Effect of heat stress on pollen development and seed set in field pea (*Pisum sativum* L.)

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Abstract

Pea (*Pisum sativum* L.) is a major legume crop grown in a semi-arid climate in western Canada, where heat stress often causes flower abortion and reduces yield. Heat specifically affects pollination and seed set, the processes associated with pollen fertilizing an ovule to form an embryo, and seed development. The goals of this research were to investigate the effect of heat stress on pollen development, function, and seed set, and to identify single nucleotide polymorphism (SNP) markers associated with reproductive development traits via association mapping.

Heat stress reduced pollen viability, the proportion of ovules that received a pollen tube, seed number per pod, and the seed-ovule ratio in a pod when exposed to 35/18°C day/night temperatures for 4-7 days. Heat stress also reduced ovule viability with $P = 0.09$. High temperature reduced *in vitro* pollen germination by approximately 30% in CDC Sage and 55% in CDC Golden when the 10-h incubation temperature increased from 24 °C to 36 °C. Pollen wall (intine) thickness increased by 46% from 222 nm to 414 nm as a result of heat stress, and anther dehiscence failed to occur following exposure to 35/18°C day/night for 7 days. The lipid region of the pollen coat and exine of CDC Sage was more stable compared to CDC Golden, which may explain the greater robustness of CDC Sage pollen to elevated temperature.

Timing of flower appearance and age of the two flowers is sequential on a nodal raceme, and nodes and flowers along the main stem are also sequential. Heat stress impacted young, barely visible floral buds in the developing inflorescence apex more than more advanced buds and open flowers. The flower abortion rate was greater when plants were exposed to heat stress and when young flower buds were visible at the first reproductive node (T1) compared to the development stage when flowers at the second reproductive node were fully open (T2). Similarly, seed-set, pod development, and seed yield were more negatively affected when high temperature exposure started at T1 compared to T2.

Seed development was negatively impacted by the pollen interaction with pistil under heat stress, such as anther indehiscence and fewer ovules evidently fertilized. Heat stress accelerated seed abortion in all ovule positions within pods. In half of the cultivars tested, ovules at the pod's medial and stylar-end positions were more likely to develop into seeds compared to basal ovules. Cultivars with small seed-size such as '40-10' and 'Naparnyk' were able to retain the most ovules and seeds per pod compared to large seed size cultivars, and large-seeded cultivars like 'MFR043' aborted seeds when exposed to heat.

Population structure analysis was conducted on a panel of 92 diverse pea varieties, and they clustered into three subpopulations mainly consistent with their geographical origins. Association analyses identified 60 single nucleotide polymorphisms (SNPs) significantly associated with reproductive development related traits including days to flowering (DTF), duration of flowering (DOF), number of reproductive nodes, number of pods on the main stem, pod set potential, percentage of pod set, and pollen germination reduction due to heat stress. Among these 60 marker-trait associations, 33 SNPs were associated with the onset of flowering, 8 SNPs with pod development, and 19 SNPs with the number of reproductive nodes. Twelve SNPs associated with DTF and 2 SNPs associated with DOF overlapped with the SNP markers associated with the number of reproductive nodes. However, markers associated with *in vitro* pollen germination were not found due to the variability in sampling 92 genotypes. The findings obtained from this research will benefit plant physiologists and plant breeders for a better understanding of successful reproductive development in field pea and other legume crops experiencing increasing temperatures due to global climate change.

Preface

This thesis is based on the findings presented in the following papers and manuscripts:

I. **Jiang, Y.**, R. Lahlali, C. Karunakaran, S. Kumar, A.R. Davis, R. Bueckert. 2015. Seed set, pollen morphology and pollen surface composition response to heat stress in field pea. Plant Cell and Environment 38:2387-2397. (Chapter 3)

II. Lahlali, R., **Y. Jiang**, S. Kumar, C. Karunakaran, X. Liu, F. Borondics, E. Hallin, R. Bueckert. 2014. ATR-FTIR spectroscopy reveals involvement of lipids and proteins of intact pea pollen grains to heat stress tolerance. Frontiers in Plant Science 5:747. (not included in the thesis)

III. **Jiang, Y.**, R.A. Bueckert, R. Lahlali, C. Karunakaran, D. Lindsay, T.D. Warkentin, A.R. Davis. Effect of heat stress on pollen-pistil interactions and patterns of seed development in field pea (*Pisum sativum*). Annals of Botany. Submitted on November 9, 2016. Manuscript number: 16816. (Chapter 5)

IV. **Jiang, Y.**, D. Lindsay, A.R. Davis, Z. Wang, T.D. Warkentin, R.A. Bueckert. Size, position and hierarchy - improving seed retention in heat stress for field pea. In preparation, to be submitted to Crop Science (Chapter 6)

V. **Jiang, Y.**, M. Diapari, B. Tar'an, R.A. Bueckert, T.D. Warkentin. Population structure and association mapping of traits related to reproductive development in field pea. In preparation, to be submitted to Euphytica. (Chapter 9)

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List of Abbreviations

ANOVA	Analysis of variance
ATR	Attenuated total reflectance
CDC	Crop Development Centre
DOF	Duration of flowering
DTF	Days to flowering
FDA	Fluorescein diacetate
FTIR	Fourier transform infrared spectroscopy
HSPs	Heat shock proteins
LSD	Fisher's least significant difference
PAM	Pea Association Mapping Panel
PMC	Pollen mother cell
QTL	Quantitative trait locus
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SK	Saskatchewan
SNPs	Single nucleotide polymorphisms
TEM	Transmission electron microscopy
TSW	Thousand seed weight

Chapter 1 Introduction

1.1 Introduction

Globally, the combined land and ocean surface temperature of Earth has increased by 0.72°C from 1951 to 2012 (IPCC Working Group II, 2013), and temperatures will likely continue to rise during this century. Emission of greenhouse gases such as carbon dioxide, methane, and nitrous oxide from agricultural systems is one of the main sources leading to the global increase of temperature (Smith and Olesen, 2010). Heat stress from the rise in ambient temperature and frequency of warm weather spells has become an agricultural issue in many areas in the world (Wahid et al., 2007).

Grain crops, including oilseeds, cereals and pulses, are vulnerable in the field to warm temperature. Reproductive growth is the most sensitive to heat, particularly at temperatures approaching 30°C (Morrison and Stewart, 2002; Bueckert and Clarke, 2013; Bueckert et al., 2015), resulting in poor pollination, flower abortion, loss of seed, and lower yield. High temperature has been reported to reduce seed yield in field pea (Lambert and Linck, 1958; Karr et al., 1959; Jeuffroy et al., 1990; Bueckert et al., 2015). Ambient temperatures over 25°C led to loss of seed yield in field pea under field conditions in Australia (Sadras et al., 2012). Temperatures above 28°C reduced seed yield as well as the period from flowering to maturity in field pea under dryland conditions in western Canada (Bueckert et al., 2015). Severe heat stress (33/30°C day/night for 2 days) resulted in immediate abortion and abscission of reproductive organs in pea under environmentally controlled conditions (Guilioni et al., 1997, 2003). High temperature reduced the number of seeds in pea (Jeuffroy et al., 1990; Guilioni et al., 2003). Similarly, hot and dry growing conditions caused abscission of already formed buds, flowers, and young ovules in field pea, leading to 60-80% seed yield loss (Makasheva, 1984).

Successful pollination needs viable pollen, which is recognized by the female stigma surface, and pollen hydrates and a pollen tube germinates into the stigma to fertilize ovules within the ovary. Understanding the process of pollination, and screening for more robust pollen and seed set in diverse pea germplasm could improve heat resistance of pea crops.

1.2 Statement of hypotheses and objectives

The goal of this research was to evaluate the effects of heat stress on pollen development and seed set in pea cultivars and germplasm. Selected cultivars from a pea germplasm collection (94 varieties) at the Crop Development Centre (CDC) of University of Saskatchewan, known as the "Pea Association Mapping Panel (PAM)", were tested in this study, as a likely source of genotypes with heat-robust pollen and pollination mechanisms. The effect of heat stress on pollen development and seed set were evaluated from floral biology, crop physiology, and plant breeding perspectives. Little is known about these pea varieties in response to abiotic stress, and a better understanding of the physiological processes can facilitate identifying sensitive steps where heat tolerance is required.

I have proposed the following hypotheses. First, elevated temperatures would negatively affect pollen development and seed set when the diurnal and night temperatures are above temperature thresholds. Second, certain reproductive stages may be more sensitive compared to other stages when exposed to heat stress. Third, cultivars would differ in heat tolerance with respect to pollen performance, embryo formation, and seed development. Fourth, genetic regions where genes control reproductive development can be identified using association mapping. In order to achieve the overall goal and test the above hypotheses, experiments with the following objectives were conducted.

Exp. 1 – Day temperature study (Chapter 3)

The main objective was to determine the critical day temperature threshold above which the temperature began to reduce *in vitro* percentage pollen germination, pollen-tube growth, and seed set of field pea under environmentally controlled conditions using two currently grown cultivars, one heat sensitive and one more heat robust in terms of yield. Secondly, the effects of heat stress on these parameters, as well as pod formation, were evaluated across five temperature regimes. Finally, the molecular composition of the pollen grain surface was examined using mid-infrared spectroscopy to investigate its significance in pea pollen tolerance to elevated temperatures.

Exp. 2 – Night temperature study (Chapter 4)

The objective of this experiment was to evaluate the effect of varying elevated night temperatures while maintaining a constant daytime temperature during the flowering stage on morphological traits in pea. In many crops, high night temperature is associated with heat stress, but little is known about effects on pea.

Exp. 3 - Relationship between pollen interaction with pistil and seed development (Chapter 5)

The aims of this study were to investigate the relationship between pollen interaction with the pistil and seed development under heat stress, to test any resource gradients within five nodes of the plant and two flowers on individual racemes per node, and to test the influence of heat stress on ovule fate with respect to ovule position within fertilized pods.

Exp. 4 – Ovule position study (Chapter 6)

The objectives of this study were to investigate whether ovule development is dependent on its position within pods and pods at different nodes when exposed to heat stress, and to screen for more heat-resistant cultivars in ovule fertilization and subsequent seed development among 16 cultivars in growth chambers and in the field.

Exp. 5 – Timing and duration study (Chapter 7)

The objectives of this experiment were to determine the sensitive reproductive stage to heat stress, and to test the effects of different durations of exposure to heat stress on pollen development and seed set in different pea cultivars.

Exp. 6 – Pollen germination screening study (Chapter 8)

The objectives of this experiment were to test the effects of heat stress on *in vitro* pollen germination and pollen-tube growth, as well as to evaluate whether 24 different cultivars differed with respect to these two traits.

Exp. 7 – Association mapping (Chapter 9)

The objectives of this experiment were to diagnose the genotype and environment interaction on days to flowering, analyze the population structure of a panel of 92 pea varieties, as well as identify DNA markers associated with reproductive development related traits using previously published gene-anchored SNP markers (Sindhu et al., 2014). These traits included days to flowering, duration of flowering, number of reproductive nodes, number of pods on the main stem, percentage of pods set on the reproductive nodes, and pollen germination reduction due to heat stress.

Chapter 2 Literature review

2.1 Origin, production, agronomic characteristics, and nutritional values of field pea

The family Fabaceae is the third largest family of angiosperm plants [after orchids (Orchidaceae) and asters (Asteraceae)] with more than 650 genera and 18,000 species (Polhill and Raven, 1981), and constitutes three subfamilies - Mimosoideae, Caesalpinioideae, and Papilionoideae (Doyle and Luckow, 2003). The subfamily Papilionoideae comprises the majority of major economically important legume crops (Zhu et al., 2005) such as field pea (*Pisum sativum*), lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*), soybean (*Glycine max*), peanut (*Arachis hypogaea*), mungbean (*Vigna radiata*), and alfalfa (*Medicago sativa*). Field pea is a diploid member (2n=2x=14) with a haploid genome size of 4.45 Giga base pairs (Dolezel and Greilhuber, 2010). Field pea is a member in the family of cool season legume crops which includes lentils, kidney beans, and chickpeas (Goodwin, 2008).

Field pea is an economically important legume crop in western Canada since it yields in a semiarid environment, and provides a rich source of food for humans and fodder for animals. Pea is of great importance to cropping systems due to its ability to fix nitrogen. Canada was the largest producer of peas with a total production of 3.4 million, followed by China, Russia, USA, and India in 2014 (FAOSTAT, 2016). The use of field pea dates back to Neolithic times (Helback, 1959). The history of the cultivation of field peas in western Canada has been over 100 years since farmers began to farm in the prairies (Goodwin, 2008). Pea cultivation started to increase from 1977 until the present day. Field pea production is currently found in Alberta, Saskatchewan, and Manitoba (Goodwin, 2008). Pea production in Canada was 1,389,000 ha in 2010, 974,000 ha in 2011, 1,475,300 ha in 2012, 1,329,000 ha in 2013, and 1,588,000 ha in 2014 with yields of 2170 kg ha^{-1} in 2010, 2570 kg ha^{-1} in 2011, 2270 kg ha^{-1} in 2012, 2980 kg ha^{-1} in 2013, and 2400 kg ha^{-1} in 2014 (Statistics Canada, 2016).

Field pea can be adapted to the moist dark brown and black soil zones, and it prefers well-drained and clay loam soils in western Canada (Goodwin, 2008). Field pea grows well when it is in rotation with cereals including barley or spring durum wheat (Goodwin, 2008). It can be seeded in spring and matures in late summer (3 months) in Canada when the spring growth habit is used. The recommended seeding rate for semi leafless field pea is 88 plants m^{-2} (Saskatchewan Pulse

Growers, 2000). Abiotic factors which restrict production include lodging, maturity and growth habit, pod shattering, fall frost, and storage conditions (Goodwin, 2008).

Seeds of field pea are beneficial for human and animal consumption due to their rich content of thiamin (Aitken, 1978) and amino acids such as lysine and tryptophan (Aitken, 1978; McKay et al., 2003). Seeds of field pea have approximately 21-25% protein, high carbohydrate content, 86-87% total digestible nutrients (McKay et al., 2003), high fiber content (20%), and low fat (2%) (Pulse Canada, 2013). The trypsin inhibitors of field pea are 5-20% less than soybean, which allows field pea to be directly fed to livestock (McKay et al., 2003). Field pea has relatively low oil content ranging from 1.5-3.7%, which suggests current cultivars are not suitable to be used as an oilseed crop (Welch and Griffiths, 1984; Yoshida et al., 2007).

2.2 Reproductive development in field pea

Most pea genotypes are facultative long-day plants (Weller et al., 1997). Both day length and temperature are critical in flowering of pea (Weller et al., 2009). Flowering time in pea is mainly controlled by flowering genes associated with photoperiod and temperature (Alcade et al., 1999; Alcalde and Larrain, 2006). Duration of flowering in field pea ranges from 3-40 days depending on temperature, humidity, and variety (Makasheva, 1984).

Pea flowers and pods develop on a raceme that is an inflorescence with an indeterminate rachis. Due to indeterminate growth patterns, flower initiation and their progressive development into pods start at the first reproductive node of the rachis and continues acropetally. Pea flowers have five sepals, five petals (two fused keel ones, two wings, and one standard), ten anthers (nine fused into a filament tube and one partially free), and a single central carpel (Tucker, 1989). The color of the corolla in grain and vegetable varieties is generally white, and in fodder or green manuring varieties the corolla has pink pigmentation with different intensity (pink, red, and purple) and rarely white (Makasheva, 1984). Field pea is predominantly self-pollinated, but cross pollination sometimes occurs under hot and dry conditions. Flower buds are visited by the larvae of thrips before flowers are fully open (Makasheva, 1984).

It takes approximately 4 h from pollination to entry of the first pollen tube into the micropyle of the ovule (Makasheva, 1984). Fertilization occurs 3-10 h after pollen contacts the stigma in field pea (Makasheva, 1984). The number of ovules is genetically determined, and varies from 4 to 12 per ovary depending on genotype and growing conditions (Makasheva, 1984). The number of seeds

set is positively correlated with the number of ovules (Makasheva, 1984). Seeds of field pea consist of an embryo (the embryo axis and two cotyledons) and the seed coat. The number of seeds is variable with a range from 3 to 12 (Makasheva, 1984). Thousand seed weight varies from less than 150 g to over 250 g (Makasheva, 1984), and ranges from 140 g to 280 g according to Saskatchewan Seed Growers Association (2016).

2.3 Sexual reproduction in flowering plants

Stamens (androecium) consist of a narrow stalk (filament) and anther (pollen-producing structure). The mature anther usually dehisces longitudinally to release mature pollen grains. The anther wall is composed of the epidermis, endothecium, middle layer, and tapetum (Greyson, 1994). The tapetum is generally assumed to have the following functions: (1) providing nutrition for the sporogenous tissue; (2) discharging callose during the breakup of the tetrads; (3) generating Ubisch bodies; (4) discharging pollenkitt, typhine, enzymes, recognition proteins, and other substance on the pollen grains (Greyson, 1994).

Each anther has four pollen sacs (microsporangia or locules). Within each pollen sac, an archesporial cell divides mitotically to produce many pollen grain mother cells (PMC). Each PMC undergoes meiosis to produce haploid tetrads that are enclosed in a callose wall (Richards, 1997; Blackmore et al., 2007). Breakdown of the callose wall releases the uninucleate microspores. These microspores are polarized with a large vacuole at one end and a nucleus at the other, preparing the microspore for asymmetric cell division. The cell plate is formed during pollen mitosis I with a distinctive hemispherical shape, and after division the smaller generative cell detaches from the cell wall and is enveloped within the vegetative cell. The generative cell undergoes another round of cell division to form the two sperm cells of the mature pollen grain (Richards, 1997; Blackmore et al., 2007). The wall layers of a pollen grain consist of the outer wall (exine), the inner wall (intine), and plasmalemma (Greyson, 1994). The main composition of exine contains lipoprotein and sporopollenin, and the intine mainly consists of cellulose (Richards, 1997).

A mature pollen grain contains a vegetative cell in which one generative cell or two sperm cells (formed from the generative cells during pollen mitosis II) are embedded. Thus, mature pollen grains are either bicellular or tricellular. The bicellular pollen grain consists of one generative and one vegetative cell. In many species, the generative cell does not undergo its mitosis until after the pollen tube starts to grow, and the metabolism rate and germination rate are slower compared with tricellular pollen grains. Mature pollen grains in field pea are bicellular (Brewbaker, 1967;

Makasheva, 1984). The tricellular type has one vegetative and two sperm cells, and the second mitosis event occurs during anther development, while pollen germination and metabolism is relatively active (Hoekstra and Bruinsma, 1975).

The gynoecium (pistil) consists of the stigma, style, and ovary. The ovary (base of the megasporophyll) consists of three layers – the outer integument, inner integument, and nucellus (the innermost tissue of the ovule that encloses the embryo sac). Ovules are enclosed within a modified sporangium-bearing leaf (megasporophyll, or carpel) that is free or fused into an ovary. There are two main types of stigmas, wet stigma and dry stigma, usually correlated with bicellular and tricellular pollen grains, respectively. A wet stigma, as in field pea, produces a stigmatic exudate composed of sugar, lipids, phenolic compounds, and traces of enzymes. The exudate plays a key role in pollen contact, hydration, and germination on the stigma, as well as preventing the cuticles on stigmatic papillae from dehydration (Richards, 1997). In contrast, a dry stigma does not produce the exudate, and the lipids and proteins in the pollen exine and pollen coat play a critical role in pollen adhesion on the stigma. Pollen grains penetrate the cuticle of stigma papilla enzymically, and absorb water from the stigma's turgid cells and become hydrated (Richards, 1997).

Pollen tubes (extending from pollen grains, the microgametophytes), filled with callose plugs (β-1,3, glucan), grow intercellularly through the transmitting tissue in the center of the style that provide water, sugar, and amino acids for pollen-tube growth (Richards, 1997). Finally, pollen tubes reach an ovule placenta, and enter a micropyle, and deliver two sperm cells to the embryo sac (female gametophyte). One sperm cell eventually fuses with the haploid egg cell to form the diploid zygote (the future embryo), and the other sperm cell fuses with the secondary endosperm nucleus, to form the primary endosperm nucleus and, eventually, the endosperm. At this point, double fertilization has occurred (Richards, 1997).

2.4 Effect of elevated temperatures in field pea

Extreme heat stress reduces crop yield (Lesk et al., 2016). The phenomenon of the reduction in seed number, reproductive nodes, and seed yield due to heat stress in field pea has been reported by different research groups (Lambert and Linck, 1958; Karr et al., 1959; Jeuffroy et al., 1990; Guilioni et al., 2003; Sadras et al., 2012; Bueckert et al., 2015). Seed yield of field pea decreases when the maximum temperature exceeded 25°C at the reproductive stage under field conditions in Australia (Sadras et al., 2012), whereas early studies in field pea reported that seed yield decreased

when temperatures were above 16/10°C day/night temperatures at growth chambers, mainly due to a reduction in the number of pods per plant (Stanfield et al., 1966). High night temperatures (24/30°C day/night temperatures with a 12 h photoperiod) caused 25% of yield loss in field pea, as opposed to 8% of yield loss for high day temperatures (32/15°C day/night temperatures with a 12 h photoperiod), and thus high night temperatures caused more yield loss compared to high day temperature (Karr et al., 1959). Field pea is the most sensitive to environmental stress at the stages between the early flowering and the beginning of seed fill for the last seed-bearing fruiting node (Guilioni et al., 2003). Seed development in pea was also sensitive to elevated temperatures approximately 6-12 days after anthesis, and heat stress decreased the number of seeds per pod, leading to seed yield loss (Jeuffroy et al., 1990).

2.5 General physiological responses of plants to elevated temperatures

The impact of heat stress varies based on the intensity of temperature, duration of exposure, and rate of temperature increase (Hall, 2012). Heat stress can impair plant function and development by direct effects of high temperature and indirect effects of high evaporative demand and water stress (Hall, 2012). Heat stress, similar to many other abiotic stresses, can reduce cell proliferation, photosynthesis, respiration, and membrane stability, and increase protein denaturation and the accumulation of reactive oxygen species (ROS) leading to cell death (Taiz and Zeiger, 2010).

High temperature increases the fluidity of plant membrane lipids, decreases the strength of hydrogen bonds, and disturbs electrostatic interactions between protein polar groups within the aqueous phase of the membrane (Taiz and Zeiger, 2010). Therefore, high temperature causes leakage of ions, and changes the structure and composition of plant membranes (Taiz and Zeiger, 2010). High temperature can also damage protein three-dimensional structure, leading to a loss of enzyme structure and activity (Taiz and Zeiger, 2010). The disruption in membrane integrity and protein stability inhibits cellular processes.

Heat stress disturbs photosynthesis, and the targeted sites of heat stress on photosynthesis include Photosystem II (PSII), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), Cytochrome b559 (Cytb559), and plastoquinone (PQ) (Mathur et al., 2014). Under hot conditions, the carbon produced from photosynthesis cannot replace the substrate carbon used for respiration, so carbohydrate reserves decrease due to the imbalance between photosynthesis and respiration, and thus fruits and vegetables lose sweetness (Taiz and Zeiger, 2010). The reduction in

photosynthate production can also be caused by stress-induced stomatal closure and decreased leaf canopy area (Taiz and Zeiger, 2010).

2.6 Impact of heat stress on plant sexual reproductive processes

Reproductive development in annual crop species is considered to be among the most sensitive phases of the life cycle in response to environmental stresses (Gross and Kigel, 1994; Hedhly et al., 2009; Devasirvatham et al., 2012). Heat stress immediately before or during anthesis induces sterility in many plant species, especially for pulse legumes (Siddique et al., 1999). High temperature during reproduction reduces pollination, and causes abscission of floral buds, flowers and pods, all of which result in substantial yield loss (Duthion and Pigeaire, 1991; Guilioni et al., 1997; Devasirvatham et al., 2012). Other legumes and crops with similar responses include common bean (*Phaseolus vulgaris* L.; Konsens et al., 1991), cowpea (Ahmed et al., 1992), tomato (*Solanum lycopersicum*; Levy et al., 1978), and cotton (*Gossypium hirsutum*; Reddy et al., 1992).

Legume crops are extremely sensitive to heat stress at the anthesis stage; exposure to high temperature (30-35°C for a few days) can cause severe yield losses due to flower and pod abortion (Siddique et al., 1999). The uninucleate stage (the microspore) of male reproductive development is the most sensitive to heat stress in many crops (Sage et al., 2015), for example garlic (Mayer et al., 2015). The most sensitive stage of reproduction in cowpea occurred 5-7 days prior to anthesis, which corresponded to the timing that tetrads were released from the microsporic mother cell sac (Warrag and Hall, 1984a). Phenology was accelerated in late-sown lentil plants that were exposed to higher temperatures during reproduction compared to early-sown plants, resulting in significant reductions in biomass, number of flowers and pods, flowering period, pod-filling duration, and seed yield (Bhandari et al., 2016).

Successful seed development is related to pollen availability. For example, heat stress (>32/20°C day/night temperatures) significantly reduced pod set in chickpea, which was associated with the reduction in pollen viability, pollen load, pollen germination (*in vivo* and *in vitro*), and stigma receptivity (Kaushal et al., 2013). A reduction in seed set and seed number in kidney bean was primarily associated with decreased pollen production and pollen viability under heat stress (Prasad et al., 2002). Similarly, lentil varieties with a higher number of filled pods per plant also had high pollen viability under heat stress (Kumar et al., 2016). Sorghum genotypes with higher ceiling temperature for pollen germination were more likely to set seeds successfully (Djanaguiraman et al., 2014). Elevated temperatures reduced seed set percentage in sorghum, and

seed set percentage was positively correlated with *in vitro* pollen germination under elevated temperatures (Singh et al., 2015).

Reproductive development in flowering plants can be divided into three stages: gametophyte development (from meiosis to pollination), the progamic stage (from pollination to double fertilization), and embryo development (from zygote to seed; Herrero and Hormaza, 1996). Therefore, evaluation of heat stress on plant sexual processes is separated into three aspects in this review: gametophyte development, progamic development, and embryo development (Table 2.1).

2.6.1 Impact of heat stress on gametophyte development (from meiosis to pollination)

Male sterility is associated with the failure of anther dehiscence and the lack of viable pollen grains (Wilson et al., 2011). Anther indehiscence, the failure of anthers to split open and release mature pollen, occurs in cowpea (Warrag and Hall, 1984a) due to degeneration of the tapetal layer when exposed to heat stress (33/30°C day/night temperatures; Ahmed et al., 1992). The degeneration of tapetum cells was also observed in common bean at 33/29°C day/night temperatures, leading to premature pollen development (Suzuki et al., 2001). Heat stress induced cytological abnormalities in the tapetum of snap bean, where the stacks of rough endoplasmic reticulum (RER) observed in control plants' tapetum were not apparent in heat stressed plants (Suzuki et al., 2001). Abnormal structure of RER may block its function in the tapetum and cause early degeneration of the tapetum, thus leading to pollen sterility (Suzuki et al., 2001). Heat stress induces the premature progression of early developments in anthers. This includes degradation of anther wall cells and progression to meiosis in pollen mother cells in barley. At the same time, transcription in anthers was changed (Sakata and Higashitani, 2008).

Heat stress causes pollen abnormalities such as small, shrunken, and empty pollen grains, and a reduction in pollen production and fertility at early flowering in legumes (Devasirvatham et al., 2012). Heat stress has decreased pollen viability in common bean (Gross and Kigel, 1994; Prasad et al., 2002), cowpea (Warrag and Hall, 1984a), chickpea (Devasirvatham et al., 2012), peanut (Boote et al., 2005), as well as in non-legume crops such as rice (*Oryza sativa* L.; Endo et al., 2009), cotton (Song et al., 2015), tomato (Pressman et al., 2002), pepper (Erickson and Markhart, 2002), and flax (Cross et al., 2003). Pollen viability is an indication of the ability of pollen grains to deliver their sperm cells to embryo sacs following pollination (Ahmed et al., 1992). Reduced pollen viability was associated with decreased sucrose content in the flower in cotton (Echer et al., 2014). Similarly, reductions of starch and soluble sugar concentrations were observed in anther walls and

pollen grains of tomato, which were likely associated with the reduction in pollen viability when exposed to 32/26°C day/night temperatures (Pressman et al., 2002).

For successful sexual reproduction, pollen grains have to survive and be able to germinate on the stigma of a flower. Heat stress has reduced *in vitro* pollen germination percentage and pollen-tube length, and these studies have been conducted in many crops including cotton (Kakani et al., 2005; Song et al., 2015), soybean (Koti et al., 2005; Salem et al., 2007), groundnut (Kakani et al., 2002), chickpea (Devasirvatham et al., 2012), sorghum (Singh et al., 2016), field pea (Petkova et al., 2009; Lahlali et al., 2014; Jiang et al., 2015), and canola (Singh et al., 2008; Morrison et al., 2016). Morrison et al. (2016) suggested that pollen germination may not be the only reason for the reproductive failure under heat stress, because canola pollen grains were still able to germinate at 33°C.

High temperature can also reduce pollen production per flower in chickpea (Devasirvatham et al., 2012). The number of pollen grains per flower decreased from approximately 2000 to almost zero when the air temperatures increased from 28/18°C to 40/30°C day/night temperatures in kidney bean (Prasad et al., 2002). Similarly, the number of pollen grains per flower was reduced from 700,000 to less than 400,000 when temperatures increased from 28/22°C to 32/26°C day/night temperatures in tomato (Pressman et al., 2002).

The abortion of pollen development is associated with a reduction in endogenous auxin concentrations in the developing anthers of barley and *Arabidopsis*, and expression of the *YUCCA* auxin biosynthesis genes was repressed due to heat stress (Sakata et al., 2010). Exposure to heat stress additionally increased the accumulation of ROS in pollen grains of grain sorghum (Prasad and Djanaguiraman, 2011) and winter wheat (Narayanan et al., 2015).

The morphology of a pollen grain can also be affected by stress. Abnormal pollen wall architecture was observed in several crops including common bean (Porch and Jahn, 2001), field pea (Jiang et al., 2015), soybean (Koti et al., 2005; Djanaguiraman et al., 2013), sorghum (Djanaguiraman et al., 2014), and pepper (Erickson and Markhart, 2002). For example, heat stress increased the exine thickness in soybean pollen grains (Djanaguiraman et al., 2013), and increased the intine thickness in pea pollen grains (Jiang et al., 2015). Pollen grains did not form a normal and thick exine in pepper under heat stress (Erickson and Markhart, 2002).

2.6.2 Impact of heat stress on the progamic stage (from pollination to double fertilization)

Heat stress causes a reduction in stigma receptivity, and pollen grains retained on the stigma surface, and ovule viability in various crops (Kaushal et al., 2016). For example, heat stress during the post-anthesis phase led to poor pollen germination on the stigma and decreased pollen-tube growth in the style of chickpea (Devasirvatham et al., 2012), common bean (Gross and Kigel, 1994), and cotton (Song et al., 2015). Similarly, pollen grains were poorly anchored on the stigma in rice under heat stress (39/30°C day/night temperatures; Endo et al., 2009). Heat stress reduced the duration of stigmatic receptivity in sweet cherry (*Prunus aviuml* L.; Hedhly et al., 2003) and ovule longevity in plum (*Prunus mume*; Cerovic et al., 2000). Heat stress also decreases ovule number and viability in common bean (Monterroso and Wien, 1990), tomato (Din et al., 2015) and *Arabidopsis thaliana* (Whittle et al., 2009).

Heat stress has negatively impacted ovule development in wheat, leading to yield loss (Saini et al., 1983). One third of heat-stressed ovaries prior to anthesis in wheat exhibited an abnormal nucellus or embryo sac (Saini et al., 1983). Some of these abnormal ovules contained small embryo sacs without complete cellular organization or lacking any recognizable cells; the development of the nucellus degenerates in some of the ovules (Saini et al., 1983). Heat stress (32/26°C) caused abnormal embryo sac development in rapeseed (*Brassica napus*) – the ovule growth was not uniform along the length of the ovaries of both pollinated and unpollinated flowers (Polowick and Sawhney, 1988). Heat stress at the post-anthesis phase led to fertilization failure in common bean (Ormrod et al., 1967). *In vitro* fertilization rate was also significantly reduced in maize in pollinated spikelets when the temperatures exceeded 36°C (Dupuis and Dumas, 1990).

2.6.3 Impact of heat stress on embryo development (from zygote to seed)

Despite successful fertilization (pollen and ovule), the embryo can still abort under certain conditions. Heat stress led to acceleration in embryo abortion in cowpea (Warrag and Hall, 1984a, b) and common bean (Gross and Kigel, 1994). Fertilized ovules may stop swelling rapidly in tomato due to high temperature (Kinet and Peet, 1997). Heat stress reduced seed size during seed filling (Kaushal et al., 2016), as well as the duration of seed filling leading to yield loss (Boote et al., 2005). Heat stress modifies source-sink relations, reduces the kernel filling stage, and produces unfilled or aborted seeds (Kaushal et al., 2016). Heat stress (>32/20°C day/night temperatures) accelerated pod abortion as well as shortened the duration of seed set, leading to yield loss in lentil (Bhandari et al., 2016).

Shortly after embryo formation, a seed proceeds through cell division to the formation of seed tissues. The later stages of seed growth (after division) are seed filling and desiccation. In seed filling, amino acids and sugars are imported. Exposure to high temperature during seed filling can accelerate senescence of seeds, decrease seed set and seed weight, and lead to yield loss due to the fact that plants tend to divert energy and resources such as photosynthates to deal with heat stress (Siddique et al., 1999). Similarly, a reduction of seed set under heat stress was associated with a decreased amount of carbohydrates and growth regulators in plant sink tissues in tomato (Kinet and Peet, 1997).

Table 2.1. A summary of the effect of heat stress on reproductive development in different crops.

Sexual organ development	Heat stress effects	High temperature regimes	Crops	References
Androecium				
Tapetum	Early degeneration of tapetum cells	33/27°C (day/night)	Common bean	Gross and Kigel, 1994
Tapetum	Early degeneration of tapetum cells	33/30°C (day/night)	Cowpea	Warrag and Hall, 1984a; Ahmed et al., 1992
Anther	Short anthers without pollen grains	Pre-meiotic stage of PMCs at 30/25°C (day/night) for 5 days	Barley	Sakata et al., 2000
Anther	A failure in anther dehiscence	32/27°C (day/night) during microsporogenesis	Common bean	Porch and Jahn, 2001
Anther	A failure in anther dehiscence	33/30°C (day/night)	Cowpea	Ahmed et al., 1992
Pollen	No or little cytoplasm in pollen grains	30/25°C (day/night) for 5 days at early differentiation of panicle	Barley	Sakata et al., 2000
Pollen	Swollen pollen grains and little starch accumulation	Meiosis of PMCs at 30/25°C (day/night) for 5 days	Barley	Sakata et al., 2000
Pollen	Reduction in pollen viability	40/25°C (day/night)	Chickpea	Devasirvatham et al., 2012
Pollen	Reduction in pollen viability	44/34°C (day/night)	Bean and peanut	Boote et al., 2005
Pollen	Reduction in pollen viability	≥33/23°C (day/night)	Kidney bean	Prasad et al., 2002
Pollen	Reduction in pollen viability	32/29°C (day/night)	Cotton	Echer et al., 2014
Pollen	Reduction in pollen viability	39/30°C (day/night)	Rice	Endo et al., 2009
Pollen	Reduction in pollen viability	32/26°C (day/night)	Tomato	Pressman et al., 2002
Pollen	Decreased pollen production per flower	≥33/23°C (day/night)	Kidney bean	Prasad et al., 2002
Pollen	Decreased pollen production per flower	32/26°C (day/night)	Tomato	Pressman et al., 2002
Pollen	Decreased pollen production per flower	40/25°C (day/night)	Chickpea	Devasirvatham et al., 2012

Pollen	Reduction in PG† and PTG† (*in vitro*)	≥43°C	Cotton	Kakani et al., 2005; Song et al., 2015
Pollen	Reduction in PG and PTG (*in vitro*)	35/20°C (day/night)	Chickpea	Devasirvatham et al., 2012
Pollen	Reduction in PG and PTG (*in vitro*)	≥47°C	Soybean	Salem et al., 2007; Koti et al., 2005; Djanaguiraman et al., 2013
Pollen	Reduction in PG and PTG (*in vitro*)	≥43°C	Groundnut	Kakani et al., 2002
Pollen	Reduction in PG and PTG (*in vitro*)	≥43°C	Sorghum	Singh et al., 2016
Pollen	Reduction in PG and PTG (*in vitro*)	45°C; 36°C	Field pea	Petkova et al., 2009; Jiang et al., 2015
Pollen	Reduction in PG and PTG (*in vitro*)	33°C	Canola	Morrison et al., 2016
Pollen	Abnormal pollen structure	32/27°C (day/night) during microsporogenesis (1-13 days before anthesis)	Common bean	Porch and Jahn, 2001
Pollen	Pollen surface composition	36/18°C (day/night) for 3 days	Field pea	Jiang et al., 2015
Pollen	Increased ROS† in pollen grains	32/28°C (day/night) for 10 days	Grain sorghum	Prasad and Djanaguiraman, 2011
Pollen	Increased ROS in pollen grains	35/24°C (day/night) for 7 days	Winter wheat	Narayanan et al., 2015
Pollen tube	Reduction in PTG in styles	> 35°C	Cotton	Song et al., 2015
Pollen wall	Disturbed pollen wall architecture	32/27°C (day/night) 12h during microsporogenesis for 12-13 days	Common bean	Porch and Jahn, 2001
Pollen wall	Disturbed pollen wall architecture	38/30°C (day/night)	Soybean	Koti et al., 2005
Pollen wall	Increased intine thickness	36/18°C (day/night)	Field pea	Jiang et al., 2015
Pollen wall	Increased exine thickness	38/28°C (day/night)	Soybean	Djanaguiraman et al., 2013
Pollen wall	Abnormal exine ornamentation	38/28°C (day/night)	Sorghum	Djanaguiraman et al., 2014
Gynoecium				
Stigmatic receptivity	Shortened stigmatic receptivity	35°C	*Annona cherimola*	Lora et al., 2011

Stigmatic receptivity	Shortened stigmatic receptivity	30°C	Sweet cherry	Hedhly et al., 2003
Stigmatic receptivity	Reduced pollen load on stigma	39/30°C (day/night) at the microspore stage	Rice	Endo et al., 2009
Ovule	Reduced ovule viability	35°C for 10 h per day for 2 days	Common bean	Monterroso and Wien, 1990
Embryo	Embryo abortion	30°C for 3 days	Wheat	Saini et al., 1983
Embryo	Embryo abortion	32/26°C	Rapeseed	Polowick and Sawhney, 1988
Embryo	Reduced embryo size	12-42°C	Sunflower	Chimenti et al., 2001
Seed	Sterile seeds	Heading stage of panicles at 30/25°C (day/night) for 5 days	Barley	Sakata et al., 2000
Seed	Reduction in seed number	40/25°C (day/night)	Chickpea	Devasirvatham et al., 2012
Seed	Reduction in seed number	28 or 31°C (day/night) for 6-12 days	Field pea	Jeuffroy et al., 1990
Seed	Reduction in seed set	≥33/23°C (day/night)	Kidney bean	Prasad et al., 2002
Seed	Reduction in seed set	38/28°C (day/night)	Sorghum	Djanaguiraman et al., 2014; Singh et al., 2015
Pod set	Reduction in pod set	40/25°C (day/night)	Chickpea	Devasirvatham et al., 2012

†PG, pollen germination; PTG, pollen-tube growth; ROS, reactive oxygen species

2.7 Plant adaptation to heat stress

Plants can escape from heat stress, or resist it by avoidance or tolerance (Bueckert and Clarke, 2013). Management and breeding approaches, such as shifting planting date and genetic manipulation of crop phenology, have been used to facilitate plants to escape from heat stress (Sadras and Dreccer, 2015). Heat avoidance is the ability of plants to avoid heat stress through some physiological mechanisms (Bueckert and Clarke, 2013). Heat tolerance is the ability of plants to survive under heat stress and usually produce lower economic yield compared to optimal conditions (Hasanuzzaman et al., 2013). The terms, heat tolerance and heat resistance, are also used in the literature interchangeably to mean a trait with stress robustness compared to heat susceptibility.

First, adaptation strategies when plants exposed to heat stress include being indeterminate and having a long flower duration, and thus flowering lasts for a long period during the growing season, and pollen is usually shed during the morning or cooler periods of the day (Hatfield and Prueger, 2015). The manipulation of flowering time regulation is of considerable importance allowing certain genotypes to avoid the most stressful phases during the growing season. Time of flowering in pea varies widely across different locations and years (Truong and Duthion, 1993). Photoperiod, temperature (vernalization and post-vernalization), and genotype are crucial factors for time of flowering in pea (Weller et al., 2009). Recently, more than 20 loci associated with flowering time and inflorescence development in pea have been identified (Weller and Ortega, 2015). Flowering time in pea is mainly controlled by genes associated with photoperiod and temperature (Alcade et al. 1999; Alcalde and Larrain, 2006). Flowering behavior in pea is controlled by six genes including *Lf, Sn, E, Dne, Ppd,* and *Hr* (Murfet, 1985; Weller et al., 1997). Flowering at different nodes is not synchronous due to the indeterminate type of growth habit in pea. Pea flowers in a sequential manner, thus flowering duration varies greatly because of the high degree of variability in the number of reproductive nodes (Roche et al., 1998) and late formed branches which can also flower.

Second, expression of heat shock proteins (HSPs) is an important defensive strategy of plants after exposure to high temperature (Feder and Hoffman, 1999). These HSPs ameliorate photosynthesis, assimilate partitioning, water and nutrient use efficiency, and membrane stability (Camejo et al., 2005; Ahn and Zimmerman, 2006; Momcilovic and Ristic, 2007). For example, expression of an *Arabidopsis thaliana* HSP 101 (*AtHSP101*) enhanced heat tolerance in cotton and

tobacco from an *in vitro* pollen germination array (Burke and Chen, 2015). Expression of the carrot (*Daucus carota*) heat shock protein gene encoding HSP17.7 (*DcHSP17.7*) enhanced heat tolerance in transgenic potato (*Solanum tuberosum*) plants (Ahn and Zimmerman, 2006).

Third, pea varieties with thicker or more epicuticular wax load have lower canopy temperatures, suggesting that incident radiation is more efficiently reflected by the increasing amount of wax that alleviates the damage of water loss and its associated heat stress (Sánchez et al., 2001). Pea varieties with a more pallid green leaf color (possibly an indicator of lower levels of chlorophyll and antenna complexes at the photosystem II reaction center) absorbed less radiation (Sánchez et al., 2001).

Fourth, heat stress is frequently related to a reduction in water availability under field conditions (Simões-Araújo et al., 2003). Under abiotic stresses such as salinity, water, and heat, various plant species may produce different osmolytes including sugars, sugar alcohols (polyols), proline, tertiary and quaternary ammonium compounds, tertiary sulphonium compounds (Sairam and Tyagi, 2004), γ-4-aminobutyric acid (Taiz and Zeiger, 2010), and glycinebetaine (Sakamoto and Murata, 2002), which in turn improve stress tolerance.

Fifth, evaporative cooling capacity allows plants with access to sufficient water to maintain leaf temperatures less than 45°C, even under hot conditions (Taiz and Zeiger, 2010). However, poor air circulation within the canopy reduces the rate of leaf evaporative cooling (Taiz and Zeiger, 2010).

The pod wall ratio, defined as the ratio between pod wall weight and whole pod weight, is partially related to seed abortion, and hence is used as a surrogate for seed abortion, which is considered to be an important trait under water deficiency and heat stress (Sadras et al., 2013). Maintenance of crop growth rate and low pod wall ratio of field pea were also considered to facilitate the adaptation to high temperature and water stress in Australian pea cultivars (Sadras et al., 2013). A negative correlation between grain yield and the pod wall ratio was reported in lupins (Lagunes-Espinoza et al., 1999; Clements et al., 2005) and field pea (Sadras et al., 2013).

From molecular perspectives, heat tolerance mechanisms including maintenance of membrane stability, scavenging of ROS, adjustment of compatible solutes, synthesis of mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) cascades, formation heat shock proteins as chaperones, and transcriptional activation, can enable plants to thrive or survive when exposed to heat stress (Wahid et al., 2007).

2.8 Issues of heat stress experiments

Heat-stress experiments conducted in the field can be difficult due to occasional uncontrollable factors affecting experimental precision and repeatability (Hall, 2012; Fritsche-Neto and Borém, 2012). First, air temperatures are positively correlated with levels of solar radiation (Hall, 2012). Second, other biotic and abiotic stresses can affect the evaluation of heat stress (Fritsche-Neto and Borém, 2012), for example, drought. Creating a uniform environment for high temperature treatment in a field using current technology remains difficult. Comparisons of thermally contrasting site-years have been used as an indirect method to evaluate temperature studies. Sowing date experiments are another practical and inexpensive approach to study the effects of elevated temperatures on crop performance, because late-seeded crops usually experience warmer conditions compared to early-sown plants (Sadras et al., 2015). However, the findings from these approaches are bound to be inconclusive, because the temperature effects are often confounded with other environmental and management factors such as solar radiation, vapour pressure deficit, precipitation, soils, and cultural practices (Bonada and Sadras, 2015).

On the other hand, although environmentally controlled experiments can isolate the effect of heat stress from other external factors, environmentally controlled experiments are unnatural, and decrease the reliability of predictions (Hall, 2012). The soil volume, roots, and shoots of plants become hot under heat treatments in growth chambers (Hall, 2012), whereas in field conditions the soil temperature below the surface is buffered (Singh et al., 2007). Pot-grown plants may experience substantially higher or lower temperatures than air temperatures when watered using tap water in growth chambers or greenhouse (Passioura, 2006). This problem can be alleviated by watering pots from a reservoir where the water is close to the air temperatures in the controlled environments (Passioura, 2006). Under environmentally controlled conditions, plants tend to have smaller root systems which affect the long-term plant responses to increased carbon dioxide concentrations (Passioura, 2002; Hall, 2012; Poorter et al., 2012; Bonada and Sadras, 2015). Small root systems also sense drought stress (reviewed in Passioura, 2002). Roots may sense restrictive environments in pots, and send inhibitory signals to the above-ground portion that in turn negatively impacts plant physiological processes, including stomatal conductance, cell expansion, cell division, leaf appearance, and biomass allocation (reviewed in Passioura, 2002; Poorter et al., 2012). Artificial lighting systems in many growth chambers do not provide radiation with the same spectra or intensity as solar radiation (Hall, 2012; Bonada and Sadras, 2015). Photothermal

Quotient (PTQ), the ratio of solar radiation to mean temperature during crop development, tends to be lower in growth chambers compared to field conditions at a given temperature. The PTQ was reported to be positively correlated with grain number per ear and grain yield in wheat (Rawson and Bagga, 1979; Fischer, 1985; Ahmed et al., 2010). Similarly, the variation in the PTQ accounted for 75% variation in seed yield in chickpea (Sadras et al., 2015).

The ideal strategy to conduct heat stress experiments is to subject different plots of plants in the same field to different temperatures (Hall, 2012). During the daytime, this can be done by using infra-red heaters which do not inhibit the direct rays of solar radiation. At night, this can be done by temporarily enclosing plants, thus the air temperatures increase. A natural soil profile rather than pot studies is recommended for heat-stress experiments conducted under greenhouse or growth chamber conditions because the shoot and roots of plants are exposed to high air temperatures (Hall, 2004; Singh et al., 2007).

2.9 Heat acclimation

Plant response to elevated temperature can be affected by acclimation, recovery, resource availability, and interactions with other stresses (Sadras and Dredder, 2015). Heat acclimation refers to the phenomenon where plants improve their ability to tolerate heat stress by directly modifying their physiology or morphology after being repeatedly exposed to increasing temperatures (Taiz and Zeiger, 2010). In other words, plants can be primed by increasing temperatures, allowing them to subsequently adapt to heat stress that is lethal to a plant in a naïve state (Bäurle, 2016). Different individuals and species possess different acclimation capacities towards heat and drought (Gutschick and BassiriRad, 2003). For example, one of the two co-occurring plants of the chaparral exhibited rapid recovery during drought acclimation, whereas the other plant experienced an extreme event (Gutschick and BassiriRad, 2003).

Thermo-priming is an active process and is genetically separable from the priming itself. The primed state lasts for several days after returning to non-stress temperatures, which is referred to as heat stress memory or maintenance of acquired thermotolerance (Bäurle, 2016). The thermo-priming is the strategy that plants have evolved to cope with recurring temperature stresses, and priming involves a lag/memory phase separating the priming event and a second stimulus (stress event). Heat stress memory is associated with the modifications of metabolites, protein stability, and chromatin complexes (Bäurle, 2016). Heat stress priming involves the activation of heat shock

transcription factors and the expression of heat shock proteins that is a key factor in protein homeostasis (Haslbeck and Vierling, 2015).

2.10 Long-term temperature trends and frequency of heat waves

Global warming involves two aspects related to agricultural production: 1) a gradual increase in long-term mean temperatures that shift phenological patterns; 2) the incidence of heat waves has become more frequent (Sadras and Dreccer, 2015). First, the increased temperature reduces yield by shortening reproduction. For example, modest heat stress accelerates plant life cycle and reduces yield, because plants spend less time in reproductive development (Guilioni et al., 1997). Second, extreme heat waves may cause the abortion and cessation of physiological processes. For example, extreme heat stress (33/30°C day/night temperatures) result in the immediate abortion of flowers and pods in field pea (Guilioni et al., 1997). During the extreme heat event, the acclimation capacities of plants are exceeded (Gutschick and BassiriRad, 2003).

Chapter 3 Seed set, pollen morphology and pollen surface composition response to high day temperatures in field pea

3.1 Introduction

Ambient temperatures are rising and altering the earth's climate systems, mainly activity and the burning of fossil fuel. Globally, the combined land and ocean surface temperature has increased by 0.72°C from 1951 to 2012 (IPCC Working Group II, 2013), and temperatures will likely continue to rise during this century. Along with an increase in temperature, disruption of climates and increased frequency of drought and storm events will reduce agricultural production. However, global warming can also enhance new cropping opportunities in some cases, particularly at high latitudes. Heat stress from the rise in ambient temperature has become an agricultural issue in many areas in the world (Wahid et al., 2007; Smith and Olesen, 2010).

One of the results of a warming climate is the increased frequency of warm temperature spells during a crop season. These warm spells can be hot enough to cause heat stress. Heat stress is well known to disrupt sexual reproductive success in legume species and other crops, and can be manifested at a myriad of stages. Generally, male reproductive development is more vulnerable to heat stress than female reproductive organs (chickpea, *Cicer arietinum* L. - Devasirvatham et al., 2012; common bean, *Phaseolus vulgaris* L. - Monterroso and Wien, 1990; cowpea, *Vigna unguiculata* - Warrag and Hall, 1983), because once pollen grains are released from anthers, they are exposed to ambient environmental conditions, whereas ovules remain enclosed and more protected within the ovary (Kakani et al., 2005). Heat stress during a plant's flowering period leads to yield loss by reducing pollen viability, pollen production per flower, and pod set in chickpea (Devasirvatham et al., 2012), common bean (Konsens et al., 1991), and cowpea (Ahmed et al., 1992), with similar effects in other crops like tomato (*Solanum lycopersicum*; Levy et al., 1978), cotton (*Gossypium hirsutum*; Reddy et al., 1992), and canola (*Brassica napus*; Young et al., 2004). Furthermore, heat stress post-anthesis leads to poor pollen germination and decreased pollen-tube growth in chickpea (Devasirvatham et al., 2012), common bean (Gross and Kigel, 1994) and soybean (*Glycine max* L. Merr.; Salem et al., 2007), as well as fertilization failure in common bean (Ormrod et al., 1967), plus embryo abortion in common bean (Gross and Kigel, 1994) and cowpea (Warrag and Hall, 1984a, b). Moreover, pollen abnormalities such as small, shrunken, and empty pollen grains occurred in chickpea under heat stress at early flowering (Devasirvatham et al., 2012).

