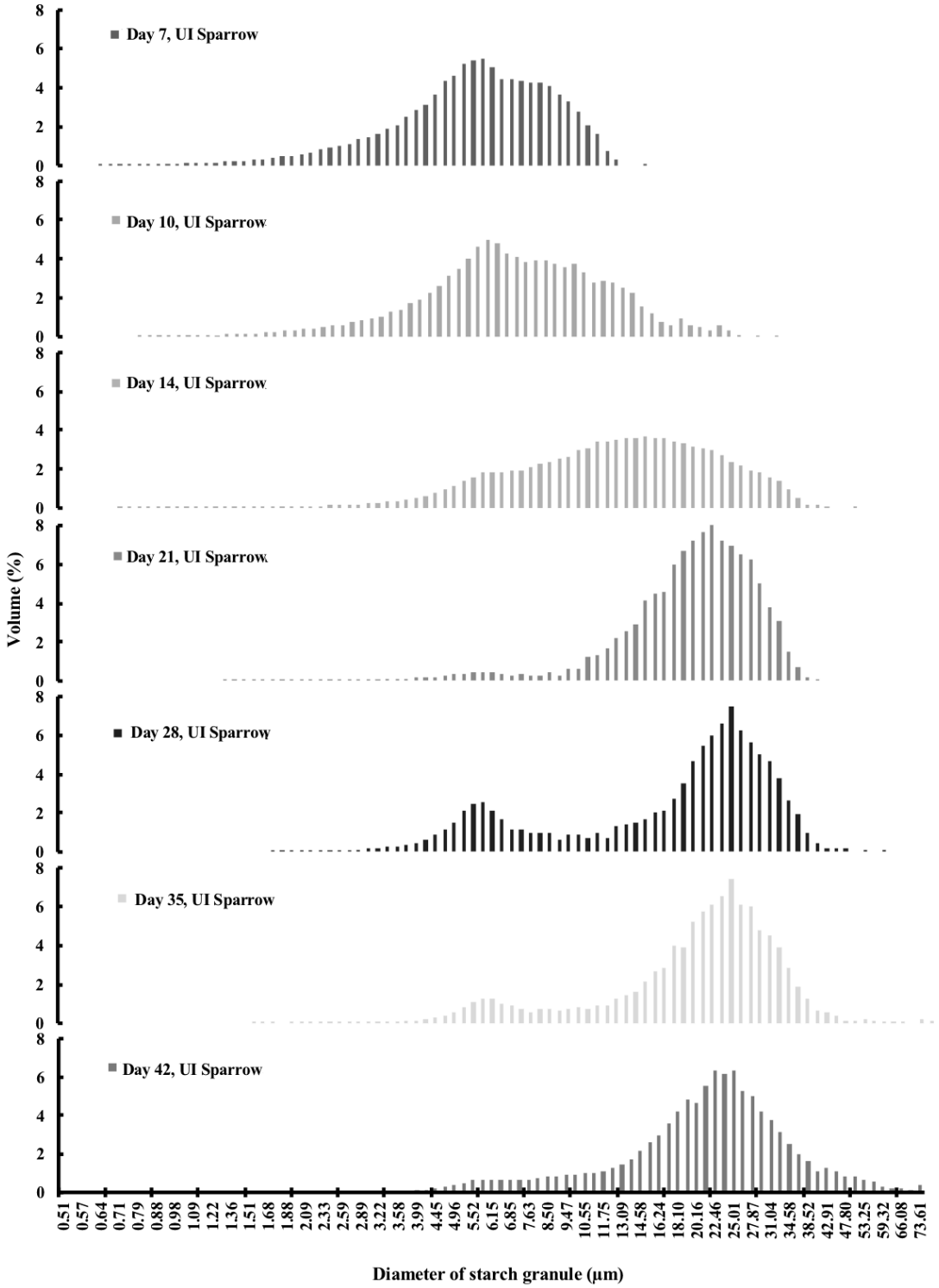

Figure 2.14 The change of starch granule size (UI Sparrow) during the grain development period from Day 7 to Day 42.