Heat stress (33/30°C day/night temperatures) during floral development of cowpea causes an absence of pods due to low pollen viability and anther indehiscence (Ahmed et al., 1992). In cool season grain legumes, exposure to high temperature during seed filling accelerates senescence, decreases seed set and seed weight, and causes yield loss due to plants diverting photosynthates to deal with heat stress (Siddique et al., 1999).

Similarly, heat-induced yield reductions have been reported in pea (*Pisum sativum* L.). Grain yield of field pea in Australia decreases with an increase in maximum temperature over 25ºC during the plant's flowering period (Sadras et al., 2012), when peas are highly sensitive to environmental stress (Guilioni et al., 2003). Serious heat stress (33/30°C day/night temperatures) leads to the immediate abortion of reproductive organs in pea (Guilioni et al., 1997), and reduces the number of seeds per pod (Jeuffroy et al., 1990). In addition, high temperature typical of heat shock (45°C for 2-3 h) reduces the functional activities of the photosynthetic apparatus, pollen viability, percentage pollen germination, and pollen-tube growth in pea (Petkova et al., 2009). However, that study did not address whether any temperature below 45°C might affect pollen function.

Fourier transform infrared (FTIR) spectroscopy is a powerful tool for biochemical analysis of pollen grains (Sowa et al., 1991; Zimmermann and Kohler, 2014; Lahlali et al., 2014). Vibrational spectra of pollen grains provide significant information on biochemical composition. The benefits of IR methodology include precision, minimal or no sample preparation, speed, ease-of-use, and non-destruction of samples (Zimmermann and Kohler, 2014). Although FTIR spectroscopy does not provide qualitative precision of the traditional cytochemical analysis, it identifies main biochemical groups and quantifies the chemical functional groups in a simple, fast, and reliable way (Sowa and Towill, 1991; Zimmermann and Kohler, 2014). This spectroscopic tool can provide complementary information compared with classical techniques including microscopy, flow cytometry, and gas chromatography (Ami et al., 2014). The wavenumber of 3100-2800 cm^{-1} has been used for lipid determination (Casal and Mantsch, 1984), with 1800-400 cm^{-1} being referred to as the fingerprint region (Fink and Chittur, 1986). The ratio of unsaturated/saturated CH_2 can be determined by dividing the absorbance at 3010 cm^{-1} (=CH vibrations) by the amount of absorbance at 2920 cm^{-1} (Sowa et al., 1991). The absorbance at 2343 cm^{-1} responds to intracellular CO_2, which is associated with cell viability (Sowa and Towill, 1991).

I hypothesized that elevated temperatures would negatively affect pollen development and seed set when daytime temperatures were above temperature thresholds. The main objective of this study was to determine the critical temperature threshold that began to reduce the percentage pollen germination, pollen-tube growth, and seed set of field pea under environmentally controlled conditions using two currently grown cultivars. Secondly, the effects of heat stress on these parameters, as well as pod formation, were evaluated across five temperature regimes. Finally, the molecular composition of the pollen grain surface was examined using mid infrared spectroscopy to investigate its significance in pea pollen tolerance to elevated temperatures.

3.2 Materials and methods

3.2.1 Plant materials

Two pea cultivars (CDC Golden and CDC Sage) adapted to field production in Saskatchewan, were tested in this study. The study was designed as a randomized complete block design (RCBD), and repeated twice with time considered as the blocking factor. For each experimental run (within the same temporal block), 40 pots (2 cultivars × 5 temperature treatments × 4 reps) of 3.8 L volume (3 plants per pot) were seeded with Sunshine Gro® mix (Seba Beach, AB, Canada) and slow-release fertilizer (14-14-14, Type 100, Nutricote®, Brampton, Ontario). Plants were thinned to two plants per pot approximately two weeks after seeding. Plants received the first application (500 ml per pot) of half strength Hoagland's culture solution (Hoagland and Arnon, 1938) at three weeks after seeding and the second application (500 ml per pot) at the early flowering stage. Soil medium moisture was monitored carefully; plants were watered with distilled water if necessary to avoid drought stress.

Plants were grown at 24/18°C day/night temperatures with 16/8 h photoperiod and irradiance of 450-500 µmol photons m^{-2} s^{-1} from cool fluorescent tubes in a growth chamber, and the relative humidity had a range of 4-8%. Plants were then transferred to four other growth chambers when the flowers at the second fruiting node of the main stem were fully open. Plants transferred to warmer regimes were exposed continually for 7 days to one of 27/18°C, 30/18°C, 33/18°C, and 36/18°C day/night regimes, respectively. The control plants remained in the 24/18°C growth chamber.

3.2.2 Seed set parameters

For both cultivars, pod length, the number of seeds per pod, and the seed-ovule ratio were monitored. Each pod from the first four fruiting nodes on the main stem from all 80 plants (2 cultivars × 5 temperature regimes × 4 replications per run × 2 runs) was opened at physiological maturity when plants became dead and brown and pods became dry and yellow, and the condition of each ovule was recorded. An aborted ovule was determined as a vestigial funiculus that failed to develop into a seed, whether the ovule had been fertilized or went unfertilized (Figure 3.1). For examples, ovules at positions 1, 3, and 6 were considered as aborted seeds in Figure 3.1. The seed-ovule ratio was calculated by dividing the number of seeds per pod by the number of ovules or funiculi positions per pod.

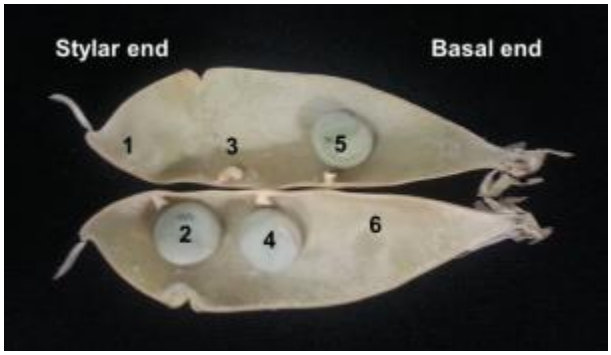

Figure 3.1. Ovule position and stages of seed development within an opened, mature pod (legume) of *Pisum sativum* cv. CDC Sage: Positions 1 and 6 – ovules without fertilization (funiculus alone or bearing only a small unfertilized ovule), Positions 2, 4, and 5 – full developed seeds; Position 3 – seed abortion following fertilization. The seed-ovule ratio is 0.5 or 50% in this example.

3.2.3 In vitro percentage pollen germination and pollen-tube growth

In vitro pollen germination and tube growth were examined using a pollen germination medium as described elsewhere (Lahlali et al., 2014). Fresh pollen grains were collected from anthers of slightly opened flowers (anther dehiscence had already occurred) in the control growth chamber (24/18°C) at 5-6 h after the 16 h photoperiod began. Pollen grains were dusted onto the germination medium on microscope slides that were then placed individually above moistened filter paper in 90-mm petri dishes. Petri dishes were sealed with Parafilm® (Greenwich, Connecticut) to maintain high humidity, and they were placed in five growth chambers with these five temperature regimes: 24, 27, 30, 33, and 36°C. After 10 h of incubation, pollen grains (100 grains per replication, 5 replications per treatment) were examined for their germination using direct microscopic

observation at 1000x-4000x. Pollen grains were considered germinated when the pollen-tube length was greater than the grain diameter (Shivanna and Rangaswamy, 1992; Salem et al., 2007). Pollen-tube length of 30 randomly selected grains was measured with the computer software Infinity Analyze (Version 6.3.0, Lumenera Corporation, Ottawa).

3.2.4 Pollen surface morphology and pollen wall structure

The morphology of entire pollen grains and their detailed surface features of CDC Golden and CDC Sage were examined using scanning electron microscopy (SEM) following plant exposure to heat stress (36°C) or controlled conditions (24°C) in growth chambers. Pollen was harvested from slightly open flowers on the fourth day after application of heat stress. Mature pollen grains were gold-coated (Edwards S150B Sputter Coater, Wilmington, MA) before examination using SEM (Phenom, G_2Pure), and imaged using a charge-coupled device (CCD) navigation camera.

Effect of heat stress on pollen wall structure was examined using a transmission electron microscope (TEM). Fresh mature pollen grains of CDC Golden in the control growth chamber (24/18°C day/night temperatures) and the high temperature chamber (36/18°C day/night temperatures) were collected from slightly open flowers on the fourth day after plants were exposed to high temperature. Sample preparation included fixation, dehydration, infiltration, and embedding of pollen grains using the procedure reported by Lee et al. (2008) with minor modifications. In brief, pollen grains were fixed in the fixative which consisted of 1% formaldehyde, 0.025% glutaraldehyde (GA), and 0.01 M Na phosphate (Na_2HPO_4/NaH_2PO_4) buffer (pH 7.2) overnight. Samples were washed with the same buffer at 4°C for 30 minutes, then dehydrated in an ethanol series at 4°C. A gradual substitution of ethanol by LR White (London Resin Company Ltd., Reading, Berkshire, UK) for sample infiltration at 4°C preceded the final embedding in pure LR White and polymerization at 55°C overnight. Ultra-thin sections (70-100 nm) were obtained using a diamond knife on a Leica ultra-microtome, floated on copper grids and then stained with uranyl acetate. Eight to ten pollen grains from each cultivar and temperature treatment combination were observed in a TEM (Philips CM100). Thickness of the intine and the exine's tectum layer at three randomly selected locations for each of the eight pollen grains (N = 24) was measured using Image J (version 51.52.0.0; National Institute of Health, Bethesda, USA).

3.2.5 Pollen surface composition

The pollen surface composition was analyzed using Fourier Transform infrared (FTIR) Attenuated Total Reflection (ATR) at the Mid Infrared beamline (01B1-1). The globar source (silicon carbide) was employed as the infrared source. This ATR method used a germanium crystal (20x objective, 100 µm crystal surface, angle of incidence = 45°) attached to the Bruker IFS 66V/S spectrometer together with the Bruker Hyperion 2000 confocal microscope (Bruker Optics, Ettlingen, Germany) with a KBr beam splitter and liquid nitrogen cooled mercury cadmium telluride (MCT) detector. Fresh mature pollen grains of CDC Golden and CDC Sage in the control growth chamber (24/18°C day/night temperatures) and the high temperature chamber (36/18°C day/night temperatures) were collected from slightly open flowers on the fourth day after plants were exposed to high temperature. Pollen grains from four flowers per treatment were pooled and dusted on CaF_2 windows (Crystran Ltd., Dorset) approximately 45 min before the measurement. Each IR spectrum was recorded in the mid infrared range of 4000-800 cm^{-1} wavenumbers at a spectral resolution of 2 cm^{-1}. The detailed methodology was documented by Lahlali et al. (2014).

3.2.6 Data analyses

Statistical analyses on seed set parameters, pollen germination, and pollen-tube length were performed using the mixed procedure of SAS statistical software (version 9.3, SAS Institute, Inc., Cary, NC). Analysis of variance (ANOVA) with the Least Significant Difference (LSD) test ($P < 0.05$) was used. The effects of cultivar and temperature treatments were considered as fixed effects, and blocks and replications within each block were considered as random effects. The Kenwardroger option was used to approximate the degrees of freedom for means. Intine and tectum thickness were analyzed using a two-sample t test ($P < 0.05$).

ATR data analyses were performed using the Unscrambler software (version 10.1, Camo Software AS., Norway) for Principal Component Analysis (PCA). Before PCA analysis, spectra were separated into lipid (3,100-2,750 cm^{-1}) and fingerprint regions (1,800-800 cm^{-1}). The present study focused on the lipid region, although our raw data included other regions and the protein region was discussed in Lahlali et al. (2014). The data were then corrected by the Extended Multiplicative Scattering Correction (EMSC) algorithm. The - EMSC corrected data were transferred into the second derivative form by using Savitzky-Golay algorithm, with 13-point window size. The PCA was performed on the data by considering each wavelength of FTIR treated

as an equally weighted variable. The major peaks of PCA graphs were determined using the Quick Peaks routine in OriginPro with the settings of local maximum at 0% threshold height, no baseline, and area at Y=0 (version 9.1, OrginLab Corporation, Northampton, MA).

3.3 Results

3.3.1 Seed set parameters

High temperature significantly reduced pod length at the different reproductive stages of pre-anthesis, anthesis, and post-anthesis in CDC Golden and CDC Sage (Table 3.1). For the advanced floral stages (Node 1 - post-anthesis; Node 2 - anthesis) when the 7-day heat treatments began, high temperature of 36°C significantly decreased pod length, but pod length did not change when temperatures increased from 24 to 33°C. At Node 3, pod length started to decrease when temperatures increased from 24 to 30°C, and pod length continued to decrease when the high temperature reached 36°C. At Node 4, pod length with the 36°C treatment was significantly shorter compared to the temperature regimes of 24, 27, and 30°C. The average pod length from Node 1 to Node 4 decreased with an increase in temperature from 24 to 36°C. Pod length of CDC Sage was significantly greater than CDC Golden except at Node 4.

High temperature had no significant effect on the number of seeds per pod (fully developed seeds shown at Positions 2, 3, 4 and 5 in Figure 3.1 were considered) at the post-anthesis stage (Node 1), the timing of which fertilization was already completed prior to heat stress (Table 3.1). However, the highest temperature treatment (36°C) significantly reduced the number of seeds per pod if plants were at the anthesis or a pre-anthesis stage when heat treatments began, as seen for pods formed at Nodes 2 and 3. At Node 4, temperatures ranging from 24 to 36°C had no effect on the number of seeds per pod. The average seed number per pod from Node 1 to Node 4 with the 36°C temperature regime was significantly fewer compared to other lower temperature regimes. CDC Sage also had significantly more seeds per pod than CDC Golden, a genetic attribute and characteristic of this cultivar.

At Nodes 1 and 2, extreme heat stress (36°C) significantly decreased the seed-ovule ratio compared to other temperature treatments (Table 3.1). However, the seed-ovule ratio at Nodes 3 and 4 was not affected by the increased temperature regimes. The average value of seed-ovule ratio

with the 36°C temperature regime was significantly lower compared to other temperature treatments. The seed-ovule ratio did not differ between CDC Golden and CDC Sage at any node.

Table 3.1. Effects of cultivar and temperature on percentage pollen germination, pollen-tube length, and seed set parameters including pod length, number of seeds per pod, and seed failure ratio. The bottom panel contains probability values from the ANOVA.

	Pollen germination (%)	Pollen tube length (mm)	Pod length (mm)					Number of seeds per pod					Seed-ovule ratio (%)				
			Node1†	Node2†	Node3†	Node4†	Mean	Node1	Node2	Node3	Node4	Mean	Node1	Node2	Node3	Node4	Mean
Cultivar																	
CDC Golden	75.3b‡	0.463a	55b	51b	52b	51a	51b	3.9a	3.9b	4.2b	3.5b	3.8b	77.7a	69.5a	78.1a	62.0a	71.9a
CDC Sage	83.6a	0.465a	68a	65a	61a	55a	62a	4.5a	5.2a	5.1a	4.5a	4.8a	80.8a	69.0a	68.7a	62.9a	69.8a
Temperature																	
24°C	90.3a	0.783a	65a	63a	62a	56ab	62a	4.6a	5.0a	4.7a	4.2a	4.6a	85.0a	74.7a	69.5a	68.8a	73.3a
27°C	89.9a	0.618b	67a	61a	62a	60a	61ab	3.9a	4.3a	5.0a	4.4a	4.4a	84.3a	65.7a	75.0a	68.4a	72.0a
30°C	87.6a	0.525c	64a	61a	57b	53ab	58bc	4.5a	5.0a	4.8a	4.2a	4.6a	84.1a	78.7a	73.4a	65.4a	76.5a
33°C	81.4b	0.291d	63a	60a	57b	50bc	57c	4.7a	5.1a	5.3a	3.7a	4.6a	83.0a	78.7a	86.6a	56.9a	76.4a
36°C	47.9c	0.102e	48b	45b	46c	44c	46d	3.6a	3.0b	3.5b	3.5a	3.4b	60.0b	48.5b	62.5a	52.3a	56.0b
P value																	
Cultivar (C)	<0.001	0.96	<0.001	<0.001	<0.001	0.07	<0.001	0.09	<0.001	0.002	0.005	<0.001	0.37	0.93	0.09	0.86	0.49
Temperature (T)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.17	<0.001	0.005	0.38	<0.001	<0.001	0.002	0.12	0.19	<0.001
C*T	<0.001	0.19	0.97	0.39	0.77	0.72	0.77	0.83	0.41	0.75	0.43	0.33	0.81	0.83	0.83	0.2	0.42

†Node 1: post-anthesis (pod development) at the onset of heat exposure; Node 2: anthesis (fully open) at the onset of heat exposure; Node 3: pre-anthesis (young bud) at the onset of heat exposure; Node 4: very small bud visible at the onset of heat exposure. Node 1 is the oldest, and Node 4 is the youngest in development. For each node, data were analyzed separately.

‡Means with a common letter within the main effect of cultivar or temperature did not differ at $P < 0.05$.

3.3.2 In vitro percentage pollen germination and pollen-tube growth

Pollen germination, which serves as an indicator of pollen viability, responded differently to elevated temperatures depending on cultivar (Figure 3.2). High temperature reduced percentage pollen germination by approximately 30% in CDC Sage and 55% in CDC Golden when the 10-h incubation temperature increased from 24 °C to 36 °C (Figure 3.3). Under the extreme heat stress (36°C) treatment tested, percentage pollen germination of CDC Sage was significantly greater than CDC Golden (Figure 3.3), for all heat treatments tested (Table 3.1).

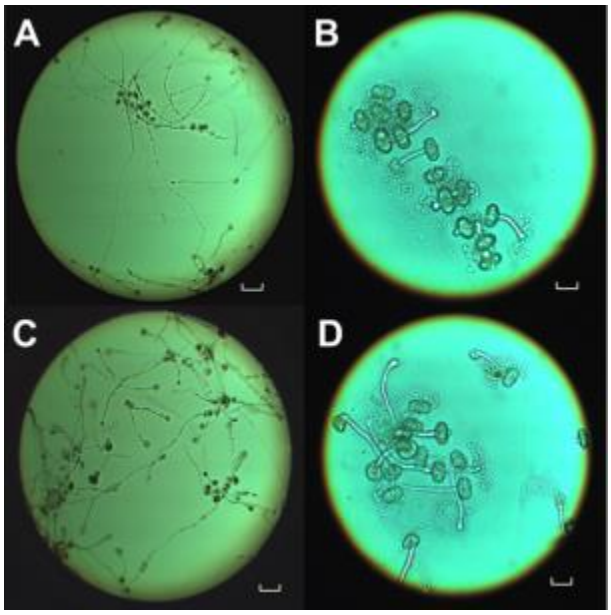

Figure 3.2. *In vitro* pollen-tube growth of *Pisum sativum* cv. CDC Golden (A, B) and CDC Sage (C, D) after 10 h incubation at 24°C (A, C) and 36°C (B, D). Bar = 200 µm in A and C, 20 µm in B and D.

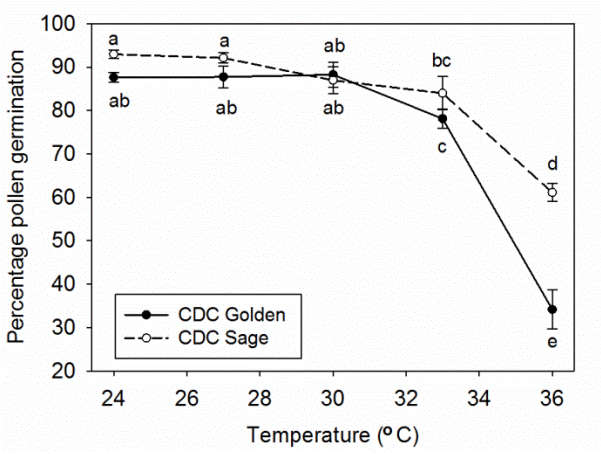

Figure 3.3. Effect of various temperatures applied for 10 h on *in vitro* percentage pollen germination in *Pisum sativum* cv. CDC Golden and CDC Sage. Each dot represents the mean value of five replications shown with standard error bars. Means with a common letter did not differ at $P < 0.05$ at each temperature regime.

After 10-h incubation, elevated temperatures had a significant reduction on pollen-tube length (a proxy of pollen vigor). For example, pollen-tube length decreased from 783 µm to 102 µm when temperature was increased from 24°C to 36°C (Table 3.1). However, pollen-tube growth of successfully germinating pollen did not differ between the two cultivars (Table 3.1). Pollen-tube length was negatively correlated with temperature in a simple linear model when temperature increased from 24 to 36°C (Figure 3.4). Thus, the pollen-tube length of 1.2 mm at 24°C decreased about 0.08 mm for each 1°C rise in temperature.

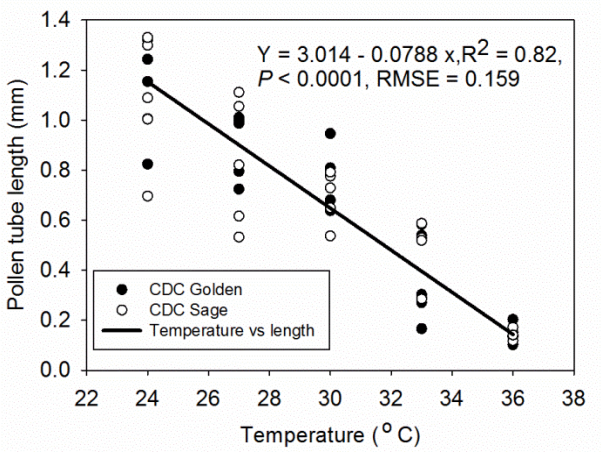

Figure 3.4. Linear regression analysis of *in vitro* pollen-tube length response in *Pisum sativum* to temperature after 10 h incubation. Each dot represents the value of each treatment per replication

which was the mean value of 30 subsamples (50 dots in total, 2 cultivars × 5 temperature regimes × 5 replications).

3.3.3 Pollen surface morphology and pollen wall structure

No morphological differences between the whole pollen grains and pollen surface (the reticulate sculpturing – ektexine of exine) of CDC Golden and CDC Sage were observed, nor any difference between the control and the heat treatments (Figures 3.5 and 3.6). Each pea pollen grain contained three furrows, each with a potential germinating pore at its mid region. *In situ*, when a pollen grain lands on the pea stigma, a pollen tube germinates from one of these pores and grows through the style to the ovary. Similarly, when cultured *in vitro*, each pollen grain germinates from just one of the three furrows present per grain (Figure 3.2B, D).

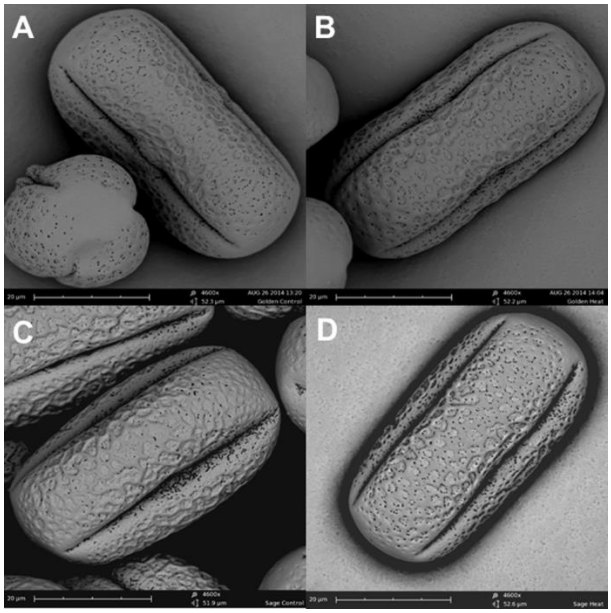

Figure 3.5. Scanning electron microscope (SEM) images of whole pollen grains at 4,600x of *Pisum sativum* cv. CDC Golden (A, B) and CDC Sage (C, D) from the 24°C (control) growth chamber (A, C) and the 36°C (heat-treated) growth chamber (B, D).

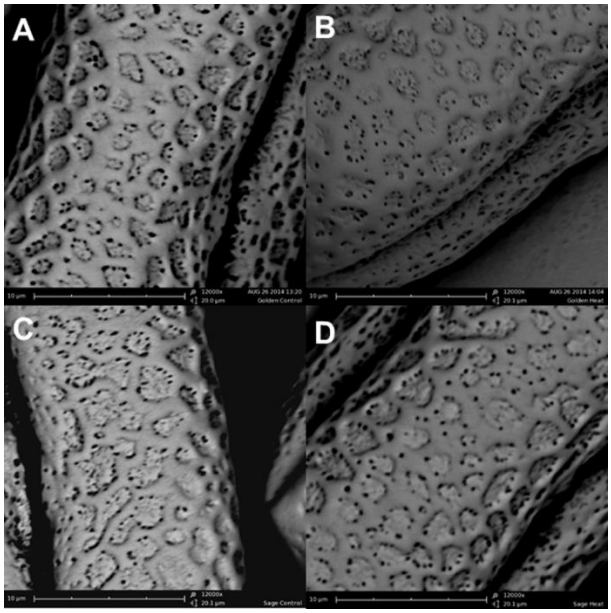

Figure 3.6. Scanning electron microscope (SEM) images of the exine surface at 12,000x of *Pisum sativum* cv. CDC Golden (A, B) and CDC Sage (C, D) from the 24°C (control) growth chamber (A, C) and the 36°C (heat-treated) growth chamber (B, D).

Effect of heat stress on the multi-layer pollen wall structure was shown in Figure 3.7. Pea pollen wall consisted of an outer layer, the exine, and an inner layer, the intine. The exine can be divided into pollen coat, sexine (tectum and bacula), and nexine [consisting of the foot layer (nexine I) and endexine (nexine II)]. The intine has two layers – the exintine and the endintine. High temperature (36°C) caused a significant increase in intine (exintine plus endintine) thickness (mean ± S.E. = 414 ± 52 nm, N = 24) compared to pollen subjected to 24°C (222 ± 30 nm; t test, $P < 0.05$). However, heat stress (36°C) did not affect tectum thickness (mean ± S.E. = 450 ± 27 nm, $N = 24$) compared to control (24°C) pollen (440 ± 23 nm; t test, $P = 0.77$).

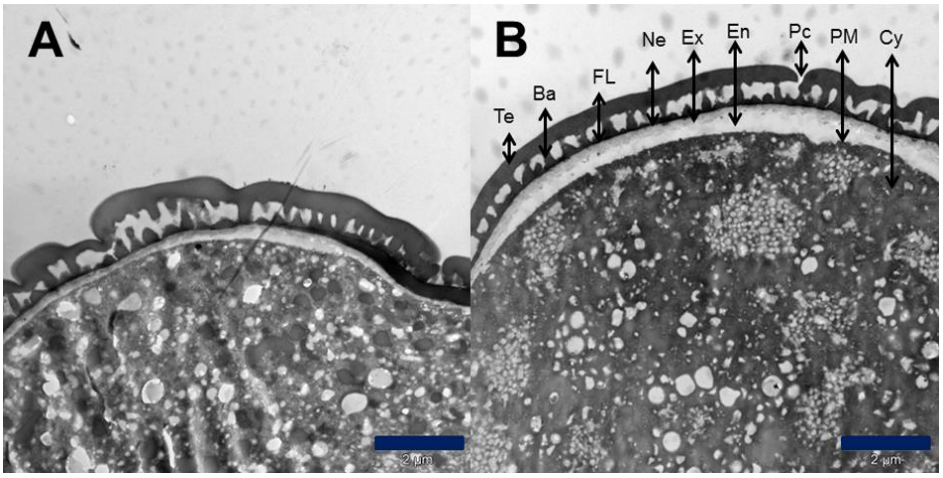

Figure 3.7. Transmission electron microscope (TEM) images of the pollen wall structure of *Pisum sativum* cv. CDC Golden from the 24°C (control) growth chamber (A) and the 36°C (heat-treated) growth chamber (B). Bar = 2 µm. Te, tectum; Ba, bacula (or columella); FL, foot layer (or Nexine I); Ne, Nexine II; Ex, exintine; En, endintine; Pm, plasma membrane; Cy, cytoplasm; Pc, pollen coat.

3.3.4 Pollen surface composition

For control and heat treated pollen grains of both cultivars, the average ATR spectra with observable differences in the intensities of some peaks are shown in Figure 3.8. The ATR spectra of the pollen grain surface were based on previous data (Wolkers and Hoekstra, 1995; Sowa and Connor, 1995). For intact and heat-stressed pollen, bands associated with different chemical groups of the pollen surface were identified. These included long-chain aliphatic waxes and outer exine layer (thin asymmetric and symmetric CH_2-stretching absorptions at 2,921 and 2,852 cm^{-1} and CH_2-bending one at 1,460 cm^{-1}), the polyester cutin (C-O-stretching vibration in an ester environment band at 1,740 cm^{-1} and the asymmetrical and symmetrical C-O-C-stretching absorptions at 1,178 and 1,107 cm^{-1}, respectively), polysaccharides (the band associated with the glycosidic bond at 1,054 cm^{-1} and the band associated with ring vibration at 995 cm^{-1}), and some unsaturated/aromatic compounds [stretching of C=C double bonds, aromatic rings conjugated with double bonds at 1,515 cm^{-1}, respectively)]. The broad and very strong band at 3354 cm^{-1}, assigned to the O-H-stretching vibration, could be related to tissue hydration. The second derivative of absorption spectra in the lipid was obtained from the area-normalized spectra of individual control and heat stressed individual pollen grains of both cultivars as shown in Figure 3.9. The second derivative spectra in the lipid region revealed the more obvious differences between the cultivars and the effect of heat stress on pollen grains. Two strong negative peaks were noticeable near 2,921 cm^{-1} (asymmetrical CH_2) and 2,852 cm^{-1} (symmetrical CH_2) in the secondary derivative mode in

the lipid region, but differences between the spectra were small in CDC Sage compared to CDC Golden with respect to heat stress treatment (Figure 3.9). The fingerprint region, which is known to be beneficial in highlighting the difference between spectra (Susi and Byler, 1986; Hori and Sugiyama, 2003), was discussed in Lahlali et al. (2014).

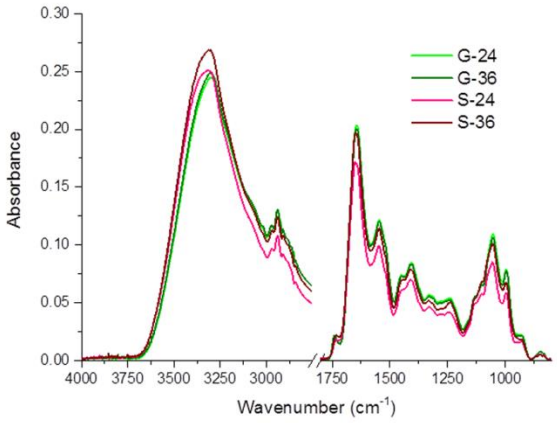

Figure 3.8. Average MIR-ATR spectra of control and heat stressed pollen grains of *Pisum sativum* cv. CDC Golden and CDC Sage. G-24 is CDC Golden with the temperature of 24°C; G-36 is CDC Golden with the temperature of 36°C; S-24 is CDC Sage with the temperature of 24°C; S-36 is CDC Sage with the temperature of 36°C. Data are means of 15 individual pollen spectra for each treatment.

In addition, studies on pollen grains of CDC Sage and CDC Golden showed a new and interesting profile of lipids (Figure 3.9). Both cultivars contained the two types of pollen lipids, but the amount of these lipids varied substantially. We observed changes in CH_2 vibration which mainly arises from lipid acyl chain in the 3000-2800 wavenumber region. This vibration correlated with the C=O vibration of lipid ester at 1740 cm^{-1}. Two CH_2 asymmetric stretching vibrations at 2,940 and 2,921 cm^{-1} and symmetric stretching vibrations at 2,862 and 2,851 cm^{-1} were observed respectively, indicative of the two different lipids. Both major types of lipids were similar in quantity; however, the other lipids usually present in minor quantities were more plentiful in CDC Golden (approximately 18%) compared to CDC Sage (approximately 5%). We observed a significant reduction of CH_2 vibration in pollen grains of CDC Golden treated with high temperature (Figure 3.9) and very minimal reduction of CH_2 vibration in pollen grains of CDC Sage. Because CDC Sage had a very small quantity of other lipids, there was a minimal effect overall. The observation of 18% minor lipids in CDC Golden and its lower percentage pollen

germination when exposed to heat stress may indicate a link between a greater concentration of minor lipids associated with heat sensitivity.

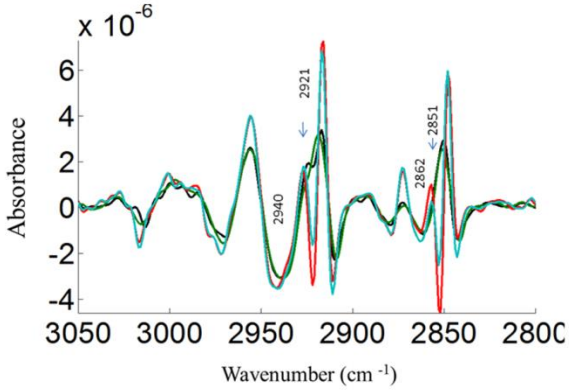

Figure 3.9. The averaged second derivative of MIR-ATR spectral range 3,050 to 2,800 cm^{-1} (the lipid region). Red, blue, black, and green lines represent CDC Golden (control, 24°C), CDC Golden (heat stress, 36°C), CDC Sage (control, 24°C), and CDC Sage (heat stress, 36°C) respectively. Data are means of 15 individual pollen spectra for each treatment.

Principal component analysis (PCA) is one of the most common multivariate methods used to deconstruct the data matrix and concentrate the source of variability in the data into the first few Principal Components (PCs). In the lipid region, a total variation of 86% of the pollen grain samples was explained by the scatter plot PC1 versus PC2 (Figure 3.10A). The PC1 axis clearly distinguished CDC Golden in a separate cluster on the negative side of PC1 from CDC Sage on the positive side of PC1. This segregation indicated that the difference between chemical compositions in lipids was larger between pollen grains of both cultivars than between heat stressed and control pollen grains. The positive influence of PC1 loadings had peak values around asymmetrical CH_2 and symmetrical CH_2 stretching at 2,921 and 2,852 cm^{-1} whereas the negative influence had a strong peak at symmetrical CH_2 (2,845 cm^{-1}) (Figure 3.10B). The scatter plot of PC1 and PC2 showed differentiation between heat-stressed and control pollen grains of CDC Golden; heat-stressed pollen grains of CDC Golden clustered mostly on the positive side of PC2, whereas control pollen samples were almost scattered over the whole of PC2 axis. The loading plot of PC2 underlined an important increase in intensity from a negative to positive direction in both asymmetrical (2,911-2,917 cm^{-1}) and symmetrical CH_2 (2,842 to 2,848 cm^{-1}) peaks in CDC Golden, which may be due to heat stress. Therefore, both PC1 and PC2 showed that the lipid composition of the CDC Golden pollen surface changed due to heat stress compared to CDC Sage

and most of the changes were related to long-chain aliphatic lipids (asymmetrical and symmetrical CH_2) (Figure 3.10).

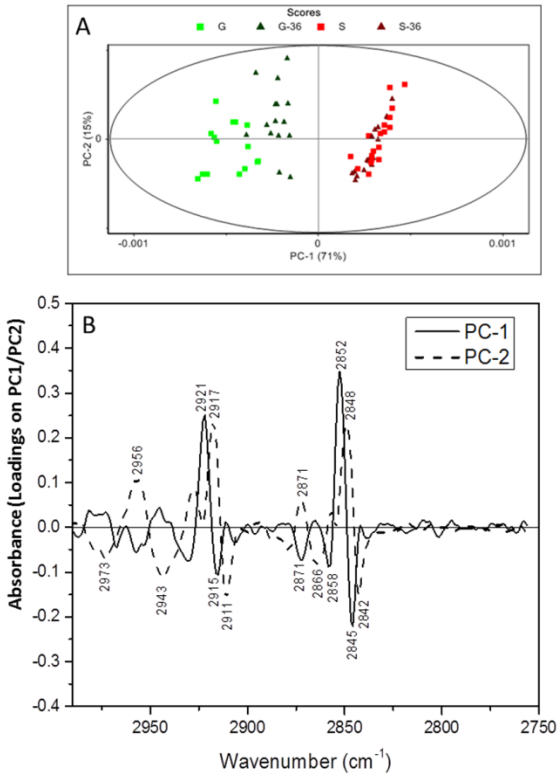

Figure 3.10. Principal component analysis of FTIR ATR spectra of pea pollen grains analyzed in the lipid region. A: Score plot; B: Loading plot for PC1 and PC2. In panel A, G and G-36 represent pollen of CDC Golden subjected to 24 and 36°C, whereas S and S-36 refer to pollen of CDC Sage in response to 24 and 36°C, respectively.

In the fingerprint region (1,450 to 500 cm^{-1}), PC1 and PC2 explained more than 70% of variability associated with heat stress in pollen grains (Figure 3.11). The PC1 axis distinguished CDC Sage and CDC Golden as two separate clusters on the positive and negative sides, respectively. The heat stressed pollen grains from CDC Sage were spread along the PC2 axis (Figure 3.11A). Specifically, the fingerprint region showed more pronounced differences in CDC Sage pollen grains compared to CDC Golden. The most important peaks in the positive PC1 loading were dominated by carbonyl compounds such as pectin (1,744 and 987 cm^{-1}) and proteins (1,642 cm^{-1}). The positive influence of PC2 loading at specific wavenumbers corresponded to

protein amide I (1,670 and 1,622 cm^{-1}) and carbohydrates (1,136 and 994 cm^{-1}) whereas the negative influence had peaks at protein amide I (1,645 cm^{-1}) and carbohydrates (1152, 1090, and 971 cm^{-1}). The trend of the scores was plotted in four groups from positive to negative value using S (CDC Sage control pollen grains), S-36 (CDC Sage heat-treated pollen grains), G (CDC Golden control pollen grains), and G-36 (CDC Golden heat-treated pollen grains) groups, which could reflect the effect of temperature on pollen grains. The results did not indicate prominent clusters in the fingerprint region between control and heat-stressed pollen grains. Therefore, the subsequent discussion will focus on the importance of aliphatic hydrocarbons of the pollen grain surface in pollen germination and its tolerance to heat stress.

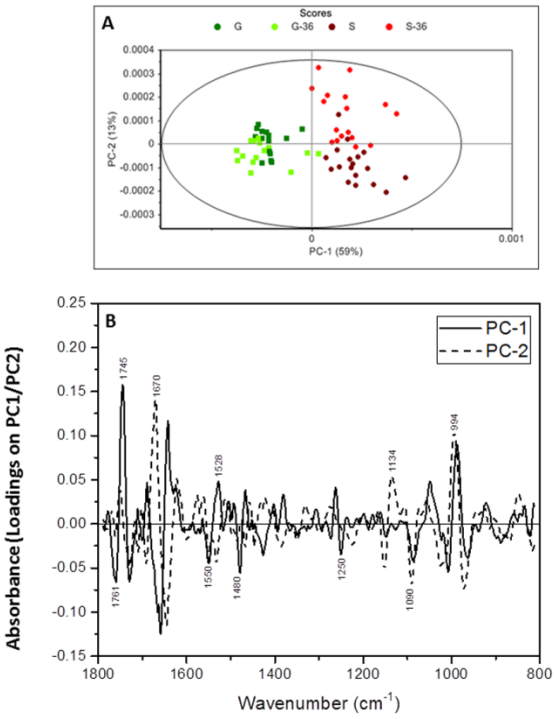

Figure 3.11. Principal component analysis of MIR-ATR spectra of pea pollen grains analyzed in the fingerprint region. A: Score plot (PC1 and PC2) and B: Loading plot for PC1 and PC2. In panel A, G and G-36 represent pollen of CDC Golden subjected to 24 and 36°C, whereas S and S-36 refer to pollen of CDC Sage in response to 24 and 36°C, respectively.

3.4 Discussion

3.4.1 Heat stress on seed set parameters

Moderate heat stress in pea (several days below 32°C) did not affect seed set severely (Guilioni et al., 1997; Jeuffroy et al., 1990; Lambert and Linck, 1958). Temperatures above 32°C did cause

abortion of reproductive structures. In the present study, the average number of seeds per pod and the average seed-ovule ratio at the four basal reproductive nodes were not affected when temperatures increased from 24°C to 33°C, but they were reduced dramatically at 36°C. Flowers at anthesis (second basal reproductive node) were the most sensitive to the extreme heat stress (7 consecutive days at 36°C) as reflected in their significant reduction in pod length, number of seeds per pod, and seed-ovule ratio. In contrast, pod and seed development at the fourth basal reproductive (Node 4) was the least sensitive to the high temperatures ranging from 24 to 36°C, because the number of seeds per pod and the seed-ovule ratio at Node 4 were not affected by the high temperature. Similarly, the sensitivity to heat stress in common bean was gradually reduced, and young pods were not affected negatively by high temperature (Gross and Kigel, 1994). High temperature had a detrimental effect on seed set due to the decrease in pollen number and pollen viability in groundnut (Prasad et al., 1999; Kakani et al., 2002). High temperature (28°C and 31°C) did not cause flowering to cease under environmentally controlled conditions in pea (Jeuffroy et al., 1990), but flowering stopped under high temperature in the field (Duthion et al., 1987 in Jeuffroy et al., 1990; Laconde et al., 1987 in Jeuffroy et al., 1990). Flower number and number of pods per plant were not affected by high temperature during flowering (28 or 31°C for 6 h during the middle of the day for 2 or 4 days; Jeuffroy et al., 1990).

The maximum threshold temperature affecting pea growth performance such as the number of seeds per pod and the seed-ovule ratio under environmentally controlled conditions in the present study was much higher than field conditions. Under field conditions, pea yield decreased when the maximum temperature was over 25°C at the reproductive stage in Australia (Sadras et al., 2012). Similarly, air temperatures exceeding 30°C resulted in pea yield loss under field conditions in Saskatchewan, Canada (Bueckert et al., 2015), with evidence of yield reductions at lower temperatures. Although environmentally controlled experiments isolate the effect of heat stress from other external factors, environmentally controlled experiments are unnatural, which decrease the reliability of predictions (Hall, 2012). Artificial lighting systems in many growth chambers do not provide radiation with the same spectra, quality or intensity as solar radiation (Hall, 2012), Air temperatures are positively correlated with levels of solar radiation (Hall, 2012), but other biotic and abiotic stress can affect the evaluation of heat stress (Fritsche-Neto and Borém, 2012), for example water-deficit stress in drought, which frequently occurs at the same time as heat stress.

3.4.2 Heat stress on in vitro pollen germination and pollen-tube growth

In the present study, *in vitro* pollen germination and pollen-tube growth of CDC Golden and CDC Sage were severely reduced at 36°C. This threshold maximum temperature was much lower than 45°C reported by Petkova et al. (2009) for pea pollen germination and pollen-tube growth, who used only one control temperature treatment and 45°C. Published optimum temperatures for pollen-tube growth were 36.1°C in soybean (Salem et al., 2007) and 30-34°C in groundnut (*Arachis hypogaea* L. - Kakani et al., 2002), higher than pea. Mean pollen-tube length in pea was reduced by 7.7 times when temperature was increased from 24°C to 36°C for a 10-h period. This result is not surprising because more heat units are required for growth of soybean and groundnut, which are warm season crops compared to pea. Similarly, *in vitro* pollen-tube growth of canola (*Brassica napus*) showed stunted morphology when exposed to 35°C for 4 hours (Young et al., 2004). Pollen-tube length of *B. juncea* was also significantly reduced by high temperature (Rao et al., 1992).

The pollen-tube length of pea was 102-783 µm in the present study. The *in vitro* pollen-tube lengths of other crops were reported to be 437 µm in soybean (Salem et al., 2007), 1000-1800 µm in corn (Binelli et al., 1985), 450-1400 µm in groundnut (Kakani et al., 2002), 20-60 µm in muskmelon (*Cucumis melo* L., Maestro and Alvarez, 1988), and 410-1400 µm in cotton (Kakani et al., 2005). A lower percentage pollen germination under high temperature conditions resulted from either under-utilization or unavailability of carbohydrates in pollen grains during pollen formation (Kakani et al., 2005). Greater pollen cell membrane stability is additionally related to higher optimum temperature for pollen germination in groundnut (Kakani et al., 2002). The reduction in tomato pollen viability was associated with a lower soluble sugar concentration in the developing pollen grains (Pressman et al., 2002). According to Hoekstra et al. (1989), sucrose may act as a desiccation protectant in pollen, because pollen dehydration survival rate had a positive association with sucrose concentration.

3.4.3 Heat stress on pollen surface morphology and pollen wall structure

Although no morphological changes in the pollen surface were observed using SEM, TEM of wall structure of pea pollen from plants exposed to heat stress (36°C/18°C day/night temperatures for 4 days) revealed the average thickness of the grain's intine layer had almost doubled. The increased intine thickness in the pea pollen wall might be involved with a protective mechanism; plants may produce a thicker pollen wall to protect the sperm cell(s) inside the pollen grains under

environmental stress conditions. Interestingly, in *Phaseolus* pollen examined by TEM from plants grown at 32°C/27°C day/night temperatures for 12 days, the grain's intine layer was not clearly discernible (Porch and Jahn 2001) and so a direct comparison with this study of pea is not possible. Further research is required to investigate the dynamics of the intine layer of pollen from legume plants subjected to high temperature. We concur with Porch and Jahn (2001) that any detrimental effect of heat stress on either the intine and exine layers of pollen could cause abnormal pollen function.

3.4.4 Heat stress on pollen surface lipid composition

The pollen surface consists of pollen coat and the exine layer. The sporopollenin on the exine layer consists of a series of polymers derived from long-chain fatty acids, phenylpropanoids, and oxygenated aromatic rings, which are at least in the Brassicaceae synthesized in the tapetum (Piffanelli et al., 1998). At extreme heat stress (36°C), CDC Sage had greater percentage pollen germination than CDC Golden, which was likely associated with lipids on the pollen coat and/or exine being more stable in CDC Sage compared to CDC Golden. The pea pollen surface lipid composition detected using ATR spectroscopy was most likely from the pollen coat, although some lipids may have come from the sporopollenin on the elaborately sculpted exine layer. The pollen surface lipid change under heat stress in pea (cv. CDC Golden) could affect a series of pollination-related events. These would include pollen contact, adhesion and attachment of pollen to the stigma, and further pollen hydration, pollen germination, pollen-tube growth through the stigma, style, and ovary, and fertilization of the ovule. Lipids on the pollen coat are required for pollen hydration which is a basic prerequisite for pollen germination (Preuss et al., 1993; Dickinson, 1993; Evans et al., 1990; Piffanelli et al., 1997; Edlund et al., 2004). Hexane washed pollen grains in spruce (*Picea pungens* Engelm.) and pine (*Pinus ponderosa* Laws.) had lower percentage pollen germination compared to pollen grains without the hexane treatment, which indicated that tryphine on the pollen coat played an important role in pollen germination (Sowa and Conner, 1995). Once pollen grains land on dry stigmas, the lipid-rich pollen coat develops an interface between two cell surfaces (Edlund et al., 2004). Lipids on the pollen coat may also play an essential factor in the ability of pollen tubes to penetrate the stigmatic papillae (Wolters-Arts et al., 1998). Cis-unsaturated triacylglycerides are essential for penetration of the stigma by pollen tubes in tobacco (Wolters-Arts et al., 1998). The pollen coat of *Brassica napus* has a rich content of non-polar

neutral esters with mostly medium-chain and long-chain saturated fatty acids (Piffanelli et al., 1998).

The content of unsaturated fatty acids in sorghum pollen grains increased while saturated fatty acids decreased under heat stress, possibly leading to an increase in membrane fluidity because of the accumulation in double bonds (Prasad and Djanaguiraman, 2011). Phospholipid species containing polyunsaturated acyl species, such as C18:3, C34:3, C36:3, and C36:6, were significantly increased by high night temperature in sorghum pollen grains (Prasad and Djanaguiraman, 2011). Similarly, a reduction of phospholipid content in soybean pollen grains was observed (Djanaguiraman et al., 2013). Our results could not explain why the greater amount of minor lipids in pollen grains of CDC Golden than CDC Sage were related to heat sensitivity, except perhaps due to the fact that the stability of the major lipids in CDC Sage played a greater role in heat tolerance compared to CDC Golden, or possibly due to the modification of protein secondary structure under heat stress. Protein richness with α-helical structures in CDC Sage under heat stress may protect pollen grains from the detrimental effects of dehydration (Lahlali et al., 2014).

3.5 Conclusions

The research hypotheses of this study were accepted that high day temperature negatively impacts pollen development and seed set when the temperature was above temperature thresholds. High temperature stress decreased percentage pollen germination *in vitro*, pollen-tube growth *in vitro*, pod length *in vivo*, and seed number per pod *in vivo*. At the extreme heat stress (36°C) tested, percentage pollen germination of CDC Sage was greater than CDC Golden. No morphological changes in the pollen surface were observed, whereas heat stress increased the intine thickness. Lipid composition on the pollen coat and exine of CDC Sage was more stable compared to CDC Golden at 36°C. A pollen surface lipid acyl chain change associated with heat treatment in CDC Golden may result in lower percentage pollen germination compared to CDC Sage.

Contributions by others to this thesis chapter

The majority of this study (seed-set parameters, *in vitro* pollen germination and pollen-tube growth, pollen surface morphology, and pollen wall structure) was performed by Yunfei Jiang. The analysis of pollen surface composition using the FTIR spectroscopy was co-performed by Dr. Rachid Lahlali (Canadian Light Source Inc. scientist), Dr. Chithra Karunakaran (Canadian Light Source Inc. scientist), and Yunfei Jiang at Canadian Light Source Inc.

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Transition section between Chapter 3 and Chapter 4

Effects of elevated day-time temperatures on pollen development and seed set in field pea were elucidated in Chapter 3. High daytime temperatures changed pollen surface composition and pollen wall structure, and heat stress reduced *in vitro* pollen germination percentage, pollen-tube length, pod length, and seed-to-ovule ratio. The aim of the next experiment in Chapter 4 was to investigate the influence of elevated night-time temperatures on pollen development and seed development in field pea.

Chapter 4 Pea pollen morphology and seed development response to high night temperatures

4.1 Introduction

Negative effects by elevated night temperatures had been observed in many crops, such as pea (*Pisum sativum*; Karr et al., 1959; Nonnecke et al., 1971), rice (*Oryza sativa*; Mohammed and Tarpley, 2009, 2011), corn (*Zea mays*; Cantarero et al., 1999), soybean (*Glycine max*; Gibson and Mullen, 1996), sunflower (*Helianthus annuus*; Izquierdo et al., 2002), wheat (*Triticum aestivum*; Prasad et al., 2008), sorghum (*Sorghum bicolor*; Prasad and Djanaguiraman, 2011), and also in cotton (Gipson and Joham, 1969; Echer et al., 2014). The detrimental effects of heat stress on seed yield are supposedly exacerbated by elevated night temperature.

The earliest published research on pea heat-stress by Linck's group showed that the negative effects of elevated night temperatures were more severe compared to elevated day temperatures in pea in an environmentally controlled environment (Karr et al., 1959). Elevated night temperature resulted in 25% yield loss, while elevated day temperature led to 8% yield loss (Karr et al., 1959). The controlled elevated day/night temperature (27/17°C day/night temperatures) through the entire plant growth period reduced the number of pods per plant in pea compared to the controlled temperature regime (17/7°C day/night temperatures), with the reduction of 46%, 65%, 66%, and 54% in the four investigated cultivars, namely Dark Skin Perfection, Nugget, Early Sweet 11, and Elf, respectively (Nonnecke et al., 1971). However, no significant effect on growth and yield was observed when plants first remained at 17/7°C until full bloom, and were then transferred to the 27/17°C regime (Nonnecke et al., 1971).

In cotton, elevated night temperature increased the rate of flower production per plant and the flower abortion rate during the floral bud stage under environmentally controlled conditions (Echer et al., 2014). Elevated night temperature decreased cotton pollen viability during floral bud development due to low sucrose content in flowers, but elevated night temperature did not reduce pollen viability during flowering. Further, elevated night temperature during flowering also reduced the number of seeds per locule and the number of seeds per boll (Echer et al., 2014). In sorghum, elevated night temperature reduced phospholipid saturation levels and changed different phospholipid levels, which may result in decreased pollen function (Prasad and Djanaguiraman,

2011). Phospholipids are essential for pollen development, including pollen viability, pollen germination (Xue et al. 2009), pollen hydration and directing pollen-tube growth to penetrate the stigma (Wolters-Arts et al., 1998). The reduction in pollen viability of sorghum under elevated night temperature resulted from loss of membrane integrity (Prasad and Djanaguiraman, 2011).

Pollen viability is defined as the ability of the pollen to deliver male gametes to the embryo sac (Shivanna, 2003). Loss of pollen viability is associated with loss of membrane integrity, lack of respiratory substrates, and loss of moisture (Shivanna, 2003). The fluorescein diacetate (FDA) test, also referred to as the fluorochromatic reaction (FCR), is commonly used to test pollen viability. Two pollen properties are evaluated using the FDA test – (1) the plasma membrane integrity of the vegetative cell, and (2) presence of active esterases in the pollen cytoplasm. The FDA, a non-polar and non-fluorescing substance, passes freely through the pollen membrane and reaches the pollen cytoplasm, which is hydrolyzed to produce fluorescein (a fluorescent substance) and acetate in the presence of active esterases. In viable pollen, fluorescein (a polar substance) cannot pass through the intact plasma membrane and it accumulates inside the pollen cytoplasm, so pollen grains show bright fluorescence under a fluorescence microscope. In nonviable pollen, fluorescein passes through disrupted nonfunctional plasma membrane, resulting in uniform background fluorescence (Shivanna, 2003).

Although many studies about elevated night temperature on different crops were conducted, none of these studies involved elevated night temperature effects on field pea. Most elevated temperature studies in pea were conducted under two set of conditions – elevated day/elevated night and low day/low night temperature. In general, effects of elevated night temperatures on pollen function and seed set in pea are poorly understood, especially for current cultivars of grain pea. Therefore, I hypothesized that elevated temperatures would negatively affect pollen development and seed set when the night temperatures were above temperature thresholds. The objective of this study was to evaluate varying elevated night temperatures while maintaining a constant daytime temperature during the flowering stage on morphological traits in pea.

4.2 Materials and methods

4.2.1 Plant material and growth conditions

Two cultivars of pea, CDC Golden and CDC Sage, were used in this study. The study was designed as a randomized complete block design (RCBD). This experiment was repeated twice and time was considered as the blocking factor. For each experimental run (within the same temporal block), 24 pots (2 cultivars × 3 treatments × 4 reps) of 3.8 L volume (3 plants per pot) were seeded with Sunshine Gro® mix (Seba Beach, AB, Canada) and slow-release fertilizer. The pot dimensions were 15.9 cm depth and 16.5 cm diameter. Plants were thinned to 2 plants per pot approximately two weeks after seeding. Plants received the first application (500 ml per pot) of half strength Hoagland's culture solution at three weeks after seeding and the second application (500 mL per pot) at the early flowering stage. Soil medium water moisture was monitored carefully to avoid drought stress.

Plants were grown at 27/18°C day/night temperatures with a 16 h photoperiod in each 24 h cycle and the irradiance of 450-500 µmol photons m^{-2} s^{-1} from cool fluorescent tubes in a growth chamber, and the relative humidity had a range of 4-8%. Plants were then transferred to the other two growth chambers to expose them to elevated night temperatures for 7 days when the flowers at the second fruiting nodes of the main stem were at the anthesis stage (the "wing" and "standard" petals reflexed back from their closed position). The control plants remained in the 27/18°C growth chamber until harvest. The temperatures in the two growth rooms were 27/22°C and 27/26°C day/night. Other environmental conditions, such as photoperiod and light intensity, remained the same as the controlled growth chamber.

4.2.2 Data collection

Plant height, the numbers of reproductive nodes and flowers on the main stem, percentage of flower abortion during the heat treatment, pollen viability, the number of seeds per plant, and seed yield (g $plant^{-1}$) were measured throughout the study. Percentage flower abortion during the heat treatment was calculated by dividing the number of aborted flowers by the total number of flowers produced on the main stem during the 7-day heat treatment duration. Pollen viability was tested using the FDA method. Viable pollen grains showed bright fluorescence under a fluorescence microscope, and nonviable pollen grains were dark which resulted in uniform background fluorescence (Figure 4.1).

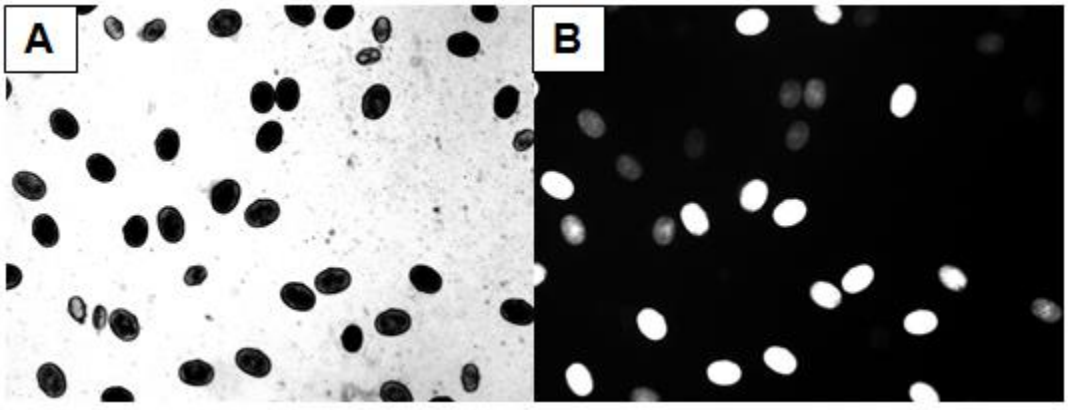

Figure 4.1. Pea pollen grains in the fluorescein diacetate (FDA) test. A: Pollen of CDC Sage (27/18°C) with the bright field microscopy; and B: Fluorescence microscopy.

4.2.3 Statistical data analysis

The statistical analyses were performed using the Mixed procedure of SAS version 9.3 statistical software (SAS Institute, Inc., Cary, NC). Analysis of variance (ANOVA) with the Least Significant Difference (LSD) test ($P < 0.05$) was used. The effects of cultivar and temperature treatments were considered as fixed effects, and blocks and replications within each block were considered as random effects. The Kenwardroger option was used for approximating the degrees of freedom for means.

4.3 Results

Genotype differences in plant height, the number of flowers on the main stem, and seed number per pod were observed (Table 4.1). CDC Sage was taller and had more seeds per pod but had fewer flowers remaining on the main stem due to the fact that not every single reproductive node produced two flowers per node compared to CDC Golden. Night temperature and its interaction with genotype had no significant effect on any of the tested parameters, namely plant height, the number of reproductive nodes on the main stem, the number of flowers on the main stem, percentage of flower abortion during the heat treatment, pollen viability, the number of seeds per pod, and seed yield (Table 4.1).

Table 4.1. Effect of genotype and night temperatures on growth performance, pollen viability, and yield in pea with a daytime temperature of 27°C.

Genotype (G)	Height (cm)	Number of flowering nodes	Total flower number	Percentage of flower abortion (%)	Pollen viability (%)	Seed number per pod	Yield (g plant^{-1})
CDC Golden	60.1 a†	5 a	9 a	22.5 a	74.5 a	3 b	8.1 a
CDC Sage	55.0 b	5 a	7 b	23.5 a	79.5 a	5 a	8.5 a
Night temperature (NT, °C)							
18	58.8 a	5 a	7 a	21.1 a	78.1 a	4 a	8.5 a
22	58.3 a	5 a	8 a	20.8 a	79.4 a	4 a	8.2 a
26	56.9 a	5 a	8 a	27.0 a	73.4 a	4 a	8.2 a
P values							
G	**0.005**‡	0.16	**0.002**	0.84	0.21	**<0.001**	0.59
NT	0.72	0.15	0.38	0.57	0.41	0.61	0.94
G×NT	0.27	0.85	0.72	0.64	0.46	0.81	0.52

†Means with a common letter within each column and each main effect did not differ at $P < 0.05$.

‡ The *P* values in bold type indicate that the main effect or interaction had a significant effect on the response.

4.4 Discussion

Night temperatures ranging from 18 to 26°C did not have a significant negative effect on pollen viability, plant growth performance, seed-set, and seed yield in pea for CDC Golden and CDC Sage. One possibility was that the night temperature of 26°C was not high enough to have had a negative influence in pea. The reason of using the maximum night temperature of 26°C was because that is typically the highest and most realistic night temperature in the field in Saskatchewan. In Saskatchewan fields, which reside in a semi-arid region, the minimum and maximum night temperatures never exceed 26°C, and are always lower than day temperatures in the summer. The maximum night temperatures occur shortly after dusk, and the minimum night temperatures usually occur at dawn or within 2 h of dawn.

Karr et al. (1959) observed that elevated night temperature caused yield reduction in pea. In that study, three different temperature regimes (32/15°C, 24/30°C, and 32/30°C day/night temperatures with 12-hour photoperiod) were studied on the first reproductive node position only. Yield with elevated temperature treatments was calculated as % of seed yield at the first fruiting nodes in the controlled plants. The results showed that elevated night temperatures were more critical than elevated day temperatures in pea, with 25% yield loss under elevated night temperature stress and 8% yield loss under elevated day temperature stress. At a day temperature just below heat stress in the field (< 28 or 29°C), elevated night temperature does not have any additional impact on yield. The potential deleterious impact of night temperature as claimed in the literature should be researched further at a selected range of greater daytime maximum temperature, and also under greater radiative (irradiance) conditions, and perhaps under a range of relative humidity.

An elevated night temperature (26/18°C, 26/22°C, 26/24°C, and 26/26°C day/night temperature) study conducted on tomato showed that there were less normal pollen grains at the night temperatures of 24 and 26°C compared to 18 and 22°C. The night temperature of 26°C reduced number of flowers and fruits on the first cluster in tomato (Peet and Bartholemew, 1996). Peet and Bartholenew (1996) mentioned that effects of night temperature on pollen function and fruit set in tomato were less negative than expected from the literature. Echer et al. (2014) observed that elevated night temperature (elevated temperature 32/29°C vs control 32/24°C) increased the flower abortion rate in cotton during the floral bud stage but no effect was observed during the flowering stage. Elevated night temperature decreased the cotton pollen viability during the floral bud stage

due to the low sucrose content in the flower, but elevated night temperature did not reduce pollen viability during the flowering stage. Further, elevated night temperature during the flowering stage also reduced the number of seeds per locule and the number of seeds per boll (Echer et al., 2014).

4.5 Conclusions

The research hypothesis of this experiment was rejected that the increased night temperatures from 22 and 26°C had no effect on pollen and seed development compared to the control night temperature of 18°C. Negative effects on reproductive growth from elevated night temperatures ranging from 18 to 26°C were much less than expected. Elevated night temperatures compared to the control had no additional effect on plant height, number of reproductive nodes on the main stem, number of flowers on the main stem, flower abortion rate, pollen viability, the number of seeds per pod, and seed yield. In future, the relationship between ovule position and seed abortion/fertilization failure will be investigated using the data collected from this study.

Transition section between Chapter 4 and Chapter 5

In Chapters 3 and 4, the effects of high day-time and night-time temperatures on pollen development and seed set were described. In Chapter 3, we observed the negative effects of elevated day-time temperatures on *in vitro* pollen germination percentage, *in vitro* pollen-tube length, pollen wall morphology, and seed set parameters including pod length and seed-to-ovule ratio. In Chapter 4, however, we did not find any negative effects of high night-time temperatures on pollen viability and seed development in field pea when night-time temperatures increased from 18 to 26°C. It is of great interest to explore the link between pollen – pistil interaction and seed development. Therefore, in Chapter 5, experiments were carried out to determine the relationship between the pollen – pistil interaction and seed development in field pea.

Chapter 5 Effect of heat stress on pollen-pistil interactions and patterns of seed development in field pea (*Pisum sativum*)

5.1 Introduction

Even in flowering plants that are self-compatible and autogamous, sexual reproduction still requires a defined sequence of significant steps in order to yield fruits with seeds. If pollen initiation, maturation, or anther dehiscence is impeded, failure to achieve reproductive success can occur even prior to pollination (Kinet and Peet, 1997). Failure at other pre-fertilization events can also occur, such as maturation of embryo sacs or interactions of pollen at the stigma necessary for pollen germination and pollen-tube growth to the ovules through the style. Moreover, fertilized ovules may not generate seeds owing to abortion, defined as the mortality of seeds between fertilization and maturation (Bawa and Webb, 1984).

Within the Fabaceae, the flower possesses one carpel (a simple pistil) whose ovary bears ovules linearly arranged within the future dry, elongate fruit, the legume or pod. Past studies of flat pea (*Lathyrus sylvestris*), perennial pea (*L. latifolius*) (Hossaert and Valero, 1988), field pea (*Pisum sativum*; Linck, 1961; Pate and Flinn, 1977), and black locust (*Robinia pseudoacacia*; Susko, 2006) have demonstrated that some ovules remain unfertilized. In general, ovules at the pistil's stylar end are fertilized first, whereas ovules at the peduncular end, farthest from the stigma, are fertilized thereafter. However, ovule fate is complex because peduncular ovules reside closest to the maternal supply of nutrients (Hossaert and Valero, 1988) and the developing legume itself exhibits some photosynthetic activity (Andrews and Svec, 1975; Atkins et al., 1977; Flinn et al., 1977). Then again, the maximum direct uptake of CO_2 by pods diminishes as they age and is considered negligible (i.e., 4% of total import of a pod's dry weight; Sambo et al. 1977). Consequently, the fate of seed maturation is regarded as a balance between early initiation and genetic quality of embryos at the ovary's stylar end, and proximity to maternal nutrition at the peduncular end (Hossaert and Valero, 1988). The latter trend is important in the Brassicaceae, for example, fostering higher seed maturation at the fruit's basal end by providing a spatial competitive advantage for photosynthate and other nutrients and water (Meinke, 1982; Pechan and Morgan, 1985; Marshall and Ellstrand, 1986). In contrast, ovules near the stylar end in pods of Fabaceae have higher probability to mature as seeds, suggesting a temporal advantage of early fertilization by the most vigorously growing pollen tubes (Rocha and Stephenson, 1991; Jakobsen and Martens,

1994; Gutiérrez et al., 1996). Furthermore, a higher frequency of selfing than outcrossing resulted in greater randomness of ovule fertilization and maturation within legumes, partly due to lower competition among potential sires (Hossaert and Valero, 1988). Finally, legume morphology itself may mechanically restrict seed growth; ovule failure in pea varieties with tapered pods exceeds that in pods with blunt ends (Linck, 1961; Pate and Flinn, 1977).

Superimposed on these sequential post-fertilization events leading to a flower's seed set within a fruit, is the influence of metabolic activities of a legume plant's other flowers. For example, in soybean where multiple flowers are borne at a node's racemes, pods of proximal flowers more likely mature than those of distal flowers, which eventually abscind (Huff and Dybing, 1980; Crosby et al., 1981; Brun and Betts, 1984; Carlson et al., 1987; Peterson et al., 1990). Greater abortion owing to competition for limited maternal resources thus varies by the number and position of previously fertilized fruits (Marcelis et al., 2004). As a result, in many angiosperms, only a proportion of ovules ever matures into seeds (Stephenson, 1981; Hossaert and Valero, 1988; Charlesworth, 1989). Post-fertilization events such as predation, unfavourable weather, and the aforementioned limiting resources of the maternal parent causing abortion, are among the post-fertilization events restricting seed maturity. Production of supernumerary ovules may represent a strategy to accommodate these constraints (Ehrlen, 1991).

Heat stress is one such environmental effect of great interest to this study. Elevated temperatures adversely affect retention of pollen grains on the stigma, pollen germination, pollen-tube growth, ovule viability, fertilization, and the growth of endosperm and embryos in various crops (Kaushal et al., 2016), thereby inducing both flower and fruit abscission (Aloni et al., 1991). Among the Fabaceae, high temperature caused anther indehiscence in cowpea (Warrag and Hall, 1984a), plus inferior pollen germination and decreased pollen-tube growth in chickpea and common bean (Gross and Kigel, 1994; Devasirvatham et al., 2012). Pea plants, in particular, grow and yield well in relatively cool temperatures (Sadras et al., 2012). Accordingly, because there is a general dearth of information on heat-stress effects on the sexual processes of female reproductive organs in angiosperms, we investigated pollen quantity, viability, and anther dehiscence leading to pollen-pistil interactions in two cultivars of field pea, and continued to follow these events through the style and developing ovary during flowering phenology. Furthermore, we examined the effect of elevated daytime temperatures on flower retention and seed set at different nodes, and on a flower's position within an inflorescence, as well as the effect of an ovule's position on the pattern of seed

maturation within pods. A standardized scale of seven stages of pea flower phenology allowed precision in characterization of sexual-reproductive events, plus the identification of bud stages most vulnerable to elevated temperatures. In part, this study utilized synchrotron-based phase contrast X-ray imaging to observe the internal structure of pea flowers and the developmental progression of ovule maturation immediately following fertilization in field pea.

5.2 Materials and methods

5.2.1 Plant material and floral stages examined

Two cultivars, CDC Golden and CDC Sage of field pea (*Pisum sativum* L.), were grown in growth chambers at specific temperature regimes (see below). In pea, two flowers are formed at each nodal raceme, staggered in age and time of opening. Seven stages of flower buds, mature flowers, and pods, were examined in CDC Golden. The first six stages designated (Figures 5.1A-I, L-N; Figure 5.2) were: (1) Stage I – small green buds approximately 10 mm long (Figure 5.1A), hidden by the stipules; (2) Stage II - buds approximately 12 mm long, with petal and sepal length being similar (Figure 5.1D), about 49 h after Stage I (Figure 5.2); (3) Stage III - buds approximately 17 mm long and attaining their maximum length, with petal tips exceeding sepal length (Figure 5.1H), about 29 h after Stage II (Figure 5.2); anther dehiscence had occurred by stage III (Figures 5.1I, J); (4) Stage IV – fully open (Figure 5.1L), flowers approximately 18 mm long, about 9 h following Stage III (Figure 5.2); (5) Stage V - closed flowers at post-anthesis (Figure 5.1M), with petals withering and ovary elongation evident, approximately 3 d following anther dehiscence; (6) Stage VI - immature pod about 28 mm long with persistent calyx but senescent corolla and androecium (Figure 5.1N); approximately 1.5 d after Stage V (Figure 5.2). Thus, at day/night temperatures of 24/18°C for a 16 h photoperiod, total duration of floral phenology from Stage I to VI required about 6.5 days (Figure 5.2). Mature pods at their physiological maturity, with filled seeds within, comprised Stage VII (Figure 5.3).

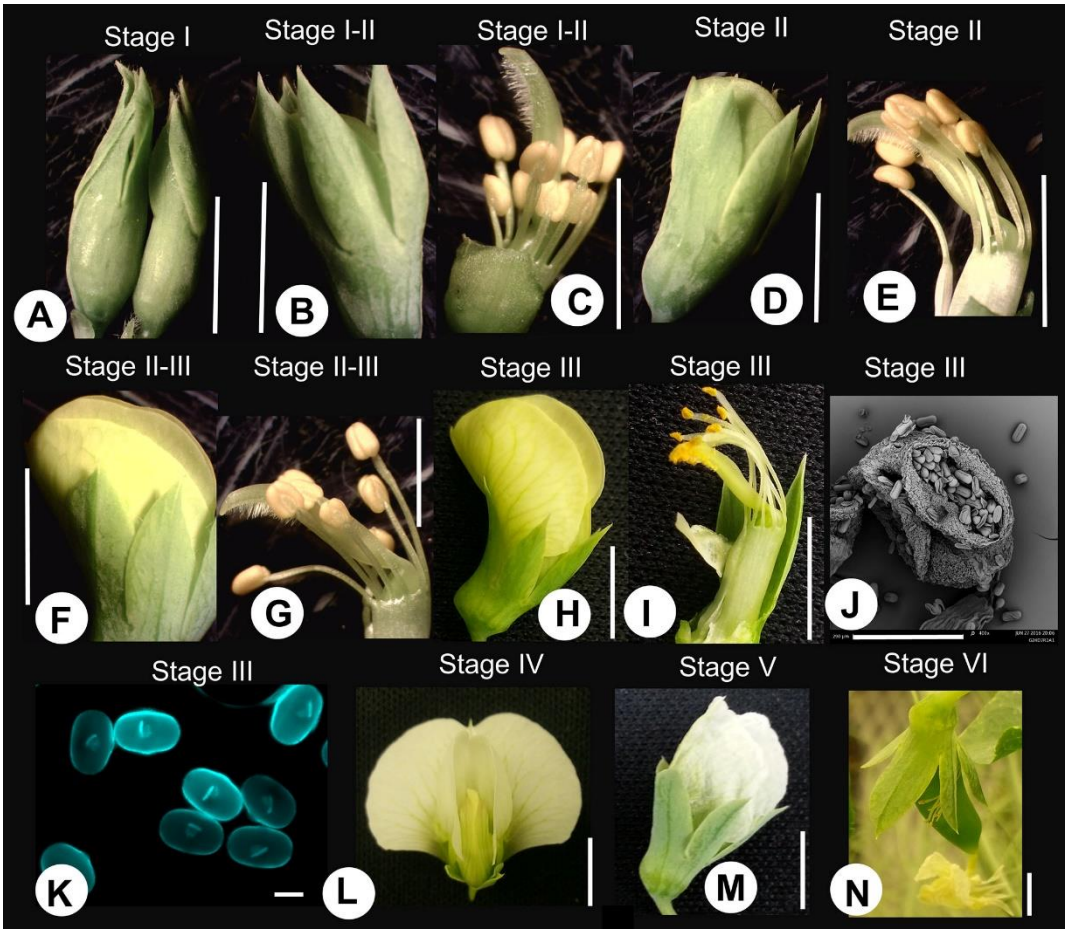

Figure 5.1. Floral organs of *Pisum sativum* cv. CDC Golden at different growth stages. A: Stage I, small green buds typically concealed by the stipules; B: Stage I-II floral bud showing separating tips of sepals; C: Stage I-II floral bud, after the perianth was removed; D: Stage II floral bud, with similar petal and sepal length; E: Stage II floral bud, after the perianth was removed; F: Stage II-III floral bud with petals beyond the calyx lobes; G: Stage II-III floral bud, after the perianth was removed; H: Stage III floral bud, with petal tips exceeding sepals and having attained their maximum length, banner (standard) petal is loosening; I: Stage III floral bud, after the corolla was removed; J: Anther dehiscence during Stage III; K: Pollen grains (stained with Hoechst 33342 and examined by fluorescence microscopy) at Stage III are bicellular; L: Stage IV, anthesis; note reflexed banner (standard) petal; M: Stage V, post-anthesis, with petals withering and ovary elongation evident; N: Stage VI immature pod, with petals falling off. Scale bars: A-I, L-N, 5 mm; scale bar = 20 µm (K) and 290 µm (J).

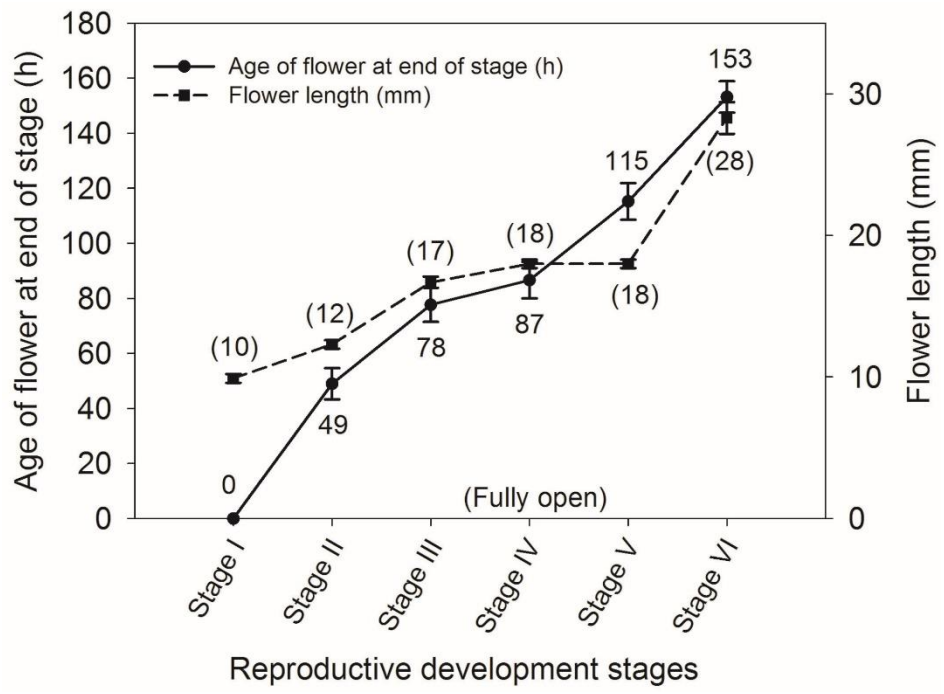

Figure 5.2. Average length (mm) of floral buds and flowers at the first and second reproductive nodes in field pea (*Pisum sativum*) cv. CDC Golden and the average duration (h) between consecutive floral stages. Each point represents the mean ± s.e. for ten flowers. The 0 on the left Y axis denotes the time when the flower bud is at Stage I. Staging is shown in Figure 5.1.

5.2.2 Quantity of pollen produced per anther

The number of pollen grains per anther was determined in cultivar CDC Golden. Floral buds (n=2) at the first reproductive node prior to anther dehiscence (Stage II-III; Figure 5.1G) were collected from two different plants in the control chamber (24/18°C). Two of the flower's 10 anthers (field pea has 9 stamens fused in a tube, with 1 adaxial stamen free) were randomly selected from the staminal tube for each floral bud. Each indehiscent anther was dissected and pollen grains released in one drop of 0.5 M sucrose solution in a 16 square grid on a microscope slide; the same anther was transferred to a second drop in a different 16 square grid to liberate any residual pollen grains. The total number of pollen grains per anther was counted using a light microscope at 100× magnification and calculated by summation of all pollen grains in the 32 squares. Estimates of total pollen produced per pea flower were calculated by multiplication of the average quantity per anther, by 10.

5.2.3 Exp. 1: Influence of cultivar, reproductive node position, flower position per raceme, and temperature, on seed set

5.2.3.1 Plant material and growth conditions

The study was arranged as a randomized complete block design (RCBD) with three replications in the first experimental run and four replications in a second run. Initially, all plants were grown at 24/18°C for 16 h light / 8 h dark in a growth chamber. The irradiance was 450-500 µmol photons m^{-2} s^{-1} from cool fluorescent tubes and the relative humidity had a range of 4-8% in growth chamber. For the first experimental run, 12 pots (two cultivars × two temperature regimes × three replications) of 3.8 L volume filled with Sunshine Gro® mix (Seba Beach, AB, Canada) and slow-release fertilizer (14-14-14, Type 100, Nutricote®, Brampton, ON, Canada) were planted with three seeds per pot. The pot dimensions were 15.9 cm depth and 16.5 cm diameter. For the second experimental run, a total of 16 pots (two cultivars × two temperature regimes × four replications) were seeded. For the purposes of availability of two plants per pot to provide material for different experiments, pots were thinned to two plants approximately two weeks after seeding. When the flowers at the second fruiting node of the main stem were fully open (Stage IV), one half of the pots were transferred to another chamber operating at a high temperature regime, for a period of 7 days: 16 h light; temperature increased over a 5 h period from 21°C to 35°C by 3°C increments; 35°C maintained for 6 h; temperature decreased over 5 h period to 18°C for 8 h dark. Control plants remained in the 24/18°C growth chamber.

5.2.3.2 Measurement of ovule and seed development

Each pod from the first five fruiting nodes on the main stem from all 28 plants (two cultivars × two temperature regimes × seven replications from two experimental runs) was opened at physiological maturity (Stage VII) and the condition of each ovule (seed) recorded. The position and developmental stage of each ovule were determined, proceeding from the stylar end (first position) to the basal end (last position) of each pod. The development of ovules was categorized into three groups according to Hossaert and Valero (1988), with modifications: 1) an "aborted ovule" was defined as a vestigial ovule that failed to develop into a seed, whether the ovule had been fertilized or not; diameter < 2 mm; rated quantitatively as 0.5; 2) an "aborted seed" represented a fertilized ovule which began to develop but failed to mature; diameter 2-5 mm; rated as 1.0; 3) an ovule that successfully developed into a seed; diameter > 5 mm; rated as 1.5 (Figure

5.3). All ovules within aborted pods were assigned a rating of zero. In total, 280 pods (two cultivars × two temperatures × five fruiting nodes × two pod positions at each node × seven replications) were examined from the main stems of the experimental plants.

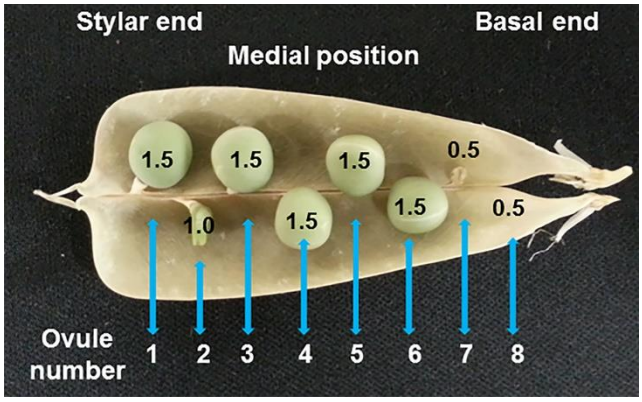

Figure 5.3. Ovule position and stages of seed development within an opened, mature pod of *Pisum sativum* cv. CDC Sage at physiological maturity (Stage VII). Positions of ovules were assigned numbers 1 to 8 starting at the stylar end. In this example, ovules at Positions 1, 3, 4-6 – fully developed seeds, rated as 1.5; Position 2 - an aborted seed with a diameter 2 - 5mm, rated as 1.0; Positions 7 and 8 - aborted ovules with a diameter < 2mm, rated as 0.5.

5.2.4 Exp. 2: Synchrotron based phase contrast X-ray imaging of flower development

Synchrotron based phase contrast X-ray imaging (PCI), a non-destructive technique, was utilized to observe structural changes of internalized floral organs of the two cultivars during the developmental progression of ovule maturation following fertilization, as affected by heat stress. The imaging experiment was conducted at the Biomedical Imaging and Therapy Beamline (BMIT-BM, 05B1-1) at the Canadian Light Source. The X-ray source of the beamline is a bend magnet in a 2.9 GeV synchrotron (Cutler et al., 2007). The accessible energy range of the beamline is 18-40 keV and the beamline produces a monochromatic and coherent X-ray beam of size 240 mm (horizontal) × 7 mm (vertical) into the experimental station, which is about 23 m from the bend magnet source (Karunakaran et al., 2015).

The beamline was set up for the PCI mode and the sample to detector distance was 0.6 m. The X-ray energy of the incident beam was 18 keV and an aluminum filter 0.5 mm thick was used to reduce the heat load on the monochromator. The transmitted X-ray images were recorded by converting the X-ray intensities into visible images by combination of a scintillator and a detector (C9300-124, 35 mm diameter, AA-60, 1344 × 1024 pixels, Hamamatsu) with pixel resolution of 8.75 µm. The dark current of the detector was recorded (dark image) when there was no incident

X-ray beam. The imperfection of the incoming X-ray beam and the inhomogeneity in the scintillator screens were captured (flat image) by recording an image when there was no sample in front of the detector.

Flowers from three pre-anthesis stages (Stages II, II-III, and III of Figure 5.1) grown in the control environment (24/18°C day/night temperatures, 16/8 h photoperiod) were collected and imaged using PCI. After dissection of the perianth and androecium, control and heat-stressed pistils at post-anthesis (Stage V; Figure 5.1M) were also imaged following exposure to 35/18°C for 4 days.

The 2D projection PCI images were normalized using flat and dark images and the X-ray intensity images converted into subsequent optical densities using the Beer-Lambert law (Karunakaran et al., 2015). All data analysis of flower images was carried out using ImageJ software (ImageJ 1.47v, National Institutes of Health, USA).

5.2.5 Exp. 3: Pollen-pistil interactions and the influence of elevated growth temperature

Two pea cultivars, CDC Golden and CDC Sage, were grown in growth chambers at the same temperature regimes outlined for Exp. 1. The study was arranged as a RCBD with four replications per experimental run, and repeated twice with run considered as the blocking factor. For each run, 16 pots (two cultivars × two temperature regimes × four replications) were prepared exactly as described in Exp. 1. Pots were thinned to two plants approximately two weeks after seeding. The procedure was similar to Exp. 1, with one modification. When small green buds (Stage I; Figure 5.1A) at the first fruiting node of the main stem were visible, half the pots were transferred to the high temperature regime as described in Exp. 1 for 7 days. Control plants remained in the 24/18°C growth chamber.

5.2.5.1 Pollen viability

Pollen viability was tested using the fluorescein diacetate (FDA) staining method, wherein viable pollen grains appear brightly fluorescent using a fluorescence microscope, and nonviable pollen grains did not fluoresce (i.e., appear dark). The FDA stock solution was prepared in acetone (2 mg ml^{-1}), and sucrose solution (0.5M, 1.7g in 100 ml distilled water) was used to prevent bursting of pollen grains (Shivanna and Rangaswamy, 1992). The FDA working solution was prepared by adding drops of the FDA solution to approximately 5 ml of sucrose solution until the mixture showed persistent turbidity (Shivanna and Rangaswamy, 1992). Mature pollen grains were

collected from flowers at Stages III and IV from both cultivars from the control and high-temperature growth chambers following 4-d exposure to high temperature. A drop of FDA working solution was added to a glass microscope slide, and pollen grains dispensed onto the drop followed by gently mixing using individual stamens from the same flower, for uniform distribution. Each preparation was covered with a cover glass before observation by fluorescence microscopy. Pollen viability was calculated by dividing the number of viable pollen grains by the total number of pollen grains (at least 100 grains per replication, five replications per treatment).

5.2.5.2 *In vivo* pollen tubes in the style

To investigate the potential impact of heat stress on pollen-tube growth *in vivo*, the simple pistils (two cultivars × two temperature regimes × seven replications) were dissected from 28 flowers (Stage V; Figure 5.1M) from reproductive nodes 2 and 3 from plants in control conditions (24/18°C) and in the high temperature chamber (35/18°C) following exposure (4 days) to high temperature. These styles were dissected from the pistils and then fixed in a solution containing 1% formaldehyde, 0.025% glutaraldehyde, and 0.01M Na phosphate (Na_2HPO_4/NaH_2PO_4) buffer (pH 7.2), overnight. The styles were then washed twice with the same buffer at 4°C for 2 h, cleared in 4M NaOH at 65°C for 5 min, washed with the same phosphate buffer, and stained with 0.1% aniline blue in 0.1M K_3PO_4 in darkness overnight (Martin, 1959; Wist and Davis, 2008). After a final wash with the same phosphate buffer, pistils were mounted on a microscope slide using a drop of 80% glycerol, and carefully squashed under a cover slip to examine for callose staining of pollen tubes using fluorescence microscopy. Areas of callose fluorescence in the style, within 0.65 mm of its junction with the stigma, were quantified using ImageJ. This regional microscopic investigation represented approx. 3/8 of the style length.

5.2.5.3 Pollen - stigma interaction after 4 and 7 days of heat exposure

The pollen - stigma interaction was investigated using scanning electron microscopy (SEM). Styles were dissected for each cultivar with eight replications per treatment at post-anthesis (Stage V; Figure 5.1M), from controls (24/18°C) and after 4 and 7 days of exposure to high temperature (35/18°C day/night temperatures). Following 7 days of heat exposure, anthers from flowers at anthesis (Stage IV; Figure 5.1L) were also collected. These samples had dried in a glass jar with silica gel as desiccant for 3 d, then were gold-coated (Edwards S150B Sputter Coater, Wilmington,

MA) before examination using SEM (Phenom, G_2Pure), and imaged using a charge-coupled device navigation camera.

5.2.5.4 Pollen tube interaction with ovules after 4 and 7 days of heat exposure

A total of 64 ovaries (eight replications × two cultivars × two temperature regimes × two heat exposure durations) were dissected from pea flowers at post-anthesis (Stage V; Figure 5.1M) after 4 and 7 days of exposure to 35/18°C day/night temperatures. These ovaries were fixed and stained with 0.1% aniline blue using the same method described above. Ovaries were mounted on a microscope slide using a drop of 80% glycerol, to examine using fluorescence microscopy whether pollen tubes entered individual ovules within a pod.

Finally, individual ovules within ovaries (32 in total – eight replications × two cultivars × two growth conditions) of CDC Golden and CDC Sage after 4 days of heat exposure were observed using a microscope (25× magnification) and total area in surface view was measured using ImageJ, to estimate ovule size during progression of seed formation following fertilization by natural self-pollination (autogamy).

5.2.6 Data analyses

Pods were linear, unicarpellate, and multiovulate. Ovule number varied (3-9) in these two cultivars of field pea, so the number of ovules was standardized to allow for comparisons of ovule positional effects. Ovule positions were categorized into three classes: (1) stylar end; (2) medial position; (3) basal end. Ovules within individual pods were numbered beginning at the stylar end (Figure 5.3, Table 5.1). For example, if the total number of ovules in an ovary was nine, the first three ovules (numbers 1-3) were considered at the stylar position, then ovules numbered 4-6 were considered medial, and ovules 7-9 were basal. Data from the two experimental runs were combined because the effects of experimental treatments were consistent in both runs. Analysis of variance (ANOVA) with the Least Significance Difference (LSD) test ($P < 0.05$) was used to analyze effects of cultivar, temperature, node position, and pod position on seed maturity and seed abortion at a specific ovule position (stylar end, medial position, or basal end) using the Mixed procedure of SAS statistical software (version 9.3, SAS Institute, Inc., Cary, NC).

Table 5.1. Categories of ovule position based on the total number of ovules within individual pods.

Total number of ovules	Stylar end position	Medial position	Basal end position
3	1†	2	3
4	1	2,3	4
5	1,2	3,4	5
6	1,2	3,4	5,6
7	1,2	3,4,5	6,7
8	1,2	3,4,5,6	7,8
9	1,2,3	4,5,6	7,8,9

†Assignment of ovule number started from the stylar to the basal end of a pod

Analysis of variance with the LSD test ($P < 0.05$) was then used to analyze the effects of ovule position (stylar end, medial position, and basal end) on ovule fate patterns within individual pods using the Mixed procedure of SAS. The effects of cultivar, temperature, and ovule position (stylar, medial, and basal) were considered as fixed effects, whereas node position, pod position in a nodal raceme, and replications and the interaction between the fixed terms and replications were all considered as random effects. Results from the first step of data analysis (preceding paragraph) showed that ovule positon effects on seed maturity and abortion with the treatments of node position and pod position were consistent, so the data from different node and pod positions were combined to simplify the data analysis.

Data of pollen viability, callose area in styles, percentage of pollen tubes that entered individual ovules within a pod, and total area of ovules (as an indicator of ovule size) were analyzed using the Mixed procedure of SAS. Analysis of variance with the LSD test ($P < 0.05$) was performed. For the data of pollen viability, the effects of cultivar, flower stage, and temperature regime were considered as fixed effects; for the data of callose area in styles and surface area of ovules, the effects of cultivar and temperature treatments were considered as fixed effects; for the data of the proportion of ovules that received a pollen tube within a pod, the effects of cultivar, temperature regime, and the duration of heat exposure were considered as fixed effects. For the above analyses, replications and the interaction between the above fixed terms and replications were considered as random effects. In all analyses, the Kenwardroger option was used for approximating the degrees of freedom for means.

5.3 Results

5.3.1 Exp. 1: Influence of cultivar, reproductive node position, flower position per raceme, and temperature, on seed set

High temperature led to seed abortion of ovules located at the pod's stylar end and at medial ovule positions; high temperature did not affect seed maturity and abortion in ovules at the pod's basal end (Table 5.2). Main effects of node position (first five reproductive nodes) and pod position (proximal versus distal within the raceme per node) were also significant factors affecting seed maturity and abortion according to ovule location within a pod (Table 5.2). Ovules at all positions within pods at the first two (older) reproductive nodes were most likely to develop successfully into seeds compared to those at the younger, fourth and fifth reproductive nodes (Table 5.2). Only at the pod's basal end was ovule development at node 3 significantly lower than at node 2 (Table 5.2). Within each two-flowered raceme, the distal (younger) pod was more likely to abort compared to the proximal (older) pod at the same node (Table 5.2). A two-way interactive effect of node position and temperature regime was significant on seed maturation from ovules situated at the stylar ends of pods (Table 5.2, Figure 5.4). High temperature increased seed abortion at the stylar ends of the first three reproductive nodes, whereas high temperatures did not impact seed maturation at the fourth and fifth reproductive nodes (Figure 5.4).

Table 5.2. Analysis of variance (ANOVA) showing effects of cultivar, day temperature (24/18°C and 35/18°C day/night temperatures with the 16 h photoperiod for 7 days), node position, and pod position within a raceme, on seed maturation according to ovule position within pods at physiological maturity (Stage VII) in field pea plants grown in growth chambers.

Source	Stylar end ovule position		Medial ovule position		Basal end ovule position	
	F value	*P* value	F value	*P* value	F value	*P* value
Cultivar (C)	1.49	0.224	1.65	0.201	0.68	0.410
Temperature (DT)	9.48	**0.002**§	7.30	**0.008**	3.15	0.078
Node (N)	11.01	**<0.001**	12.83	**<0.001**	13.48	**<0.001**
Pod position (PP)	20.54	**<0.001**	11.80	**0.001**	15.29	**<0.001**
C*DT	0.35	0.557	0.23	0.636	2.23	0.137
C*N	1.72	0.418	0.12	0.975	0.49	0.746
C*PP	0.19	0.666	2.73	0.100	0.74	0.391
N*DT	2.81	**0.027**	2.37	0.055	1.97	0.101
DT*PP	0.07	0.793	0.03	0.853	0.40	0.527
N*PP	1.01	0.403	1.97	0.101	0.44	0.782
C*N*DT	0.71	0.584	0.39	0.816	0.21	0.932
C*N*PP	1.04	0.390	2.17	0.075	1.84	0.124
N*DT*PP	1.35	0.255	1.09	0.364	1.00	0.411
C*N*DT*PP	1.17	0.328	0.56	0.731	0.23	0.949
	Mean (± s.e.) ratings for seed development within pods					
Cultivar	Stylar end ovule position		Medial ovule position		Basal end ovule position	
CDC Golden	1.05† ± 0.043 a‡		1.13 ± 0.042 a		0.84 ± 0.053 a	
CDC Sage	0.97 ± 0.050 a		1.05 ± 0.049 a		0.78 ± 0.059 a	
Day temperature (°C)	Stylar end ovule position		Medial ovule position		Basal end ovule position	
24	1.11 ± 0.051 a		1.18 ± 0.050 a		0.86 ± 0.059 a	
35	0.91 ± 0.042 b		1.00 ± 0.041 b		0.75 ± 0.052 a	
Node position	Stylar end ovule position		Medial ovule position		Basal end ovule position	
1	1.19 ± 0.069 a		1.32 ± 0.064 a		1.00 ± 0.071 ab	
2	1.24 ± 0.066 a		1.31 ± 0.067 a		1.10 ± 0.074 a	
3	1.08 ± 0.071 ab		1.16 ± 0.069 ab		0.88 ± 0.075 b	
4	0.98 ± 0.071 b		1.06 ± 0.69 b		0.63 ± 0.076 c	
5	0.55 ± 0.090 c		0.61 ± 0.088 c		0.43 ± 0.093 c	
Pod position	Stylar end ovule position		Medial ovule position		Basal end ovule position	
Proximal	1.16 ± 0.039 a		1.20 ± 0.037 a		0.93 ± 0.050 a	
Distal	0.86 ± 0.053 b		0.98 ± 0.052 b		0.68 ± 0.061 b	

†Rating scale: a full seed = 1.5; an aborted seed = 1.0; an aborted ovule = 0.5; an aborted pod = 0. See Figure 5.3, and text.

‡Means with a common letter within a column under the main effects of cultivar, day temperature, node, or pod position, did not differ significantly at $P < 0.05$.

§The *P* values in bold type indicate that the main effect or interaction had a significant effect on the response.

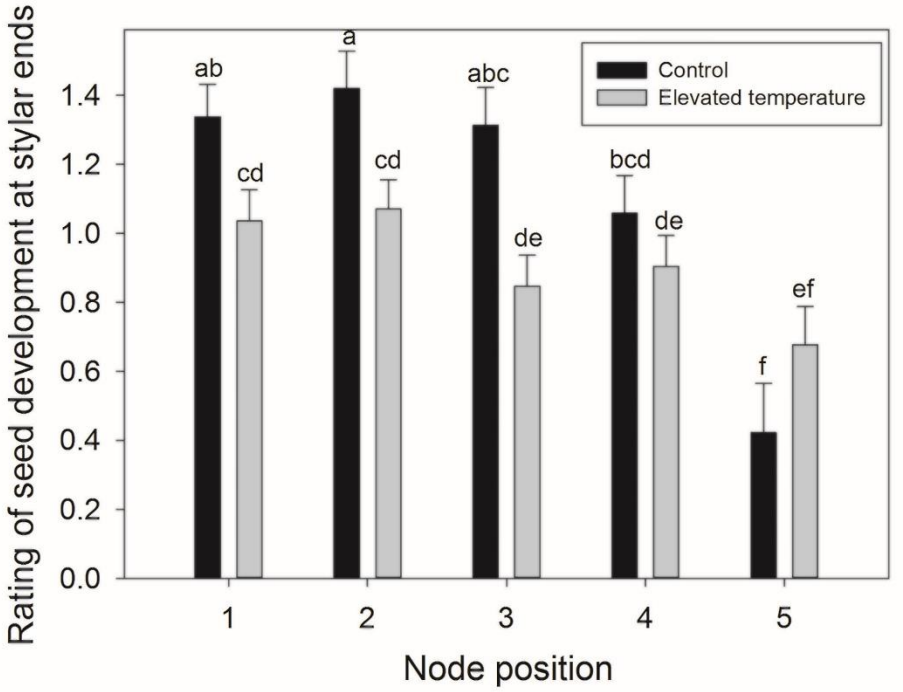

Figure 5.4. Two-way interactive effects of fruiting node position and day temperature on seed development at stylar ends of pods at physiological maturity (Stage VII) from plants of field pea grown in growth chambers. The rating scale of seed development is shown in Figure 5.3. Each bar represents the mean ± s.e. across seven replications (one plant as one replication), two cultivars (CDC Golden, CDC Sage), and two pod positions (proximal, distal) per node. Means with a common letter did not differ significantly at $P < 0.05$.

Daytime temperature and ovule position within a pod also affected seed maturity and abortion (Table 5.3). In general, exposure to the daytime temperature of 35°C for 7 days increased ovule abortion in both cultivars compared to control conditions of 24°C (Table 5.3). Ovules at the pod's medial and stylar-end positions had a similar tendency to mature as seeds compared to ovules at the basal end (Table 5.3).

Table 5.3. Mean (± s.e.) ratings for seed development in pods at physiological maturity (Stage VII) in field pea plants grown in growth chambers as affected by cultivar, day temperature for 7 days, and ovule position within pods.

Cultivar (C)	Rating of seed development†
CDC Golden	1.01 ± 0.028 a‡
CDC Sage	0.96 ± 0.030 a
LSD	0.067
P value	0.128
Day temperature (DT)	Rating of seed development
24°C	1.09 ± 0.028 a
35°C	0.88 ± 0.028 b
LSD	0.068
P value	<0.001
Ovule position (OP)	Rating of seed development
Stylar end	1.03 ± 0.035 a
Medial position	1.11 ± 0.034 a
Basal end	0.82 ± 0.034 b
LSD	0.082
P value	<0.001
P values of other effects	
C*DT	0.097
C*OP	0.880
DT*OP	0.453
C*DT*OP	0.474

†Rating scale: a full seed = 1.5; an aborted seed = 1.0; an aborted ovule = 0.5; an aborted pod = 0.
‡Means with a common letter for the main effects of cultivar, day temperature, and ovule position did not differ significantly at $P < 0.05$. Seven replications (one plant as one replication), five reproductive nodes on each main stem, and two pod positions (proximal, distal) on the raceme at each node, were considered random effects.

5.3.2 Exp. 2: Synchrotron based phase contrast X-ray imaging of different flower stages during their development

Phase contrast imaging illustrated intricate details of internal floral organs, especially the stamens and ovaries, within intact buds (Figures 5.5A-F) and ovules within pistils of dissected Stage IV flowers (Figures 5.5G-J), details not possible using conventional scanning electron microscopy. The PCI images did not show substantial differences in the internal structures of these living flowers of CDC Golden and CDC Sage. The average number of ovules per pod is 6 for CDC Golden (Figures 5.5G, H, 5.9A), and 8 for CDC Sage (Figures 5.3, 5.5I, J). After 4 days at elevated

temperatures, no obvious differences were observed in ovule number or fate (compare Figure 5.5G and H; Figure 5.5I and J) per ovary, indicating that any visible abortion of fertilized ovules occurs beyond Stage IV.

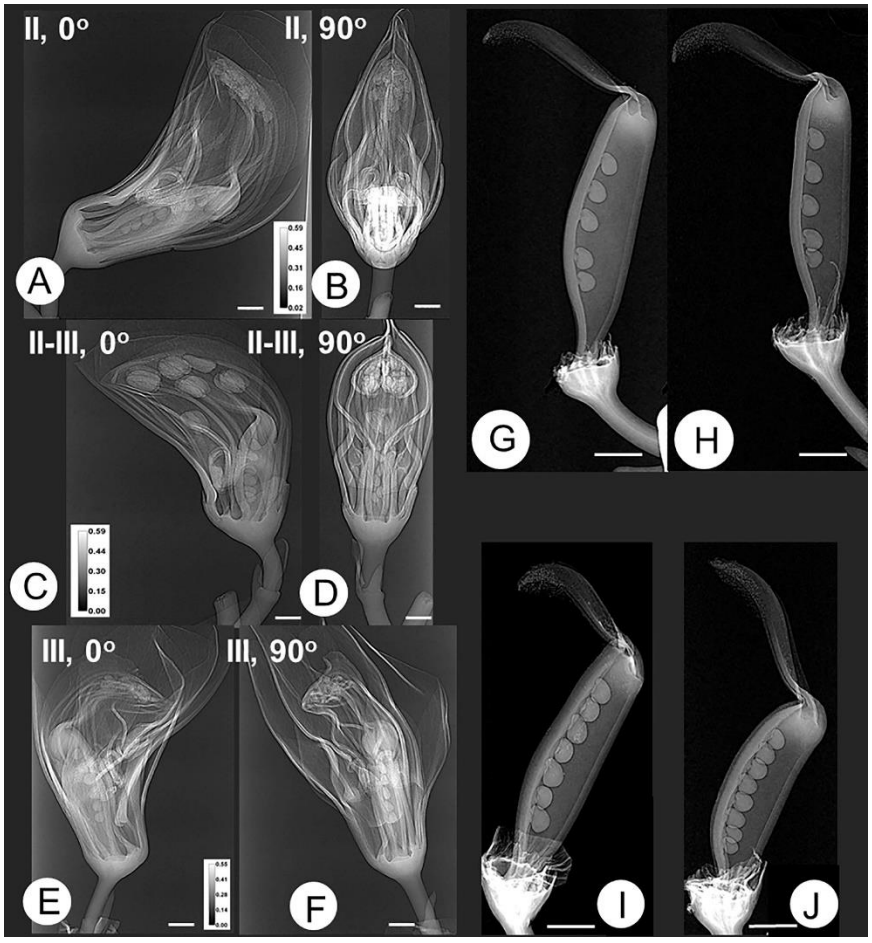

Figure 5.5. X-ray images of fresh floral tissues of CDC Golden (A-H) and CDC Sage (I, J). Images illustrate intact floral buds at Stage II (A, B), II-III (C, D), and III (E, F) from control (24/18°C) plants, and dissected pistils from Stage IV flowers (G-J) taken from controls (G, I) or plants exposed to elevated temperature (35/18°C) for 4 days (H, J). Paired images per bud stage were recorded at projection angles of 0° (A, C, E) and 90° (B, D, F). Scale bar = 1mm.