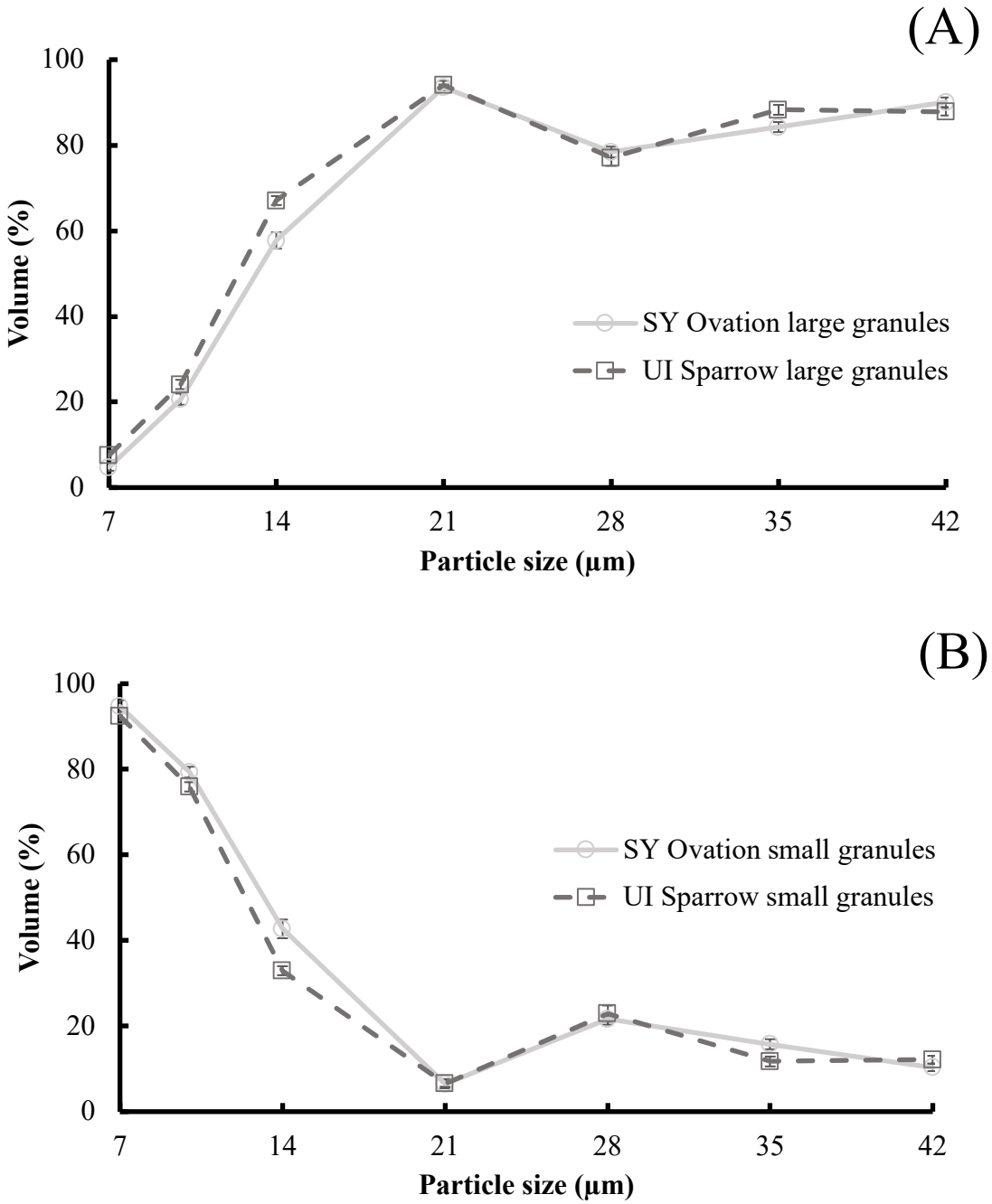

Figure 2.15 The change of the volume percentage of large (A) and small (B) starch granules of SY Ovation (solid line) and UI Sparrow (dash line) during the grain development period