5.3.3 Exp. 3: Pollen-pistil interactions and the influence of elevated growth temperature

5.3.3.1 Pollen viability

Using the FDA test, pollen grains that fluoresced brightly were considered viable (Figures 5.6A, B). Effects of high temperature on pollen viability depended on cultivar and flowering stage (Table 5.4, Figure 5.6C). In CDC Golden, 4 days of elevated day temperature reduced the viability of pollen grains collected from flower buds (Stage III) by approximately 50% (Figure 5.6C).

Moreover, pollen grains from control flower buds of CDC Golden had a higher viability compared to pollen grains collected from flowers at the anthesis stage (Stage IV). However, no significant differences in viability were observed in pollen grains from the anthesis stage between the control temperature and the high temperature treatments. In contrast, at control temperature, viability of pollen grains of CDC Sage from flower buds (Stage III) and open flowers (Stage IV) were similar and exceeded 85% (Figure 5.6C). High day temperatures did not reduce pollen grain viability at the flower bud stage, whereas elevated day temperature significantly decreased pollen viability at anthesis in CDC Sage (Figure 5.6C). Therefore, elevated day temperature did not affect pollen grains of CDC Sage in flower buds of Stage III, compared to Stage III buds of CDC Golden (Figure 5.6C).

Table 5.4. Analysis of Variance (ANOVA) showing effects of cultivar, day temperature (24/18°C and 35/18°C day/night temperatures with the 16 h photoperiod for 4 days), and flowering stage (pre-anthesis - Stage III and anthesis - Stage IV) on pollen viability. Prior to analysis, the data for pollen viability were square transformed.

Source	F value	*P* value
Cultivar (C)	40.44	<0.001
Day temperature (DT)	76.13	<0.001
Flowering stage (FS)	24.57	<0.001
C*DT	0.41	0.527
G*FS	1.31	0.260
DT*FS	3.36	0.076
C*DT*FS	45.65	<0.001

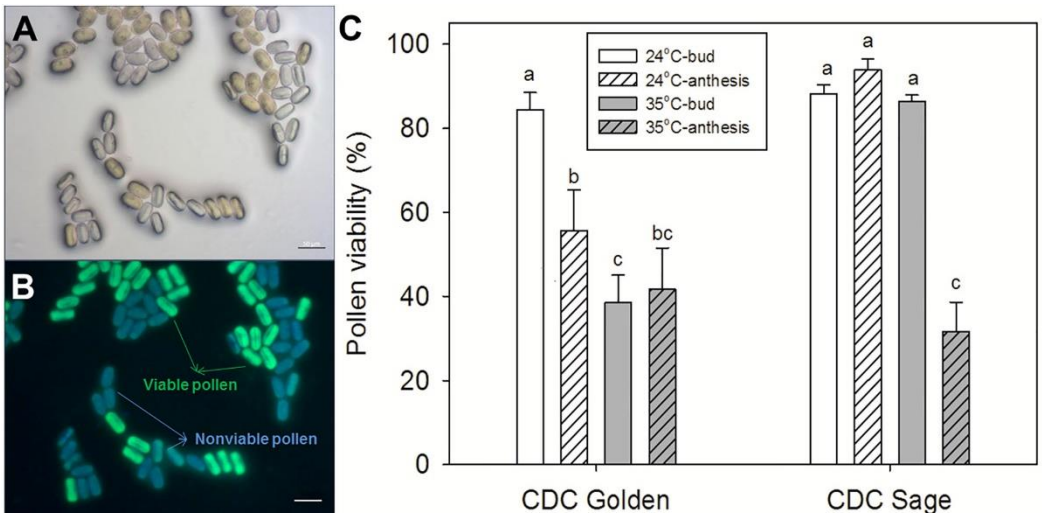

Figure 5.6. Viability of pollen grains collected from two flowering stages (flower bud – Stage III, and anthesis – Stage IV) in two cultivars (CDC Golden, CDC Sage) of *Pisum sativum* after exposure to 24/18°C and 35/18°C day/night temperatures for 4 days. A: Total number of pollen grains viewed under merged bright-field and fluorescence microscopy; B: Viable vs nonviable pollen grains stained with fluorescein diacetate before viewed with fluorescence microscopy; C: Each bar represents pollen grain viability (mean ± s.e.) of five replications (five flowers from five different plants for each treatment). Mean values followed by the same letter did not differ significantly at $P < 0.05$. Scale bar = 50µm.

5.3.3.2 *In vivo* pollen tubes in the style

Effects of day temperature (4 days at 35/18°C day/night vs 24/18°C) on *in vivo* pollen-tube growth in styles are shown in Figures 5.7A-E. Many hundreds of linear pollen tubes were detected in the transmitting tissue per style, based on fluorescence microscopic examination of callose (β-1,3 glucan) in pollen tubes, highlighted by aniline blue staining (Figures 5.7A-D). In general, the density of pollen tubes in CDC Sage (Figures 5.7C, D) was greater compared to CDC Golden (Figures 5.7A, B) for both control and high-temperature regimes. Elevated day temperature had a negligible effect on pollen-tube growth in styles of CDC Sage (Figures 5.7C, D). High day temperature (Figures 5.7B), however, reduced the number of pollen tubes in CDC Golden compared to the control plants (Figure 5.7A). These representative observations (Figures 5.7A-D) were based on at least seven replications; further images are available in Figures S5.1 (CDC Golden) and S5.2 (CDC Sage). Although numbers of pollen tubes per style were so plentiful to prevent accurate quantification, these qualitative, inter-cultivar differences yielded significant quantitative differences, in terms of areas of callose fluorescence (an indicator of pollen tube walls and plugs) in the anterior region (a length of 0.65 mm from the stigma end) of styles in CDC Sage being

significantly greater than those of CDC Golden (Figure 5.7E). However, elevated day temperature had no effect on callose areas in comparison to styles of control plants within each cultivar (Figure 5.7E).

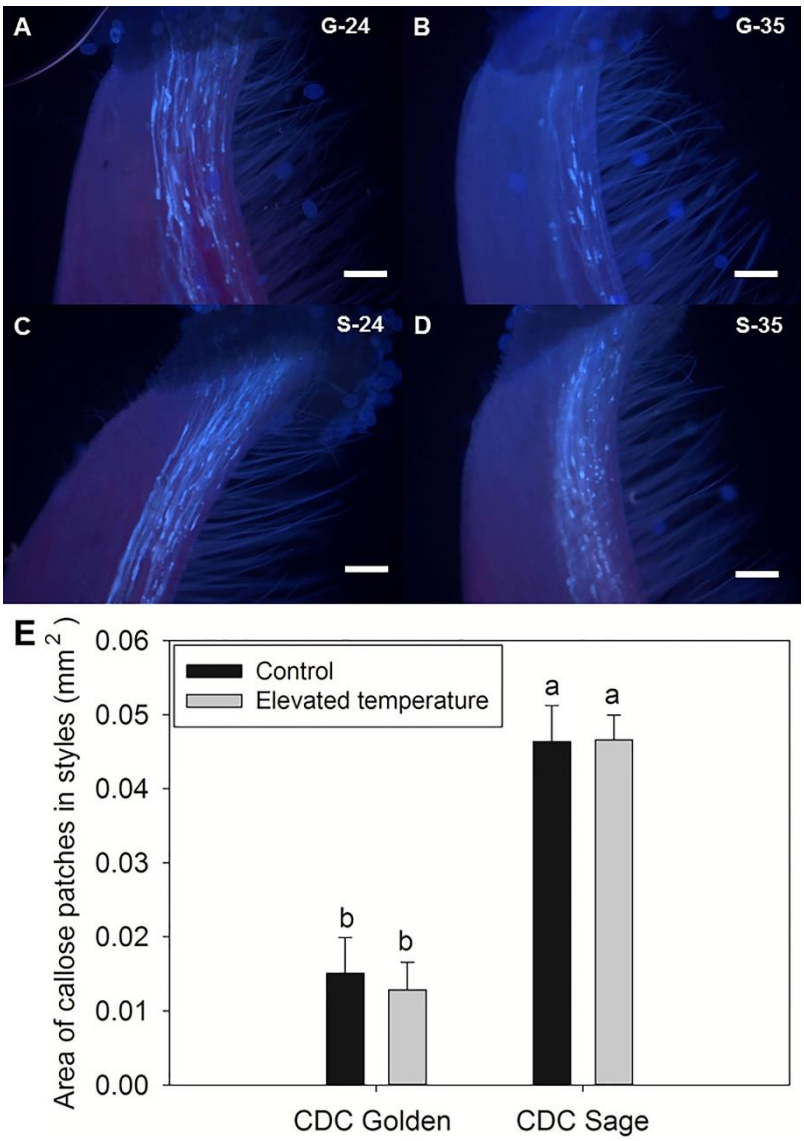

Figure 5.7. Pollen tubes in styles of *Pisum sativum* after staining for callose with aniline blue using fluorescence microscopy from representative pods examined at Stage V (young pod development) taken from control (24/18°C day/night temperatures for 4 days) and heat-stressed (35/18°C day/night temperatures for 4 days) plants. A: CDC Golden exposed to day temperature of 24°C; B: CDC Golden at 35°C; C: CDC Sage at 24°C; D: CDC Sage at 35°C; E: Area of callose fluorescence in styles quantified by ImageJ software. Each bar represents the mean ± s.e. from four replications per treatment. Means with a common letter did not differ significantly at $P < 0.05$. Scale bar = 100 µm.

5.3.3.3 Effects of day temperature on anther dehiscence and stigma receipt of pollen

Elevated day temperature negatively affected the pollen-pistil interaction in pea, the impact being more severe after 7 days compared to 4 days of heat exposure (Figures 5.8A-L). When exposed to 35/18°C day/night temperature for 7 days, there were no or few pollen grains retained on the stigma in both cultivars (Figures 5.8E, K), which may have reflected a failure in anther dehiscence (Figures 5.8F, L). After 4 days of heat exposure, however, large numbers of pollen grains were evident on the stigma surface of both cultivars (Figures 5.8C, I), with several having germinated pollen tubes (Figures 5.8D, J). Stigmas from control plants (24/18°C day/night for 7 days) of both cultivars exhibited a few hundred pollen grains each (Figures 5.8A, G), corresponding to the large number of bicellular grains (Figure 5.1K) per dehiscent anther (Figures 5.1J, 5.8B), which averaged 2,256±45 per mature, indehiscent anther of cultivar CDC Golden. A high proportion of these pollen grains germinated on the stigma (Figure 5.8H). Whereas Figure 5.8A-L were deemed representative of these SEM observations, further images are available in Figures S5.3 (anthers and stigmas of Stage IV flowers) and S5.4 (stigmas of young pods at Stage V), for both cultivars.

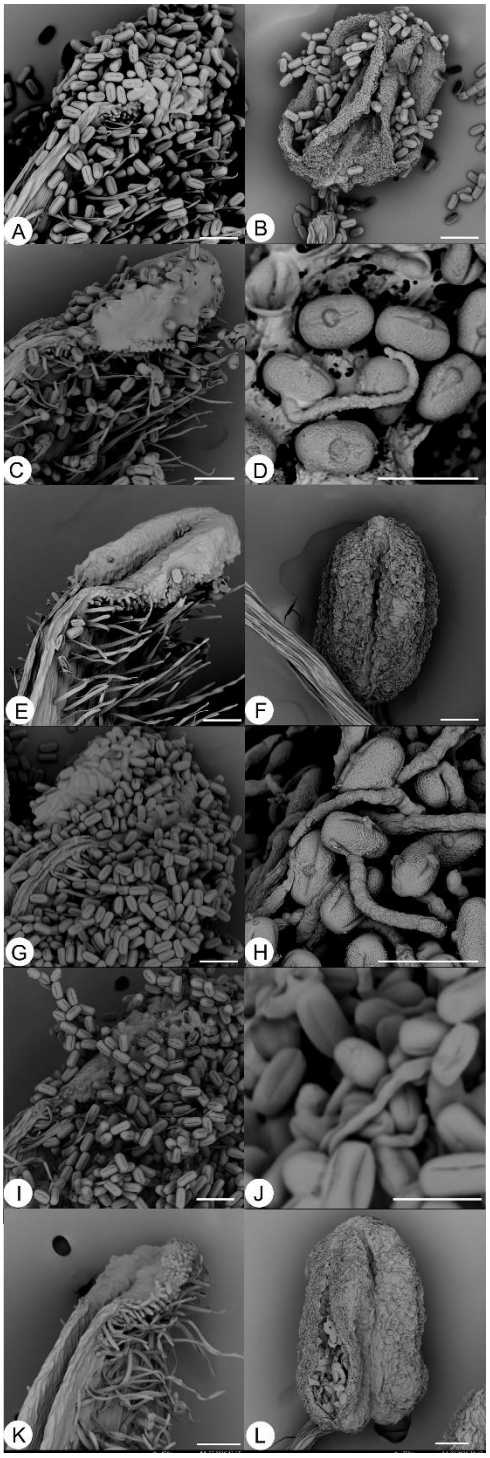

Figure 5.8. Representative scanning electron micrographs of stigmas (left column), anthers (right, B, F, L), and germinated pollen tubes on stigmas (right, D, H, J) of *Pisum sativum* cv. CDC Golden (A-F) and CDC Sage (G-L) from flowers of Stage IV (C, D, I, J), and V (A, B, E-H, K, L), following exposure of plants to day temperatures of 24°C for 7 days (A, B, G, H), 35°C for 4 days (C, D, I, J), or 35°C for 7 days (E, F, K, L). Scale bar = 100 µm (A-C, E-G, I, K-L), 50 µm (D, H, J).

5.3.3.4 Effects of day temperature on ovule fertilization

In pea flowers at post-anthesis (Stage V), when the banner (standard) and other petals begin withering (Figure 5.1M) and ovary elongation is perceived following ovule expansion, the proportion of ovules that had received a pollen tube after plants had been exposed to elevated day temperature for 4 days and 7 days was examined by fluorescence microscopy after staining for callose (Figures 5.9A-H). In the featured example, apparent failure of fertilization was observed at ovule position 5, near the basal end of the pod (Figure 5.9F). Ovule 5 size appears smaller than the other 5 fertilized ovules in that pod, including ovule 6 more basal to it (Figure 5.9A). Overall, the effect of heat stress on the proportion of ovules that had evidently been fertilized varied depending on the duration of heat exposure (Table 5.5). After 4 days of heat exposure, the proportion of ovules that received a pollen tube was similar to control plants (Figure 5.9H). However, in plants exposed to high temperature for 7 days, the proportion of ovules that received a pollen tube diminished significantly (<15%) compared to control plants (>80%; Figure 5.9H).

Table 5.5. Analysis of variance (ANOVA) showing effects of cultivar, day temperature (24/18°C and 35/18°C day/night temperatures with the 16 h photoperiod), and duration of heat exposure (4 and 7 days) on proportion of ovules in young pods (Stage V) that received a pollen tube.

Effect	F value	*P* value
Cultivar (C)	1.65	0.204
Day temperature (DT)	64.63	<0.001
Heat duration (HD)	107.68	<0.001
C*HD	0.20	0.655
C*DT	0.00	0.984
HD*DT	59.90	<0.001
C*HD*DT	1.18	0.281
Cultivar	Percentage of ovules that received a pollen tube	
CDC Golden	69.1 a†	
CDC Sage	74.7 a	
Day temperature	Percentage of ovules that received a pollen tube	
24°C	89.4 a	
35°C	54.4 b	
Heat duration	Percentage of ovules that received a pollen tube	
4 days	94.5 a†	
7 days	49.3 b	

†Means with a common letter under each section did not differ at $P < 0.05$.

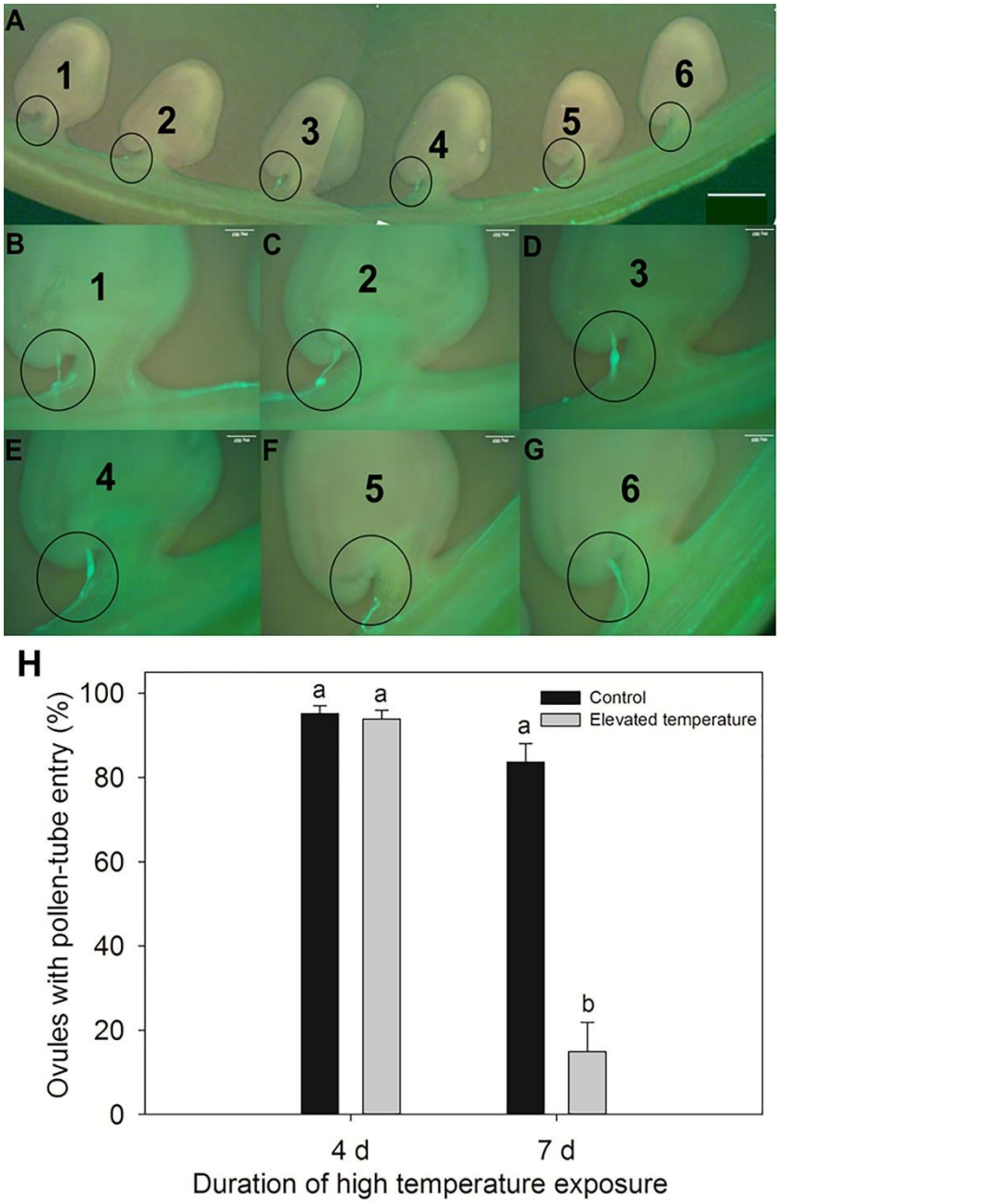

Figure 5.9. Effect of elevated day temperature and its duration on the proportion of ovules in *Pisum sativum* cv. CDC Golden and CDC Sage that received a pollen tube. A: Intact pod with stylar end to the left, showing ovules numbered 1-6. The pod was stained with aniline blue prior to examination by fluorescence microscopy. B-G: Individual ovules of A, photographed at their micropyle regions, demonstrating pollen tube entry (B-E, G) or in vicinity (F), following exposure of plants to elevated temperature for 4 days. H: Effect of temperature regimes (24/18°C vs 35/18°C day/night temperatures with the photoperiod of 16/8 hr) and heat exposure duration (4 and 7 days) on the percentage of *in situ* ovules to which a pollen tube had entered. Each bar represents the mean ± s.e. from eight replications per treatment. Means with a common letter did not differ significantly at $P < 0.05$. Scale bar at A = 500 µm; Scale bars = 100 µm (B-G).

5.3.3.5 Effects of day temperature on ovule size (growth)

Imaging (PCI) of ovules within intact gynoecia demonstrated consistent trends in ovule size between plants grown at control and elevated daytime temperatures for 4 days, even soon after anticipated fertilization in Stage IV flowers (Figures 5.5G-J). Thus, this potential acceleration in ovule growth due to elevated temperature, was investigated. Indeed, the main effect of day temperature and the interactive effect of cultivar and day temperature had significant effects on ovule growth in young pods (Stage V), resulting in ovule surface size of 0.35 mm^2 compared to 0.26 mm^2 at the control conditions (Table 5.6). Specifically, compared to the control temperature regime, exposure to elevated temperature for 4 days increased ovule surface size by 16% in CDC Golden, and by 51% in CDC Sage (Figure 5.10).

Table 5.6. Analysis of variance (ANOVA) showing effect of cultivar and day temperature (24/18°C and 35/18°C day/night with a 16 h photoperiod for 4 d) on ovule size in young pods (Stage V) of *Pisum sativum*.

Effect	F value	*P* value
Cultivar (C)	3.67	0.060
Day temperature (DT)	89.37	<0.001
C*DT	19.22	<0.001
Cultivar	Ovule size (mm^2)	
CDC Golden	0.30 a†	
CDC Sage	0.31 a	
Day temperature	Ovule size (mm^2)	
24°C	0.26 b	
35°C	0.35 a	

†Means with a common letter under each section did not differ significantly at $P < 0.05$.

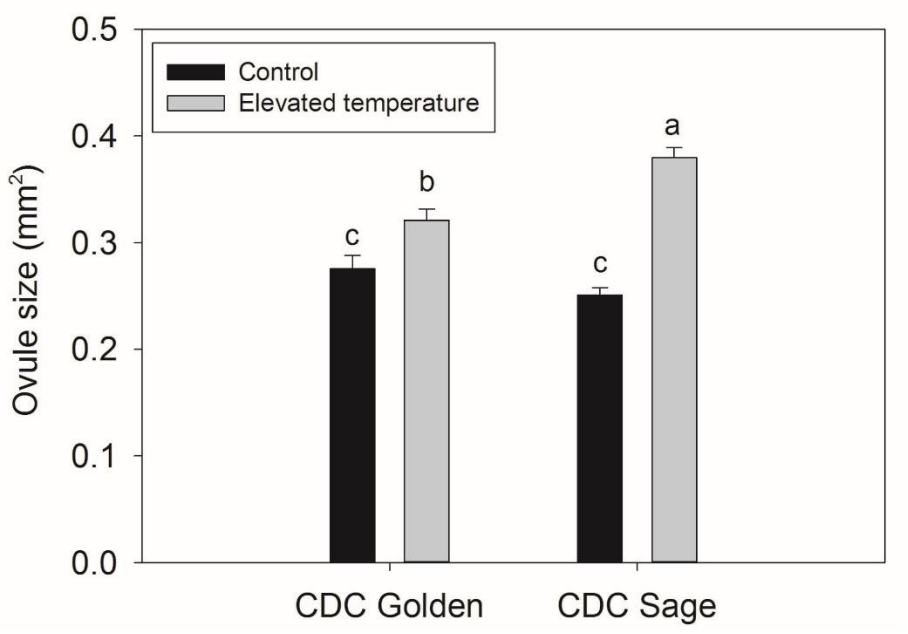

Figure 5.10. Effect of day temperature regime (24/18°C vs 35/18°C day/night with a 16 h photoperiod for 4 d) on ovule size in *Pisum sativum* cv. CDC Golden and CDC Sage. Each bar represents the mean ± s.e. from eight replications per treatment (one ovary as one replication). Means with a common letter did not differ significantly at $P < 0.05$.

5.4 Discussion

Results from this study suggest that a hierarchy of factors related to a flower's position influenced seed maturation in plants of field pea. For instance, node location of an inflorescence, a flower's position within an inflorescence, and even an ovule's location within an ovary, all contributed significantly to the quantity of mature seeds and fruits achieved. Availability of a plant's maternal resources may participate in all these events. For example, in control plants (24/18°C), the first-formed racemes (i.e., at the lowest reproductive nodes) outyielded those at node 5. In experimental plants that received elevated daytime temperature (35/18°C) for 7 consecutive days, the ratings of seed maturation were more uniform at nodes 1-5, although significantly lower than controls only for the first three racemes formed (Figure 5.4). However, at nodes 4 and 5, and particularly the latter, seed maturation in heat-stressed plants may have trended higher than in control plants as a compensatory mechanism for their significantly lower degree of seed maturation at nodes 1-3.

Similarly, even within the two-flowered racemes of field pea, subsequent seed-set and growth of young pods (i.e., distal flower) was significantly inhibited by the presence of older developing

pods (i.e., proximal flower). This positional effect was strong and not altered by daytime temperatures in this study. That flowering sequence within an inflorescence governs the probability of fruit set is well established for legumes such as *Glycine max* (McAlister and Krober, 1958), *Phaseolus vulgaris* (Tamas et al., 1979), *Vicia faba* (Peat, 1983), as well as many non-legume species (Addicott and Lynch, 1955; Wyatt, 1980), indicative that immature young fruits and new flowers compete for limited maternal resources (Stephenson, 1981; Bangerth, 1989; Marcelis et al., 2004). Competition for maternal resources perhaps caused the differences in seed maturity and abortion in distal and proximal pods at the same node in our study. The likelihood of ovule abortion in distal pods was higher compared to the proximal pod within an inflorescence, suggesting the proximal pod position was more privileged to maternal resources.

Furthermore, progeny originating at specific ovule positions within individual pods have variable probabilities of reaching maturity. For both cultivars (CDC Golden, CDC Sage), ovules located closer to the stylar end and medial positions were more likely to reach maturity compared to ovules at the basal (peduncle) end. However, a previous study of *P. sativum* cv. Alaska showed that ovules in the medial ovary position are more likely to develop into seeds, owing to pod (carpel) shape fostering spatial advantages in the medial position (Linck, 1961). However, ovules at a pod's stylar end were more likely to reach seed maturity compared to ovules at the pod base in *Anagyris foetida* (Valtueña et al., 2012), *Bauhinia ungulata* (Mena-Ali and Rocha, 2005a), *Poincianella pyramidalis* (Silveira and Fuzessy, 2015), and *Robinia pseudoacacia* (Susko, 2006), which is interpreted as a response to a fertilization gradient. Ovules located closer to the stylar end were more likely to be fertilized by more rapidly germinating male gametophytes and more vigorously growing pollen tubes (Rocha and Stephenson, 1991; Jakobsen and Martens, 1994; Gutiérrez et al., 1996; Susko, 2006). Silveria and Fuzessy (2015) ascribed differences in ovule fate within individual pods to varying pollination systems between species. In *Poincianella pyramidalis*, which is self-compatible and autogamous, pods set a high frequency of seeds at the stylar end. However, a higher probability of seed predation was observed at the pods' stylar ends of *Anandenanthera colunrina* that is a self-incompatible plant and pollinated by generalists and small insects, leading to a greater number of aborted ovules at the stylar ends (Silveria and Fuzessy, 2015).

By introducing a standardized scale of six stages (I-VI) and their intervals to represent flower phenology (i.e., extremely young buds to immature pods with senescing perianth) spanning about 6.5 days, it became possible to undertake this comprehensive focus on pollen-pistil interactions to elucidate the normal course of events, plus to identify floral stages particularly vulnerable to elevated day temperature (35°C), in pea. This focus began by determination of male elements of sexual reproduction, namely the pollen quantity per flower, and the influence of daytime temperature on the viability of pollen grains and their release from anthers. During these experiments, it became evident that cultivar (genotype) is an important factor determining the androecium's role in pea sexual reproduction.

For *P. sativum* cv. CDC Golden, pollen grains are bicellular at anther dehiscence, a trait common for Fabeceae (Brewbaker, 1967; Makasheva, 1984). Moreover, the quantity of pollen per flower was estimated at 22,560 for control plants grown at the 24/18°C regime. These plants produced 5.9 ovules per ovary, establishing a pollen-ovule (P-O) ratio of 3,823 for this cultivar, according with the average (3,312; range 142 – 21,660) for several members of Fabaceae (Goetzenberger et al., 2006). Cruden (1977) categorized such angiosperms with moderate P-O ratios (797±88) as facultatively xenogamous to xenogamous (5,859±937). Although *P. sativum* is self-compatible and autogamous, outcrossing may occur in hot, dry weather, possibly by immature thrips before anthesis (Makasheva, 1984), and the pea flower possesses a nectar-secreting floral nectary (Razem and Davis, 1999), which may attract bees as potential pollinators. Interestingly, this quantity of pollen per flower and the P-O ratio for CDC Golden greatly exceed those for *P. sativum* var. *hortense* (391 per anther) and var. *arvense* (446) detected for field-grown plants in India (Aney, 2014). The 95-fold increase in the P-O ratios (39.5, 43.5) reported for control plants by Aney (2014) are influenced slightly by the greater denominator (i.e., 9.0-11.3 ovules per ovary, compared to 5.9 for CDC Golden); however, this large disparity in P-O ratio between studies probably reflects differences in genotype and growth conditions on the pollen quantity per anther.

Our study suggests that seed maturity in field pea may be associated with pollen availability depending on the duration of exposure to high temperatures. When exposed to high daytime temperatures for a relatively short duration (35/18°C for 4 d), viability of pollen grains collected from the anthesis stage (Stage IV) decreased in both cultivars tested. However, exposure to high temperatures for 4 d at the pre-anthesis stage (Stage III) only reduced pollen viability in CDC

Golden, but not in CDC Sage. Moreover, pollen grains of CDC Sage retained a longer longevity compared to CDC Golden under control conditions.

The disparity in pollen viability between cultivars directly explains why more *in vivo* pollen tubes were observed in the styles in CDC Sage than CDC Golden. However, the number of pollen tubes in styles still exceeded ovule number within individual pods under both control and heat-stress conditions, although a reduction of pollen viability may have occurred after 4 d of exposure to high temperatures. To ensure successful reproduction, male organs should invest in quantity, while female organs should invest in quality (Richards, 1997). Heat stress is reported to not only impact the quantity of pollen load on stigmas, but also reduce pollen quality. Heat stress causes changes in pollen wall structure and pollen surface lipid and protein composition in field pea (Jiang et al., 2015; Lahlali et al., 2014). Heat stress reduced *in vitro* pollen germination percentage by approximately 55% and 30% in CDC Golden and CDC Sage, respectively, when temperatures increased from 24 to 36°C (Jiang et al., 2015). Since ovules at the basal end position of the ovary had a higher probability of seed abortion in our study (Figure 5.3, Table 5.3), pollen-tube growth and fertilization at the later stages could be a constraint in seed maturity in field pea. More research is needed specifically on heat stress effects on the process of pollen-tube growth in styles leading to double fertilization.

Successful seed development in field pea is negatively affected by pollen availability after a 7-d period of exposure to high daytime temperature (35/18°C). When exposed to high temperatures for one entire week, anther dehiscence (which normally occurs during Stage III) did not occur in the majority of anthers in both cultivars examined, leading to a significant reduction of pollen load on the stigma and in the percentage of *in situ* ovules penetrated by a pollen tube. Callose deposition and lignification of hypostase cell walls were observed in aborted ovules in *P. sativum* (Briggs et al., 1987). Therefore, unsuccessful double fertilization and seed development are directly associated with the reduced amount of pollen tubes that entered ovules after 7 d of heat exposure in field pea. Similarly, anther indehiscence caused by exposure to high temperatures (33/30°C day/night) for 2 d was observed in cowpea (Warrag and Hall, 1984a; Ahmed et al., 1992) and common bean (Porch and Jahn, 2001). By tracking back in pea flower phenology for the Stage IV-V flowers examined, the lack of impact on anther dehiscence following 4 d (96 h) of daytime exposure to 35°C suggests that floral buds already attaining Stage I at the onset of this heat

treatment (Figure 5.2), are spared. However, floral buds younger than Stage I – the smallest buds discernible and tracked in this study – at the commencement of 7 d (168 h) of daytime exposure to 35°C, are clearly impacted for their anther dehiscence. Consequently, studies to ascertain the physiological and structural mechanisms that regulate anther dehiscence in pea, are warranted.

Our results demonstrated that elevated temperatures also negatively impacted post-fertilization events of the reproductive process in field pea. Ovule abortion was accelerated at the stylar end and medial position within individual pods, supporting our hypothesis. Heat stress led to acceleration in embryo abortion in cowpea (Warrag and Hall, 1984a) and common bean (Gross and Kigel, 1994). Exposure to high temperatures during seed filling can accelerate senescence of seeds, decrease seed set and seed weight, and lead to yield loss in cool season grain legumes due to the fact that plants tend to divert energy and resources such as photosynthates to deal with heat stress (Siddique et al., 1999). High temperatures have been reported to cause fruit abscission, but whether high temperatures abort seeds directly or indirectly remains unclear (Addicott and Lynch, 1955). Seed abortion may be caused by the expression of lethal or deleterious alleles (Yuan et al., 2014). Further, high seed abortion rates perhaps result from high levels of homozygosity due to inbreeding, as seen in *Epilobium angustifolium* (Wiens et al., 1987).

Heat stress accelerated the growth of pea ovules in both cultivars tested following exposure to 35/18°C day/night temperatures for 4 d, because ovule size of heat-exposed plants was greater compared to ovules from control plants. Although I am not aware of any previous reports regarding the acceleration of ovule growth shortly after fertilization in the response to heat stress in field pea, high night temperatures during flowering has been reported to increase the rate of flower production per plant in cotton (Echer et al., 2014). Similarly, a shorter life cycle caused by faster development has been observed in non-perennial crops under high temperature stress (Hatfield and Prueger, 2015). In addition, a reduction in final grain size was also observed in wheat under heat stress (Calderini et al., 1999a, b; Ugarte et al., 2007).

Synchrotron-based PCI did not show any observable differences in the flowers at different developmental stages between CDC Golden and CDC Sage. With the detector resolution and the X-ray energy used in this study, it was difficult to visualize pollen-tube growth through the stigma and entrance of pollen tubes to the ovules in pollinated flowers. On the other hand, using PCI, we could document seed abortion due to heat stress during development and to study differences

between cultivars by tracking the same pea pod non-destructively during events following pollination leading to seed set within a pod. Such non-destructive tracking of ovules is not possible with traditional electron microscopy of fixed material, the alternative technique for obtaining high magnification and resolution.

5.5 Conclusions

Successful seed development was negatively impacted by pollen availability depending on the duration of heat exposure. Anther dehiscence did not occur to the majority of anthers and the number of ovules that received a pollen tube was significantly reduced when exposed to high daytime temperatures for 7 days. The likelihood of seed maturation versus abortion was dependent on ovule position within the multi-seeded fruits in field pea. Ovules at the pod's medial and stylar-end positions were likely to become mature seeds compared to ovules at the pod's base, indicative of a combined effect of spatial advantages and fertilization gradients. We speculate that the pods generated first, in lower racemes at reproductive nodes or even the proximal pod position out of the two in a nodal raceme, established a dominance over maternal resources. Thus, ovules within these pods have a higher probability of maturing into seeds compared to inflorescences at nodes developing later along the elongating stem of field pea plants.

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Contributions by others to this thesis chapter

The majority of this study (floral development, the effect of ovule position on seed development, and pollen-pistil interactions) was conducted by Yunfei Jiang. The x-ray imaging work was co-performed by Dr. Rachid Lahlali (Canadian Light Source Inc. scientist), Dr. Chithra Karunakaran (Canadian Light Source Inc. scientist), and Yunfei Jiang at Canadian Light Source Inc.

Supplementary materials

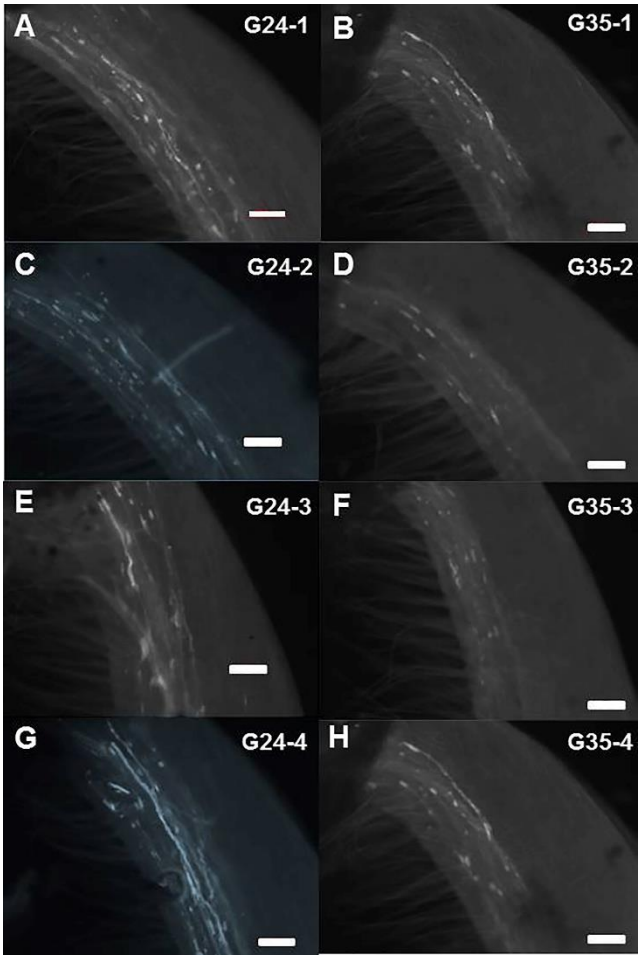

Figure S5.1. Squashed-style preparations examined by fluorescence microscopy to reveal callose of pollen tubes evident in the styles of *Pisum sativum* var. CDC Golden stained with aniline blue, in pods of Stage IV harvested from control (24/18°C day/night temperatures for 4 days) and heat-stressed (35/18 °C day/night temperatures for 4 days) plants. G-24 represents 24°C; G-35 represents 35°C. The numbers 1-4 represent four different replications. Scale bars = 100 µm.

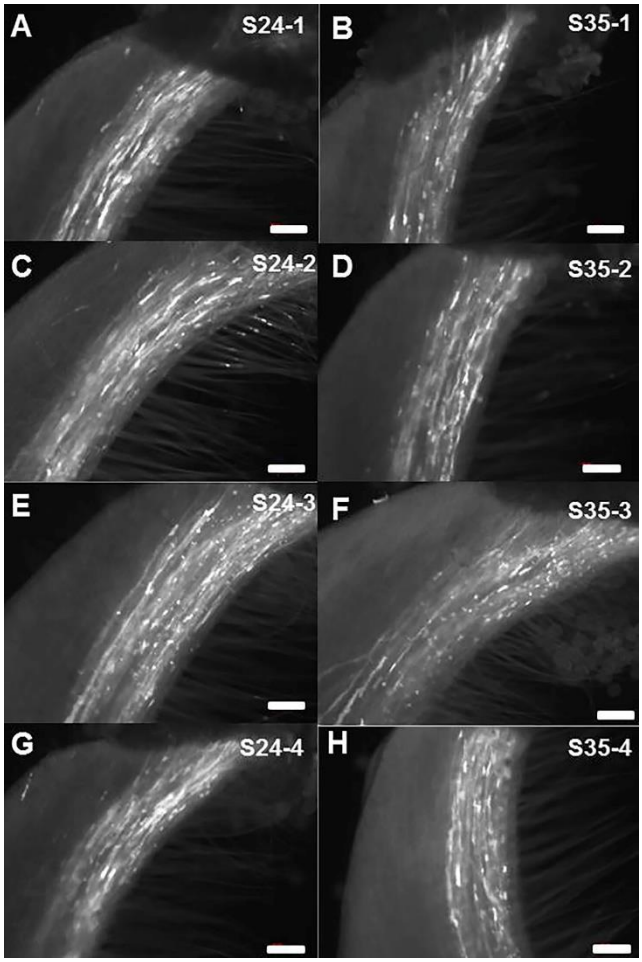

Figure S5.2. Squashed-style preparations examined by fluorescence microscopy to reveal callose of pollen tubes evident in the styles of *Pisum sativum* var. CDC Sage stained with aniline blue, in pods of Stage IV harvested from control (24/18°C day/night temperatures for 4 days) and heat-stressed (35/18 °C day/night temperatures for 4 days) plants. S-24 represents 24°C; S-35 represents 35°C. The numbers 1-4 represent four different replications. Scale bars = 100 µm.

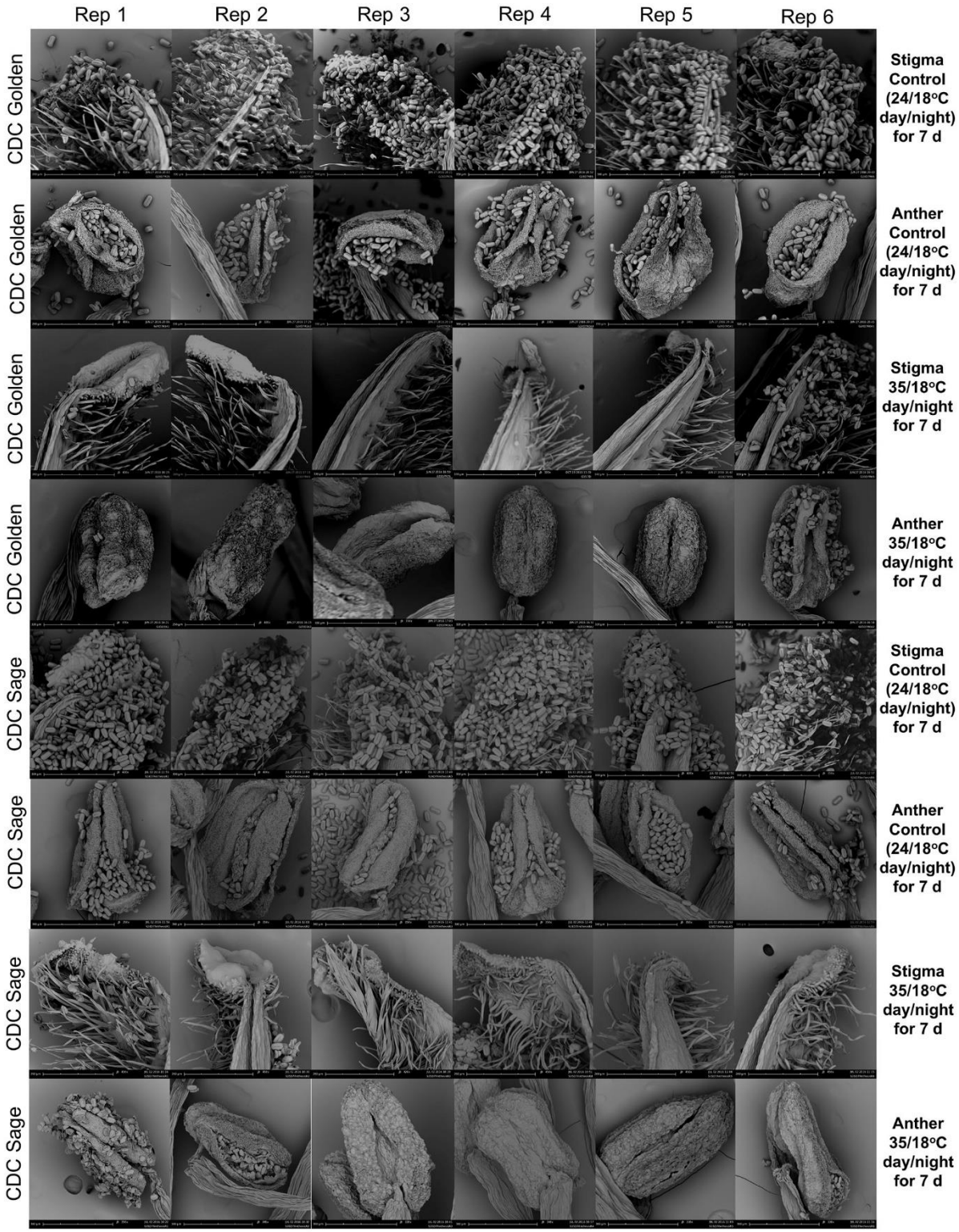

Figure S5.3. Scanning electron micrographs of stigmas and anthers sampled from Stage IV flowers from pea plants exposed to control (24/18°C day/night temperatures with the photoperiod of 16/8 h) and elevated day temperature (35/18°C day/night temperatures with the photoperiod of 16/8 h) for 7 days. Six replications are illustrated for each cultivar (CDC Golden, CDC Sage).

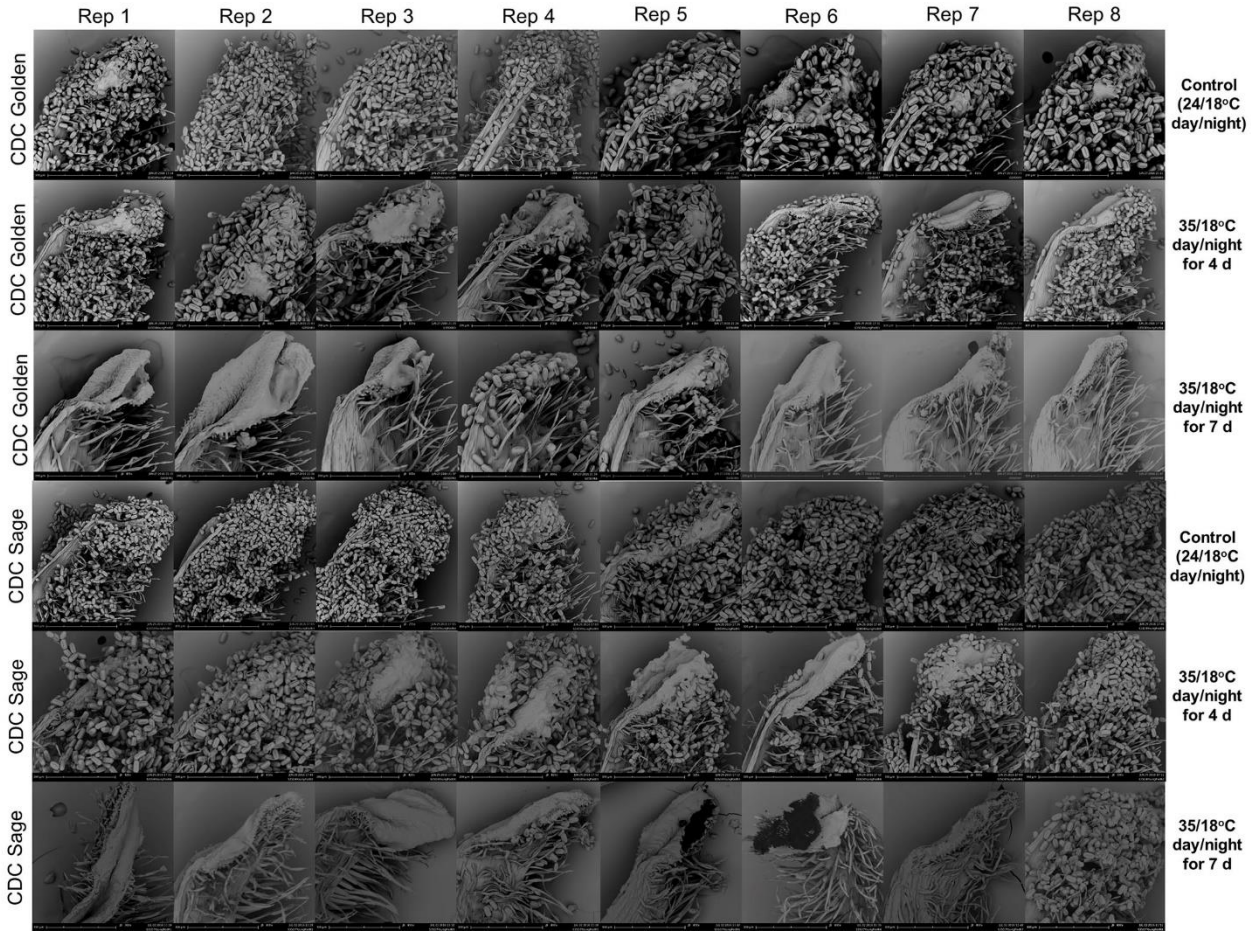

Figure S5.4. Scanning electron micrographs of stigmas of *Pisum sativum* recorded from young pods (Stage V) sampled from control (24/18°C day/night temperatures with the photoperiod of 16/8 h) and elevated day temperature (35/18°C day/night temperatures with the photoperiod of 16/8 h) plants of cv. CDC Golden and CDC Sage after 4 and 7 days. Eight replications are shown per treatment.

Transition section between Chapter 5 and Chapter 6

In Chapter 5, the relationship between the pollen – pistil interaction and seed development was explored. I concluded that successful seed development is dependent on pollen availability (i.e. anther dehiscence and pollen tubes reaching ovules) and the positions of ovule, pod, and node in two pea cultivars, CDC Golden and CDC Sage. In the two cultivars tested, I observed that ovules at the basal end within a pod are most likely to abort. I would like to evaluate more pea cultivars to confirm the patterns of seed development in relation to ovule positions. In Chapter 6, the effects of ovule, pod, and node positions were tested in 16 pea cultivars grown at two different environments - growth chambers and field.

Chapter 6 Size, position and hierarchy - improving seed retention in heat stress for field pea

6.1 Introduction

Global warming has detrimental impacts on some crops, whereas it can enhance new cropping opportunities in others. Many cool-season legume crops suffer yield loss from various environmental stresses, including temperature. Negative effects of elevated temperature on reproductive development have been reported in legumes such as chickpea (*Cicer arietinum*; Devasirvatham et al., 2013; Kaushal et al., 2013), field pea (*Pisum sativum*; Jiang et al., 2015), and lentil (*Lens culinaris*; Bhandari et al., 2016). Field pea is particularly sensitive to several days of elevated air temperature in the field, with reports of flower, fruit and seed abortion and reduced seed size (Lambert and Linck, 1958; Guilioni et al., 1997; Bueckert et al., 2015). To maintain or improve yield performance in a warming climate, new cultivars need to 1) be resistant to several more degrees or days of heat exposure, 2) produce more reproductive nodes, and 3) abort fewer pods and seeds per pod. This means that we need to both understand the physiological processes associated with reproductive development and use major findings to screen for more robust retention of reproductive organs.

Most studies related to the effects of heat stress on plant growth focus on pollen, the male or microgametophyte, because pollen development has been reported as more sensitive to elevated temperature compared to the ovules, the female or megagametophytes in crops such as chickpea (Devasirvatham et al., 2013), barley (Sakata and Higashitani, 2008), and bean (Monterroso and Wien, 1990). In reality, both male and female reproductive organs are exposed to heat stress simultaneously, and both male and female flower organs are vulnerable to high temperature stress before pollination and during the post-pollination stage (Hedhly, 2011). For example, Saini and Aspinall (1982) observed that heat stress led to both male sterility and damaged female sexual function in wheat (*Triticum aestivum*).

Seed and fruit set consists of sequenced processes such as dehiscence of pollen, pollen hydration and germination on the stigmatic surface, pollen-tube growth into the stigma and style, fertilization of ovules within an ovary, and seed initiation and ovary swelling (Kinet and Peet, 1997). Even after successful pollination, many additional processes can interrupt successful seed set. Successful

fertilization requires precise navigation by the pollen tubes to find the ovules, as well as communication between cells and within cellular processes (Zinn et al., 2010).

The ovules are susceptible to heat stress (Kaushal et al., 2016). Heat stress causes a reduction in ovule viability, stigma receptivity, and pollen grains retained on the stigma surface in various crops (Kaushal et al., 2016). Heat stress has negatively impacted ovary development in wheat, leading to yield loss (Saini et al., 1983). In that crop, one third of ovaries heat-stressed prior to anthesis exhibited an abnormal nucellus or embryo sac (Saini et al., 1983). Some of these abnormal ovaries contained small embryo sacs without complete cellular organization or lacking recognizable cells; the development of the nucellus was defective in some of the ovaries (Saini et al., 1983). In addition, heat stress (32/26°C day/night temperature) caused abnormal ovary development in rapeseed (*Brassica napus*), resulting in non-uniform ovule growth along the length of the ovaries of both pollinated and unpollinated flowers (Polowick and Sawhney, 1988). Heat stress also decreased ovule number and viability in common bean (*Phaseolus vulgaris*) and *Arabidopsis thaliana* (Whittle et al., 2009).

Fertilized embryos, not just the unfertilized ovules, are also susceptible to abortion. In *Arabidopsis*, approximately 50% reduction in seed set was caused by either early zygotic embryo lethality or ovule abortion under high temperature (Zinn et al., 2010). Fertilized ovaries reduced their rates of swelling in tomato (*Solanum lycopersicum*) due to high temperature (Kinet and Peet, 1997). Heat stress reduces the genetically predetermined seed size by limiting cell division, and later by limiting the duration of seed filling or rate of seed filling, leading to yield loss (Boote et al., 2005; Kaushal et al., 2016). The upper limit of seed size is also affected by pre-pollination growth of maternal tissue (Sadras and Denson, 2009; Yang et al., 2009). For example, kernel dry weight in wheat was positively correlated with ovary volume at anthesis (Yang et al., 2009). Heat stress modifies source-sink relations, reduces the kernel filling stage, and produces unfilled or aborted seeds in various crops (Kaushal et al., 2016).

Tropical legume trees have been used as ecological and botanical models for plant fecundity. These trees have long linear seed pods with many seeds, the pods are easy to measure, and the patterns in ovule and seed development are more obvious. Plant fecundity is reduced by combinations of pollen limitations, resource limitations, and herbivory pressure (Jacobi et al., 2000). Ovule development is dependent on ovule position within pods in *Poincianella pyramidalis*

(catingueira, used for firewood) and *Anandenanthera colubrina* (a mimosa-like tree used for its bark; Silveira and Fuzessy, 2015). Seed survival is also affected by the ovule position within pods of the small tree, *Bauhinia ungulata* (Fabaceae; Mena-Ali and Rocha, 2005a, b). Ovules situated at stylar ends of pods are more likely to produce seed that reach maturity compared to basal (peduncle) ends in *Poincianella pyramidalis* (Silveira and Fuzessy, 2015) and *Bauhinia ungulata* (Mena-Ali and Rocha, 2005b). Ovules situated at stylar ends are more likely to be fertilized by fast-growing pollen tubes, and therefore are more capable of competing for maternal resources compared to ovules located at the basal ends (Mena-Ali and Rocha, 2005b). In contrast, seed predation occurs in the pods's stylar ends in *Anandenanthera colubrina* (Silveira and Fuzessy, 2015). Pollination systems also differ between species and is a major factor determining the different patterns of ovule development (Silveira and Fuzessy, 2015).

Little is known about the effects of heat stress on female reproductive organs in field pea. Studies have yet to be documented on how any synergistic effects of ovule position within pods can be used to reduce seed yield losses in legume grain crops. We hypothesized that abortions of flowers and pods were dependent on node position and age of a plant. Younger (small) flower buds would be more sensitive to stress than older pods (ovaries) that had already had their ovules fertilized prior to stress. Ovule and seed development within a pod would be a measure of ovule hierarchy, and dependent on the ovule position within a pod. Basal ovules within a pod may produce larger seed because ovules and their resulting seeds are closest to maternal assimilate supply. Or conversely, basal ovules could be smaller on average if these ovule positions fail due to lack of pollination; pollen tubes would have further to travel to fertilize ovules at basal positions. In addition, there may be some genotypic variation in nodal, pod and ovule position hierarchy in heat stress that can be discovered and exploited for developing future pea cultivars with greater pod and seed retention following exposure to high temperatures. Therefore, our objectives were to (1) measure genotypic variation in pod and seed retention at four node positions in pea; (2) measure genotypic seed retention by ovule position within a pod; and (3) screen heat-resistant genotypes from 16 cultivars that could maintain seed number and seed size following exposure to high temperatures in both growth chambers and in the field.

6.2 Materials and methods

6.2.1 Exp. 1: Growth chamber experiment

Sixteen pea cultivars, namely '40-10', 'Agassiz', 'Carneval', 'CDC Bronco', 'CDC Centennial', 'CDC Golden', 'CDC Meadow', 'CDC Mozart', 'CDC Patrick', 'CDC Sage', 'CDC Treasure', 'Cutlass', 'MFR043', 'Naparnyk', 'Nitouche', and 'TMP15213', were tested in a growth chamber experiment. These cultivars are adapted for growth in western Canada, where CDC Meadow is currently the most widely produced cultivar in commercial production in this region. Thousand seed weight (TSW) or seed size, leaf types, flower colors, cotyledon colors, and origins of these cultivars are listed in Table 6.1. The study was arranged as a randomized complete block design with three replications. Ninety-six pots (16 cultivars × two temperature treatments × three replications) of 3.8 L volume (three plants per pot) were seeded with Sunshine Gro® mix (Seba Beach, AB, Canada) and slow-release fertilizer (approximately 20 g pot^{-1}; 14-14-14, Type 100, Nutricote®, Brampton, ON, Canada). The pot dimensions were 15.9 cm depth and 16.5 cm diameter. Plants were thinned to two seedlings per pot about two weeks after seeding. Pots received a first application (500 mL per pot) of half strength modified Hoagland's culture solution (Hoagland and Arnon, 1938) at three weeks after seeding and a second application (500 mL per pot) approximately five weeks after seeding, which marked the flower initiation stage. Moisture content of the soil medium was checked daily and plants were watered every 1-2d to avoid drought stress.

Plants were grown at 24/18°C day/night temperature with a 16 h photoperiod in each 24 h cycle at irradiance of 450-500 µmol photons m^{-2} s^{-1} from cool fluorescent tubes in a growth chamber, and the relative humidity had a range of 4-8%. Plants were then transferred to a warm temperature regime (35/18°C day/night temperature) for 7 days when the flowers at the second reproductive node of the main stem had their petals fully open (anther dehiscence occurs in large flower buds the day before flowers are fully open; Makasheva, 1984). Usually commercial pea cultivars have two flowers on a raceme at each reproductive node; the proximal flower is about one day older than the second, distal flower. Conditions in the high temperature growth chamber were 16 h light with temperature increasing from 18°C by 3°C increments to 35°C over 5 h, maintained at 35°C for 6 h, and decreased over 5 h to 18°C in the dark, for 7 consecutive days. The control plants remained in the 24/18°C growth chamber.

Table 6.1. Description of thousand seed weight (TSW), leaf type, flower color, cotyledon color, and origin of 16 pea cultivars tested. TSW was calculated by multiplying the average of single seed weight at the first four reproductive nodes by 1000.

Genotype	TSW (g)	Leaf type	Flower color	Cotyledon color	Origin
40-10	176	normal leaf	purple	yellow	Germany
Agassiz	306	semi-leafless	white	yellow	AAFC†, Canada
Carneval	256	semi-leafless	white	yellow	Sweden
CDC Bronco	251	semi-leafless	white	yellow	CDC‡, Canada
CDC Centennial	285	semi-leafless	white	yellow	CDC, Canada
CDC Golden	197	semi-leafless	white	yellow	CDC, Canada
CDC Meadow	220	semi-leafless	white	yellow	CDC, Canada
CDC Mozart	252	semi-leafless	white	yellow	CDC, Canada
CDC Patrick	208	semi-leafless	white	green	CDC, Canada
CDC Sage	224	semi-leafless	white	green	CDC, Canada
CDC Treasure	226	semi-leafless	white	yellow	CDC, Canada
Cutlass	231	semi-leafless	white	yellow	CDC, Canada
MFR043	334	normal leaf	white	green	CDC, Canada
Naparnyk	251	normal leaf	white	yellow	eastern Europe
Nitouche	267	semi-leafless	white	green	Denmark
TMP 15213	272	normal leaf	red	yellow	eastern Europe

†AAFC, Agriculture and Agri-Food Canada;
‡CDC, Crop Development Centre, University of Saskatchewan

Pods at each of the first four reproductive nodes on the main stem from all 96 plants (16 cultivars × two temperature regimes × three replications) were opened at physiological maturity and the condition of each ovule or seed within a pod was measured. The position and developmental stage of each ovule were recorded, starting from the stigmatic end (first position) to the basal end (last position) within the pods. The developmental stages of ovules were classified into four groups based on Hossaert and Valero (1988) with minor modifications: 1) a "successfully developed seed" with a diameter > 5 mm was rated as 1.5; 2) an "aborted seed" represented a fertilized ovule that never reached maturity with a diameter between 2 – 5 mm was rated as 1.0; 3) an "aborted ovule" was a vestigial funiculus that failed to develop into a seed, whether the ovule had been fertilized or went unfertilized, with a diameter < 2 mm rated as 0.5; 4) ovules within aborted pods, and aborted pods were all assigned a rating of zero. A total of 768 pods (16 cultivars × two temperature regimes × four fruiting nodes × two pod positions at each node × three replications) were examined from the main stems of these experimental plants.

Additionally, total seed weight on the main stem, duration of flowering, TSW, and the number of reproductive nodes, flowers, pods, seeds, and ovules on the main stem were measured. Pod length, the number of seeds per pod, the number of ovules per pod, seed-to-ovule ratio within a pod, and seed weight per pod were also measured based on the first four reproductive nodes. Pod retention was calculated by dividing the number of pods by the number of flowers. Seed retention was calculated by dividing the total number of seeds on the main stem by the total number of ovules on the main stem. The seed-to-ovule ratio was calculated by dividing the number of seeds per pod by the total number of ovules per pod across the first four reproductive nodes. TSW was calculated by multiplying the average of single seed weight on the main stem by 1000.

6.2.2 Exp. 2: Field experiment

The same 16 pea cultivars as the above growth chamber experiment were identified from a field trial with a panel of 94 diverse pea varieties grown at Rosthern, Saskatchewan (lat. 52˚40'N, long. 106˚20'W; black soil zone) during the growing season of 2015. The experiment was a randomized completed block design with three blocks. Seventy-five seeds of each cultivar were planted in a 1 m ×1 m microplot with 4 rows and 0.25 m spacing between rows. The seeding date was May 13 in 2015, and plants were harvested at the end of August, 2015. The monthly mean temperatures were 9.4, 16.4, 18.8, and 16.9°C in May, June, July, and August, respectively. The average maximum temperatures were 17.8, 24.1, 25.8, and 23.8°C in May, June, July, and August, respectively. The number of days when the maximum temperatures exceeded 28°C was 28 days during the growing season in 2015. The total precipitation was 10.9, 37.4, 80.5, 75.9, and 204.7 mm for May, June, July, August, and the period from May to August, respectively.

Two subsamples (one subsample = one plant) from each plot of the 16 cultivars were collected randomly at physiological maturity. Pods from these plants were opened, and the ovules/seeds within pods for each of the first four nodes were measured similarly to the growth chamber study. Only pods present at maturity were measured during this experiment, with three ovule/seed rating scales - 1) a "successfully developed seed", which was rated as 1.5; 2) an "aborted seed", which was rated as 1.0; 3) an "aborted ovule", which was rated as 0.5. Missing pods at maturity were treated in two different ways: 1) as missing data; 2) as aborted pods, and were assigned the value 0.

6.2.3 Data analysis

To determine ovule position within pods, ovule position per pod was categorized into three parts: (1) stylar end; (2) medial position; (3) basal end. The number of ovules per pod ranged from 3 to 9 among all pods collected from the cultivars tested. For the total number of ovules indivisible by three (4, 5, 7, and 8), the grouping of ovule positions were determined as follows: 1) if the total ovule number was 4, ovule 1 was located at the stylar end, and the medial position included ovules 2 and 3, and the basal end included ovule 4; 2) if the total ovule number was 5, ovules 1 and 2 were located at the stylar end, and the medial position included ovules 3 and 4, and the basal end included ovule 5; 3) if the total ovule number was 7, ovules 1 and 2 were located at the stylar end, and the medial position included ovules 3, 4 and 5, and the basal end included ovules 6 and 7; 4) if the total ovule number was 8, ovules 1 and 2 were located at the stylar end, and the medial position included ovules 3, 4, 5 and 6, and the basal end included ovules 7 and 8.

Analysis of variance (ANOVA) with a multi-nested design was used to analyze effects of cultivar, temperature, node position, and pod position within specific nodes on seed maturity and seed abortion at a specific ovule position (stylar end, medial position, or basal end) using the generalized linear model (GLM) procedure of SAS statistical software (version 9.3, SAS Institute, Inc., Cary, NC). The experiment was arranged as a split-plot design with fixed factors. The main plot factors were cultivar and node position in factorial combination; and the sub-plot factors included temperature, pod position, temperature and pod position interaction, and interactions with the main plot factors. For Exp. 1, analysis of growth chamber data included ratings of ovules and pods that were aborted on the plant as zero. For Exp. 2, field data were analyzed in two different ways: 1) missing pods were considered as aborted pods, assigned 0; 2) only pods present on plants, not accounting for missing pods.

For Exp. 1 and Exp. 2, ANOVA was then used to analyze the effects of ovule position (stylar end, medial position, and basal end) on ovule fate patterns within individual pods using the mixed procedure of SAS statistical software. The effects of cultivar, temperature regime, and ovule position (stylar, medial, and basal) were considered as fixed effects, whereas node position, pod position, and replication were all considered as random effects. Results from the first step of data analysis (preceding paragraph) showed that ovule positon effects on seed development with the treatments of node position, and pod position nested in node position were consistent, so the data from node and pod positions were combined.

Simple linear regression analysis was conducted to test the relationship between TSW and the rating scale of seed development in Exp. 1. Correlation coefficients among total seed weight on the main stem, duration of flowering, TSW, and the number of reproductive nodes, flowers, pods, seeds, and ovules on the main stem were analyzed for the control and heat-stressed treatments separately. The averages of the above seed-set parameters were calculated across 3 replications for the control and heat-stressed treatments.

6.3 Results

6.3.1 Exp. 1: Seed-set parameters in the growth chamber experiment

Seed development at the three specific ovule positions (stylar, medial, basal) within pods was significantly affected by cultivar, temperature regime, node position, and pod position (Table 6.2). High temperature (35/18°C for 7 days with the 16/8 h photoperiod) increased seed abortion of ovules located at all three positions (Table 6.3). Node hierarchy also influenced seed development. Ovules at all three ovule positions in pods on the first (oldest) reproductive node were most likely to develop into successful seeds compared to the younger reproductive nodes higher on the plant, such as the fourth (youngest) reproductive node (Table 6.3). Of the two pods (proximal and distal) at each reproductive node on plants in the growth chambers, the stylar ovule position was not affected by pod position per node, but medial and basal ovule positions were (Table 6.3). Medial and basal (peduncle) end ovule positions in the distal (young) pods were more likely to abort compared to those positions in proximal (older) pods at the same node (Table 6.3). This means that the proximal pods benefitted more from limited maternal resources than distal pods, and within pods seed abortion or limited seed development was evident in the medial and basal positions. In contrast, stylar end seed development was similar in proximal and distal pods at each node.

Among the 16 cultivars tested in the growth chambers, when the seed scale was averaged over all three ovule positions within a pod, those cultivars most likely to develop mature seeds were 40-10 and Naparnyk (Figure 6.1A, B). In contrast, cultivars most likely to abort ovules were Nitouche, CDC Treasure, Carneval, and especially MFR043 (Figure 6.1A, B).

Table 6.2. Probabilities from analysis of variance (ANOVA) showing effects of cultivar, temperature, node position, and pod position on seed development according to their ovule position (stylar, medial, basal) within pods at physiological maturity in 16 field pea cultivars in growth chambers with two temperature regimes (24/18 and 35/18°C day/night temperatures during the reproductive development for 7 days) and under field conditions (Rosthern, SK 2015). All 3-way and 4-way interaction terms were not significant.

Effect	Growth chamber						Field (only pods present at maturity)						Field (missing pods considered as aborted pods)					
	Stylar		Medial		Basal		Stylar		Medial		Basal		Stylar		Medial		Basal	
	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value
Cultivar (C)	6.04	**<0.001**†	4.77	**<0.001**	6.67	**<0.001**	9.63	**<0.001**	4.87	**<0.001**	3.28	**<0.001**	6.19	**<0.001**	4.00	**<0.001**	2.88	**<0.001**
Day temperature (DT)	28.85	**<0.001**	23.38	**<0.001**	6.74	**0.010**	-	-	-	-	-	-	-	-	-	-	-	-
Node (N)	12.04	**<0.001**	13.87	**<0.001**	18.40	**<0.001**	6.87	**<0.001**	0.29	0.830	9.77	**<0.001**	26.43	**<0.001**	16.16	**<0.001**	28.06	**<0.001**
Pod position (PP)	2.40	0.120	6.98	**0.009**	9.90	**0.002**	2.73	0.100	0.31	0.580	3.44	0.070	167.55	**<0.001**	161.23	**<0.001**	138.83	**<0.001**
C*DT	2.66	0.001	2.40	0.003	1.15	0.310	-	-	-	-	-	-	-	-	-	-	-	-
C*N	1.43	0.047	1.94	0.001	1.26	0.140	1.68	0.009	1.82	0.003	1.18	0.220	2.03	<0.001	1.44	0.039	1.50	0.026
C*PP	0.91	0.550	0.87	0.600	1.01	0.440	0.52	0.930	2.24	0.007	1.07	0.390	6.00	<0.001	5.97	<0.001	4.61	<0.001
N*DT	2.06	0.110	1.05	0.370	0.77	0.510	-	-	-	-	-	-	-	-	-	-	-	-
DT*PP	0.00	0.980	0.63	0.430	0.01	0.920	-	-	-	-	-	-	-	-	-	-	-	-
N*PP	0.20	0.900	2.85	0.040	0.56	0.640	0.89	0.450	2.54	0.060	0.50	0.680	1.51	0.023	2.69	0.047	0.84	0.474

†The *P* values in bold type indicate that the main effect had a significant effect on the response.

Table 6.3. Effects of cultivar, node, and pod position on seed development according to their ovule position (stylar, medial, basal) within pods at physiological maturity in 16 field pea cultivars in growth chambers with two temperature regimes (24/18 and 35/18°C day/night temperatures during the reproductive development for 7 days) and under field conditions (Rosthern, SK 2015).

	Exp. 1 Growth chamber†			Exp. 2 Field‡ (only pods present at maturity)			Exp. 2 Field§ (missing pods considered as aborted pods)		
	Stylar	Medial	Basal	Stylar	Medial	Basal	Stylar	Medial	Basal
Cultivar									
40-10	1.34 a ¶	1.39 a	1.35 a	1.39 a-c	1.47 ab	1.25 ab	0.76 d	0.80 d	0.68 e
Naparnyk	1.24 ab	1.38 ab	1.10 b	1.35 a-d	1.40 a-c	1.29 a	1.09 a-c	1.14 a-c	1.04 a
CDC Mozart	1.26 ab	1.38 ab	0.83 c	1.47 a	1.49 a	1.03 c-f	1.22 a	1.24 a-c	0.86 a-e
CDC Golden	1.26 ab	1.27 a-c	0.84 c	1.34 b-d	1.46 ab	0.97 d-f	1.06 a-c	1.16 a-c	0.77 b-e
CDC Patrick	1.18 a-c	1.23 a-c	0.88 c	1.42 ab	1.47 ab	1.18 a-c	1.09 a-c	1.13 a-c	0.91 a-c
CDC Meadow	1.07 b-d	1.31 a-c	0.85 c	1.34 a-d	1.33 de	0.91 ef	1.12 a-c	1.11 a-c	0.76 b-e
CDC Sage	1.08 bc	1.15 c	0.88 c	1.25 de	1.42 a-c	1.06 c-e	0.89 cd	1.01 cd	0.75 c-e
Cutlass	1.04 a-e	1.26 a-c	0.88 c	1.26 de	1.46 a-c	0.97 d-f	1.03 a-c	1.18 a-c	0.79 b-e
CDC Centennial	1.23 ab	1.13 cd	0.80 c	1.38 a-d	1.42 a-c	1.03 c-f	1.03 a-c	1.07 a-c	0.77 b-e
Agassiz	1.19 a-c	1.20 a-c	0.73 cd	1.29 c-e	1.40 a-c	1.10 b-d	1.13 ab	1.22 a-c	0.96 ab
TMP 15213	1.06 b-d	1.18 cd	0.82 c	1.33 b-d	1.44 a-c	1.07 c-e	0.92 cd	0.99 cd	0.73 c-e
CDC Bronco	1.05 b-d	1.18 bc	0.61 de	1.35 a-d	1.46 ab	1.01 d-f	1.21 a	1.31 a	0.91 a-d
CDC Treasure	0.85 ed	1.13 cd	0.71 cd	1.31 b-e	1.44 a-c	1.01 d-f	1.09 a-c	1.20 a-c	0.84 b-e
Carneval	0.81 ef	1.13cd	0.73 cd	1.20 e	1.47 ab	0.91 ef	0.92 b-d	1.14 a-c	0.70 de
Nitouche	0.99 c-e	0.93 d	0.48 e	1.28 c-e	1.44 a-c	0.88 f	1.12 a-c	1.26 ab	0.77 b-e
MFR043	0.62 f	0.66 e	0.51 e	0.90 f	1.27 e	0.94 ef	0.73 d	1.03 b-d	0.78 b-e
Temperature regime									
Control	1.17 a	1.27 a	0.87 a	-	-	-	-	-	-
High temperature	0.98 b	1.10 b	0.76 b	-	-	-	-	-	-
Node position									
1	1.17 ab	1.29 a	0.97 a	1.33 a	1.41 a	1.13 a	1.12 a	1.19 ab	0.96 a
2	1.18 a	1.26 ab	0.88 b	1.34 a	1.44 a	1.03 b	1.17 a	1.24 a	0.91 a
3	1.06 b	1.19 b	0.75 c	1.26 b	1.43 a	0.99 b	0.98 b	1.11 b	0.77 b
4	0.86 c	0.98 c	0.61 d	1.23 b	1.42 a	0.91 c	0.93 c	0.95 c	0.61 c
Pod position at a given node									
Proximal	1.10 a	1.21 a	0.85 a	1.28 a	1.43 a	1.06 a	1.22 a	1.32 a	0.99 a
Distal	1.03 a	1.15 b	0.76 b	1.31 a	1.42 a	0.97 b	0.84 b	0.92 b	0.64 b

†Rating scale of growth chamber data: a full seed = 1.5; an aborted seed = 1.0; an aborted ovule = 0.5; an aborted pod = 0.

‡Rating scale of field data: a full seed = 1.5; an aborted seed = 1.0; an aborted ovule = 0.5.

§Means with a common letter within each column under each section of cultivar, temperature regime, node position, and pod position did not differ at $P < 0.05$.

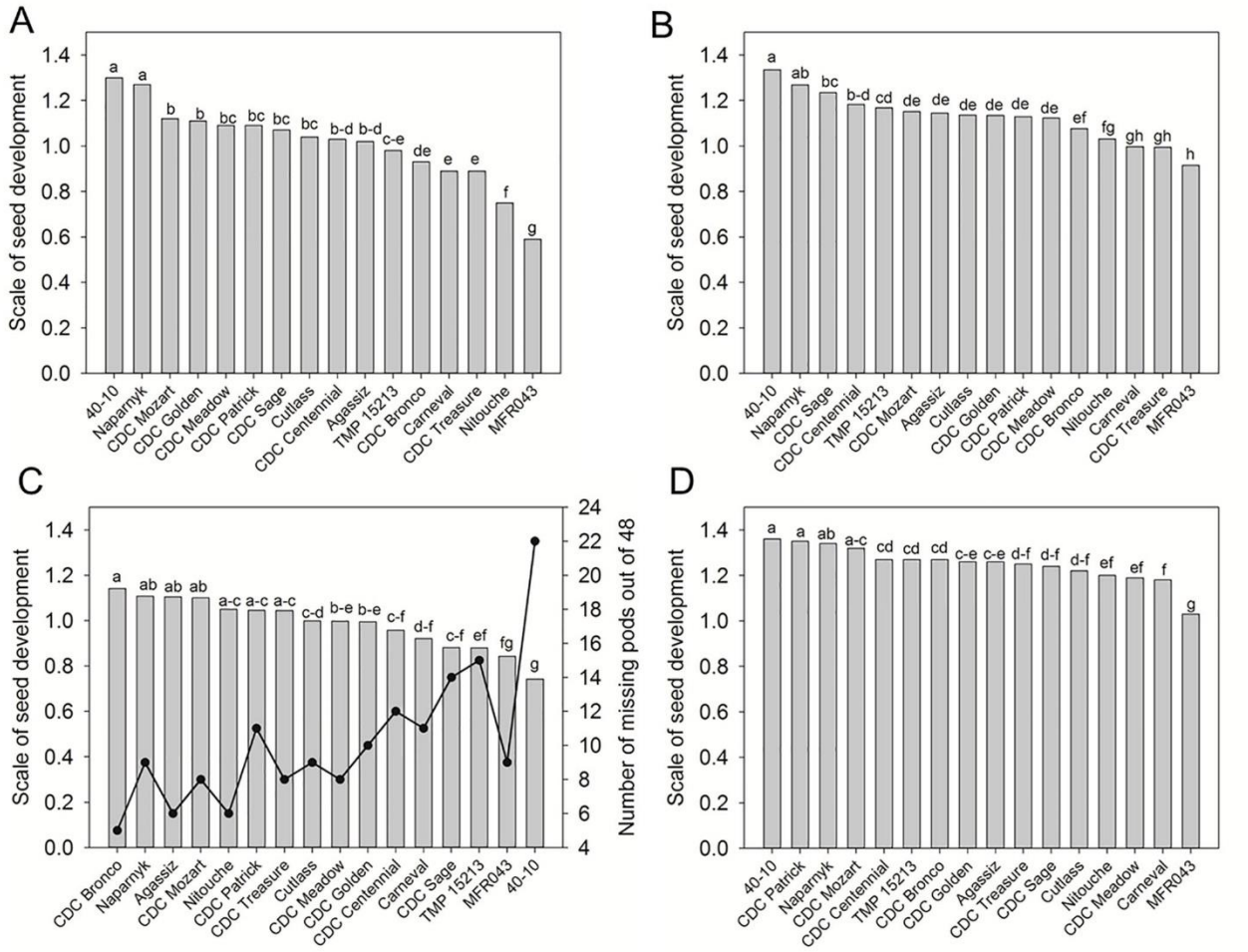

Figure 6.1. Average rating scale of seed development within pods at the first four reproductive nodes in 16 pea cultivars under controlled environment (A, B) and field conditions (C, D). Rating scale of seed development in A: a full seed = 1.5, an aborted seed = 1.0, an aborted ovule = 0.5, an aborted pod = 0; rating scale of seed development in C: a full seed = 1.5; an aborted seed = 1.0; an aborted ovule = 0.5, a missing pod = 0; rating scale of seed development in B and D: a full seed = 1.5; an aborted seed = 1.0; an aborted ovule = 0.5; only pods present at maturity were measured. The bars in A and B represent mean values of 48 pods with three replications (one plant per pot as one replication), two temperature regimes (24/18 °C and 35/18°C day/night temperature for 7 days), four nodes per plant, and two pods per node. The bars in C and D represent mean values of 48 pods with three replications (two sub-sampled plants per replication), four nodes per plant, and two pods per node. The points in the line in C represent the number of missing pods out of 48 pods in each cultivar. Means with a common letter did not differ at $P < 0.05$.

Ovule position within pods played a critical role in seed development, and the patterns of seed development responding to ovule positions differed among these 16 cultivars (Table 6.4). In general, ovules at the medial position within a pod had the highest chance to develop successfully into mature seeds, followed by ovules at the stylar end position, whereas ovules at the basal end were most likely to abort (Figure 6.2).

Table 6.4. Probabilities from analysis of variance (ANOVA) showing effects of cultivar, day temperature, and ovule position within pods on seed development at physiological maturity across the first four reproductive nodes in 16 field pea cultivars in growth chambers with two temperature regimes (24/18 and 35/18°C day/night temperatures during the reproductive development for 7 days) and under field conditions (Rosthern, SK 2015).

Effect	Growth chamber		Field (Rosthern 2015)	
	F value	*P* value	F value	*P* value
Cultivar (C)	19.07	**<0.001**†	10.39	**<0.001**
Day temperature (DT)	48.46	**<0.001**	-	-
Ovule position (OP)	140.41	**<0.001**	345.96	**<0.001**
C*DT	4.32	**<0.001**	-	-
C*OP	2.39	**<0.001**	3.94	**<0.001**
DT*OP	1.68	0.19	-	-
C*DT*OP	0.63	0.94	-	-

†The *P* values in bold type indicate that the main effect or interaction had a significant effect on the response.

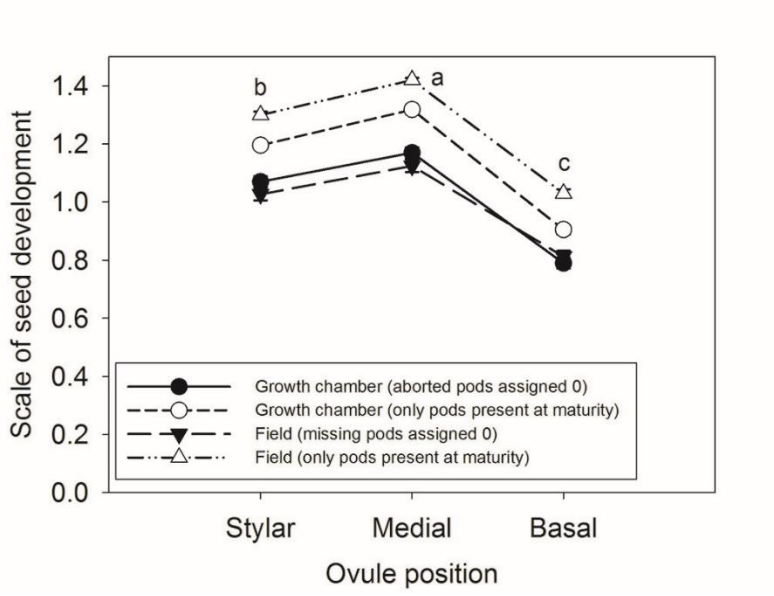

Figure 6.2. Effect of ovule positions (stylar, medial, and basal) on seed development under controlled environment (growth chambers) and field conditions (Rosthern, SK 2015). Means with a common letter did not differ at $P < 0.05$. For growth chamber data, each dot represents the mean value of 768 samples (16 cultivars × 2 temperature regimes × 4 nodes × 2 pods per node × 3 replications); for field data, each dot represents the mean value of 768 samples (16 cultivars × 4 nodes × 2 pods per node × 2 subsamples per plot × 3 blocks).

The 16 cultivars were further categorized into five groups based on patterns of seed development by ovule position (Figure 6.3A-E). For over half (nine out of 16) of the cultivars

tested, ovules at the pod's medial position and stylar end positions developed equally into mature seeds with a frequency that exceeded ovules at the basal end (Figure 6.3A); these nine cultivars were Agassiz, CDC Bronco, CDC Centennial, CDC Golden, CDC Mozart, CDC Patrick, CDC Sage, Nitouche, and TMP15213. One cultivar, CDC Meadow, developed seeds from the medial ovule position, followed by the stylar end position, but ovules at the pod's basal end aborted most often (Figure 6.3B). Three cultivars, Carneval, CDC Treasure, and Cutlass, had successful seed development at the medial position compared to ovules at either end of the pod; ovule abortion was more likely in, and similar in frequency, for both the stylar and basal ends (Figure 6.3C). Ovule position *per se* did not affect seed maturity and abortion in 40-10 and MFR043. These two cultivars, however, had quite different strategies. Ovules at all positions in 40-10 were most likely to develop into successful seeds, and ovules at all positions within pods of MFR043 were most likely to abort (Figure 6.3D). In Naparnyk, ovules at stylar and medial positions were most likely to reach seed maturity, while the basal ovules with their lowered seed development were still above 1.1 (Figure 6.3E). Only two cultivars, 40-10 and Naparnyk, could maintain seed development scores above 1.1 for basal position ovules. Both 40-10 and Naparnyk seed development scores were among the highest in both growth chamber and field (Figure 6.1; Table 6.3), and were the two cultivars most likely to maintain seed development scales of 1.0 or greater at all ovule positions, and maintain high numbers of mature seeds per pod of retained pods in stress (Figure 6.1).

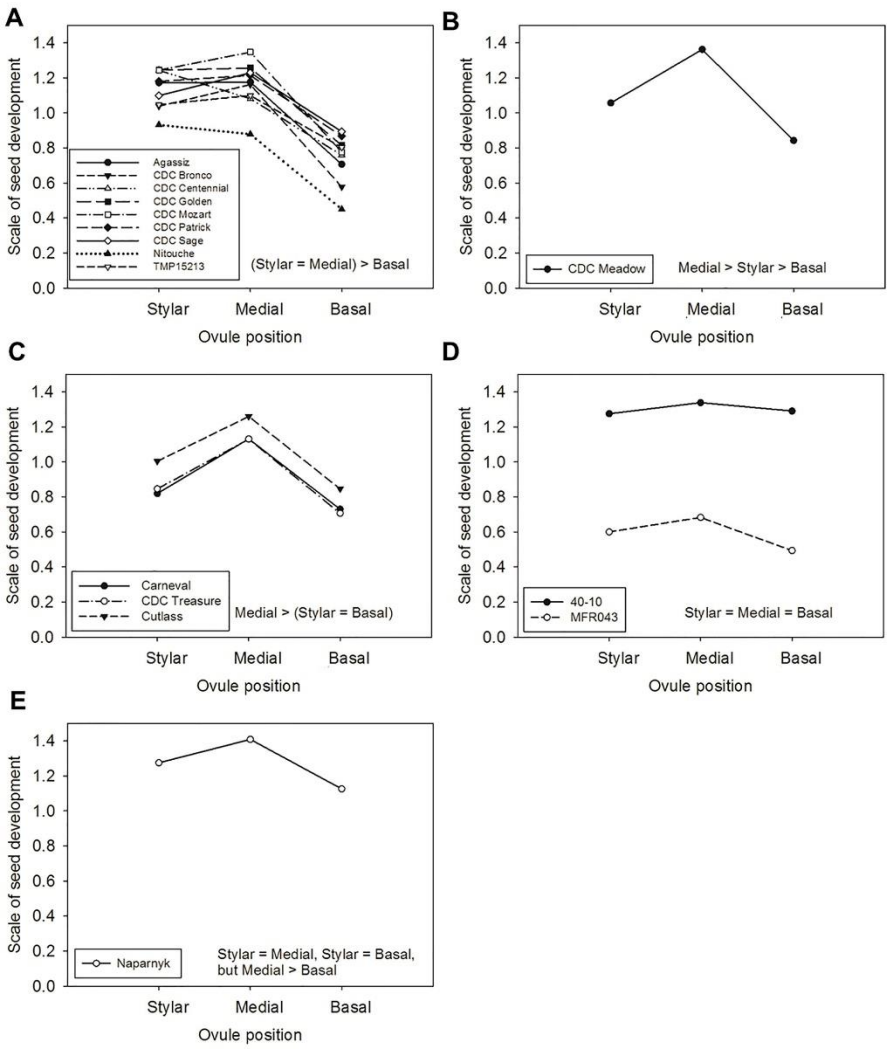

Figure 6.3. Effects of ovule position on seed development in 16 cultivars of field pea in growth chambers. Rating scale: a full seed = 1.5; an aborted seed = 1.0; an aborted ovule = 0.5; an aborted pod = 0. (A) seed development at (stylar position = medial end) > basal end; (B) seed development at medial position > stylar end > basal end; (C) seed development at medial position > (stylar end = basal end); (D) seed development at stylar end = medial position = basal end; (E) seed development at stylar end = medial position, stylar end = basal end, but medial position > basal end. Each dot represents the mean value of 48 samples (two temperature regimes × four nodes per plant × two pods per node × three replications).

The ability of ovules developing into seeds using the scale of seed development was inversely related to TSW (Figure 6.4). Ovules and seeds for each of the categories of ovule position indicated that cultivars with small seeds were less likely to abort seeds, and cultivars with large seed were more likely to abort seed. Ovules at the medial and stylar pod ends were also more likely to develop into seeds compared to ovules at the basal pod end.

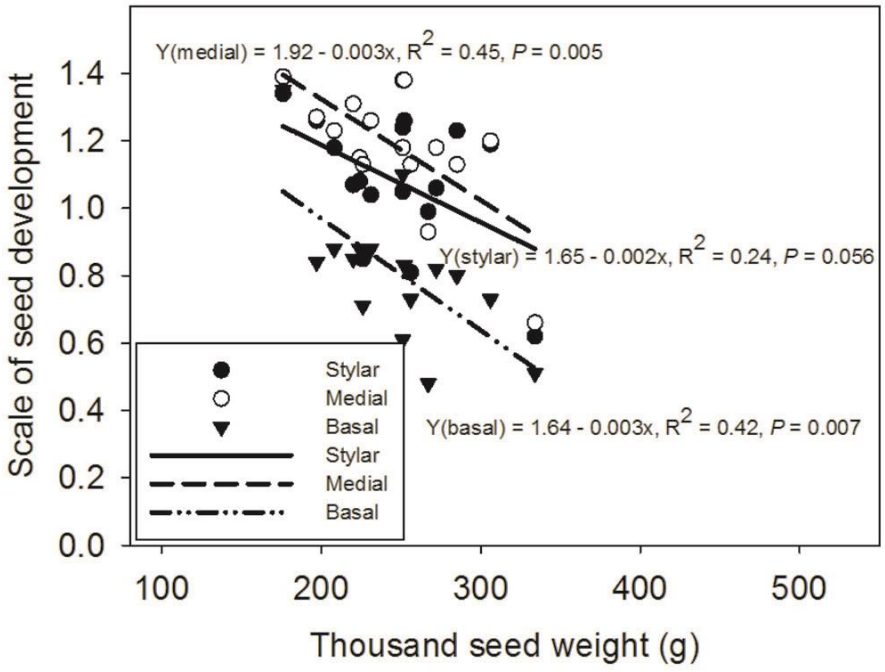

Figure 6.4. Simple linear regression analysis for scale of seed development and thousand seed weight. Rating scale for each seed: a full seed = 1.5; an aborted seed = 1.0; an aborted ovule = 0.5; an aborted pod = 0 for all seed positions. Each dot represents the average of 48 values (16 cultivars × three replications) measured over ovules of two pods at each of four nodes.

Under control conditions, total seed weight on the main stem was positively correlated with duration of flowering, the number of flowers, pods, seeds, and ovules on the main stem, pod length, the number of seeds and ovules per pod, seed-to-ovule ratio, and seed weight per pod (Table 6.5). In contrast under heat-stressed conditions, total seed weight on the main stem was positively correlated with the number of pods, seeds, and ovules on the main stem, pod length, the number of seeds and ovules per pod, and seed weight per pod (Table 6.5). The TSW was negatively correlated with pod retention, the number of seeds per pod, and seed-to-ovule ratio under both control and heat-stressed conditions (Table 6.5). Additionally, TSW was negatively correlated with total number of seeds on the main stem and seed retention under heat-stressed conditions (Table 6.5). In other words, large seed-size cultivars had more aborted pods and seeds.

Table 6.5. Correlation matrix of the relationships among total seed weight, duration of flowering, the number of reproductive nodes (Rnode), the number of flowers, the number of pods, pod retention, total number of seeds, total number of ovules, seed retention, thousand seed weight, pod length, the number of seeds per pod, the number of ovules per pod, seed-to-ovule ratio, and seed weight in 16 pea cultivars under control (24/18°C day/night) and heat-stressed (35/18°C day/night for 7 days) conditions.

Control \ Heat stress	Total seed weight	Duration of flowering	Number of Rnode	Number of flowers	Number of pods	Pod retention	Total number of seeds	Total number of ovules	Seed retention	Thousand seed weight	Pod length	Number of seeds per pod	Number of ovules per pod	Seed-to-ovule ratio	Seed weight per pod
Total seed weight†		-0.008ns	-0.082ns	0.368ns	0.768**	0.339ns	0.893***	0.863***	0.324ns	-0.140ns	0.717**	0.582*	0.575*	0.460ns	0.627**
Duration of flowering†	0.548*§		0.539*	0.304ns	0.124ns	-0.168ns	-0.077ns	0.040ns	-0.416ns	0.332ns	-0.242ns	-0.322ns	-0.090ns	-0.388ns	-0.274ns
Number of Rnode†	0.400ns	0.602*		0.730**	0.304ns	-0.451ns	-0.166ns	0.108ns	-0.728**	0.509*	-0.524*	-0.527*	-0.148ns	-0.639**	-0.561*
Number of flowers†	0.610*	0.589*	0.802***		0.631**	-0.487ns	0.316ns	0.501*	-0.345ns	0.281ns	-0.232ns	-0.113ns	0.144ns	-0.255ns	-0.182ns
Number of pods†	0.777***	0.521*	0.583*	0.911***		0.352ns	0.784***	0.920***	-0.015ns	-0.201ns	-0.196ns	0.238ns	0.246ns	0.189ns	0.025ns
Pod retention†	0.175ns	-0.286ns	-0.626**	-0.326ns	0.062ns		0.434ns	0.370ns	0.351ns	-0.528*	0.375ns	0.326ns	-0.012ns	0.469ns	0.131ns
Total number of seeds†	0.938***	0.550*	0.287ns	0.552*	0.750**	0.322ns		0.900***	0.545*	-0.521*	0.625*	0.772***	0.611*	0.701**	0.480ns
Total number of ovules†	0.868***	0.534*	0.525*	0.839***	0.959***	0.149ns	0.863***		0.158ns	-0.370ns	0.472ns	0.483ns	0.580*	0.357ns	0.252ns
Seed retention†	0.307ns	0.173ns	-0.283ns	-0.293ns	-0.180ns	0.222ns	0.440ns	-0.041ns		-0.664**	0.529*	0.891***	0.376ns	0.962***	0.595*
Thousand seed weight†	-0.141ns	-0.011ns	0.245ns	-0.012ns	-0.167ns	-0.509*	-0.459ns	-0.255ns	-0.477ns		-0.263ns	-0.662**	-0.354ns	-0.736**	-0.069ns
Pod length‡	0.790***	0.456ns	0.138ns	0.236ns	0.407ns	0.214ns	0.750**	0.587*	0.369ns	-0.102ns		0.702**	0.698**	0.576*	0.847***
Number of seeds per pod‡	0.607*	0.244ns	-0.148ns	-0.034ns	0.180ns	0.443ns	0.746**	0.382ns	0.821***	-0.625*	0.656**		0.713**	0.942***	0.664**
Number of ovules per pod‡	0.672**	0.318ns	0.279ns	0.499*	0.609*	0.216ns	0.724**	0.782***	0.121ns	-0.375ns	0.680**	0.595*		0.447ns	0.546*
Seed-to-ovule ratio‡	0.424ns	0.140ns	-0.295ns	-0.238ns	-0.036ns	0.425ns	0.573*	0.125ns	0.929***	-0.611*	0.471ns	0.942***	0.298ns		0.567*
Seed weight per pod‡	0.644**	0.181ns	-0.039ns	-0.033ns	0.100ns	0.149ns	0.594*	0.291ns	0.695**	-0.146ns	0.713**	0.821***	0.488ns	0.767**	

†Data were based on the main stem;

‡Average across the first four reproductive nodes.

§Significance levels for the correlation coefficient (r) is denoted by the symbols *, **, ***, for $P < 0.05$, $P < 0.01$, $P < 0.001$ or not significant (ns), respectively.

6.3.2 Exp. 2: Seed-set parameters in the field experiment

In summer 2015, data gathered from the field represented natural growing conditions for commercial pea production. The first four nodes of plants that were collected had flowered during the first two weeks of July, and experienced midday (11:00-17:00) air temperature between 15.1°C and 32.5°C with an average of 24.3°C. Seed development at the three specific ovule positions within pods was significantly affected by cultivar, and for stylar and basal ovule positions, and also by node (Table 6.2).

Ovules for the stylar end and basal end ovule positions in pods at the first (oldest) reproductive node were most likely to develop successfully into mature seeds compared to the younger reproductive nodes higher on a plant, such as reproductive node 4 (youngest; Table 6.3). For data where only pods present at maturity were measured, the basal ovule position was additionally affected at each node by pod position (proximal versus distal; Table 6.2), meaning basal ovules of distal pods were likely to abort compared to that position within proximal pods (Table 6.3). For data where missing pods were treated as aborted pods, ovules at the distal pod position were more likely to abort compared to proximal pod ovules, regardless of ovule position within a pod.

For data where only pods present at maturity were measured, among the 16 cultivars tested, those with the highest possibility to develop into mature seeds were CDC Patrick and 40-10, Naparnyk and CDC Mozart (Figure 6.1D). Cultivars with the most ovule abortions were MFR043 and Carneval (Figure 6.1D). Among these two, MFR043 had the most ovule abortion, consistent with the growth chamber results (Figure 6.1A, B, D). For data where missing pods were treated as aborted pods, cultivars CDC Bronco, Naparnyk, Agassiz, CDC Mozart, Nitouche, CDC Patrick, and CDC Treasure were more likely to yield a high number of mature seeds (Figure 6.1C). In contrast, 40-10 and MFR043 were most likely to abort seeds (Figure 6.1C, D). Among these 16 cultivars, 40-10 had the greatest amount of missing data (22 out of 48 pods measured), indicating only one flower on average was formed at each node (Figure 6.1C).

In general in the field, ovules at the medial position within a pod had the highest chance to develop successfully into mature seeds, followed by ovules at the stylar end position, whereas ovules at the basal end were most likely to abort, a pattern similar to the growth chamber experiment (Figure 6.2). In Figure 5.5A, seven cultivars had similar seed development in stylar and medial ovule positions (40-10, CDC Bronco, CDC Centennial, CDC Meadow, CDC Mozart,

CDC Patrick, and TMP 15213). Of these cultivars, 40-10 had the smallest basal seed development reduction. In Figure 5.5B, seven other cultivars (Agassiz, Carneval, CDC Golden, CDC Sage, CDC Treasure, Cutlass, and Nitouche) favored medial over stylar seed development. Naparnyk maintained seed equally at all three ovule positions (Figure 5.5D), and again MFR043 aborted seed equally at stylar and basal ovule positions (Figure 5.5C).

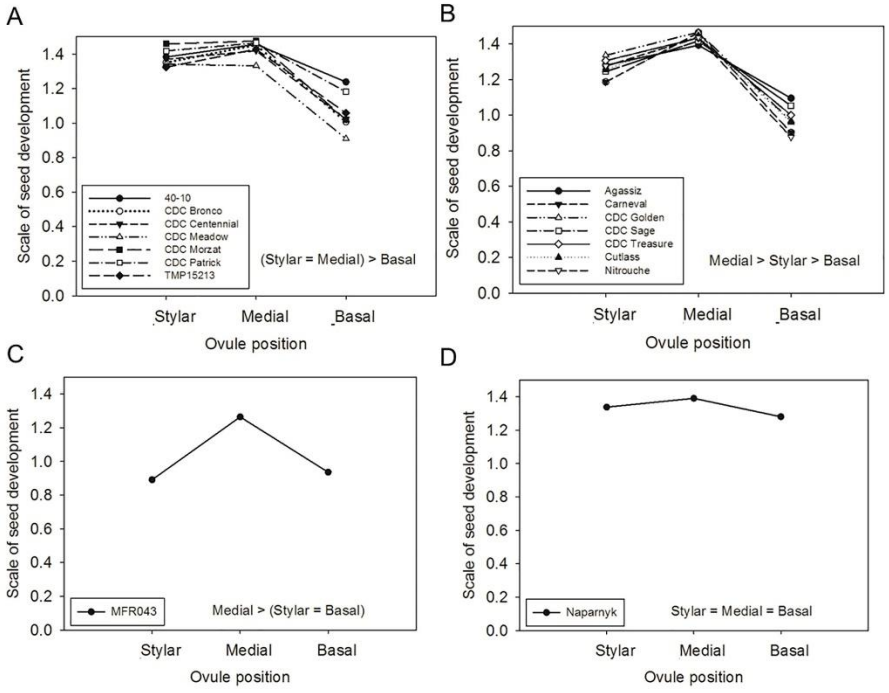

Figure 6.5. Effects of ovule position on seed development in 16 cultivars of field pea under field conditions at Rosthern, SK in 2015. Rating scale: a full seed = 1.5; an aborted seed = 1.0; an aborted ovule = 0.5. Only pods present at plant senescence were considered. (A) seed development at (stylar end = medial position) > basal end; (B) seed development at medial position > stylar end > basal end; (C) seed development at medial position > (stylar end = basal end); (D) seed development at stylar end = medial position = basal end. Each dot represents the mean value of 48 samples (four nodes per plant × two pods per node × six replications) and measured over all ovules present in pods retained by plants.

6.4 Discussion

We observed that ovules located in the medial and stylar end positions were most likely to develop into mature seed compared to ovules at the basal (peduncle) ends in most of the tested cultivars (nine out of 16) in growth chambers, indicating a combined effect of spatial advantages and early fertilization with fast-growing pollen tubes. In the same species (*P. sativum*), about 33% of sterile ovules were found in the stylar ends of pods and over 50% of sterile ovules were located in the basal ends of pods (Makasheva, 1984). Similarly, an earlier study of ovule fate and seed

maturity in pea cv. Alaska showed that ovules at the medial position were most likely to end with seeds compared to both stylar and basal ends, owing to fruit shape favoring spatial advantages in the medial position (Linck, 1961), a finding consistent with three cultivars (Carvenal, CDC Treasure, and Cutlass) in the growth chamber and one cultivar, MFR043, in the field. Ovules at the medial position in seven out of 16 cultivars were likely to develop into seeds, followed by ovules at the stylar end, but ovules at the basal end were likely to abort in the field.

Ovule fate patterns in field pea can be explained by several hypotheses - fertilization gradient within a pod and fruit shape as mentioned above, as well as genetic quality. Ovules at any position including stylar and basal ends and medial position tended to develop into successful seeds in 40-10 in growth chambers and Naparnyk in the field. In contrast, ovules at any position most likely to fail were in MFR043 grown in the growth chamber. A few cultivars had greater tendencies for either seed maturity or seed abortion independent of ovule position, but most cultivars showed differential development and abortion according to ovule position.