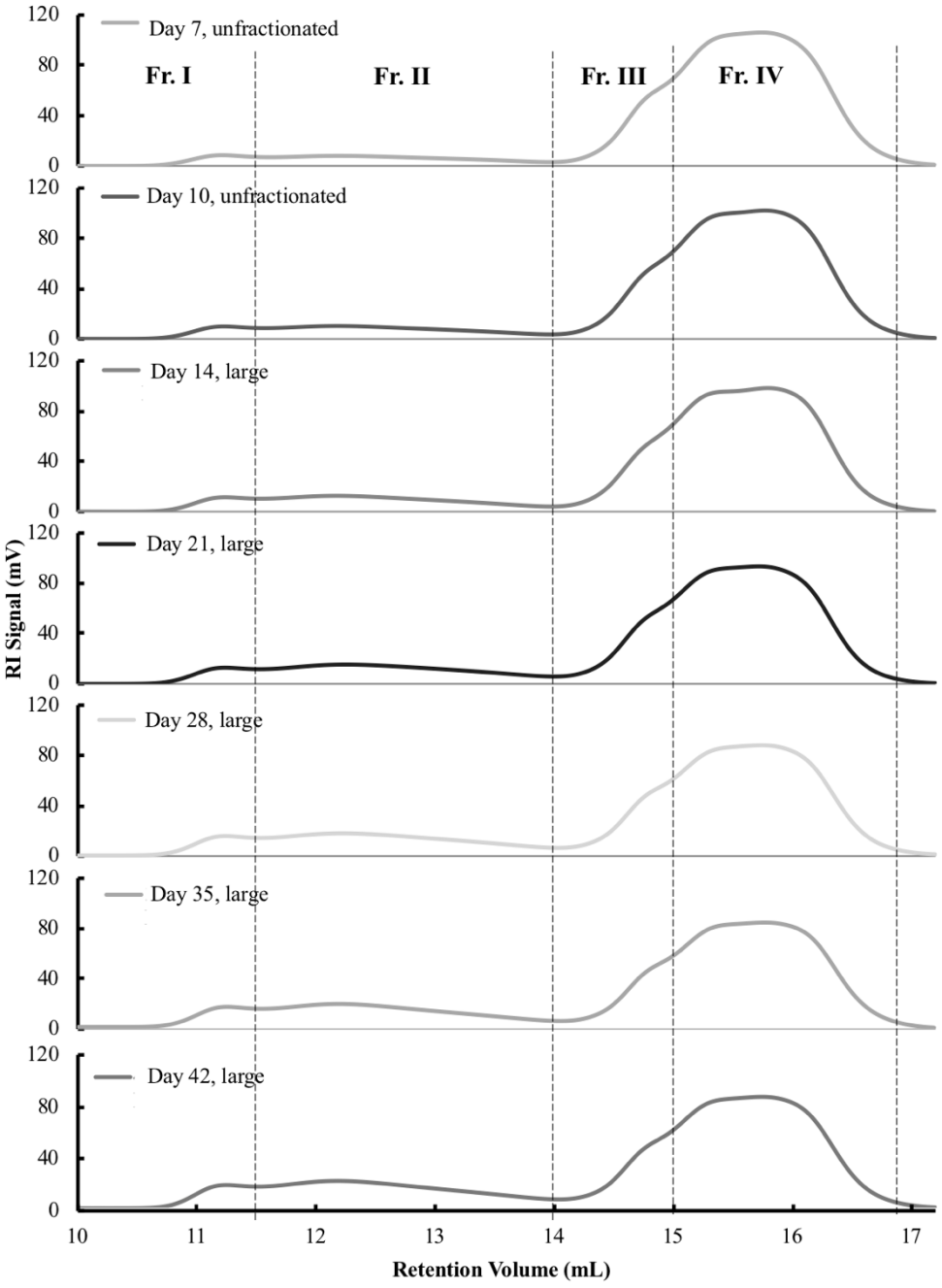

Figure 2.16 HPSEC chromatograms of debranched large SY Ovation starch.
Fraction (Fr.) I eluted at 10.4 – 11.5 mL, Fr. II eluted at 11.5 – 14.0 mL, Fr. III eluted at 14.0 mL – 15.0 mL, and Fr. IV eluted at 15.0 – 16.8 mL. Starch collected on Day 7 and Day 10 was not separated into large and small granules because the primary starch granules were smaller than 10 μm.