Nutrient resources may not be the major limiting factor for seed development in field pea, because the pod wall of field pea is photosynthetically active (Atkins et al., 1977; Flinn et al., 1977). The pod wall of field pea has two photosynthetic layers: 1) the outer layer contains chlorenchyma of the mesocarp, and is able to capture CO_2 from the atmosphere via the stomata of the outer epidermis; 2) the inner epidermis comprises chloroplasts, and the layer connects the pod gas cavity and takes in the CO_2 released from respiring seeds (Atkins et al., 1977). From our own unpublished field measurements of stomatal conductance of pods in field pea plants, developing pods are able to transpire and presumably have functional stomata. Pod photosynthesis and maternal assimilate supply can only partially explain the ovule success at the basal ends of pods, because basal positions are situated closest to the maternal nutrients but they were the most likely to abort in our data.

Competition-driven ovule abortion in younger reproductive nodes indicates that pea plants lack sufficient maternal resources for allocation among different fruiting nodes in our study. Makasheva (1984) also documented a higher proportion of productive ovules found in the earlier formed pods in pea. The inhibition of distal pods by the proximal pod at the same node was also observed in tomato fruiting hierarchy in the same nodal inflorescence or from different trusses (nodes; Kinet and Peet, 1997), and in soybean (Weibold, 1990). Approximately 20-30% of ovules were likely to

abort during seed development depending on growing conditions and genotype in pea (Makasheva, 1984). One reason for this ovule abortion is that plants regulate the sink for maternal resources or photoassimilate by adjusting the number of flowers and gametophytes to maximize their fitness and survival (Barnabás et al., 2008). In the event of environmental stress, plants conserve maternal resources by reducing fertility, and shunt them into mechanisms to combat stress (Barnabás et al., 2008). Therefore, seed development in field pea is likely to be dependent on assimilate distribution controlled by the activity of source and sink. The second reason for ovule abortion may be due to lack of successful pollination. The pollen tube is less likely to reach ovules in the basal ovule position, and abiotic stress exacerbates this tube failure.

We observed that heat stress (35/18°C day/night temperature for 7 days) changed seed development in ovule positions in field pea pods. In the literature, stress has a negative effect on reproductive development and subsequent seed growth in many crops including grain legumes. A moderate heat stress caused a reduction in carbon metabolism leading to the abortion of reproductive organs in the distal flowers in field pea (Guilioni et al., 1997). Similarly, female infertility was induced by heat stress in wheat (Saini et al., 1983). Heat stress decreased ovule viability in bean (Monterroso and Wien, 1990). Heat stress accelerated embryo abortion in cowpea (*Vigna unguiculata*; Warrag and Hall, 1983) and bean (Monterroso and Wien, 1990). Heat stress (>32/20°C day/night temperature) significantly reduced pod set in chickpea, which was associated with the reduction in pollen viability, pollen load, pollen germination (*in vivo* and *in vitro*), and stigma receptivity (Kaushal et al., 2013). Heat stress (>32/20°C day/night temperature) accelerated pod abortion as well as shortened the duration of seed set leading to a reduction in biomass accumulation, flowers, and pods, thus lowering yield production in lentil (Bhandari et al., 2016).

Large seed size was a potential limiting factor for successful seed development in field pea, because cultivars with genetically inherited large seed size were more likely to be impacted by heat stress and abort pods, or abort developing seeds in pods. Seed size had a weak relationship with the total seed weight on the main stem under both control and heat-stressed conditions, which was consistent with previous research in field pea (Sadras et al., 2013). However, cultivars with large seed size tended to have fewer seeds per pod. Annual crops change seed number rather than seed size in response to environmental variations (Jiang and Egli, 1995; Sadras et al., 2015). However, the analysis of yield components from single plants in growth chambers overlooked several critical

factors such as plant density and branching and plant competition in the field. Pot-grown plants are almost useless for population-level traits such as seed yield and plant density (Sadras and Dreccer, 2015).

The trade-off between seed size and seed number has been documented in many crops such as wheat and soybean (Sadras, 2007; Sadras and Slafer, 2012). The number of offspring that parents can produce is reduced when the energy spent on individual offspring is increased, resulting in an increase in the fitness of individual offspring (Smith and Fretwell, 1974). For a given species and environment, seed number has high plasticity, whereas seed size is conservative and heritable due to stabilizing natural selection (Sadras, 2007; Sadras and Slafer, 2012). On the other hand, certain crops such as sunflower and maize may have increased variability of seed size and decreased plasticity in seed number due to the selection for one or few inflorescences (Sadras, 2007).

The number of seeds per fruit can also be affected by parent-offspring conflict, sibling rivalry, and self-organization involving simple rules of resource allocation among ovules (Geneshaiah and Shannker, 1988; Shaanker et al., 1988; Geneshaiah and Shannker, 1992, 1994; Geneshaiah et al., 1995; Shannker and Ganeshaiah, 1995; Ravishankar et al., 1995; Shannker et al., 1996; Mock and Parker, 1997). Parent-offspring conflicts are manifested by the production of growth hormones – 1) hormones (e.g. auxins and gibberellic acids) involving mobilization of resources being synthesized by offspring, and 2) hormones (e.g. abscisic acid) inhibiting resources flow into seeds being synthesized by the maternal tissue (Ravishankar et al., 1995). Sibling rivalry is increased within a fruit, because the genetic relatedness among offspring within a fruit is decreased (Shaanker et al., 1988). Parent-offspring conflict, sibling rivalry, and self-organization could also be potential factors affecting the seed development in the present study, but are beyond the scope of this study.

6.5 Conclusions

In summary, seed set in field pea is impacted by cultivar, ovule position within pods, node position within each stem, pod position within each node, and environmental conditions such as high temperature prevailing during the reproductive development. Some of the 16 cultivars tested, including 40-10 and Naparnyk, performed better compared to other cultivars in terms of ovule and seed retention, whereas others such as MFR043 (large seed size) failed to retain many ovules and seeds. The association between seed number, seed size and seed retention requires additional validation because the best performing cultivars exposed to high temperatures in this study, 40-10

and Naparnyk, produce many small seeds genetically. Selecting for high yield with smaller seed size and more seeds per pod may be one way to improve yields of field pea, and other legume crops, in heat-stressed seasons.

Acknowledgements

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Contributions by others to this thesis chapter

Experiment of effect of heat stress on seed development at different ovule, pod, and node positions in 16 pea cultivars at growth chambers was performed by Yunfei Jiang, and all of the data from growth chamber experiments was collected by Yunfei Jiang. Dataset of ovule position (the same 16 cultivars) from the field (Rosthern in 2015) were collected by the crew at the Crop Physiology Lab led by Dr. Rosalind Bueckert, and designed by Dr. Rosalind Bueckert and the previous postdoctoral researcher, Dr. Donna Lindsay. Data analysis and interpretation was performed by Yunfei Jiang and Dr. Rosalind Bueckert, and Yunfei Jiang wrote this thesis chapter.

Transition section between Chapter 6 and Chapter 7

In Chapter 6, the relationship between seed development and positions of ovules, pods, and nodes under heat stress was unravelled. One of the main results was consistent with Chapter 5 – ovules at the basal ends are most likely to abort. At this stage of the thesis, we explored the effects of high day-time (Chapter 3) and night-time (Chapter 4) temperatures on pollen development and seed set; we also investigated the factors affecting successful seed development including pollen – pistil interaction and the positions of ovules, pod, and nodes in Chapter 5 and Chapter 6. As mentioned in Chapter 2 (literature review), the effects of heat stress can be varied, depending on intensity of temperature, duration of exposure, rate of temperature increase, and timing of heat exposure. Therefore, the experiment in Chapter 7 was designed to evaluate the impact of timing and duration of heat exposure on pollen and seed development.

Chapter 7 Impact of heat stress exposure timing and duration on reproductive organs in field pea

7.1 Introduction

Flowering plants are highly sensitive to high temperature stress during the reproductive phase. The reproductive phase involves the development of flowers containing ovules (female) and pollen (male) gametes, pollination, double fertilization, embryo formation, and fruit and seed development (Carlson and Lersten, 1987). Vulnerability of plants to heat stress varies depending on the developmental stage when exposed to heat stress and the intensity of stress (Wahid et al., 2007). The critical period for seed yield determination was documented to be between 10 days before flowering and 40 days after flowering in field pea (Sandaña and Calderini, 2012). The early stage of anther development has been reported to be extremely susceptible to heat stress in wheat, barley, and many other commercially important crops (Sakata et al., 2010). For example, pollen grains produced normal exine but no or little cytoplasm in barley (*Hordeum vulgare*) when exposed to 30/25°C day/night temperatures for 5 days at the early differentiation stage of the panicle, but the same high temperature regime led to the development of short anthers without pollen grains during heat exposure at the pre-meiotic stage of pollen mother cells (PMCs). Pollen grains were swollen and little starch was accumulated in pollen grains with heat exposure during meiosis of PMCs, and seeds were virtually sterile when panicles at the heading stage were exposed to heat (Sakata et al., 2000). Similarly, the uninucleate stage during pollen development is highly sensitive to elevated temperatures in many crops such as chilli pepper (*Capsicum annuum* and *Capsicum baccatum*), coyote melon (*Cucurbita palmate*), tepary bean (*Phaseolus acutifolius*), runner bean (*Phaseolus coccineus*), and maize (*Zea mays*) (Sage et al., 2015 and therein; Dolferus et al., 2011).

In addition to which developmental stages are exposed to heat stress, the duration of heat exposure and the amplitude of temperature are factors determining stress intensity and the reproductive output under heat stress (Prasad et al., 2015). For example, a longer heat duration of 14 days significantly reduced floret fertility in sorghum (*Sorghum bicolor*) compared to 7 days of heat exposure (Prasad et al., 2015). The knowledge of the impact of heat exposure duration on pollen and ovule function such as number of pollen grains per anther, pollen morphology, and ovule viability is needed for many crops, and specifically pea. Ovule viability at an early development stage can be determined if callose deposition is absent in ovules. Callose deposition

has been observed in sterile ovules in 13 species of angiosperms in four families, namely Fabaceae, Brassicaceae, Solanaceae, and Poaceae (Vishnyakova, 1991). Here, over 8,000 ovules were observed and most pollen tubes failed to penetrate the fluorescent ovules, with resulting callose deposition (Vishnyakova, 1991). Callose is often synthesized in cells when exposed to stress conditions (Vishnyakova, 1991). Previously, both callose deposition and lignification of hypostase cell walls were observed in aborted ovules in peas (*Pisum sativum*) (Briggs et al., 1987).

Reactive oxygen species (ROS) are precisely regulated by enzymatic and non-enzymatic antioxidant defense systems (You and Chan, 2015). The accumulation of ROS is due to perturbations of cellular redox equilibrium and cellular metabolism, which further results in oxidative damage and cell death (Zhang et al., 2009; You and Chan, 2015). An increase in ROS was observed in pollen grains of sorghum exposed to high night temperatures (Prasad and Djanaguiraman, 2011). The fluorogenic marker, 5-(and-6)-carboxy-2',7'-dichloro-dihydro-fluorescein diacetate (carboxy-H_2DCFDA) has been used for *in vivo* ROS detection in root cells and root hair response to nutrient deficiency (Shin and Schachtman, 2004; Shin et al., 2005), and to diagnose programmed cell death in protoplasts in an Arabidopsis mutant (Yao and Greenberg, 2006). Furthermore, wounding induces the formation of ROS, and can be visualized by molecular and immunological proteins (Kristiansen et al., 2009).

Although several studies of high temperature stress on reproduction in field pea have been documented, the critically sensitive growth stages, and the duration of stress exposure have not been thoroughly identified and quantified. I hypothesized that certain reproductive stages would be more sensitive to heat stress compared to other stages, and longer heat exposure duration would cause more damage to reproductive development compared to a shorter heat exposure. Accordingly, the objectives of this study were to test the effects of varying the duration of heat stress exposure on specific stages of flower development, on pollen and ovule function, and seed set in field pea.

7.2 Materials and methods

7.2.1 Exp. 1: Impact of heat stress on pollen and ovule function

7.2.1.1 Plant materials and growth conditions

Two pea cultivars, CDC Golden and CDC Sage, were tested in growth chambers. The study was designed as a randomized complete block design (RCBD). The experiment was repeated twice

and experimental run was considered as a blocking factor. For each run, 16 pots (two cultivars × two temperature regimes × four replications) of 3.8 L volume filled with Sunshine Gro® mix (Seba Beach, AB, Canada) and slow-release fertilizer (14-14-14, Type 100, Nutricote®, Brampton, ON, Canada) were planted with three seeds per pot. The pot dimensions were 15.9 cm depth and 16.5 cm diameter. Pots were thinned to two plants approximately two weeks after seeding. Plants received the first application (500 mL per pot) of half strength modified Hoagland's culture solution (Hoagland and Arnon, 1938) at three weeks after seeding and the second application (500 mL per pot) at the early flowering stage. Soil medium moisture was monitored carefully; plants were watered if necessary to avoid drought stress. Plants were grown at 24/18°C day/night temperatures with 16/8 h photoperiod and irradiance of 450-500 µmol photons m^{-2} s^{-1} from cool fluorescent tubes in a growth chamber, and the relative humidity had a range of 4-8%. Plant were then transferred to the high temperature growth chamber when small green buds (Stage I of Chapter 5) at the first reproductive nodes were visible. Conditions in the high temperature growth chamber were 16 hr light with temperature increasing from 21°C by 3°C increments to 35°C over 5 hr, maintained at 35°C for 6 hr, and decreased over 5 hr to 18°C in the dark for 8 hrs. The ramping temperature setting was used to mimic outdoor conditions where heat stress was evident in the field. The control plants remained in a constant 24/18°C growth chamber.

7.2.1.2 Number of pollen grains per anther after 4 and 7 days of heat exposure

The number of pollen grains per anther was determined using the method of Prieto-Baena et al. (2003) with modifications. Floral buds of CDC Golden prior to anther dehiscence (Stage II-III of Chapter 5) were collected from the control and high temperature chambers (20 buds in total, 2 temperature regimes × 2 heat exposure durations × 5 buds per treatment). Two out of 10 anthers per flower (field pea has 9 fused in a staminal tube and 1 stamen free) were randomly selected from the 9 stamens in the staminal tube for each floral bud. Each anther was placed near the lid of an Eppendorf tube and then cut open using a narrow-surgical blade, and 200 µL of 0.5 M sucrose solution was added to the tube to prevent pollen bursting from turgor pressure. The tube was agitated with vortex for 60 seconds. At least three proportional samples of 1 µL of the sucrose solution with pollen grains were deposited on a microscope slide, and the total number of pollen grains per 1 µL aliquot was counted under a light microscope with the magnification of 25× to 100×. The number of pollen grains per anther was calculated as the number of pollen grains per volume from an average of the three 1 µL aliquots and multiplying by 200.

7.2.1.3 Morphological changes in pollen grains after 4 and 7 days of heat exposure

Pollen morphology of control and heat-stressed pollen grains collected from floral buds (Stage II-III of Chapter 5) was observed using a light microscope. CDC Golden pollen was collected just prior to anther dehiscence after 4 or 7 days of exposure to high temperature. Observations were conducted simultaneously with the measurement of the number of pollen grains per anther (see section 5.2.5.3).

7.2.1.4 Accumulation of reactive oxygen species after 5 days of heat exposure

An Image-iT^{TM} Live Green ROS Detection Kit (Molecular Probes Inc., Eugene, OR, USA) was used to detect ROS in viable pollen grains. This method is based on a reliable fluorogenic marker, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) for ROS detection (Kristiansen et al., 2009; Zhang et al., 2009; Prasad and Djanaguiraman, 2011; Narayanan et al., 2015). The carboxy-H_2DCFDA, a non-fluorescent molecule, is able to permeate live cells and becomes converted to a green-fluorescent form when the acetate groups are removed by intracellular esterases and the oxidation process (by the activity of ROS) occurs. Pollen grains with ROS accumulation show bright fluorescence under a fluorescence microscope, and pollen grains with a low level of ROS synthesis show less bright fluorescence, and pollen grains without ROS accumulation do not fluoresce. The 10 mM carboxy-H_2DCFDA stock solution was prepared by adding 50 µL of dimethylsulfoxide (DMSO) into a vial of 275 µg of carboxy-H_2DCFDA. The 15 µM carboxy-H_2DCFDA working solution was made by adding 1.5 µL of 10 mM carboxy-H_2DCFDA stock solution in 1 mL of 0.1M piperazine-N, N'-bis (2-ethanesulfonic acid) (PIPES) buffer according to the manafacturer's instructions.

Fresh pollen grains of CDC Golden were collected from flowers at a mature bud stage (right after anther dehiscence and before anthesis; Stage III of Chapter 5) from the control growth chamber (24/18°C day/night temperatures with the 16/8 hr photoperiod) and the high temperature growth chamber (35/18°C day/night temperatures with the 16/8 hr photoperiod) after a 5-d exposure to high temperature. A drop of carboxy-H_2DCFDA solution was added to a glass microscope slide, and pollen grains were dusted onto the carboxy-H_2DCFDA solution and incubated at room temperature for 30 minutes in darkness before being observed under a fluorescence microscope. The experiment was carried out with six replications for each temperature regime with an individual flower as one replication, and an average of over 150 pollen

grains were counted in one microscope slide. The carboxy-H_2DCFDA has the excitation wavelength of 495 nm and the emission wavelength at 529 nm. Additionally, positive controls were conducted using 100 µM *tert*-butyl hydroperoxide (TBHP) to induce ROS production in pollen grains incubated at 35°C for 1 h, and then pollen grains were stained using the carboxy-H_2DCFDA solution, as described above.

7.2.1.5 Ovule viability

Thirty-two ovaries of CDC Golden and CDC Sage (two cultivars × two temperature regimes × eight replications) were collected from flowers at a mature bud stage, right after anther dehiscence (Stage III of Chapter 5), from plants in control conditions (24/18°C) and from plants exposed to high temperature (35/18°C day/night) for 4 or 7 days. Ovaries were dissected from these buds, then fixed in a solution containing 1% formaldehyde, 0.025% glutaraldehyde, and 0.01M Na phosphate (Na_2HPO_4/NaH_2PO_4) buffer (pH 7.2) overnight. The ovaries were then washed with the same buffer at 4°C for 2 h twice, cleared in 4M NaOH at 65°C for five minutes, washed again with the same phosphate buffer, then stained with 0.1% aniline blue in 0.1M K_3PO_4 overnight in darkness (Martin, 1959; Wist and Davis, 2008). After a final wash with phosphate buffer, stained ovaries were mounted on a microscope slide using a drop of 80% glycerol and examined for callose depositions inside individual ovules within ovaries using fluorescence microscopy.

7.2.2 Exp. 2: Impact of heat stress on different developmental stages

7.2.2.1 Plant materials and growth conditions

Two pea cultivars, CDC Golden and CDC Sage, were seeded in pots with 3.8 L volume (3 plants per pot) with Sunshine Gro® mix (Seba Beach, AB, Canada) and slow-release fertilizer (14-14-14, Type 100, Nutricote®, Brampton, Ontario). Plants were thinned to two plants per pot approximately two weeks after seeding. Plants received the first application (500 mL per pot) of half strength Hoagland's culture solution (Hoagland and Arnon, 1938) at three weeks after seeding and the second application (500 mL per pot) at the early flowering stage. Soil media moisture was monitored carefully; plants were watered if necessary to avoid drought stress (soil media was damp but not saturated). Plants were grown at 24/18°C day/night temperatures with 16/8 hr photoperiod and irradiance of 450-500 µmol photons m^{-2} s^{-1} in a growth chamber.

Three different regimes of high temperature duration and timing were evaluated in this study: heat exposure started when 1) small green buds (Stage I of Chapter 5) were visible at the first reproductive node, for 14 days; 2) flowers at the second reproductive node were fully open (Stage IV of Chapter 5), for 7 days; 3) flowers at the second reproductive node were fully open, for 14 days. Treatments 1 and 2 had eight replications, and 3 had five replications. One selected plant at each pot was considered one replication. The difference between the early timing when small green buds at the first reproductive node were visible and the later timing when flowers at the second reproductive node were fully open, was 5.5 days. All of these high temperature treatments had a corresponding control temperature regime where plants were grown at 24/18°C day/night temperatures throughout their life cycle. Conditions in the high temperature growth chamber were 16 h light with temperature increasing from 18°C by 3°C increments to 35°C over 5 h, maintained at 35°C for 6 h, and decreased over 5 h to 18°C in the dark. The control plants remained in the 24/18°C growth chamber. After the high temperature treatment periods of 7 or 14 days, heat-stressed plants were moved back to the control chamber to continue their growth.

7.2.2.2 Seed set parameters

For all three different temperature treatment regimes, the numbers of flowers and pods on the main stem were counted. Flower abortion rate was calculated by dividing the number of pods on the main stem by the number of flowers on the main stem. For the temperature regime of heat exposure for 14 days starting when small green buds at the first reproductive node were visible, measurements were the number of reproductive nodes, the number of lateral pods on the main stem, seed weight of the main stem, and seed yield per plant.

7.2.3 Data analyses

Statistical analyses of the number of pollen grains per anther, percentage of ROS accumulation in pollen grains, ovule viability, and seed set parameters were performed using the mixed procedure of SAS statistical software (version 9.3, SAS Institute, Inc., Cary, NC). Analysis of variance (ANOVA) with the Least Significant Difference (LSD) test ($P < 0.05$) was used. For the data of number of pollen grains per anther, temperature regime and heat exposure duration were considered as fixed effects, and replication and its interactions with fixed effects were considered as random effects. For the data of percentage of ROS accumulation in pollen grains, temperature regime was considered as a fixed effect, and replication and its interaction with temperature regime were

considered as random effects. For the data of ovule viability, cultivar, temperature regime, and heat exposure duration were considered as fixed effects, and replication and its interactions with fixed effects were considered as random effects. For flower number, pod number, flower abortion and other seed set parameters, cultivar and temperature regime were considered as fixed effects, and replication and its interaction with fixed effects were considered as random effects. The Kenwardroger was used to approximate the degrees of freedom for means.

7.3 Results

7.3.1 Exp. 1: Heat stress and heat exposure duration on pollen and ovule development

7.3.1.1 Number of pollen grains per anther after 4 and 7 days of heat exposure

High temperature and heat exposure duration had significant effects on the number of pollen grains per anther in CDC Golden (Table 7.1). The temperature regime of 35/18°C day/night temperatures reduced the number of pollen grains per anther by 35.6% from 3740 pollen grains per anther under control conditions to 2410 pollen grains per anther under heat stress (Table 7.1). The number of pollen grains per anther was significantly reduced after 7 days of heat exposure compared to 4 days of heat exposure (Table 7.1). The number of pollen grains per anther of flower buds at an upper (younger) reproductive node position was lower compared to a lower (more advanced) reproductive node position under both control and heat-stressed conditions. To be more specific, the number of pollen grains per anther was reduced from 4707 (buds from an upper node position) after 4 days of control conditions to 2773 (buds from a lower node position) after 7 days of the same control conditions (Table 7.1). Similarly, the number of pollen grains per anther was reduced from 3333 after 4 days of heat exposure to 1487 after 7 days of heat exposure (Table 7.1).

Table 7.1. Effects of high temperature and heat exposure duration on total number of pollen grains per anther of *Pisum sativum* cv. CDC Golden.

Effect	F value	*P* value
Day temperature (DT)	11.27	<0.01
Heat duration (HD)	22.76	<0.01
HD*DT	0.01	0.91
Day temperature	Number of pollen grains per anther	
24°C (control)	3740 a†	
35°C (heat)	2410 b	
Heat duration	Number of pollen grains per anther	
4 days	4020 a	
7 days	2130 b	
HD*DT	Number of pollen grains per anther	
4 days of control	4707 a	
4 days of heat	3333 b	
7 days of control	2773 b	
7 days of heat	1487 c	

†Means with a common letter within each section did not differ at $P < 0.05$.

7.3.1.2 Morphological changes in pollen grains after 7 days of heat exposure

Exposure to 35/18°C day/night temperatures for 7 days resulted in no or little cytoplasm in pollen grains in field pea (Figure 7.1). Exposure to heat stress for 4 days only did not cause any change in morphology of pollen grains (image not shown; heat exposed pollen grains were similar to that in Figure 7.1A) compared to the control pollen grains (Figure 7.1A).

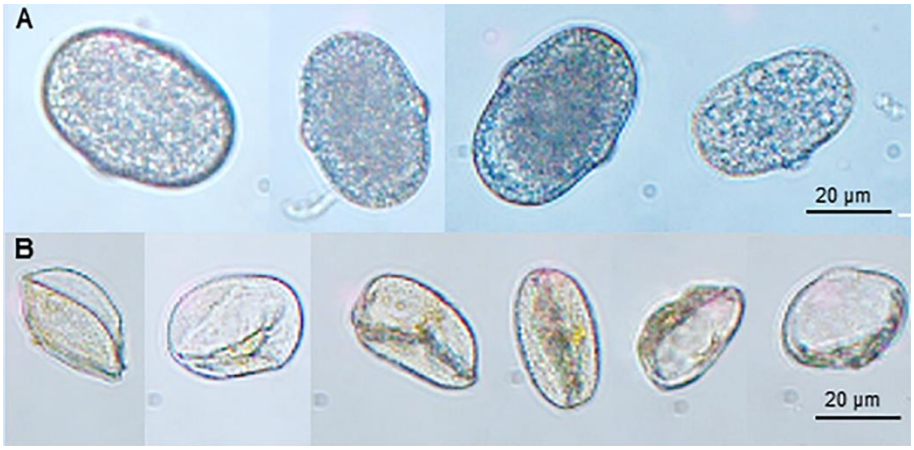

Figure 7.1. Comparison of pollen grains of *Pisum sativum* cv. CDC Golden sampled from Stage II-III buds produced by plants grown at A) control conditions (24/18°C day/night temperatures for 7 days) and B) heat-stressed conditions (35/18°C day/night temperatures for 7 days).

7.3.1.3 Accumulation of reactive oxygen species after 5 days of heat exposure

Despite the lack of visual changes in pollen of Stage III buds taken from heat-treated pollen after 5 days, heat stress significantly increased the accumulation of ROS in these pollen grains (Figure 7.2). The 35/18°C 5-d regime significantly increased pollen grains with bright fluorescence (Figure 7.2B) indicating the accumulation of ROS, with an average of 43% pollen grains detected with high ROS levels compared to 8% pollen grains detected with high ROS levels at the control temperature regime (24/18°C day/night temperatures for 5 days) in Figure 7.2C. The proportion of pollen grains displaying low levels of ROS accumulation averaged 40% in both control and high-temperature treated plants (Figure 7.2C), which was not significantly different. However, when all pollen grains displaying some ROS fluorescence activity were combined, the proportion of 82% from heat-treated plants significantly exceeded the proportion (50%) from control plants. The positive control was conducted using TBHP to induce ROS production in pollen grains and the result was similar to Figure 7.2B, with bright fluorescence detected in pollen grains.

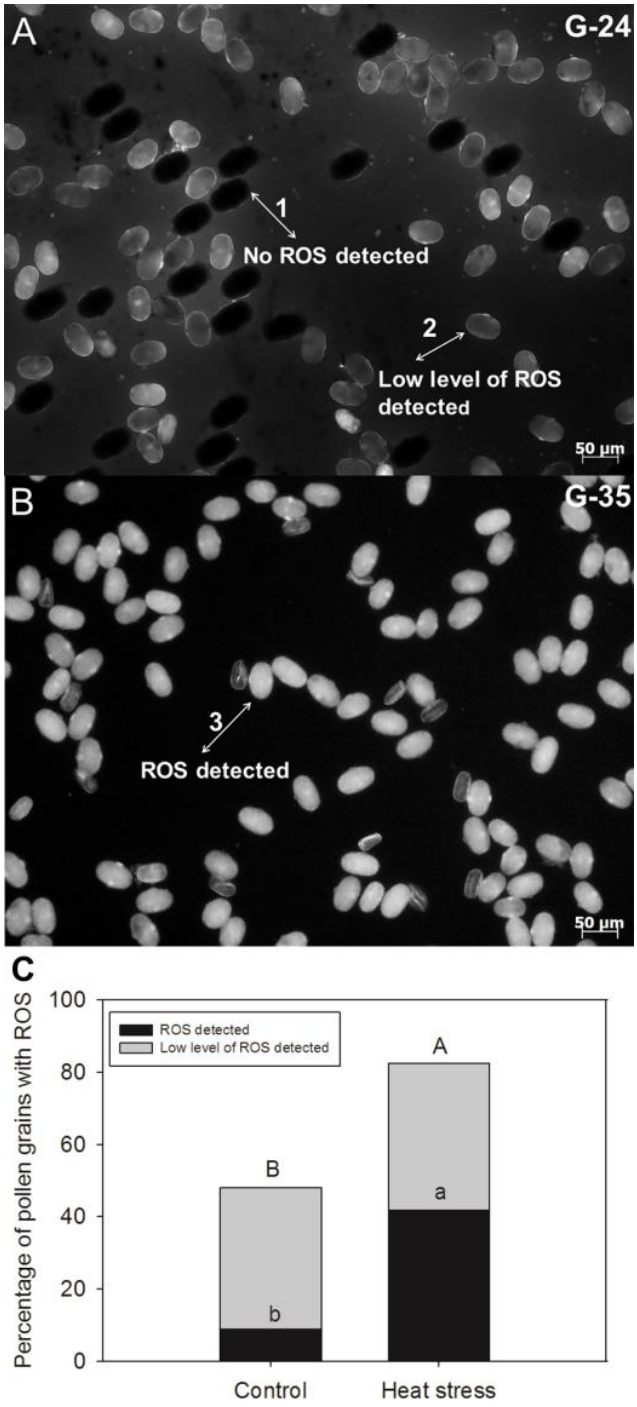

Figure 7.2. Effect of heat stress (35/18°C vs 24/18°C day/night temperatures for 5 days) on the accumulation of reactive oxygen species (ROS) detected within pollen grains from Stage III buds of field pea cv. CDC Golden using the carboxy-H_2DCFDA method. (A) Synthesis of ROS under control conditions; (B) Synthesis of ROS within pollen grains sampled from plants exposed to 5 days of high temperature; (C) Percentage of pollen grains with ROS accumulation. The bars are represented with mean values of six replications. Means with a common letter did not differ at $P < 0.05$.

7.3.1.4 Ovule viability

Ovule viability was determined by whether callose deposition was observed in ovules (Figure 7.3B) or not (Figure 7.3A). The duration of heat exposure had a significant effect on ovule viability (Table 7.2). Ovule viability was significantly reduced when the viability test was conducted using ovules of Stage III buds following 7 days of experimental treatment (Table 7.2). Heat stress for 4 and 7 days tended to reduce ovule viability, but the effect was not significant with the *P* value of 0.09 (Table 7.2). Among the total of 451 ovules examined, 25 ovules were observed with callose deposition and considered as non-viable ovules. Among these 25 non-viable ovules, 12%, 4%, and 84% of them were located at the stylar end, medial position, and basal end within a pod, respectively.

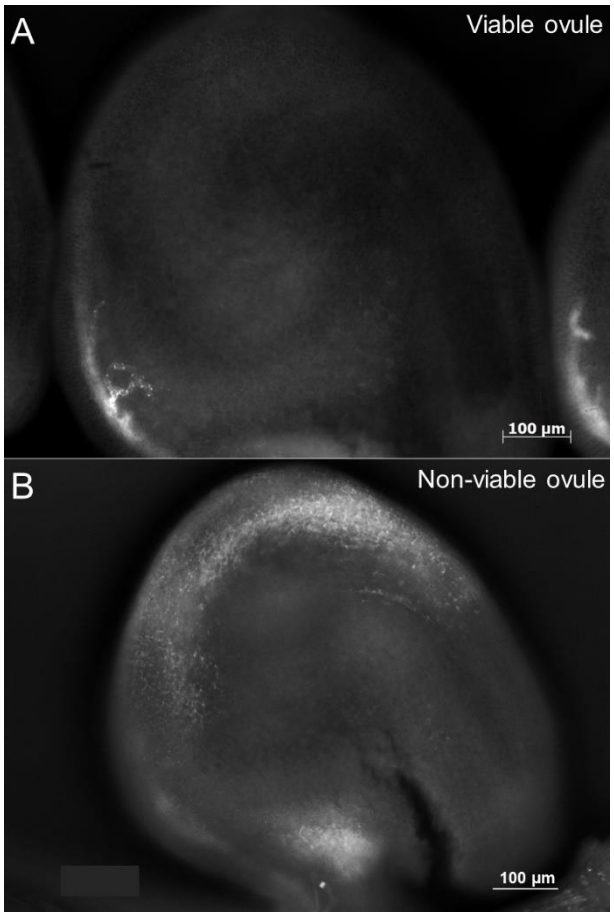

Figure 7.3. Determination of ovule viability in *Pisum sativum* after staining for callose with aniline blue using fluorescence microscopy. (A) Viable ovule without callose deposition in the ovule; (B) Non-viable ovule with callose deposition in the ovule. Scale bars = 100 µm.

Table 7.2. Effect of cultivar, heat stress, and heat exposure duration on ovule viability determined in ovules of Stage III buds following 4 or 7 days of exposure of high temperatures to pea plants (CDC Golden and CDC Sage) in growth chambers.

Effect	F value	*P* value
Cultivar (C)	0.03	0.87
Heat duration (HD)	5.93	0.02
Day temperature (DT)	2.91	0.09
C*HD	0.90	0.35
C*DT	2.60	0.11
HD*DT	0.03	0.86
C*HD*DT	0.46	0.50
Heat duration	Ovule viability (%)	
4 days	96.8 a†	
7 days	91.7 b	
Day temperature	Ovule viability (%)	
24°C	96.0 a	
35°C	92.4 a	

†Means with a common letter under each section did not differ at $P < 0.05$.

7.3.2 Exp. 2: Impact of heat stress on different developmental stages

Exposure to high temperature when small green buds were visible at the first reproductive node was more detrimental to reproductive output compared to heat exposure started later when flowers at the second reproductive node were fully open (Table 7.3). Exposure to heat stress when young floral buds were visible at node 1 caused greater flower abortion compared to the control flowers, with flower abortion rates of 60.1% and 12.9% for high temperature and control temperature regimes, respectively (Table 7.3). Similarly, the number of flowers and pods on the main stem were more negatively affected when high temperature exposure started earlier compared to when flowers at node 2 were fully open (Table 7.3). Exposure to heat stress for either 7 or 14 days when flowers at node 2 were fully open did not result in less flowers and pods on the main stem compared to control plants (Table 7.3). CDC Golden had more pods than CDC Sage when heat stress began at node 1 or 2 for 14 days (Table 7.3).

Table 7.3. Effect of different heat exposure timing and duration on reproductive development in pea plants (CDC Golden and CDC Sage).

Effect	Heat stress began at 1st fruiting node for 14 days†			Heat stress began at 2nd fruiting node for 7 days†			Heat stress began at 2nd fruiting node for 14 days‡		
	Flower number	Pod number	Flower abortion rate (%)	Flower number	Pod number	Flower abortion rate (%)	Flower number	Pod number	Flower abortion rate (%)
Cultivar									
CDC Golden	9	6a§	33.7	8	6	21.4	8	6a	25.0
CDC Sage	9	5b	40.0	8	6	13.3	6	4b	31.6
Day temperature (DT)									
24°C	7b	6a	12.9b	8	6	16.8	7	5	28.5
35.5±0.5°C	11a	4b	60.1a	8	7	17.8	7	5	25.4
P values from ANOVA									
Cultivar (C)	0.94	**0.047¶**	0.10	0.46	0.38	0.30	0.15	**0.049**	0.45
Day temperature (DT)	**<0.001**	**<0.001**	**<0.001**	0.77	1.00	0.90	0.15	0.39	0.83
C*DT	0.14	0.55	0.16	0.24	0.51	0.25	0.49	0.18	0.57

†Data of columns 2-7 were analyzed based on eight replications;
‡Data of columns 8-10 were analyzed based on five replications;
§Means across eight replications with a common letter within each column under each section did not differ at $P < 0.05$.
¶The *P* values in bold type indicate that the main effect or interaction was statistically significant ($P < 0.05$).

Heat-stressed plants exhibited a compensatory response, producing more reproductive nodes and lateral branch pods compared to control plants on removal of heat stress (Table 7.4). Although heat-stressed plants produced more reproductive nodes and lateral branch pods, seed yield loss was observed due to more flower abortion under heat stress (Table 7.3, 7.4). Pea plants aborted flowers under high temperature stress and set seeds when adverse growth conditions ended owing to its indeterminate type risk strategy.

Table 7.4. Effect of heat stress for 14 days on reproductive development in pea plants (CDC Golden, CDC Sage) first exposed when small green buds at the first reproductive node were visible.

	Number of reproductive nodes	Lateral pods on main branch	Seed weight of main branch (g)	Seed yield (g plant^{-1})
Cultivars				
CDC Golden	8a†	6a	6.9b	14.3b
CDC Sage	8a	6a	8.6a	18.1a
Day temperature (DT)				
24°C	6b	4b	9.1a	20.0a
35°C	10a	7a	6.4b	12.1b
P values from ANOVA				
Cultivars (C)	0.20	0.60	0.13	**0.001**
Day temperature (DT)	**<0.001**	**0.04**	**0.002**	**<0.001**
C*DT	0.09	0.79	0.81	0.79

†Means across eight replications with a common letter within each column under each section did not differ at $P < 0.05$.
‡The *P* values in bold type indicate that the main effect or interaction had a significant effect on the response.

7.4 Discussion

Heat stress reduced the number of pollen grains per anther in field pea. A similar phenomenon of less pollen produced due to heat stress has been reported in other crops. For example, high temperature reduced pollen production per flower in chickpea (Devasirvatham et al., 2012). The number of pollen grains per flower decreased from approximately 2000 to almost zero when air temperatures increased from 28/18°C to 40/30°C day/night temperatures in kidney bean (Prasad et al., 2002). Similarly, the number of pollen grains per flower reduced from 700,000 to less than 400,000 when temperatures increased from 28/22 to 32/26°C day/night temperatures in tomato (Pressman et al., 2002).

In field pea, heat stress induced smaller pollen grains with little cytoplasm when exposed to elevated daytime temperature for 7 days in the current research. Similarly, barley exposure to 30/25°C day/night temperatures for 5 days at the stage of early panicle differentiation resulted in no or little cytoplasm in pollen grains (Sakata et al., 2000). Moreover, heat stress caused pollen abnormalities such as small, shrunken, and empty pollen grains in legume crops (Devasirvatham et al., 2012). In contrast, my previous study indicated that exposure to high temperature at 35/18°C day/night temperatures for 4 days failed to cause any morphological changes in pollen grains and pollen surface structure in field pea (Jiang et al., 2015). One potential explanation was due to the difference in heat exposure duration. Heat exposure duration in plants reported in Jiang et al. (2015) was 4 days at 35/18°C day/night temperatures, whereas abnormal pollen morphology observed in this chapter was due to 7 days of exposure to 35/18°C day/night temperatures. Another possible explanation is that the plant phenology when heat stress started was different in these two studies. In this chapter, pea plants were exposed to high temperature when small green buds were visible at the first reproductive node, node 1. In the previous research by Jiang et al. (2015), the high temperature treatment started when flowers at node 2 were fully open. Possibly, early flower initiation was more sensitive to heat stress compared to more advanced flower and pollen development in field pea. The uninucleate stage of pollen development has been reported to be highly sensitive to elevated temperatures in many crop species (Sage et al., 2015 and references therein). Pre-fertilization stages are more sensitive to heat stress compared to post-fertilization stages (Gross and Kigel, 1994). Gametogenesis, some 8-9 days before anthesis, and fertilization, some 1-3 days after anthesis, are the most sensitive stages to heat stress in various plants (Foolad, 2005).

Callose deposition was displayed in aborted ovules in field pea (Briggs et al., 1987), which indicated that examining callose deposition in ovules is a useful tool of testing ovule viability in this chapter. Callose synthesis was probably due to a shift in carbohydrate metabolism owing to the disturbance of normal ovule development (Vishnyakova, 1991), and callose deposition is viewed as an initial symptom of ovule abortion and embryo senescence at an early stage (Vishnyakova, 1991; Sun et al., 2004; Teng et al., 2006). Callose synthesis can be induced by pathogen infection, wounding, mechanical stress, and abiotic stress (Stass and Horst, 2009). Heat stress tended to reduce ovule viability of field pea. This observation was in line with the previous publications that male floral organs are more sensitive to heat stress compared to their female

counterparts in chickpea (Devasirvatham et al., 2013), common bean (Monterroso and Wien, 1990), and barley (Sakata and Higashitani, 2008). In contrast, a reduction in ovule viability due to heat stress has been reported in various crops (Kaushal et al., 2016). Heat stress has decreased ovule number and viability in common bean (Monterroso and Wien, 1990), tomato (Din et al., 2015) and *Arabidopsis thaliana* (Whittle et al., 2009).

Heat stress increased the accumulation of ROS in pollen grains in field pea. The same phenomenon of ROS synthesis in pollen grains due to heat stress has been reported in sorghum (Prasad and Djanaguiraman, 2011) and winter wheat (Narayanan et al., 2015). Prasad and Djanaguiraman (2011) suggested that intensive oxidative damage in pollen grains due to heat stress was likely to decrease pollen viability and seed set in sorghum. Heat stress also increased ROS accumulation in leaves in sorghum (Djanaguiraman et al. 2010; Prasad and Djanaguiraman, 2011) and winter wheat (Narayanan et al., 2015). The increased accumulation of ROS in leaves may itself reduce photosynthetic rate via the inhibition of photosynthetic enzyme activity (Takeda et al., 1995).

In the present study, heat stress for 14 days increased flower abortion in field pea when small green buds at the first reproductive node were only just visible. Similarly, an increase in abortion frequency of pea floral buds and flowers due to heat stress has been documented (Guilioni et al., 1997). High night temperatures during flowering increased the rate of flower production per plant in cotton, whereas no increase was seen in the final number of reproductive organs because abortion of flowers was high compared to the control regime (Echer et al., 2014). However, I did not observe an increase in flower abortion when pea plants were exposed to high temperature when flowers were already fully open at node 2. It took 5.5 days for pea plants to develop from small green buds at node 1 to fully open at node 2. More advanced flowers were less sensitive to heat stress compared to young floral buds in field pea seen in my work and Guilioni et al. (1997).

I also observed a compensatory strategy of pea plants producing more reproductive nodes and lateral pods after heat stress ended. Due to increased flower abortion under heat stress, however, seed yield per plant under heat stress was lower compared to seed yield under control conditions. Heat-stressed flax plants also showed a compensatory response via producing flowers and bolls at a greater rate compared to control plants upon removal of heat stress (Cross et al., 2003). The compensatory growth after stresses such as herbivory and defoliation has been well documented

(McNaughton, 1983; Lone et al., 2008). For example, photosynthetic compensation was observed in mustard (*Brassica juncea*) in response to defoliation (removal of leaves); the photosynthetic capacity was improved by removing lower and senescing leaves (Lone et al., 2008).

7.5 Conclusions

The research hypotheses of this study were accepted that an early flowering stage was more sensitive to heat stress compared to a later flowering stage, and longer duration of heat exposure would cause more detrimental effects on reproductive development. Heat stress reduced the number of pollen grains per anther, induced smaller pollen grains with little or no cytoplasm, and increased the accumulation of ROS in pollen grains. Heat stress also tended to decrease ovule viability. High temperature exposure when young floral buds were visible at the first reproductive node was more damaging compared to heat exposure started later when flowers at the second reproductive node were fully open. Negative effects were 1) the flower abortion was greater; 2) seed-set, pod development, and yield were reduced more when high temperature exposure started earlier. In summary, due to the staggered timing of flower appearance and age on a nodal raceme and along the main stem, heat stress impacted young, barely visible floral buds in the developing apex the most compared with more advanced buds and open flowers. Further research refining the interaction between timing and intensity of stress such as heat and drought can be added to crop simulation models to make them applicable to predict yield reductions from stress.

Transition section between Chapter 7 and Chapter 8

The majority of previous experiments (from Chapter 3 to Chapter 7) in my PhD project were focused on heat stress effects on two pea cultivars, CDC Golden and CDC Sage, although 16 cultivars were evaluated in Chapter 6 to explore the relationship between seed development and ovule position, and to screen for heat-tolerant cultivars. In Chapter 8, I continued to screen heat-tolerance by including 24 cultivars using an *in vitro* pollen germination assay.

Chapter 8 Differences in *in vitro* pollen germination and pollen-tube growth of pea cultivars in response to high temperature

8.1 Introduction

Extreme weather events due to global warming are one of the major limitations to crop production worldwide. To maintain yields, the importance of developing heat-tolerant cultivars for rising ambient temperatures cannot be underestimated. Elevated temperatures adversely affect pollen germination, pollen-tube growth, ovule viability, the number of pollen grains retained by stigma, fertilization, and the growth of endosperm, pre-embryo, and fertilized embryo in various crops (Kaushal et al., 2016). Although both male and female reproductive organs are sensitive to heat stress in tomato, ovules could be less sensitive to heat stress compared to pollen in other crops (Peet et al., 1998).

In vitro pollen germination percentage has been used as a screening criterion for heat tolerance (Kakani et al., 2002, 2005; Salem et al., 2007; Song et al., 2015; Singh et al., 2016). Seed set percentage was positively correlated with *in vitro* pollen germination in sorghum (*Sorghum bicolor*; Singh et al., 2016). Studies of *in vitro* pollen germination have been conducted on soybean (*Glycine max*; Salem et al., 2007), groundnut (*Arachis hypogaea*; Kakani et al., 2002), chickpea (*Cicer arietinum*; Devasirvatham et al., 2012), field pea (*Pisum sativum*; Petkova et al., 2009; Jiang et al., 2015), and non-legumes like cotton (*Gossypium*; Kakani et al., 2005), to test high temperature effects on pollen germination and pollen-tube length. The method of assessing *in vitro* pollen germination uses pollen growth on agar, supplemented with sugar, boron, and calcium concentrations that are essential for *in vitro* pollen germination and pollen-tube growth. Pollen germination is closely related to the stability of pollen cell membranes under high temperature (Kakani et al., 2002). Cardinal temperatures (T_{min}, T_{opt}, and T_{max}) for *in vitro* pollen germination in cotton were 15.0, 31.8, and 43.3°C, respectively (Kakani et al., 2005). Similarly, the cardinal temperatures (T_{min}, T_{opt}, and T_{max}) were observed to be 14.1, 34.4, and 43.0°C for *in vitro* pollen germination in groundnut (Kakani et al., 2002). The maximum temperature for *in vitro* pollen germination in sorghum was 43.2°C (Singh et al., 2016).

Understanding the genotype × environment (G×E) interaction would facilitate breeders to select superior and stable genotypes across a wide range of environments (DeWitt and Scheiner, 2004;

Marjanović-Jeromela et al., 2011; Sabaghnia et al., 2008; Sadras and Richards, 2014). The additive main effects and multiplicative interaction (AMMI) model is a combination of analysis of variance (ANOVA) and principal component analysis (PCA) that partitions the variability of genotype, environment, and the G × E interaction (Marjanović-Jeromela et al., 2011; Sabaghnia et al., 2008). The AMMI biplot analysis is an effective method to determine patterns of the G × E interaction graphically (Thillainathan and Fernandez, 2001). Stability describes the stable performance of a specific genotype over a wide range of environments. A desirable genotype is expected to have a uniform and superior phenotypic performance. The phenotypic value of an individual can be described as: $P_{ij} = \mu + g_i + e_j + ge_{ij} + r_{ij}$, where μ is the grand mean, g_i is the genotypic effect of the i^{th} family, and e_j is the effect of macro environment j, and ge_{ij} is the effect of the interaction between genotype and the j^{th} macro environment. The value r_{ij} represents the residual deviation that cannot be explained by genotype, environment, and the G × E interaction.

I hypothesized that heat stress would reduce in vitro pollen germination and pollen-tube growth, and pea cultivars would differ with respect to these two traits. The objectives of this study were to test the effects of high temperature on *in vitro* pollen germination and pollen-tube growth, as well as to evaluate genotypic differences among 24 pea cultivars grown in growth chambers and under field conditions (Sutherland in 2014, Rosthern in 2015). The goal was also to determine the impact of the G × E interactions on *in vitro* pollen germination to facilitate the selection of heat-tolerant and stable cultivars.

8.2 Materials and methods

8.2.1 In vitro pollen germination

Twenty-four diverse pea cultivars with different leaf types, flower colors, and cotyledon colors from different origins were tested in this study (Table 8.1). The plants were grown in experimental fields at Sutherland, Saskatchewan in 2014 (with four replications from the POYT-HEAT trial) and Rosthern, Saskatchewan in 2015 (with three replications from the PAM trial) with a randomized complete block design (RCBD), as well as in a growth chamber in 2015 (University of Saskatchewan Phytotron). At Sutherland in 2014, plots were seeded in early May, and plants were harvested at the end of August. The monthly mean temperatures were 10.1, 14.1, 18.3, and 17.9°C in May, June, July, and August in 2014, respectively. The average maximum temperatures were 17.3, 19.4, 24.5, and 24.6°C in May, June, July, and August in 2014, respectively. The number

of days when the maximum temperatures exceeded 28°C was 17 days during the growing season in 2014. The total precipitation was 61.1, 94.8, 44.5, 18.5, and 218.9 mm for May, June, July, August, and the period from May to August in 2014, respectively. At Rosthern in 2015, the seeding date was May 13, and plants were harvested at the end of August. The monthly mean temperatures were 9.4, 16.4, 18.8, and 16.9°C in May, June, July, and August in 2015, respectively. The average maximum temperatures were 17.8, 24.1, 25.8, and 23.8°C in May, June, July, and August in 2015, respectively. The number of days when the maximum temperatures exceeded 28°C was 28 days during the growing season in 2015. The total precipitation was 10.9, 37.4, 80.5, 75.9, and 204.7 mm for May, June, July, August, and the period from May to August in 2015, respectively.

For the growth chamber experiment, seeds were planted in 3.8 L volume pots (three plants per pot) with Sunshine Gro® mix (Seba Beach, AB, Canada) and slow-release fertilizer (14-14-14, Type 100, Nutricote®, Brampton, Ontario). The pot dimensions were 15.9 cm depth and 16.5 cm diameter. Pots were thinned to two plants per pot approximately two weeks after seeding. Plants received the first application (500 ml per pot) of half strength modified Hoagland's culture solution (Hoagland and Arnon, 1938) at two weeks after seeding and the second application (500 ml per pot) at the early flowering stage. Soil medium moisture was monitored carefully; plants were watered if necessary to avoid drought stress. Plants were grown at 24/18°C day/night temperatures with 16/8 hr photoperiod and irradiance of 450-500 µmol photons m^{-2} s^{-1} from cool fluorescent tubes. The relative humidity had a range of 4-8% in growth chambers.

Table 8.1. List of pea cultivars and their origins, leaf types, flower color, and cotyledon color. Data are provided by the Pulse Breeding Program at the Crop Development Centre at the University of Saskatchewan.