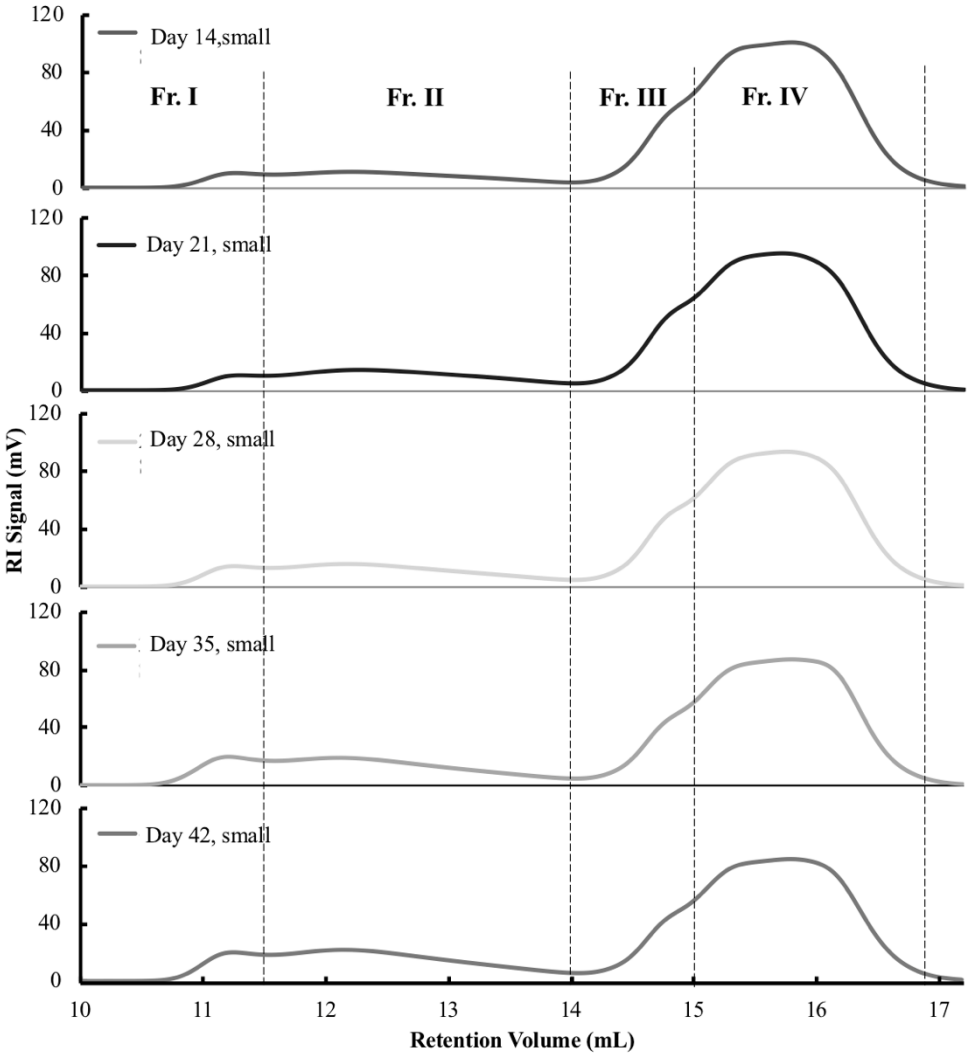

Figure 2.17 HPSEC chromatograms of debranched small SY Ovation starch.

Fraction (Fr.) I eluted at 10.4 – 11.5 mL, Fr. II eluted at 11.5 – 14.0 mL, Fr. III eluted at 14.0 mL – 15.0 mL, and Fr. IV eluted at 15.0 – 16.8 mL.