Genotype	Origin	Leaf type	Flower color	Cotyledon color
40-10	Germany	N§	Purple	Yellow
TMP 15213	eastern Europe	N	Red	Yellow
MFR043	CDC†, Canada	N	White	Green
Mini	USA	N	White	Green
MPG87	AAFC‡, Canada	N	White	Green
Naparnyk	eastern Europe	N	White	Yellow
Rally	USA	N	White	Green
Superscout	USA	N	White	Yellow
TMP 15116	eastern Europe	N	White	Yellow
TMP 15181	eastern Europe	N	White	Mix
TMP 15202	eastern Europe	N	White	Green
Torsdag	western Europe	N	White	Yellow
Kaspa	Australia	S¶	Cream	Yellow
03H107P04HO2026	Australia	S	Pink	Yellow
03H26704HO2006	Australia	S	Pink	Yellow
CDC Vienna	CDC, Canada	S	Red	Yellow
Aragorn	USA	S	White	Green
CDC Golden	CDC, Canada	S	White	Yellow
CDC Meadow	CDC, Canada	S	White	Yellow
CDC Sage	CDC, Canada	S	White	Green
Delta	western Europe	S	White	Yellow
Eclipse	western Europe	S	White	Yellow
TMP 15179	eastern Europe	S	White	Yellow
TMP 15206	eastern Europe	S	White	Yellow

†Crop Development Centre at University of Saskatchewan;
‡Agriculture and Agri-Food Canada;
§Normal leaf;
¶Semi-leafless or afilia trait;

At Sutherland in 2014, four replications of pollen germination and pollen-tube growth were conducted during four consecutive days with one replication (24 cultivars × two temperature regimes) completed each day. Fresh pollen grains were collected from anthers of slightly open flowers (Stage III; Figure 5.1H), just after anther dehiscence but prior to anthesis, between 7:00 - 9:00 am. *In vitro* pollen germination and pollen-tube growth were evaluated using a pollen

germination medium. The medium consisted of 15 g sucrose ($C_{12}H_{22}O_{11}$), 0.03 g calcium nitrate [$Ca(NO_3)_2 \cdot 4H_2O$], and 0.01 g boric acid (H_3BO_3) dissolved in 100 mL of deionized water (Salem et al., 2007; Lahlali et al., 2014; Jiang et al., 2015). To solidify the medium, 0.5 g of agar per 100 mL solution was added. Fresh pollen grains were dusted onto the germination medium on microscope slides, and then microscope slides were placed individually in 9 cm diameter petri dishes with moistened filter paper. A piece of filter paper was placed in the inner layer of the lid of the petri dish to absorb the moisture. The lids of petri dishes were sealed with Parafilm® to maintain high humidity. The slides were placed in two growth chambers under constant light conditions with the irradiance of 450-500 µmol photons m^{-2} s^{-1} where one chamber was set at 24°C and the other set at 36°C. After 24 hours of incubation, pollen grains (100 grains × 4 replications × 2 temperature incubation treatments) were counted for germination rate using direct microscopic observation at 100-200× magnification. Germinated pollen grains were determined when the length of the pollen tube was greater than the diameter of the pollen grain (Salem et al., 2007). Pollen germination was calculated by dividing the number of germinated pollen grains by the total number of pollen observed and expressed as a percentage. Pollen-tube length of 30 randomly selected pollen grains was measured with the computer software Carl Zeiss AxioVision (Release 4.8.2, Zeiss International, Toronto, ON, Canada).

For samples collected from field plots at Rosthern, SK, in 2015, two replications for each treatment (24 cultivars × two temperature regimes, i.e., 24°C and 42°C) were conducted using the same method above with one modification: pollen grains were incubated in darkness instead of under light for 24 hours. For samples collected from plots grown in the growth chambers, two replications for each treatment (24 cultivars for each of three temperature treatments, i.e., 24°C, 37°C, and 42°C) were conducted under dark conditions for 24 hours.

8.2.2 Seed-set parameters

Seven of these 24 cultivars, including 40-10, CDC Golden, CDC Meadow, CDC Sage, MFR043, Naparnyk, and TMP 15213, were also tested under control and heat-stressed conditions with 3 replications in growth chambers in Experiment 4 of Chapter 6. The detailed description of the experimental design was documented of Chapter 6. Total seed weight on the main stem, duration of flowering, thousand seed weight, and the number of reproductive nodes, flowers, pods, seeds, and ovules on the main stem were measured. Pod length, the number of seeds per pod, the number

of ovules per pod, seed-to-ovule ratio within a pod, and seed weight per pod were also measured based on the first four reproductive nodes. Pod retention was calculated by dividing the number of pods by the number of flowers. Seed retention was calculated by dividing the total number of seeds on the main stem by the total number of ovules on the main stem.

8.2.3 Data analyses

Data from the three environments (2014 Sutherland, 2015 Rosthern, and the 2015 growth chamber) were analyzed separately because genotypic performance was not consistent in these three environments and the high temperature regimes with these three environments were different. Statistical analyses were performed using the Mixed procedure of SAS version 9.3 statistical software (SAS Institute, Inc., Cary, NC). Analysis of variance (ANOVA) was used, and means declared different at $P < 0.05$. The effects of cultivar and temperature treatments were considered as fixed effects, and replication and its interactions with the fixed effects were considered as random effects.

Analysis of cultivar differences at the control and high temperature regimes was then conducted separately, because the main objective of this study was to screen cultivars in terms of pollen germination percentage under high temperature. The G × E interaction (21 cultivars and four temperature regimes: 2014 field samples with the 36°C incubation temperature under light conditions; 2015 growth chamber samples with the 37°C incubation temperature in the dark; 2015 growth chamber samples with the 42°C incubation temperature in the dark; and 2015 field samples with the 42°C incubation temperature in the dark) was assessed using the AMMI model using SAS, as described in Thillainathan and Fernandez (2011). Due to missing data for three cultivars, data from 21 out of 24 cultivars were used in the analysis of the G × E interaction.

Correlation coefficients (r) between the average of *in vitro* pollen germination and the average of seed set parameters were analyzed for the control and heat-stressed treatments separately in seven cultivars tested in both Experiment 6 (Chapter 8) and Experiment 4 (Chapter 6). The average of *in vitro* pollen germination was calculated across 4 replications for the control and heat-stressed treatments for flower samples collected from Sutherland in 2014, and the average of seed-set parameters were calculated across 3 replications for the control and heat-stressed treatments in growth chambers.

8.3 Results

8.3.1 In vitro pollen germination and pollen-tube length

Temperature regimes (control and high temperature) and cultivars had significant effects on *in vitro* pollen germination (Table 8.2). *In vitro* pollen germination decreased substantially with an increase in temperature for samples collected from three different environments (Table 8.2). Genotypic difference in *in vitro* pollen germination among the 24 pea cultivars is shown in Figures 8.1, 8.3, and 8.4.

Table 8.2. Effect of temperature regimes (24, 36, 37, 42°C for 24 h) and cultivars on *in vitro* pollen germination (PG) percentage.

	P value in 2014 field	*P* value in 2015 field		*P* value in 2015 growth chamber	
Temperature (T)	<0.01	T	<0.01	T	<0.01
Cultivar (C)	<0.01	C	<0.04	C	<0.01
T*C	0.11	T*C	0.05	T*C	0.05
T (°C)	PG (%)	T (°C)	PG (%)	T (°C)	PG (%)
24	81.6 a†	24	94.7 a	24	89.6 a
36	30.8 b	42	34.2 b	37	36.9 b
				42	6.0 c
LSD	5.8	LSD	9.3	LSD	8.2

†Means with a common letter within each column did not differ at $P < 0.05$.

For pollen samples collected from Sutherland in 2014, the high temperature regime (36°C for 24 hours in the light) significantly reduced *in vitro* pollen germination percentage (Figure 8.1) and pollen-tube length (Figure 8.2) in all 24 pea cultivars. Cultivar 40-10 had the highest pollen germination percentage at 36°C among these 24 cultivars, similar to TMP15181, 03H26704HO2006, CDC Sage, Torsdag, TMP15206, Rally, Delta, 03H107P04HO2026, Naparnyk, CDC Meadow, and TMP15202. CDC Vienna had the lowest percentage pollen germination among these 24 genotypes at 36°C (Figure 8.1). Under the control conditions (24°C for 24 h incubation), Superscout had a low *in vitro* pollen germination percentage, similar to Kaspa and Torsdag but these three were significantly lower than the other 21 cultivars (Figure 8.1). Under the control temperature regime (24°C for 24 h incubation), CDC Sage had the longest pollen-tube length, similar to Mini, 40-10, Eclipse, CDC Meadow, TMP15213, Aragorn, TMP15179, and

MFR043, and significantly greater than the other 15 cultivars (Figure 8.2). Pollen-tube length was substantially reduced at 36°C incubation compared to 24°C incubation. Pollen-tube length of successfully germinated pollen held at 36°C did not differ among these 24 cultivars (Figure 8.2), therefore, pollen-tube length was not measured for flower samples collected from Rosthern and from the growth chamber in 2015.

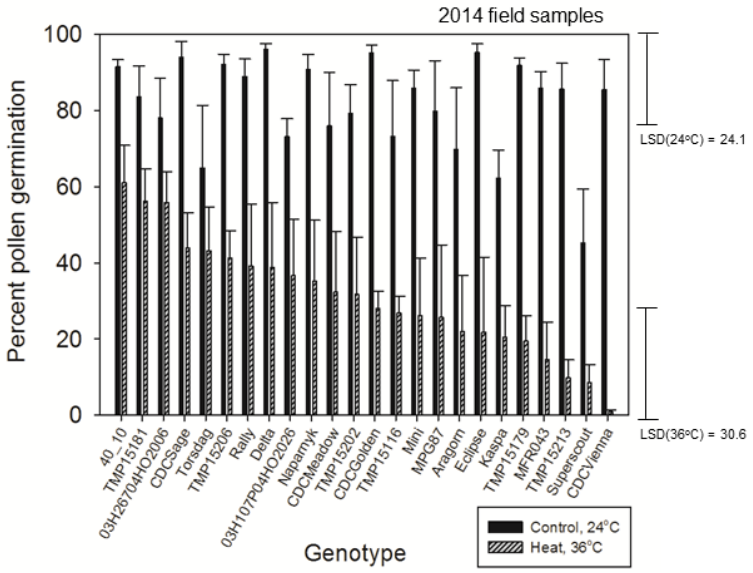

Figure 8.1. Percentage *in vitro* pollen germination ($\bar{x}$±S.E.) of pea cultivars grown at Sutherland in 2014 after 24 hours of incubation at 24°C and 36°C under light conditions. Each bar represents the mean value of four replications with standard error bars. Data were arranged by genotype from the highest pollen germination percentage to the lowest value at 36°C. The LSD values were 24.1 and 30.6 for the 24°C and 36°C treatments, respectively.

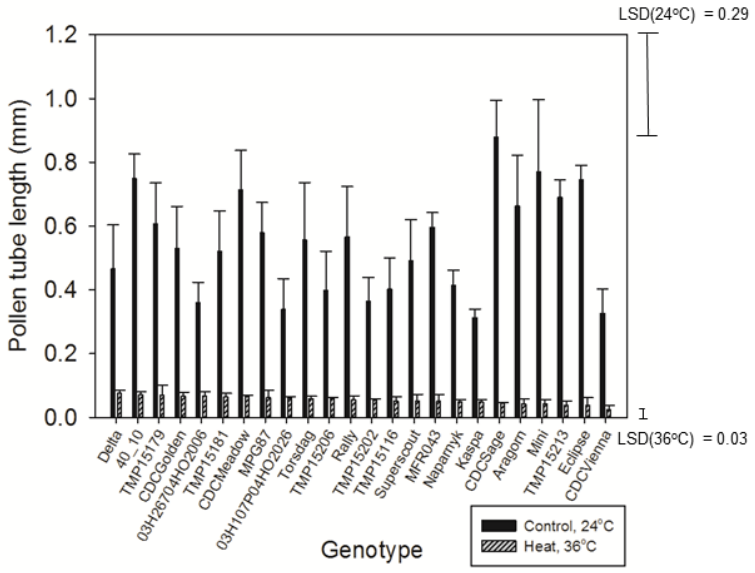

Figure 8.2. Pollen-tube length (x̄±S.E.) of pea cultivars grown at Sutherland in 2014 after 24 hours of incubation at 24°C and 36°C under light conditions. Each bar represents the mean value of four replications with standard error bars. Data were arranged by genotype from the highest pollen-tube length to the lowest value at 36°C. The LSD values were 0.29 and 0.03 for the 24°C and 36°C treatments, respectively.

Genotypic difference in *in vitro* pollen germination percentage under high temperature regimes varied greatly for pollen samples collected from these three environments (Figures 8.1, 8.3, and 8.4). For pollen samples collected from the growth chamber, 40-10 had the highest pollen germination percentage, similar to Rally, TMP 15179, CDC Sage, MFR043, Mini, Delta, and 03H107P04HO2026. In contrast, Eclipse, Naparnyk, CDC Vienna, and TMP 15202 had the lowest pollen germination percentages, similar to CDC Golden, CDC Meadow, TMP 15213, TMP 15116, MPG87, TMP 15181, Aragon, 03H26704HO2006, Kaspa, TMP 15206, Torsdag, 03H107P04HO2026, Delta, and Mini (Figure 8.3). For pollen samples collected from Rosthern in 2015, CDC Sage had the highest pollen germination percentage at 42°C, similar to Delta, Naparnyk, CDC Golden, CDC Vienna, Mini, 03H107P04HO2026, TMP 15206, 40-10, MFR043, and TMP 15181 (Figure 8.4). In contrast, TMP179, TMP15116, Kaspa, and TMP 15202 had the lowest pollen germination percentage at 42°C, the same as Superscout, Torsdag, MPG87, TMP 15213, Eclipse, Aragorn, Rally, TMP 15181, MFR043, 40-10, TMP 15206, 03H107P04HO2026, Mini, and CDC Vienna (Figure 8.4).

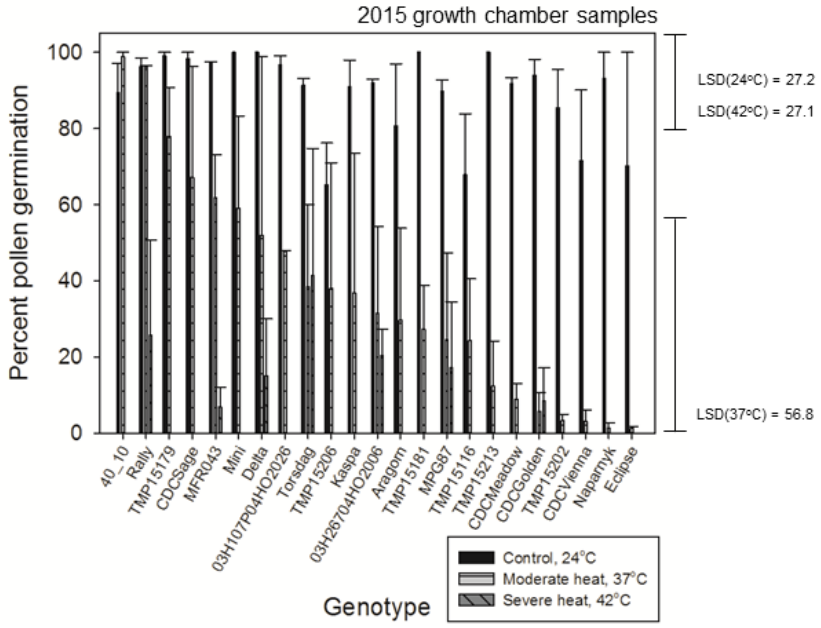

Figure 8.3. Percentage *in vitro* pollen germination (x̄±S.E.) of pea cultivars grown in the growth chamber after 24 hours of incubation at 24°C, 37°C, and 42°C in dark. Each bar represents the mean value of two replications with standard error bars. Data were arranged by genotype from the highest pollen germination percentage to the lowest value at 37°C. The LSD values were 27.2, 56.8, and 27.2 for the 24°C, 37°C, and 42°C treatments, respectively.

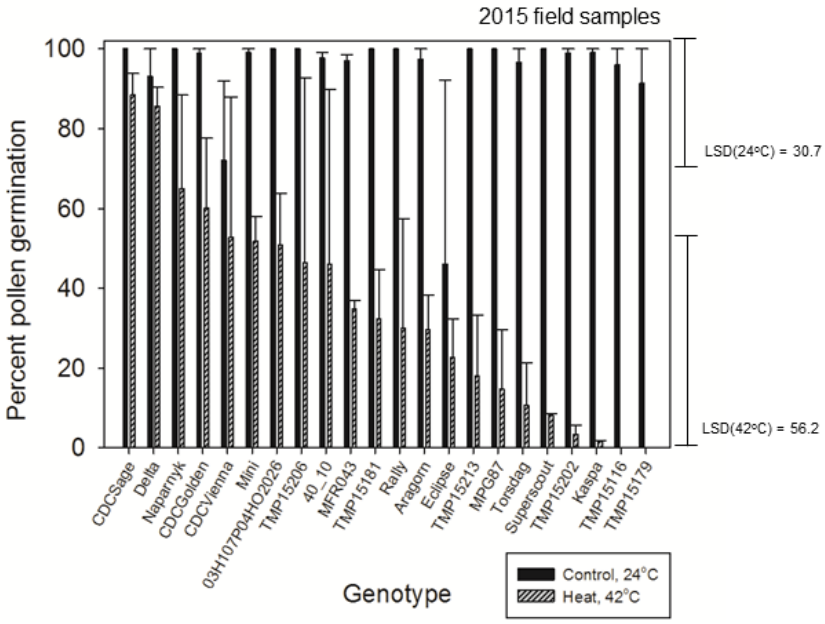

Figure 8.4. Percentage *in vitro* pollen germination (x̄±S.E.) of pea cultivars grown at Rosthern in 2015 after 24 hours of incubation at 24°C and 42°C in dark. Each bar represents the mean value of two replications with standard error bars. Data were arranged by genotype from the highest pollen

germination percentage to the lowest value at 42°C. The LSD values were 30.7 and 56.2 for the 24°C and 42°C treatments, respectively.

8.3.2 The G × E interaction for pollen germination under different temperature regimes

The first and second principal component (PC1 and PC2) scores for the G × E interaction for *in vitro* pollen germination percentage under high temperature regimes are shown in the Biplot (Figure 8.5). The positions of genotypes and environments in the Biplot played an important role in the G × E interaction. The genotypes and environments near the horizontal and vertical reference lines, x=0 and y=0, were considered to have small interactions and high stability in pollen germination percentage. Pollen germination percentage for samples collected from the growth chamber in 2015 and incubated at 42°C ("2015chamber, 42°C" in Figure 8.5) and from Sutherland in 2014 and incubated at 36°C ("2014field, 36°C" in Figure 8.5) was relatively stable compared to pollen samples collected from Rosthern in 2015 and incubated at 42°C ("2015field, 42°C" in Figure 8.5), and from the growth chamber in 2015 and incubated at 37°C ("2015chamber, 37°C" in Figure 8.5). Aragorn was the most stable cultivar among the cultivars tested, because it was located close to the center of the Biplot (x=0, y=0) in Figure 8.5. However, the pollen germination percentage of Aragorn was not superior among these 24 cultivars with an average of 30% under high temperature regimes (Figures 8.1, 8.3, and 8.4). TMP15206 was the second closest to the center of the Biplot (Figure 8.5), and it had approximately 40% of pollen germination under high temperature (Figures 8.1, 8.3, and 8.4). Pollen germination of Torsdag, TMP 15116, and Kaspa was negatively correlated with the environment of "2015 field, 42°C" since they were opposite from each other in the Biplot (Figure 8.5), which means that the environmental conditions "2015 field, 42°C" were unfavorable for pollen germination percentage in these three cultivars. In general, this Biplot provided information on relative stability and trends of similarity or dissimilarity in genotypes and environments.

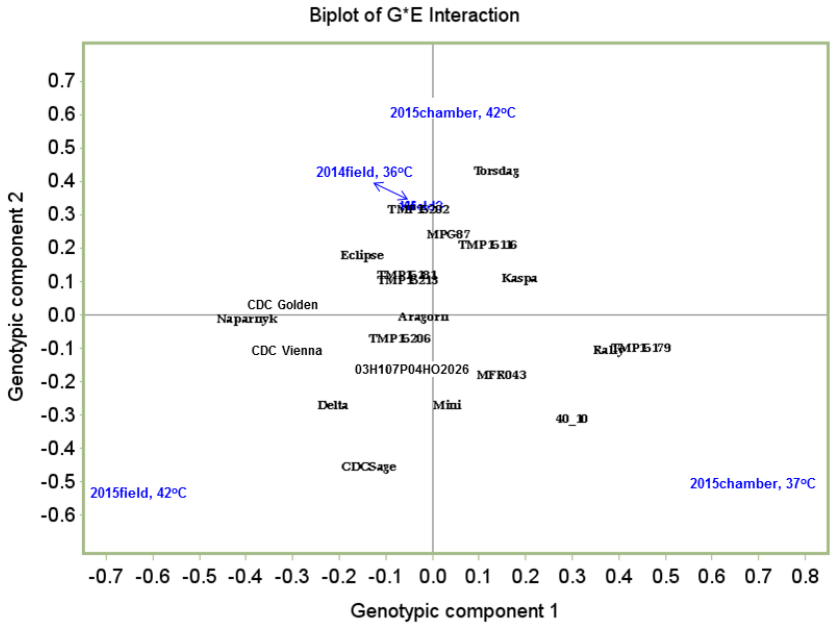

Figure 8.5. Biplot display of additive main effects and multiplicative interaction (AMMI) analysis for principal component (PC) 1 versus PC 2 for the identification of genotypic response to high temperature in *in vitro* pollen germination percentage.

Four pea cultivars, namely 40-10, CDC Sage, Rally, and Delta, had the highest means of *in vitro* pollen germination across different high temperature regimes (Figure 8.6). However, these four cultivars were not stable across these high temperature regimes since they were located far from the center of the Biplot (Figures 8.5 and 8.6). For example, pollen germination percentages in Rally were 40%, 30%, 95%, and 25% from Sutherland in 2014 incubated at 36°C, Rosthern in 2015 incubated at 42°C, growth chamber in 2015 incubated at 37°C, and from growth chamber in 2015 incubated at 42°C, respectively. Six pea cultivars including TMP 15202, TMP 15213, Eclipse, TMP 15116, CDC Vienna, and Kaspa had less than 15% pollen germination across the four different high temperature regimes (Figure 8.6). However, these six cultivars were not located at the center of the Biplot, indicating that they had poor performance under certain high temperature regimes but performed better under other high temperature regimes. Taking CDC Vienna as an example, it had the lowest pollen germination percentage for pollen collected from Sutherland in 2014 incubated at 36°C (Figure 8.1), as well as the growth chamber in 2015 incubated at 37°C and 42°C (Figure 8.3), whereas it had 50% pollen germination for pollen collected from Rosthern in 2015 and incubated at 42°C (Figure 8.4).

The overall averages of pollen germination percentage were 30.6% for samples collected from Sutherland in 2014 and incubated at 36°C, 35.4% for samples collected from Rosthern in 2015 and incubated at 42°C, and 38.4% and 5.5% for samples collected from growth chamber in 2015 and incubated at 37°C and 42°C, respectively (Figure 8.6).

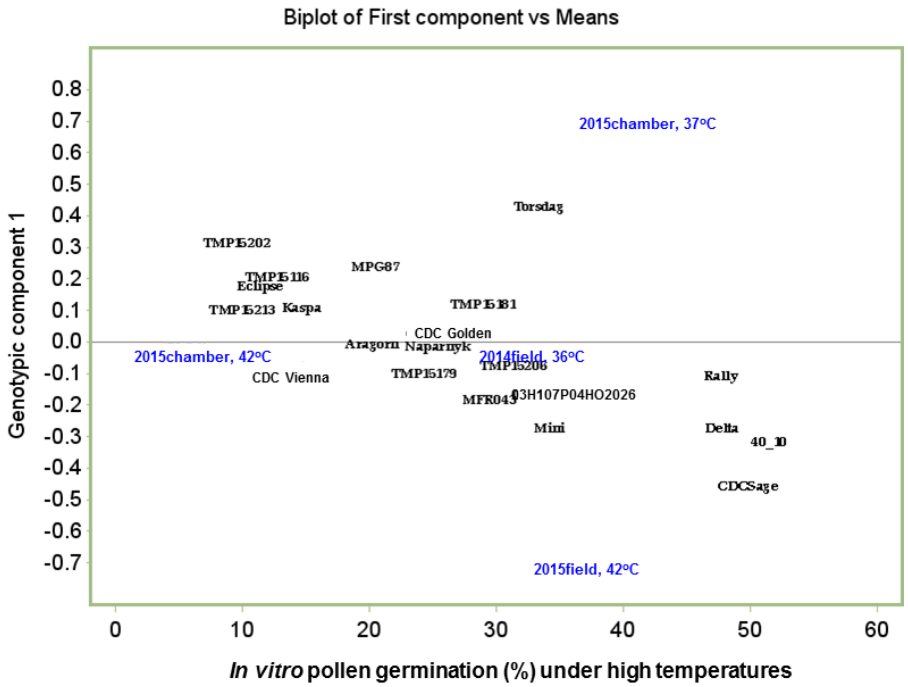

Figure 8.6. Biplot display of additive main effects and multiplicative interaction (AMMI) analysis for Principal Component (PC) 1 versus mean *in vitro* germination percentage of pollen subjected to high temperature regimes (36, 37, 42°C) for 24 h, for 21 pea cultivars.

8.3.3 Correlations between in vitro pollen germination and seed-set parameters

Under control conditions, *in vitro* pollen germination had no correlation with seed-set parameters in seven pea cultivars including 40-10, CDC Golden, CDC Meadow, CDC Sage, MFR043, Naparnyk, and TMP 15213 (Table 8.3). In contrast, *in vitro* pollen germination was positively correlated with seed retention on the main stem, the number of seeds per pod, and seed-to-ovule ratio within a pod under elevated temperatures (Table 8.3).

Table 8.3. Correlation coefficients between *in vitro* pollen germination and seed-set parameters in seven pea cultivars

Seed-set parameters	Control temperature	Elevated temperature
Total seed weight†	-0.040ns§	0.130ns
Duration of flowering†	-0.409ns	-0.481ns
Number of reproductive nodes†	-0.630ns	-0.559ns
Number of flowers†	-0.446ns	-0.553ns
Number of pods†	0.159ns	-0.053ns
Pod retention†	0.596ns	0.379ns
Total number of seeds†	0.079ns	0.408ns
Total number of ovules†	-0.164ns	0.173ns
Seed retention†	0.430ns	0.815*
Thousand seed weight†	-0.340ns	-0.665ns
Pod length‡	0.023ns	0.337ns
Number of seeds per pod ‡	0.261ns	0.807*
Number of ovules per pod‡	-0.161ns	0.530ns
Seed-to-ovule ratio within a pod‡	0.389ns	0.804*
Seed weight per pod ‡	0.085ns	0.306ns

†Data were based on the main stem;
‡Average across the first four reproductive nodes.
§Significance levels for the correlation coefficient are denoted by the symbols * and ns, for $P < 0.05$, or not significant, respectively.

8.4 Discussion

In vitro pollen germination has been used to screen heat-tolerance in many crops (Kakani et al., 2002; Kakani et al., 2005; Salem et al., 2007; Devasirvatham et al., 2012; Song et al., 2015; Singh et al., 2016). On the other hand, Petkova et al. (2009) suggested that *in vitro* pollen-tube length is more reliable to screen heat-tolerant cultivars compared to pollen germination percentage, because successful fertilization depends on whether pollen tubes reach the ovules. We observed heat stress reduced *in vitro* pollen-tube length in pea, which was consistent with the previous studies in field pea (Petkova et al., 2009; Jiang et al., 2015). However, the pollen-tube length of successfully germinated pollen held at 36°C did not differ among these 24 pea cultivars collected from Sutherland in 2014, which was in agreement with my previous study which documented that pollen-tube length of CDC Golden and CDC Sage incubated at 36°C for 10 hours did not differ (Jiang et al., 2015). Therefore, pollen-tube length from samples collected from the field and growth chamber in 2015 was not measured. Further, viable pollen alone may not be sufficient for

successful fertilization under elevated temperatures (Peet et al., 1997), because the role of megagametophytes could be of similar importance for heat tolerance (Kakani et al., 2002).

The temperature regime of 45°C for 2-3 hours was used to screen *in vitro* pollen germination and pollen-tube length in field pea for heat-tolerant genotypes (Petkova et al., 2009). However, Petkova's group did not indicate if the experiment was conducted in the light or dark. In general, *in vitro* pollen germination and pollen-tube growth performed better under dark conditions compared to light conditions (Hoyo et al., 2014; Parsons, 2006). For example, *in vitro* pollen germination percentage was higher under dark conditions compared to light conditions in *Grevillea* species (Parsons, 2006). *In vitro* pollen-tube length was greater in darkness compared to light in *Cyrtanthus mackenii* (Hoyo et al., 2014). This is one potential explanation why *in vitro* pollen germination percentage in pollen samples collected from Sutherland in 2014 and incubated at 36°C for 24 hours in light was similar to pollen germination percentage in samples collected from Rosthern in 2015 and incubated at 42°C for 24 hours in darkness (Figure 8.6). Plants grown in the field are generally more robust compared to those grown in a growth chamber, more likely an explanation of why the *in vitro* pollen germination percentage for samples collected from the field in 2015 was much greater compared to samples collected from the growth chamber and incubated under the same temperature regime of 42°C for 24 hours in darkness (Figure 8.6).

Results of the G × E interaction using the AMMI model is helpful to screen *in vitro* pollen germination for samples collected from different environments and incubated at different high temperature regimes. Results obtained from conducting the *in vitro* pollen germination experiment using samples from only one growth condition can be biased and not reliable. For example, CDC Vienna had low pollen germination percentage (less than 5%) for samples collected from Sutherland in 2014 and incubated at 36°C and collected from the growth chamber in 2015 and incubated at 37°C and 42°C, but the same cultivar had pollen germination of 50% for samples collected from Rosthern in 2015 and incubated at 42°C. Ideally, cultivars with high *in vitro* pollen germination percentage are stable across all different high temperature regimes. In reality, Aragon was the most stable cultivar among cultivars tested across all of the different high temperature regimes (Figures 8.5 and 8.6) but its percentage of pollen germination was only 30% (Figures 8.1, 8.3, and 8.4). However, even 30% germination theoretically would still exceed the total number of ovules to be fertilized in the ovaries. The phenomenon of genotypes with superior phenotypic

performance and low stability is not uncommon, and it is always a challenge for plant breeders that genotypes with superior phenotypic performance are not stable across different environments. For example, yield stability in 16 faba bean genotypes across 13 environments retained a high variability (Temesgen et al., 2015).

Although *in vitro* pollen germination was not correlated with seed-set parameters in field pea under control conditions, pollen germination was positively correlated with seed retention on the main stem, the number of seed per pod, and seed-to-ovule ratio within a pod under heat-stressed conditions. Similarly, seed set percentage was positively correlated with *in vitro* pollen germination in sorghum (Singh et al., 2016). Therefore, my findings also confirmed that *in vitro* pollen germination can be used as a screening criterion for heat tolerance, which was consistent with some previous studies in various crops (Kakani et al., 2002, 2005; Salem et al., 2007; Song et al., 2015; Singh et al., 2016).

8.5 Conclusions

The research hypotheses of this study were accepted that heat stress reduced *in vitro* pollen germination and pollen-tube length, and different pea cultivars differ with respect to these two traits. For successfully germinated pollen tubes under high temperature, the pollen-tube length did not differ among these 24 pea cultivars. In contrast, a genotypic difference was observed for *in vitro* pollen germination. Four cultivars, including 40-10, CDC Sage, Rally, and Delta, had relatively high *in vitro* pollen germination under high temperature, but they were not stable across different high temperature regimes. CDC Vienna had relatively low (<5%) *in vitro* pollen germination in most of the high temperature regimes, but the percentage was not stable with pollen germination of 50% under one high temperature regime. The cultivar, Aragorn, had highest stability in *in vitro* pollen germination under different high temperature regimes, but pollen germination was not superior with an average of 30% across different high temperature regimes. *In vitro* pollen germination was positively correlated with seed retention on the main stem, the number of seeds per pod, and seed-to-ovule ratio within a pod under elevated temperatures. Therefore, *in vitro* pollen germination was recommended to screen heat-tolerance in field pea.

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Contributions by others in the chapter

Flower sample collection, *in vitro* pollen germination experiments, microscope observations, and microscope image analysis for samples collected from Sutherland in 2014 was completed by Yunfei Jiang. Flower sample collection, *in vitro* pollen germination experiments, and microscope observations for samples collected from the growth chamber in 2015 were performed by Yunfei Jiang, and Yunfei Jiang trained the technician Zhifa Wang to measure pollen germination using the microscope images provide by Yunfei Jiang. Flower sample collection, *in vitro* pollen germination experiment, and microscope observation for samples collected from Rosthern in 2015 were performed by Yunfei Jiang with the help from the summer student Seung Bum Ryu, and Zhifa Wang measured pollen germination using the microscope images provided by Yunfei Jiang. Data analysis and interpretation was performed by Yunfei Jiang, who also wrote this thesis chapter.

Transition section between Chapter 8 and Chapter 9

From Chapter 3 to Chapter 8, six experiments were carried out to evaluate the effect of heat stress on pollen development and seed set in field pea, and these six experiments were focused on the aspects of plant physiology. Contemporary plant breeding approaches are required to dissect the quantitative traits related to reproductive development, and to identify the significant molecular markers associated to these reproductive development related traits. In Chapter 9, population structure analysis was conducted for a panel of 92 pea varieties, and association mapping was performed to identify any significant molecular markers associated to the traits related to reproduction.

Chapter 9 Population structure and association mapping of traits related to reproductive development in field pea

9.1 Introduction

Field pea (*Pisum sativum*) belongs to the family Fabaceae, subfamily Papilionoideae and is one of the economically most important legume crops. It is a diploid (2n=2x=14) with a haploid genome size of 4.45 Giga base pairs (Gbp; Dolezel and Greilhuber, 2010). Field pea is an economically important legume crop in western Canada and is a rich source of food for humans and fodder for animals. Pea is of great importance to cropping systems due to its ability to fix nitrogen. Canada was the largest producer of field pea in the world in 2014 with a total production of 3.4 million tonnes, followed by China, Russia, USA, and India (FAOSTAT, 2016).

More than 20 loci associated with flowering time and inflorescence development in pea have been identified (Weller and Ortega, 2015). Time of flowering in pea varies widely across different locations and years (Truong and Duthion, 1993). Photoperiod, temperature (vernalization and post-vernalization), and genotype are crucial factors for time of flowering in pea (Weller et al., 2009). Flowering time in pea is mainly controlled by genes associated with photoperiod and temperature (Alcade et al., 1999; Alcalde and Larrain, 2006). Flowering is controlled by six genes including *Lf*, *Sn*, *E, Dne, Ppd,* and *Hr* (Murfet, 1985; Weller et al., 1997). Flowering at different nodes is not synchronous due to the indeterminate growth habit in pea, thus flowering duration varies greatly because of the high degree of variability in the number of reproductive nodes (Roche et al., 1998). The number of reproductive nodes in pea is variable under field conditions and is an important component affecting seed yield (Roche et al., 1998). Additionally, the number of pods m^{-2} was strongly and positively correlated with yield in pea (French, 1990), but yield was reduced by loss of pollen viability in chickpea (*Cicer arietinum*; Devasirvatham et al., 2012), common bean (*Phaseolus vulgaris*; Konsens et al., 1991), and cowpea (*Vigna unguiculata*; Ahmed et al., 1992).

Association mapping, also known as association analysis or "linkage disequilibrium mapping", uses ancestral recombination events and natural genetic diversity within a population to dissect quantitative traits (Brazauskas et al., 2011). Linkage disequilibrium (LD) based association mapping has higher resolution compared to linkage mapping based on bi-parental mapping populations (Thudi et al., 2014). Association mapping has been conducted in several legume crops

including chickpea (Thudi et al., 2014; Diapari at al. 2014), soybean (*Glycine max*; Li et al., 2011), alfalfa (*Medicago sativa*; Sakiroglu et al., 2012), and common bean (Shi et al., 2011; Nemli et al., 2014). Until now, only six association mapping studies have been reported in pea: (1) 49 phenotypic traits related to seed nutrients (Kwon et al., 2012); (2) iron, zinc and selenium concentrations in seeds (Diapari et al., 2015); (3) seed lipid content (Ahmad et al., 2015); (4) agronomic and quality traits (Cheng et al., 2015; Ferrari et al., 2016); and (5) partial resistance to *Aphanomyces euteiches* (Desgroux et al., 2016).

However, little is known about the genetic dissection of traits related to reproductive development such as days to flowering (DTF), duration of flowering (DOF), the number of reproductive nodes, the number of pods, percentage of pod set, or *in vitro* pollen germination reduction due to high temperature stress. The objectives for this study were to examine the G × E interaction in DTF, analyze the population structure of a panel of 92 pea varieties, and to identify DNA markers associated with reproductive development related traits using previously published gene-anchored SNP markers (Sindhu et al., 2014).

9.2 Materials and methods

9.2.1 Plant materials and growing conditions

A panel of 92 diverse field pea varieties assembled at the Crop Development Centre, University of Saskatchewan and referred to as the Pea Association Mapping (PAM) panel was grown at nine site-years (environments) and one controlled environment. Among these 92 varieties, 31 were from western European breeding programs, 17 were from the Crop Development Centre (CDC), University of Saskatchewan, 16 were from eastern European breeding programs, 14 were from Agriculture and Agri-Food Canada (AAFC), 10 were from USA breeding programs, and 4 were from Australia. Association studies were also previously conducted in PAM for iron, zinc, and selenium concentrations (Diapari et al., 2015), with the addition of two wild relative accessions [P651 (*Pisum fulvum*) and PI344538 (*Pisum sativum* subsp. elatius)].

Field trials were arranged using a randomized completed block design (RCBD) and grown at Sutherland (lat. 52˚10’N, long. 106˚41’W; dark brown chernozemic soil zone) and Rosthern (lat. 52˚40’N, long. 106˚20’W; black soil zone), Saskatchewan in 2011, 2012, and 2013 and Rosthern in 2015. Seventy-five seeds of each variety were planted in a 1 m ×1 m microplot with 4 rows and

0.25 m spacing between rows. Six blocks were grown at each site-year at Sutherland and Rosthern in 2011 and 2012, and three blocks for each site-year at Rosthern in 2013 and 2015. The PAM panel was also evaluated near Yuma, Arizona with two seeding dates in spring and early summer 2012. The weather conditions were listed in Table 9.1. Daily maximum temperatures were used as an indicator of heat stress (Bueckert et al., 2015), because visible observations of fruit and flower abortion were observed under the field conditions when the daily maximum temperatures exceeded 28^{o}C (Bueckert et al., 2015).

The PAM panel was also tested in a growth chamber (Conviron®) with a completely randomized design (CRD). A total of 184 pots (92 pea varieties × 2 pots per variety) of 3.8 L volume (3 plants per pot) were seeded with Sunshine Gro® mix (Seba Beach, AB, Canada) and slow-release fertilizer (14-14-14, Type 100, Nutricote®, Brampton, ON, Canada). The pot dimensions were 15.9 cm depth and 16.5 cm diameter. Plants were thinned to two plants per pot approximately two weeks after seeding. Plants received the first application (500 mL per pot) of half strength modified Hoagland's culture solution (Hoagland and Arnon, 1938) at three weeks after seeding and the second application (500 mL per pot) at the early flowering stage. Soil medium moisture was monitored carefully and plants were watered as necessary to avoid drought stress. Plants were grown at 24/18°C day/night temperatures with the 16/8 hr photoperiod and illumination levels of 450-500 µmol m^{-2} s^{-1} from cool fluorescent tubes. The relative humidity had a range of 4-8% in growth chambers.

Table 9.1. Seeding date, monthly mean temperature, monthly average maximum temperature, the number of days when the daily maximum temperatures were greater than 28°C, and total precipitation at multiple site-years.

Site-year	Seeding date	Monthly mean temperature (°C)				Average maximum temperature (MT, °C)				Number of days when MT > 28°C	Total precipitation (mm)				
		May	June	July	August	May	June	July	August	From May to August	May	June	July	August	Total
Rosthern† - 2011	May 15	10.8	15.5	18.0	16.3	17.9	21.1	23.8	22.9	7	24.6	131.4	83.4	31.5	270.8
Sutherland‡ - 2011	May 3	10.9	15.5	18.4	17.2	17.9	21.3	24.6	24.9	6	17.5	94.4	68.6	16.5	197.0
Rosthern - 2012	May 11	10.2	15.9	20.0	17.4	16.4	21.6	25.6	23.9	11	92.5	116.0	92.3	63.5	364.2
Sutherland - 2012	May 16	10.1	15.8	19.7	17.3	16.4	21.5	25.3	23.9	7	108.0	121.1	80.9	48.5	358.5
Yuma§ - 2012	-	29.0	33.0	34.0	35.0	37.1	40.7	40.1	41.3	123	1302	1139	1639	1873	5953
Rosthern - 2013	May 15	12.8	15.3	17.2	17.4	20.5	20.6	23.3	24.6	15	13.5	148.8	62.4	17.4	242.0
Rosthern - 2015	May 13	9.4	16.4	18.8	16.9	17.8	24.1	25.8	23.8	28	10.9	37.4	80.5	75.9	204.7

†Data were calculated based on the average of Saskatoon and Prince Albert from Environment Canada, http://climate.weather.gc.ca;
‡Data were obtained from Environment Canada;
§Data were obtained from Weather Underground, https://www.wunderground.com.

9.2.2 Phenotyping

Seven phenotypic traits including DTF, DOF, the number of reproductive nodes, the number of pods on the main stem, percentage of pod set, potential pod set, and *in vitro* pollen germination reduction due to high temperature stress were collected. DTF was determined as the number of days from planting until 50% of the plants per plot were at the flowering stage at Rosthern in 2011, 2012, 2013 and 2015, Sutherland in 2011 and 2012, and Yuma in 2012 with two different seeding dates. For the controlled environment, DTF were determined when flower buds at the first reproductive node on the main stem were visible. The number of reproductive nodes and pods on main stems were also counted. DOF was calculated as days to flowering termination (DTFT) minus DTF, where DTFT was determined as the number of days from sowing until 50% of plants per plot reached flower termination. Percentage of pod set was calculated by dividing the number of pods present at maturity to the number of flowers formed at Yuma in 2012 with two seeding dates. Potential pod set was calculated according to Equation 1 at Rosthern and Sutherland in 2012. *In vitro* pollen germination reduction caused by high temperature stress was calculated according to Equation 2. Pollen grains collected from the field conditions (Rosthern in 2015) and the controlled environment (University of Saskatchewan phytotron in 2015) were treated as controls. Pollen samples were incubated in the dark for 24 hours in two incubators with one at 24°C and the other at 42°C. Two replications were utilized for each variety at each temperature regime in each environment (736 samples in total - 92 varieties × 2 temperatures × 2 replications at each environment × 2 environments). The detailed information about the *in vitro* pollen germination assay was previously described (Lahlali et al., 2014; Jiang et al., 2015).

Potential pod set = Pod / (Rnode×2) (Equation 1)

Pollen germination reduction = (control PG – heat stressed PG) / control PG (Equation 2)

where Pod is the number of pods, Rnode is the number of reproductive nodes, control PG is the number of germinated pollen grains at controlled temperature, and heat stress PG is the number of germinated pollen grains at high temperature.

9.2.3 Phenotypic data analysis

Homogeneity of variances for location effects was tested using Levene's test. The *P* values from the Levene's test for location effects on DTF and *in vitro* pollen germination reduction due to high

temperature stress were less than 0.05, so the datasets were analyzed separately based on different environments. The *P* values from the Levene's test for location effects on the number of reproductive nodes, the number of pods on main stem, and potential pod set were greater than 0.05, whereas the G × E interaction was significant, so the data were analyzed separately based on different environments. Variance components of genotype, environment, the G × E interaction, block within environment, and the residual were analyzed using the generalized linear model (GLM) and all factors were considered random effects. Broad sense heritability (H^2) was calculated as: $H^2= \sigma_g^2/(\sigma_g^2+\sigma_{ge}^2/n+\sigma^2/nb)$, where σ_g^2 is the genetic variance, σ_{ge}^2 is the variance of genotype and environment interaction, σ^2 is the error variance, n is the number of environments, and b is the number of replications within each environment (Wang et al., 2016).

The G × E interaction for DTF was assessed using the additive main effects and multiplicative interaction (AMMI) model using SAS (SAS Institute Inc.) as described in Kang et al. (2004). The AMMI model is a combination of analysis of variance (ANOVA) and principal component analysis (PCA) that partitions the variability of genotype, environment, and the G × E interaction model (Marjanović-Jeromela et al., 2011; Sabaghnia et al., 2008).

9.2.4 Association mapping

DNA extraction and genotyping using an Illumina GoldenGate array (Illumina Inc., San Diego, CA, USA) with a 1536-SNP pea OPA (Ps 1536) were previously described (Sindhu et al., 2014; Diapari et al., 2015). Among these 1536 SNPs, 1233 polymorphic SNPs were retained for further analysis, and the other 303 SNPs were removed due to being monomorphic or having a high ratio (35% or higher) of missing data (Diapari et al., 2015). The location of these SNPs on the pea genome was reported by Sindhu et al. (2014). Two varieties, 40-10 and CDC Dundurn, were also removed from further analysis due to high proportions of missing data (82% missing data for 40-10 and 85% missing data for CDC Dundurn), thus 92 varieties were retained in the population structure and association analysis. Population structure was analyzed using two different methods: (1) the Bayesian-based clustering approach with an admixture model using the Structure 2.3.4 software (Prichard et al., 2000); (2) discriminant analysis of principal components (DAPC), a multivariate method, using the R package "Adegenet" (Jombart, 2008; Jombart et al., 2010). DAPC partitions genetic variation into a between-group component and a within-group component. DAPC utilizes data transformation using Principle Component Analysis (PCA) before discriminant

analysis (DA) is applied. In the Bayesian method using the Structure software, the number of sub-populations (k) was set to be from 1 to 10 with 5 simulations, 100,000 burn-in phase, and 100,000 Monte Carlo Markov Chain replicates (MCMC). Based on the maximum likelihood and delta K (ΔK) values, the number of sub-populations was determined using Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/; Evanno et al., 2005; Earl and VonHoldt, 2012). For the DAPC, the SNP dataset was first converted to the genepop format using the R package "diveRsity" before using the "Adegenet" R package. The optimum number of sub-populations was determined with the smallest number of Bayesian information criterion (BIC). Both these methods allow for a probabilistic assignment of individuals to each group.

Among these 1,233 SNP markers, 943 markers were retained by considering a minor allele frequency (MAF) greater than 0.05 to remove low coverage marker sites. Estimates for clustering membership (Q matrix) from the DAPC method using the "Adegenet" R package were used as covariate in the general linear model (GLM) in association analysis using the software of Trait Analysis by Association, Evolution and Linkage (TASSEL version 5.2.19; Bradbury et al., 2007). The significant threshold ($-\log_{10}P = 4.3$) was adjusted using Bonferroni multiple test correction (0.05/943), for $P < 0.05$ and the denomination 943 was the total number of SNPs tested after marker filter alignment.

9.3 Results

9.3.1 Population structure

The optimum number of sub-populations (K) was determined using the highest value of Delta K using the Bayesian clustering method in the Structure software. The optimum k was ambiguous between 2 and 3, since the values of Delta K were similar when K was equal to 2 and 3, and Delta K decreased dramatically when K continues to increase (Figure 9.1A). Therefore, the identification of the clusters in this population was validated using the function of 'find.clusters' with the Adegenet R package. The optimum number of clusters corresponds to the lowest BIC value. The optimum number of clusters in this population was 3, because BIC values decreased until k = 3 clusters and then BIC rebounded (Figure 9.1B). These 92 pea varieties were grouped into 3 clusters as shown in the DAPC scatterplot (Figure 9.1C). The varieties and their origins under individual clusters are listed in Table 9.2. Subpopulation 1 included the majority of cultivars released by Crop Development Centre, University of Saskatchewan and Agriculture and Agri-Food Canada (AAFC),

as well as two varieties (Highlight and SW-Marquee) from western Europe (Table 9.2). Subpopulation 2 consisted of all of the Australian varieties, the majority of varieties from eastern Europe and the USA, five AAFC cultivars, five varieties from western Europe, and two CDC cultivars (CDC-Vienna and MFR043) (Table 9.2). The majority of varieties from western Europe belonged to subpopulation 3, and this cluster also included five AAFC cultivars, two CDC cultivars (CDC Sage and CDC Striker), three varieties from eastern Europe, and three varieties from the USA (Table 9.2).

Table 9.2. Pea varieties and their origins under three individual clusters in a panel of 92 diverse pea varieties.