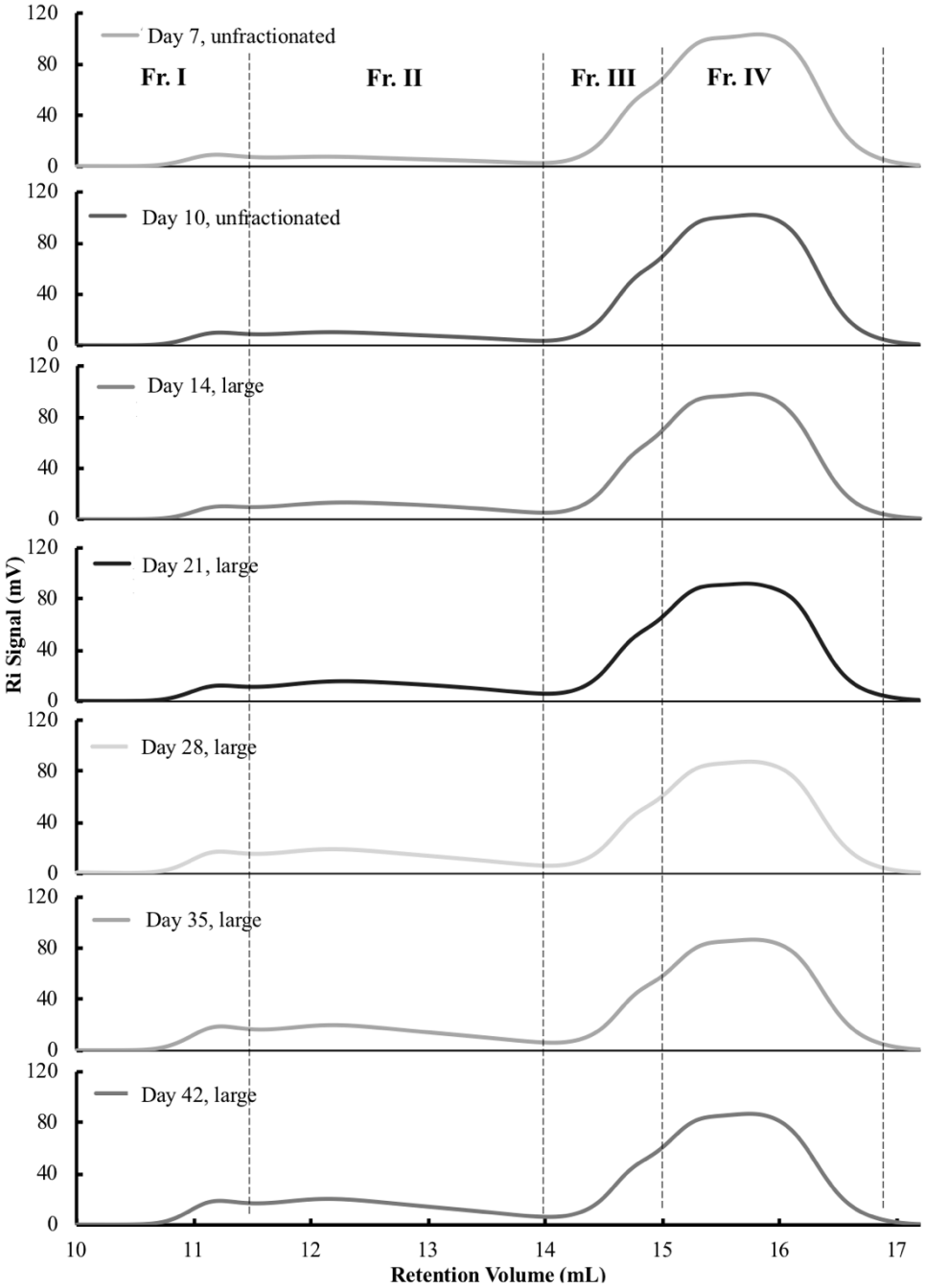

Figure 2.18 HPSEC chromatograms of debranched large UI Sparrow starch.
Fraction (Fr.) I eluted at 10.4 – 11.5 mL, Fr. II eluted at 11.5 – 14.0 mL, Fr. III eluted at 14.0 mL – 15.0 mL, and Fr. IV eluted at 15.0 – 16.8 mL. Starch collected on Day 7 and Day 10 were not separated into large and small granules because the primary starch granules were smaller than 10 μm.

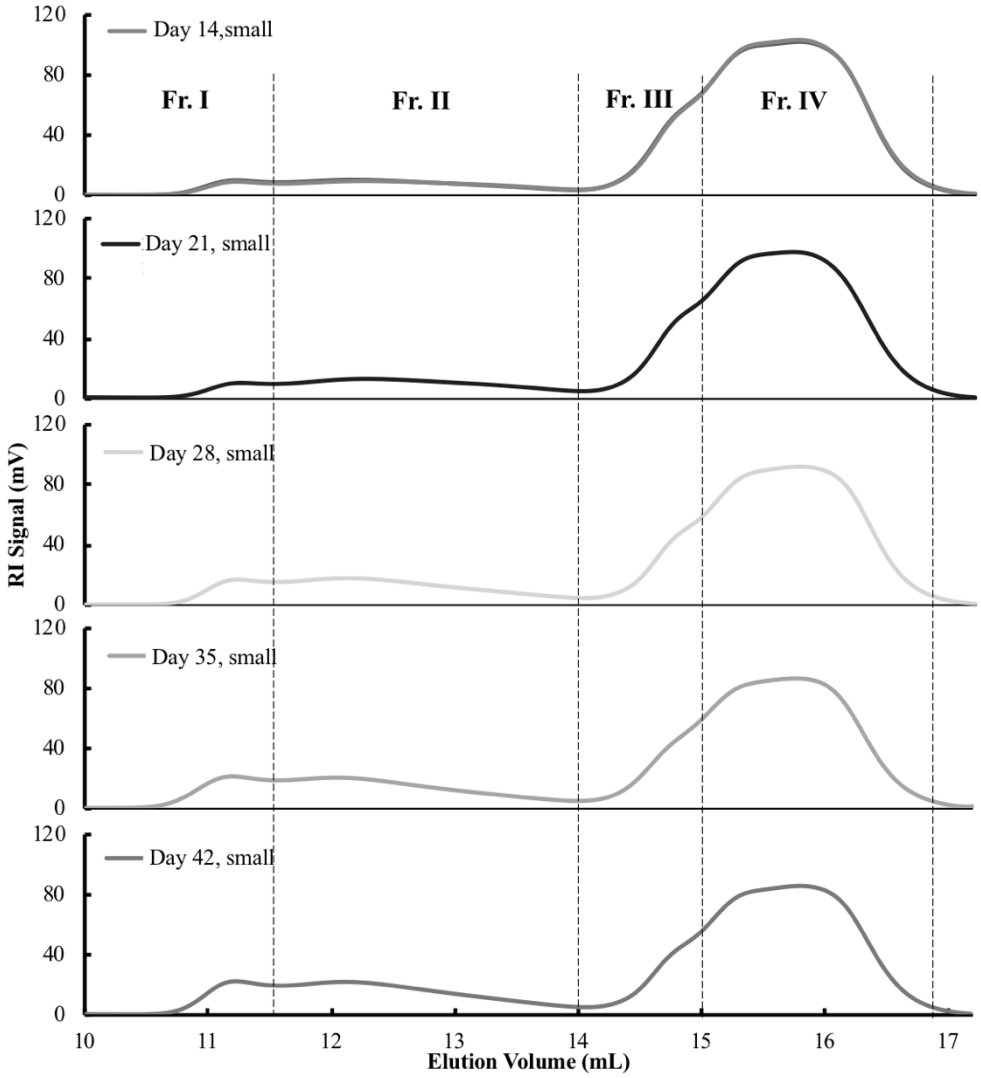

Figure 2.19 HPSEC chromatograms of debranched small UI Sparrow starch.
Fraction (Fr.) I eluted at 10.4 – 11.5 mL, Fr. II eluted at 11.5 – 14.0 mL, Fr. III at eluted 14.0 mL – 15.0 mL, and Fr. IV eluted at (15.0 – 16.8 mL).