Cluster 1		Cluster 2		Cluster 3	
Variety (entry)	Origin	Variety (entry)	Origin	Variety (entry)	Origin
Hugo (72)	AAFC[a], Canada	Argus (71)	AAFC, Canada	Agassiz (58)	AAFC, Canada
P0309-09 (88)	AAFC, Canada	MI3360 (54)	AAFC, Canada	MP1401 (36)	AAFC, Canada
P0316-04 (90)	AAFC, Canada	MI3391 (53)	AAFC, Canada	Reward (26)	AAFC, Canada
P0321-08 (89)	AAFC, Canada	MPG87 (15)	AAFC, Canada	Stella (73)	AAFC, Canada
P0322-01 (91)	AAFC, Canada	Trapper (49)	AAFC, Canada	CDC Sage (41)	CDC, Canada
CDC 1-150-81 (56)	CDC[a], Canada	02H016P-03HO2004-06TGVP004 (74)	Australia	CDC Striker (27)	CDC, Canada
CDC 1-2347-144 (57)	CDC, Canada	03H107P-04HO2026 (75)	Australia	SGL-2024 (87)	eastern Europe
CDC Acer (46)	CDC, Canada	03H267-04HO2006 (76)	Australia	TMP 15131 (4)	eastern Europe
CDC Bronco (19)	CDC, Canada	Kaspa (32)	Australia	TMP 15213 (16)	eastern Europe
CDC Centennial (20)	CDC, Canada	CDC Vienna (51)	CDC, Canada	Aragorn (94)	USA
CDC Golden (21)	CDC, Canada	MFR043 (34)	CDC, Canada	PS05100632 (77)	USA
CDC Leroy (47)	CDC, Canada	Naparnyk (48)	eastern Europe	PS05100840 (78)	USA
CDC Meadow (23)	CDC, Canada	TMP 15116 (1)	eastern Europe	Alfetta (38)	western Europe
CDC Montero (40)	CDC, Canada	TMP 15121 (3)	eastern Europe	Belote (65)	western Europe
CDC Mozart (22)	CDC, Canada	TMP 15133 (5)	eastern Europe	Bilboquet (64)	western Europe
CDC Patrick (6)	CDC, Canada	TMP 15155 (7)	eastern Europe	Carneval (35)	western Europe
CDC Treasure (2)	CDC, Canada	TMP 15159 (8)	eastern Europe	Carrera (55)	western Europe
Cutlass (18)	CDC, Canada	TMP 15164 (9)	eastern Europe	Cooper (28)	western Europe
Highlight (52)	western Europe	TMP 15169 (10)	eastern Europe	Crackerjack (61)	western Europe
SW-Marquee (39)	western Europe	TMP 15179 (11)	eastern Europe	Delta (45)	western Europe
		TMP 15181 (12)	eastern Europe	DS-Admiral (24)	western Europe
		TMP 15202 (13)	eastern Europe	Eclipse (25)	western Europe
		TMP 15206 (14)	eastern Europe	Espace (42)	western Europe
		TMP 15221 (17)	eastern Europe	FDP2010 (86)	western Europe
		Fallon (84)	USA	Hardy (68)	western Europe
		Lacy-Lady (82)	USA	Lido (92)	western Europe
		Laxtons-Superb (80)	USA	Nitouche (29)	western Europe

Mini (83)	USA	Orb (37)	western Europe
PS05101142 (79)	USA	Polstead (59)	western Europe
Rally (85)	USA	Prelude (69)	western Europe
Superscout (81)	USA	Rialto (93)	western Europe
Cartouche (70)	western Europe	Rocket (67)	western Europe
Lucy (63)	western Europe	Rose (66)	western Europe
Radley (50)	western Europe	SW-Sergeant (30)	western Europe
Rambo (33)	western Europe	Terese (43)	western Europe
Torsdag (44)	western Europe	Woody (62)	western Europe

†AAFC, Agriculture and Agri-Food Canada; CDC, Crop Development Center

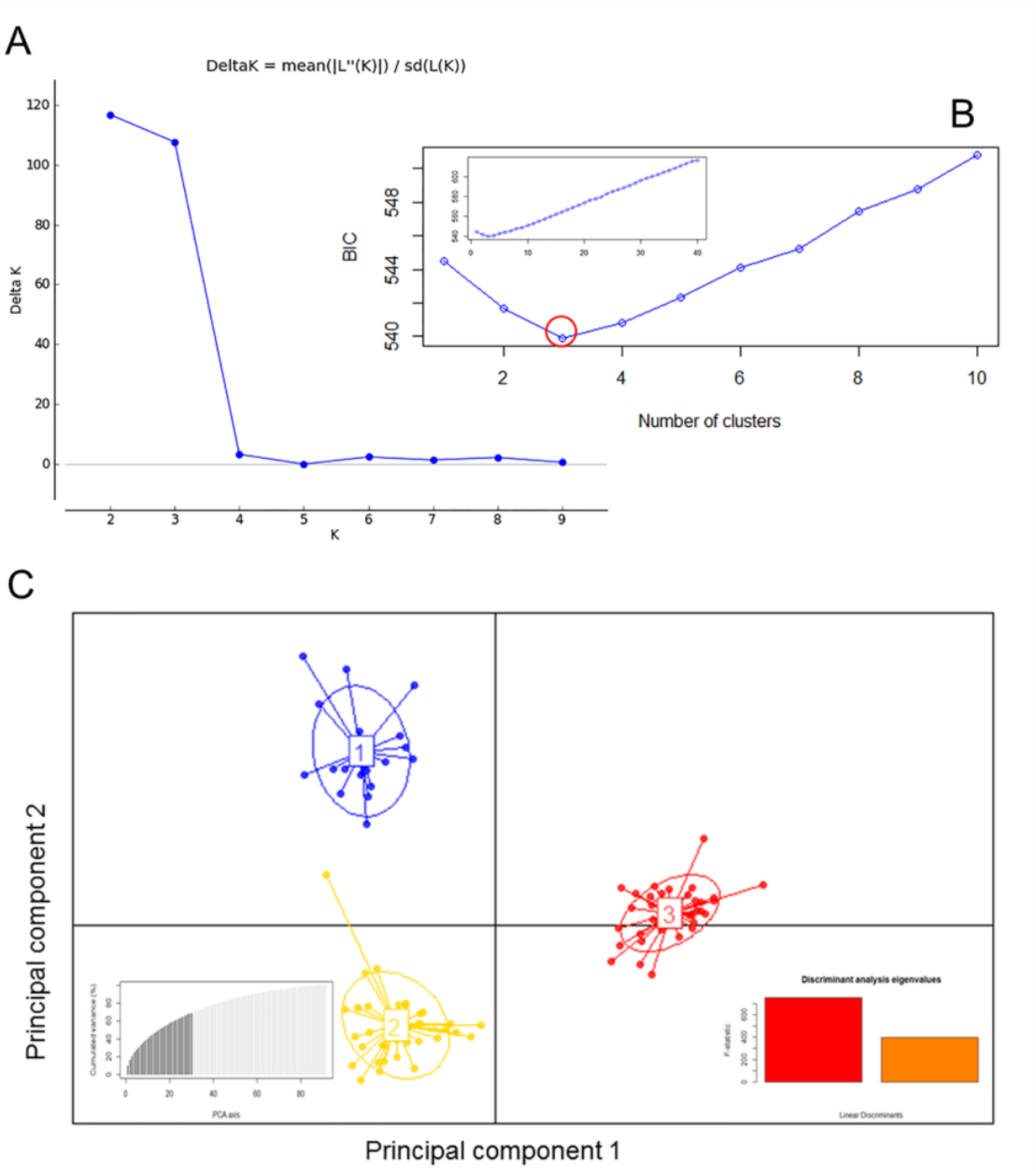

Figure 9.1. Population structure analysis in a diversity panel of 92 pea varieties. (A) Δk was used to determine the optimum k value for population structure using the Bayesian clustering method. (B) Inference of the number of clusters based on values of Bayesian information criterion (BIC). The selected number of clusters (3), corresponding to the lowest BIC, is circled in red. The inset represents the global results with the number of clusters up to 40, whereas the main figure indicates the detail of clusters up to 10. (C) Scatterplot of the discriminant analysis of principal components (DAPC) using the first two principal components (PCs) in a diversity panel of 92 pea varieties. Ninety-two dots represent pea varieties. The inset at the bottom left corner represents the number of principal components retained (35) in the DAPC; the inset at the bottom right corner represents the bar plot of eigenvalues for the discriminant analysis and two discriminant functions used in the DAPC.

9.3.2 Days to flowering

DTF varied depending on the growing environment (Figure 9.2 and Table 9.3). In general, pea plants under environmentally controlled conditions took less time to reach flowering compared to plants grown under field conditions (Figure 9.2 and Table 9.3). The measurement of DTF in the controlled environment was different from the field conditions. Under the controlled conditions, DTF was determined from seeding until flowering bud(s) at the first reproductive node were visible, whereas in the field conditions, DTF was determined as the number of days from planting until 50% of the plants had an open flower. The range of DTF (the maximum DTF minus the minimum DTF) varied from 7 to 19 days under field conditions (Table 9.3). However, the range of DTF was 56 days in controlled conditions, reflected by the largest variance and coefficient of variance among the nine environments tested (Table 9.3). In general, plants at Sutherland took approximately 5-7 days longer to reach flowering compared to Rosthern (Table 9.3). Acceleration of DTF due to late seeding was observed at Yuma in 2012, with the averages of DTF of 63 and 57 days for early seeding and late seeding, respectively (Table 9.3). The broad sense heritability of DTF was 0.67 (Table 9.4).

Table 9.3. Descriptive statistics for seven reproductive development related traits in a panel of 92 diverse pea varieties.

	Mean	StDev†	Variance	CV (%)†	Mini-mum	Maxi-mum	Range‡
Days to flowering							
Rosthern - 2011 (6 reps)	51	0.9	0.8	1.7	48	55	7
Sutherland - 2011 (3 reps)	58	1.9	3.5	3.2	56	63	7
Rosthern - 2012 (3 reps)	56	2.5	6.3	4.5	47	62	15
Sutherland - 2012 (3 reps)	61	2.2	4.8	3.6	57	70	13
Yuma - 2012, early seeding (2 reps)	63	2.8	7.8	4.4	54	70	16
Yuma - 2012, late seeding (2 reps)	57	4.3	18.1	7.5	48	64	16
Rosthern - 2013 (3 reps)	50	2.7	7.6	5.5	43	58	15
Growth chamber - 2015 (3 reps)	40	8.2	67.1	20.4	22	78	56
Rosthern - 2015 (3 reps)	52	2.8	8.0	5.5	45	64	19
Duration of flowering (days)							
Rosthern-2012 (2 reps)	25	7.7	59.6	31.3	13	46	33
Sutherland - 2012 (2 reps)	14	9.0	80.3	66.2	3	42	39
Rosthern - 2015 (3 reps)	22	7.0	49.0	32.4	10	43	33
Number of reproductive nodes							
Rosthern - 2012 (4 reps)	8	1.9	3.6	24.4	4	16	12
Sutherland - 2012 (4 reps)	7	2.1	4.5	32.0	2	18	16
Rosthern - 2015 (3 reps)	7	2.1	4.4	28.6	4	21	17
Number of pods							
Rosthern - 2012 (4 reps)	7	2.8	8.0	39.8	1	20	19
Sutherland - 2012 (4 reps)	6	2.8	7.9	47.7	1	18	17
Yuma-2012, early seeding (2 reps)	8	3.3	10.9	39.6	0	18	18
Yuma-2012, late seeding (2 reps)	4	2.1	4.4	46.8	0	11	11
Potential pod set (%)							
Rosthern - 2012 (4 reps)	46.0	14.82	219.63	32.19	13	97	85
Sutherland - 2012 (4 reps)	44.6	15.44	238.54	34.63	7	88	80
Percentage of pod set							
Yuma-2012, early seeding (2 reps)	91.2	9.85	96.98	10.80	42	100	58
Yuma-2012, late seeding (2 reps)	84.9	15.30	234.15	18.03	20	100	80
Reduction of pollen germination (%)							
Growth chamber - 2015 (2 reps)	0.90	0.192	0.037	21.22	0	1	1
Rosthern - 2015 (2 reps)	0.74	0.290	0.084	39.05	0	1	1

†StDev, standard deviation; CV, coefficient of variance;
‡Range represents the range between the minimum value and the maximum value.

Table 9.4. Variance components of environment, genotype, and their interaction and broad sense heritability (H^2) on seven reproductive development related traits in a panel of 92 diverse pea varieties.

Source	Days to flowering		Duration of flowering (days)		Number of reproductive nodes		Number of pods on main stem		Potential pod set (%)		Percent of pod set		Pollen germination reduction (%)	
	Variance	% of Total	Variance	% of Total	Variance	% of Total	Variance	% of Total	Variance	% of Total	Variance	% of Total	Variance	% of Total
Environment (E)	42.83***	76.47	32.18***	33.68	0.25ns	5.39	1.93***	19.56	0.00ns	0.00	16.24ns	8.80	0.013***	17.27
Genotype (G)	2.03***	3.62	25.50***	26.68	1.23***	27.00	0.90***	9.18	24.38**	10.42	14.37ns	7.79	0.002ns	2.08
G*E	7.98***	14.24	9.63***	10.08	0.20ns	4.37	1.90***	19.34	18.03*	7.71	13.19ns	7.15	0.012*	16.09
Block(E)	0.10***	0.19	0.23ns	0.24	0.33***	7.19	0.27***	2.78	18.02***	7.70	5.18*	2.81	0.000ns	0.00
Error	3.07	5.48	28.02	29.32	2.56	56.05	4.84	49.15	173.47	74.17	135.55	73.46	0.047	64.57
Total	56.01		95.57		4.56		9.85		233.9		184.53		0.073	
Heritability (H^2)	0.67		0.78		0.8		0.51		0.44		0.26		0.1	

*Significant at the 0.05 level of probability; **Significant at the 0.01 level of probability; ***Significant at the 0.001 level of probability; ns, not significant at the 0.05 level.

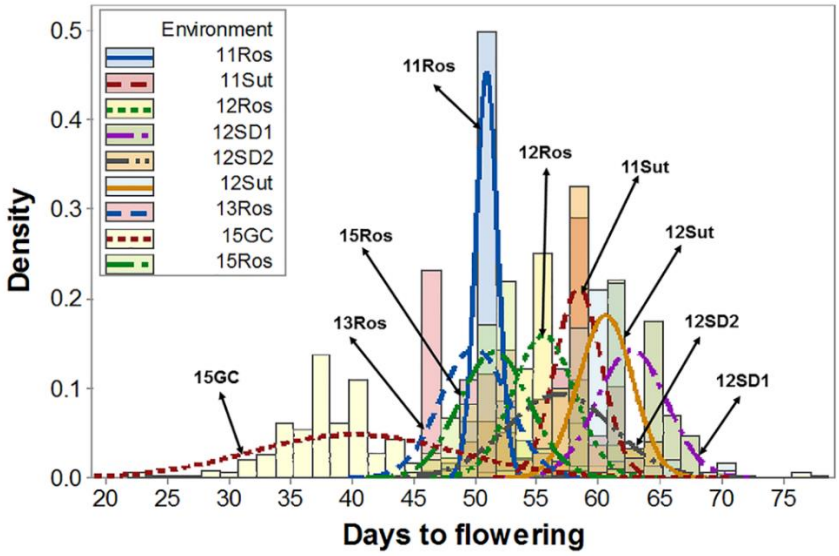

Figure 9.2. Histogram of days to flowering in a diversity panel of 92 pea varieties in nine different environments. The environments 11Ros, 12Ros, 13Ros, and 15Ros represent Rosthern in 2011, 2012, 2013, and 2015; 11Sut and 12Sut represent Sutherland in 2011 and 2012; 12SD1 and 12SD2 represent Yuma in 2012 with early seeding and late seeding; 15GC represents growth chamber in 2015.

With three multiplicative components, this AMMI model explained 77.5% of the total residual variability contributed to the G × E interaction (Figure 9.3B and C). Variability among environments was greater than variability among genotypes. The top ten early flowering varieties included Rally (entry 85), Torsdag (entry 44), TMP 15169 (entry 10), Superscout (entry 81), Lido (entry 92), PS05100632 (entry 77), TMP15181 (entry 12), Hardy (entry 68), MI3360 (entry 54), and Alfetta (entry 38) across 8 site-years tested. Varieties 68, 10, 44, 92, 85, 77, and 81, were early-flowering but unstable in this trait (PC1 not close to zero). Among these top ten early flowering varieties, entries 85, 44, 10, 81, 92, 77, 12, 68 and 54 showed negative correlations with environments 4 (Yuma in 2012, early seeding), 5 (Yuma in 2012, late seeding), 7 (Rosthern in 2013), and 8 (Rosthern in 2015), indicating these 9 varieties reached the flowering stage earlier at these 4 environments (Figure 9.3B). The positions of varieties and/or environments in the biplot played an important role in the G × E interaction – the genotypes and/or environments close to the horizontal and vertical reference lines x=0 and y=0 were considered to have small interactions and higher stability.

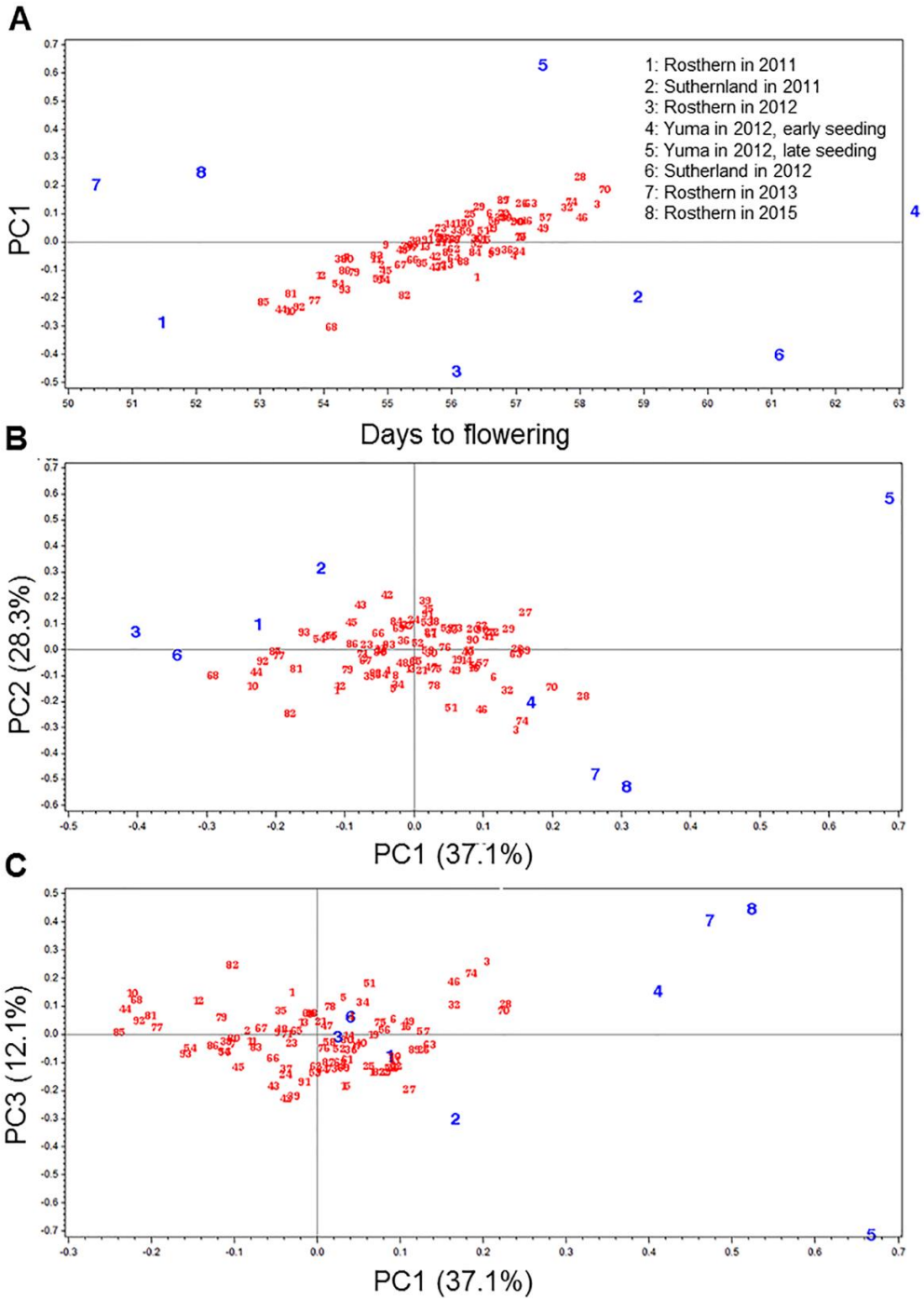

Figure 9.3. Analysis of the genotype-by-environment interaction on days to flowering (DTF) using Additive Main effects and Multiplicative Interaction (AMMI) model in field pea in 8 site-years. (A) Biplot of AMMI analysis for Principal Component Analysis (PCA) component 1 versus average of DTF for 92 genotypes at 8 environments. (B) the second versus the first multiplicative component plot (PC2 VS PC1); (C) the third versus the second multiplicative component plot (PC3 VS PC2); Numbers in blue represent environments and numbers in red denote genotype code shown in Table 9.2.

A total of 22 markers across seven linkage groups were significantly associated with DTF under environmentally controlled conditions (Table 9.5). The marker, PsC21767p87 was significantly associated with DTF at Rosthern in 2013, and explained 20% of the variation for DTF (Table 9.5). The same marker was also significantly associated with DTF under environmentally controlled conditions, and this marker explained 24% of the variation in the trait.

Table 9.5. Summary of significant ($-\log_{10}P \geq 4.3$) marker-trait associations identified by association analyses in a diversity panel of 92 pea varieties.

Trait	Location		Marker	Linkage group	Position (cM)	$-\log_{10}P$	Marker r^2†	Same marker for different traits
Days to flowering (DTF)	Rosthern-2011	1	PsC14322p98	8	22	4.5	0.20	-
	Rosthern-2012	2	PsC8005p478	8	80	4.3	0.20	-
	Rosthern-2013	3	PsC21767p87‡	4	20	4.4	0.20	-
	Growth chamber-2015	4	PsC20566p234	1	13	5.0	0.23	Rnode
		5	PsC22477p202	2	10	5.1	0.23	-
		6	PsC487p422	2	13	9.2	0.35	-
		7	PsC17431p188	2	32	5.3	0.24	Rnode
		8	PsC5013p645	2	70	4.3	0.21	-
		9	PsC5597p362	2	70	5.6	0.25	-
		10	PsC19344p128	3	17	4.6	0.23	Rnode
		11	PsC7220p181	3	91	8.3	0.35	-
		12	PsC27004p102	3	115	5.7	0.23	Rnode
		13	PsC15940p208	3	120		0.24	Rnode
		14	PsC3200p191	4	19	5.7	0.22	Rnode
		15	PsC21767p87[a]	4	20	5.1	0.24	Rnode
		16	PsC14392p100	4	59	4.5	0.21	-
		17	PsC908p622	7	46	4.5	0.21	-
		18	PsC10060p242	8	2	4.8	0.22	-
		19	PsC12051p325	8	12	8.7	0.36	
		20	PsC13289p266	8	18	5.5	0.26	Rnode
		21	PsC13955p450	8	21	9.3	0.38	Rnode
		22	PsC14155p82	8	21	6.3	0.28	Rnode
		23	PsC20385p319	8	42	5.7	0.22	Rnode
		24	PsC21134p263	8	43	5.7	0.22	Rnode
		25	PsC5231p296	8	66	4.6	0.21	-
	Rosthern-2015	26	PsC7497p542	4	64	6.1	0.26	-
		27	PsC6902p242	8	73	5.3	0.20	-
Duration of flowering (DOF)	Sutherland-2012	28	PsC18479p162	2	6	5.5	0.23	-
		29	PsC19105p141	3	98	4.3	0.18	-
		30	PsC12831p152	3	124	6.6	0.26	-
		31	PsC17990p348	3	131	5.4	0.23	Rnode
		32	PsC4940p155	6	36	4.3	0.19	Rnode
		33	PsC6187p183	6	68	5.7	0.23	-
Number of pods	Yuma-2012, early seeding	34	PsC19517p115	8	40	5.2	0.24	-
	Sutherland-2012	35	PsC27644p242	8	55	6.5	0.23	-
		36	PsC6387p181	8	71	4.3	0.20	-
Potential pod set (%)	Rosthern-2012	37	PsC8016p73	3	16	4.8	0.21	-

Percent of pod set	Yuma-2012, early seeding	38	PsC11375p247	1	87	4.6	0.20	-
		39	PsC7631p74	4	106	5.0	0.19	-
		40	PsC2509p330	5	83	4.5	0.26	-
		41	PsC1106p196	8	8	4.9	0.21	-
Number of reproductive nodes (Rnode)	Rosthern-2012	42	PsC17990p348	3	131	4.3	0.20	DOF
		43	PsC4940p155	6	36	4.9	0.23	DOF
		44	PsC12883p342	8	16	6.4	0.25	-
	Rosthern-2015	45	PsC2491p801	1	1	5.0	0.24	-
		46	PsC20566p234	1	13	6.4	0.28	DTF
		47	PsC17776p66	2	15	5.2	0.24	-
		48	PsC17431p188	2	32	6.6	0.29	DTF
		49	PsC19344p128	3	17	6.4	0.30	DTF
		50	PsC27004p102	3	115	7.1	0.28	DTF
		51	PsC15940p208	3	120	6.1	0.28	DTF
		52	PsC3200p191	4	19	7.2	0.28	DTF
		53	PsC5316p234	5	75	8.2	0.34	-
		54	PsC13188p293	6	83	5.7	0.25	-
		55	PsC10016p165	8	2	5.1	0.23	-
		56	PsC13289p266	8	18	6.0	0.28	DTF
		57	PsC13955p450	8	21	7.2	0.31	DTF
		58	PsC14155p82	8	21	6.5	0.29	DTF
		59	PsC20385p319	8	42	7.2	0.28	DTF
		60	PsC21134p263	8	43	7.2	0.28	DTF

†r^2 (coefficient of determination) for the marker after fitting other model terms (population structure);

‡Same significant marker for days to flowering at two different environments.

9.3.3 Duration of flowering

DOF varied depending on the growing environment (Table 9.3). DOF at Rosthern in 2012 and 2015 was longer than DOF at Sutherland in 2012, with the averages of DOF being 25, 22, and 14 days at Rosthern in 2012, Rosthern in 2015, and Sutherland in 2012, respectively (Table 9.3). The broad sense heritability of DOF was 0.78 (Table 9.4). A total of 6 markers in linkage groups 2, 3, and 6 were significantly associated with DOF at Sutherland in 2012 (Table 9.5).

9.3.4 Number of reproductive nodes

The average number of reproductive nodes was 7 to 8 with a range of 2 to16 at Rosthern in 2012 and 2015 and Sutherland in 2012 (Table 9.3). The coefficient of variation for number of reproductive nodes (24.4-32.0%) was relatively high (Table 9.3), reflected in the relatively high error variance (55.05%) for this trait in Table 9.4. The broad sense heritability of the number of reproductive nodes was 0.80 (Table 9.4). A total of 19 markers were significantly associated with the number of reproductive nodes at Rosthern in 2012 and 2015 (Table 9.5). The markers, PsC17990p348 at LG III, PsC4940p155 at LG VI, and PsC12883p342, were significantly associated with the number of reproductive nodes at Rosthern in 2012 and these three markers explained 20%, 23%, and 25% variation in this trait, respectively. Among these three detected markers at Rosthern in 2012, markers PsC17990p348 and PsC4940p155 were also significantly associated with DOF (Table 9.5). A total of 16 markers was significantly associated with the number of reproductive nodes at Rosthern in 2015 and these markers explained 23-31% of the phenotypic variation in this trait (Table 9.5 and Figure 9.4). Among these 16 markers detected at Rosthern in 2015, 11 out of 16 markers were co-localized with DTF (Figure 9.4 and Table 9.5).

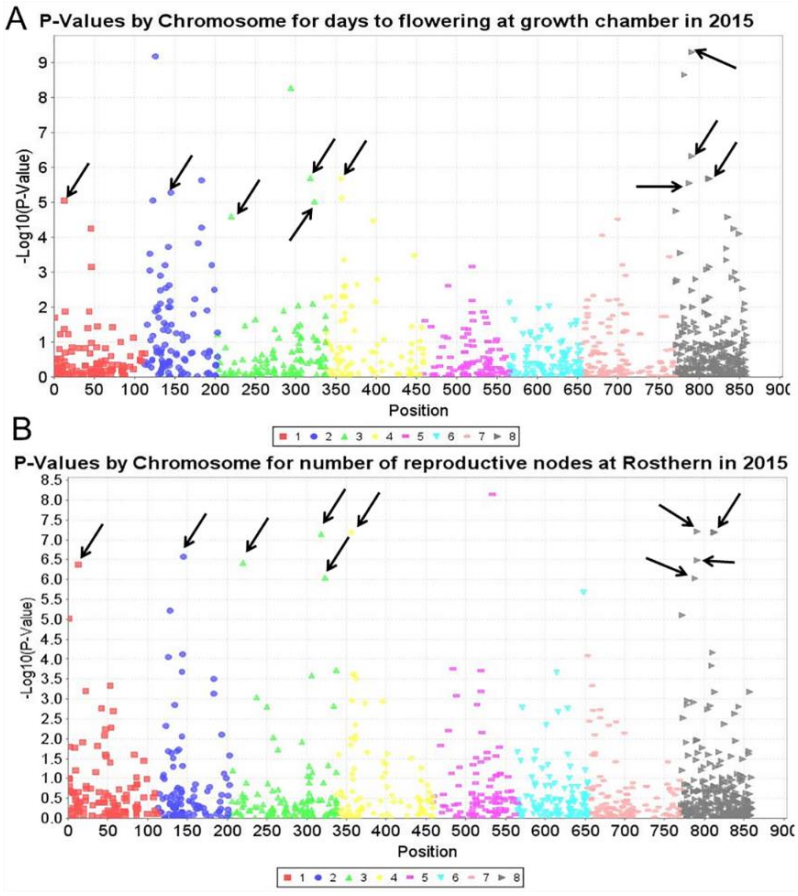

Figure 9.4. Manhattan plots of $-\log_{10}$(*P*-value) of association mapping for days to flowering at growth chamber in 2015 and the number of reproductive nodes at Rosthern in 2015 in *Pisum sativum* in a diversity panel of 92 pea varieties using the general linear model (GLM). The significant threshold is adjusted using Bonferroni correction at $-\log_{10}(P<0.05) = 4.3$. The x-axis represented seven linkage groups (LG I – LG VII) and unmapped markers are grouped in Group 8. Arrows indicated the same significant markers in both traits as shown in Table 9.5.

9.3.5 Pod number

The number of pods on the main stem varied greatly among varieties in individual environments (Table 9.3). The number of pods ranged from 1 to 20 with an average of 7 at Rosthern in 2012, and varied from 1 to 18 with an average of 6 at Sutherland in 2012 (Table 9.3). Late seeding reduced the number of pods, with the average pod numbers being 8 and 4 for early seeding and late seeding at Yuma in 2012, respectively (Table 9.3). The broad sense heritability of the number of pods was 0.51 (Table 9.4). The coefficient of variation for number of pods on main stem was relatively high (39.6-47.7%; Table 9.3), reflected in the relatively high error variance (49.15%) for this trait in Table 9.4. The markers, PsC27644p242 and PsC6387p181, located on the unmapped linkage group, were significantly associated with the number of pods at Sutherland

2012, and these markers explained 23% and 20% of the variation in this trait (Table 9.5). The marker, PsC19517p115 located on the unmapped linkage group, was significantly associated with pod number at Yuma in 2012 with early seeding (Table 9.5).

9.3.6 Potential pod set

Potential pod set (%) had a wide range of variation among varieties at individual environments. It ranged from 7% to 97% with a mean of 45% among these 92 varieties at two locations (Table 9.3). The coefficient of variation for potential pod set was relatively high (32.2-34.6%; Table 9.3), reflected in the relatively high error variance (74.2%) for this trait in Table 9.4. The broad sense heritability of potential pod set was 0.44 (Table 9.4). The marker, PsC8016p73, located on LG III, was significantly associated with potential pod set at Rosthern in 2012, and this marker explained 21% of the variation in this trait (Table 9.5). However, no marker was associated with potential pod set at Sutherland in 2012 (Table 9.5).

9.3.7 Percentage of pod set

Percentage of pod set had a range of 42 -100% with an average of 91.2% with early seeding at Yuma in 2012, which was higher than late seeding with an average of 84.9% in pod set percentage (Table 9.3). The broad sense heritability of percentage of pod set was 0.26 (Table 9.4). Four markers, PsC11375p247 located on LG I, PsC7631p74 located on LG IV, PsC2509p330 located on LG V, and PsC1106p196 located on the unmapped LG, were significantly associated with percentage of pod set (Table 9.5). These four markers explained 19-26% of the phenotypic variation in this trait (Table 9.5).

9.3.8 Pollen germination reduction

Data of pollen germination reduction for samples collected from growth chambers were square transformed. Pollen samples collected from the field conditions seemed to be more robust compared to pollen samples collected from the controlled environment, because the reduction percentage of *in vitro* pollen germination due to high temperature stress of pollen samples collected from the controlled environment was higher compared to the field conditions, with mean values of 90% and 74% at growth chamber conditions and Rosthern in 2015, respectively (Table 9.3). The broad sense heritability of pollen germination reduction was only 0.10 (Table 9.4). The coefficient of variation for reduction in percentage of *in vitro* pollen germination (21.2-39.05%) was relatively high (Table 9.3), which was reflected in the relatively high error variance (64.57%)

for this trait in Table 9.4. No marker was associated with the reduction of *in vitro* pollen germination percentage due to heat stress for flower samples collected from both the field (Rosthern in 2015) and environmentally controlled conditions (Table 9.5).

9.4 Discussion

Both Bayesian and multivariate methods were employed to infer population structure in the present study. Three subpopulations were observed in this population using the Bayesian and DAPC methods, and these three subpopulations roughly corresponded to the geographical origins of these varieties. However, these clustering results differed to some extent from the 8 subpopulations identified in a similar population (Diapari et al., 2015). Population structure of these same 92 pea varieties plus two additional wild relatives [P651 (*Pisum fulvum*) and PI344538 (*Pisum sativum* subsp. elatius)] were analyzed using the Bayesian method, and these 94 varieties were clustered into 8 subpopulations (Diapari et al., 2015). The subpopulation assignments are consistent with Diapari et al. (2015) for the majority of individuals. There is no "true K", but some values are more efficient in interpreting the population structure data than others (Jombart and Collins, 2015).

Molecular markers associated with important agronomic traits facilitate selection for interesting gene variants (Sudheesh et al., 2015). Comparing the QTLs associated with flowering time (DTF and DOF) with the QTLs associated with the number of reproductive nodes, we found that 12 DTF QTLs and 2 DOF QTLs overlapped with the QTLs associated with the number of reproductive nodes. In other words, flowering time QTLs may play a crucial role in the number of reproductive nodes in field pea. Genes and environmental cues affecting the vegetative-to-reproductive transition can continue to affect post-flowering processes related to fertility and pod development (Weller and Ortega, 2015). The trait of flowering time is associated with plant fundamental decisions such as when and how to allocate resources (Weller and Ortega, 2015). Weller and Ortega (2015) published a thorough review on genetic control in flowering time in legume crops, and the results show that flowering control is closely related to light perception/signaling, circadian clock, photoperiod response, and signal integration and inflorescence development. The pea genome is not yet sequenced, so candidate genes associated with these reproductive development related traits cannot be exploited in the present study. In addition, two strategies proposed by Gauch et al. (2011), the combination of 1) greater accuracy for the phenotypic data and 2) using

consistent patterns and systematic trends for the environments, are recommended to detect and understand QTL × environment interactions in future studies.

A total of 943 SNP markers from 1536 SNPs with known genetic positions from the consensus map were used in the present study (Sindhu et al., 2014). An average SNP frequency is 1 SNP per 667 bp in pea varieties (Sindhu et al., 2014). The synteny-based comparison between field pea and *Medicago truncatula* showed that the seven linkage groups, LG I, LG II, LG III, LG IV, LG V, and LG VII, were collinear along their length with *Medicago* chromosomes 5, 1, 3, 8, 7, and 4, respectively; and the pea LG VI corresponded to the *Medicago* chromosomes 2 and 6 (Bordat et al., 2011; Sindhu et al., 2014). Flowering time QTLs in *Medicago* have been reported to be located at chromosomes 1, 4, 5, 7, and 8, which correspond to LG II, LG VII, LG I, LG V, and LG IV of pea, respectively (Pierre et al., 2008). In the present study, significant markers related to DTF were identified on LG I, LG II, LG III, LG IV, and LG VII.

The broad sense heritability of DTF, DOF, the number of reproductive nodes, and the number of pods were 0.67, 0.78, 0.80, and 0.51 in field pea in the present study. The broad sense heritability of DTF was documented to be 0.69, 0.77, and 0.87 in three bi-parental populations in field pea (Ferrari et al., 2016). Similarly, the broad sense heritability of DTF, DOF, the number of reproductive nodes, and the number of pods were 0.90, 0.49, 0.38, and 0.57, respectively in the same plant species in a bi-parental population across five site-years at Rosthern in 2012, 2013, and 2014, and Sutherland in 2013 and 2014 (Huang et al., unpublished, personal communication).

DTF was reduced for plants seeded late at the Arizona location in 2012, probably due to day length being longer for plants with the late seeding date and long photoperiod promoting flowering time in most pea varieties. Similarly, flowering date for *Medicago* plants grown in spring were sooner compared to plants grown in autumn (Pierre et al., 2008). Moreover, the ambient temperatures were higher for plants with the later seeding date, and life cycles of plants were shortened due to rising temperatures. Environmental stresses could also induce flowering, with water stress being the most effective at inducing flowering (Glover, 2007). The regulation of flowering time via different seeding dates is of great interest since plants are able to avoid the most stressful phases during the growing season with proper seeding dates. The range of DTF under controlled conditions was larger than any other field conditions, due to certain late flowering varieties being sensitive to light spectral quality in growth chambers. Artificial light in growth

chambers usually has a higher red (660 nm) to far-red (735 nm) ratio (R:FR) compared to natural light with the R:FR ratio of ~1.2 (Holmes and Smith, 1975). For example, lentil plants flowered earlier when exposed to a lower R:FR light ratio (Mobini et al., 2016). Results from the AMMI model demonstrated DTF was unstable in certain varieties, whereas others had a higher stability in DTF, which suggested that stable flowering varieties may be less photoperiod-sensitive. Photoperiod and temperature affect time of flowering, and different genotypes respond to photoperiod and temperature differently (Truong and Duthion, 1993). Late flowering genotypes are generally more sensitive to photoperiod and temperature compared to early flowering genotypes in field pea (Truong and Duthion, 1993; references therein).

In previous studies, Kwon et al. (2012) used 285 pea genotypes from the USDA pea core collection for association mapping emphasizing seed nutrients, 35 diverse pea genotypes were used for association mapping of seed oil content by Ahmad et al. (2015), and Cheng et al. (2015) used 330 pea genotypes for association analyses for agronomic and quality traits. Therefore, a panel of 92 diverse varieties in the present study is a medium sized population that is sufficient to provide useful information for association mapping. The threshold for significant markers may have been set too high with the value of $-\log_{10}P$ 4.3, thus relatively few significant makers were detected in the present study. For example, the *P* value of 0.01 (equivalent to the value of $-\log_{10}P$ of 2) was used to determine if a QTL was associated with a marker (Pillen et al., 2003; Agrama et al., 2007; Brazauskas et al., 2011; Nemli et al., 2014), compared to the significance threshold level of $-\log_{10}P$ 4.3 used in the present study.

Additionally, the kinship analysis on a similar population conducted by Diapari et al. (2015) suggested that family relatedness among the majority of the varieties (97.2% of 94 varieties – the same 92 varieties in the present plus two wild *Pisum* accessions) is loose or they have no relationship, therefore the results from the GLM model are more reasonable compared to the results from the MLM model in the present study. Significantly associated markers are more reliable if the same markers are detected in two or more separate environments compared to significant markers only detected at a single environment. Therefore, significant markers associated with the number of reproductive nodes, the number of pods on the main stem, and percentage of pod set are less convincing compared to the marker associated with DTF in two separate environments. Thus datasets of these traits (the number of reproductive nodes and the

number of pods on the main stem) from more environments would be recommended to validate markers detected in only a single environment.

9.5 Conclusions

In conclusion, these 92 diverse pea varieties clustered into three subpopulations roughly corresponding to the geographic origin of the individuals. A total of 60 SNPs showed marker-trait associations with these seven reproductive development related traits. Fourteen flowering time QTLs overlapped with the QTLs associated with the number of reproductive nodes in field pea. One marker located at LG IV was significantly associated with DTF at two separate environments. Several markers were found to be significantly associated with other reproductive development related traits including the number of reproductive nodes, the number of pods, and percentage of pod set at one of the tested environments. Collectively, our findings unveiled the genetic basis of reproductive development related traits in pea through marker-assisted breeding, serving as an avenue for the genetic improvement through maker-assisted breeding.

Acknowledgements

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Contributions by others to this thesis chapter

This chapter incorporates the outcome of joint research by the Pulse Breeding Research Group and the Crop Physiology Lab at the University of Saskatchewan. Genotyping of this mapping population was previously conducted by Dr. Marwan Diapari, Dr. Bunyamin Tar'an and Dr. Tom Warkentin. The dataset of days to flowering at Rosthern and Sutherland from 2011 to 2013 were collected by Dr. Marwan Diapari, and the dataset for days to flowering at Yuma, Arizona in 2012 and at Rosthern in 2015 was collected by Dr. Rosalind Bueckert. The dataset of days to flowering in the controlled environment facility was collected by Yunfei Jiang. The dataset of duration of flowering, the number of reproductive nodes, the number of pods, potential pod set, and percent of pod set collected at all of the site-years were collected by the Crop Physiology lab led by Dr. Rosalind Bueckert. The experiment which assessed the reduction of *in vitro* pollen germination due to high temperature stress was conducted by Yunfei Jiang. Data analysis and interpretation was performed by Yunfei Jiang, who also wrote this chapter. Dr. Tom Warkentin, Dr. Bunyamin Tar'an, and Dr. Rosalind Bueckert have reviewed the chapter and provided valuable comments to improve the chapter.

Chapter 10 General discussion and conclusions

High temperature has been shown to reduce seed yield in field pea (Lambert and Linck, 1958; Karr et al., 1959; Jeuffroy et al., 1990; Bueckert et al., 2015). Temperatures over 25°C caused seed yield loss in field pea under field conditions in Australia (Sadras et al., 2012). Temperatures exceeding 28ºC over a period of 20 days reduced seed yield as well as the period from flowering to maturity in field pea under dryland conditions in western Canada (Bueckert et al., 2015). Severe heat stress (33/30°C day/night for 2 days) caused rapid abortion and abscission of reproductive organs in pea under controlled conditions (Guilioni et al., 1997, 2003). High temperature has also been shown to reduce seed number in pea (Jeuffroy et al., 1990; Guilioni et al., 2003). Developing seeds of field pea were reported to be sensitive to high temperature approximately 6-12 days after anthesis (Jeuffroy et al., 1990). Pea cultivars with early and extended flowering duration are more heat-tolerant (Bueckert et al., 2015).

10.1 A failure in anther dehiscence in response to heat stress

The findings from my PhD project have provided a deeper understanding of the effects of high temperature stress on reproductive development in pea, which advances the current knowledge of high temperature stress in this crop. For example, failure in anther dehiscence was observed in field pea when exposed to high temperature for 7 days. A similar phenomenon of anther indehiscence caused by heat stress was observed in cowpea (Ahmed et al., 1992) and common bean (Porch and Jahn, 2001). In certain plant species, anther dehiscence occurs when calcium is removed from the cell walls of the stomium in anthers and stored as insoluble calcium crystals, weakening the stomium cell walls and promoting separation of cells in the stomium (Shivanna, 2003; Lersten, 2004). High temperature may disturb the process of calcium removal in the cell walls of the stomium, leading to a failure in anther dehiscence. The other commonly reported mechanism of anther dehiscence is differential dehydration of multiple layers within the anther wall (Shivanna, 2003; Lersten, 2004). High temperature may minimize the differential drying of multiple layers within each anther, resulting in anther indehiscence. Accordingly, it would be informative to investigate whether exposure to high temperature interferes with the formation and function of the anther's endothecium in field pea.

10.2 Decreased pollen viability due to high temperature

My findings demonstrated that high temperature reduced *in vitro* percentage pollen germination, *in vitro* pollen-tube length, pollen viability (as revealed using the fluorochromatic reaction test), pollen load on the stigma surface, and the proportion of ovules evidently fertilized. The phenomenon of decreased pollen viability under high temperature conditions has been reported in many crop species including chickpea (Devasirvatham et al., 2012), bean and peanut (Boote et al., 2005), kidney bean (Prasad et al., 2002), cotton (Echer et al., 2014), rice (Endo et al., 2009), and tomato (Pressman et al., 2002). Loss of pollen viability under heat stress may be due to the following reasons. First, the reduction of pollen viability is caused by a loss of membrane integrity in some species such as *Crotalaria* (Jain and Shivanna, 1989; Shivanna, 2003). Second, sugar concentrations in pollen grains decrease under heat stress, leading to decreased pollen viability in tomato (Pressman et al., 2002). Third, a reduction in pollen viability in cowpea under high temperature may be associated with premature degeneration of the tapetal layer and the lack of an endothecial layer during anther development (Ahmed et al., 1992). A well-formed secondary wall within cells of the endothecium layer residing below the epidermis is typically critical for anthers to dehisce during desiccation of the anther's other layers. Fourth, high temperature advances pollen development in field pea, resulting in pollen grains becoming three-celled and mature prior to anther dehiscence when the corolla is still greenish, in comparison to pollen grains that remain two-celled and immature under normal temperatures at the same stage (Makasheva, 1984). Two-celled pollen grains usually have greater longevity compared to three-celled pollen grains (Shivanna, 2003). The rate of respiration in three-celled pollen grains is 2-3 times higher compared to two-celled pollen grains, which may be related to a lower viability in three-celled pollen (Shivanna, 2003). My results indicated that pollen viability was also related to the changes of lipids and proteins on the pollen coat and exine under heat stress, a novel finding for pea, and the first for legume crops, in heat stress.

10.3 Modifications of pollen surface composition in response to heat stress

In vitro pollen germination percentage of CDC Sage was significantly greater than CDC Golden after 10 h incubation at 36°C. Similarly, pollen viability of CDC Sage at the anthesis stage was significantly higher than that of CDC Golden when exposed to high temperature (35/18°C day/night) for 4 days. A greater *in vitro* pollen germination percentage and pollen viability in CDC Sage may be associated with more stable lipid composition in the pollen coat and exine compared

to CDC Golden. The lipids and proteins in the pollen coat and exine affect pollen hydration and germination success, allowing pollen tubes to penetrate the stigmatic papillae (Dickinson et al., 2000, Wolters-Arts 1998). This discrepancy between CDC Sage and CDC Golden may also be due to a higher ratio of α-helical structures to β-sheets in CDC Sage compared with CDC Golden (Lahlali et al., 2014), as α-helical structures prevent bulk protein in pollen grains from the destructive effects of dehydration (Wolkers and Hoekstra, 1995).

10.4 Pollen morphology change in response to heat stress

Pea pollen wall (intine) thickness increased when exposed to high temperature for 4 days. However, pollen exine morphology remained unchanged when pea plants were exposed to high temperature for 4 days. Disturbed pollen wall architecture due to heat stress has also been reported in common bean (Porch and Jahn, 2011), soybean (Koti et al., 2005; Djanaguiraman et al., 2013), and sorghum (Djanaguiraman et al., 2014). After exposure to high temperature for 7 days, pea pollen grains were smaller and had little cytoplasm compared to pollen grains sampled from control plants. Similarly, heat stress induced small pollen grains with little or no cytoplasm in barley (Sakata et al., 2000) and chickpea (Devasirvatham et al., 2012).

10.5 Comparison of heat sensitivity between male floral organs and female counterparts

My results suggest that male floral organs in field pea were more sensitive to heat stress compared to female floral organs, because high temperature reduced pollen viability and pollen vigor (as revealed by *in vitro* pollen germination and pollen-tube growth), but high temperature did not reduce ovule viability at $P < 0.05$ (instead, heat stress decreased ovule viability with $P = 0.09$). Moreover, heat stress increased the accumulation of ROS in pea pollen grains, whereas heat stress did not increase the formation of ROS in ovules compared to ROS under controlled conditions (Jiang et al., unpublished). The fact that male floral organs were more sensitive to heat stress compared to female floral organs has been reported in various crops including chickpea (Devasirvatham et al., 2013), bean (Monterroso and Wien, 1990), and barley (Sakata and Higashitani, 2008). One explanation is that once pollen grains are released from anthers, they are exposed to high ambient temperatures, whereas ovules are better protected within ovaries.

10.6 Seed development in response to heat stress

High temperature decreased pod length, seed number per pod, and the seed-ovule ratio, and increased ovule and seed abortion in field pea. Heat caused flower bud abscission in the younger

nodes, and accelerated seed abortion in all ovule positions within pods. Due to the staggered opening of the two flowers per node, ovules within the more advanced (older, proximal) pod were more likely to mature as seeds compared to ovules in the younger pod (distal) at that node. Abscission of reproductive organs is caused by a reduction in availability of photosynthates. High temperature inhibits sucrose uptake by flower buds in pepper (*Capsicum annum*; Aloni et al., 1991). Ovule and seed abortion were also related to a reduction in quality and quantity of pollen grains in field pea under heat stress. A failure in anther dehiscence, a reduction in pollen viability, and a disruption in pollen surface composition and morphology reduced the ability of pollen to deliver male gametes to ovules for successful fertilization.

In half of the 16 cultivars tested, ovules at both the pod's medial and stylar-end positions were more likely to develop into seeds. Ovules at the basal end aborted. For seven of the field pea cultivars, ovules at the medial pod position also produced mature seed. The results indicated that a combined effect of spatial advantages and early fertilization with fast-growing pollen tubes played a dominant role in successful seed development, whereas nutrient resources may not be a limiting factor because the pod wall of field pea is photosynthetically active (Atkins et al., 1977; Flinn et al., 1977). Small seed pea cultivars are most likely to successfully develop seeds.

10.7 Effect of elevated night temperatures on reproductive development

Negative effects of high night temperature (30°C) were more pronounced compared to high day temperature, with maximal yield loss of 25% and 8% for high night temperature and high day temperature regimes in field pea, respectively (Karr et al., 1959). High night temperature (30°C) induced male sterility (low pollen viability) and a failure in anther dehiscence in cowpea (Ahmed et al., 1992). However, no negative effects of high night temperature were observed in my thesis study. Elevated night-time temperatures (27/18, 27/22, and 27/26°C day/night temperatures for 7 days) during the reproductive stage had no significant effect on the number of flowers and reproductive nodes on the main stem, percentage of flower abortion, pollen viability, number of seeds per pod, and seed yield per plant. Negative effects of elevated night temperatures from 18 to 26°C on reproductive growth were much less than expected. One potential explanation was that night temperatures of 26°C were not high enough to have had a negative influence in field pea. A maximum night temperature of 26°C was evaluated in this project, because 26°C is typically the highest and most realistic night temperature under field conditions in western Canada.

10.8 Comparison between CDC Golden and CDC Sage in response to high temperature

Two pea cultivars, released by the Crop Development Centre at the University of Saskatchewan, were initially selected to compare their response with respect to reproductive development under heat stress, because CDC Golden had higher seed yield compared to CDC Sage under field conditions in western Canada. However, my findings demonstrated that CDC Sage did not have lower quality pollen or seed development compared to CDC Golden under heat stress in a controlled environment. *In vitro* pollen germination percentage of CDC Sage was significantly greater compared to CDC Golden after incubation at 36°C for 10 hrs. Similarly, viability of pollen grains collected from fully open flowers in CDC Sage was significantly greater compared to CDC Golden when exposed to high temperature for 4 days. No significant difference in pollen viability at the flower bud stage (Stage III; Figure 5.1H) was seen between the two cultivars following 4 days of heat exposure. All these results indicated that after pollen grains were released from anthers, pollen grains of CDC Sage were able to maintain their viability for longer compared to CDC Golden pollen in controlled conditions, which explained the significantly greater amount of pollen tubes detected in the styles of CDC Sage compared to CDC Golden. After exposure to high temperature for 7 days, a failure in anther dehiscence occurred in both cultivars, and so the proportion of ovules fertilized dropped dramatically. No differences in flower abortion, number of reproductive nodes, ovule viability, or seed development rating between these two cultivars were observed under heat stress. The flowering stage in CDC Golden was reached in less time compared to CDC Sage. Early flowering may facilitate CDC Golden to escape drought and heat under field conditions. Other factors such as differences in disease resistance, and not abiotic resistance, may contribute to a higher seed yield in CDC Golden compared to CDC Sage under field conditions.

10.9 Comparison of methods of quantification of total pollen grains per anther

A discrepancy of the number of pollen grains per anther was observed using two different methods described in Experiment 3 on Chapter 5 and Experiment 5 on Chapter 7. In Experiment 3, the total number of pollen grains per anther was counted. In contrast in Experiment 5, an aliquot method was used, similar to the method described in Prieto-Baena et al. (2003) with modifications. Although the method used in Experiment 3 was laborious and time-consuming, the results were more accurate and reliable, whereas the aliquot method used in Experiment 5 was more practical and efficient. In the future work, it is recommended to use the aliquot method for a study with many experimental treatments, and at the same time to use the method described in Experiment 3

as the standard to calibrate the results from the aliquot method. In addition, the number of pollen grains per anther can vary based on the position of reproductive nodes. The number of pollen grains per anther decreased from the more advanced reproductive nodes to younger ones (Table 7.1). Therefore, the node position is a confounding factor for temperature effects on pollen quantification.

10.10 Association mapping study

Genomic regions, as revealed by 12 SNP markers, governing flowering time are co-located with genomic regions controlling the number of reproductive nodes. Further, 2 SNP markers associated with duration of flowering also overlapped with a region governing the number of reproductive nodes. The above information indicates that genes controlling days to flowering and duration of flowering govern the number of reproductive nodes, and are possibly related to genes controlling the determinate/indeterminate type of growth habits in field pea.

The association mapping study in my thesis failed to yield any significant marker associated with *in vitro* pollen germination, because phenotyping of this trait was both technically challenging and time-sensitive. These problems might be alleviated with a repeated check cultivar in the field. In this way, I could run the *in vitro* pollen germination assay on the check cultivar after every ten samples, so I could compare the repeated check to standardize and calibrate my data to improve the accuracy of phenotyping. It took approximately 4-5 h to run the *in vitro* pollen germination test on 94 varieties with two temperature treatments (one replication). Pollen viability and vigor may be reduced between the first few varieties compared to the last few varieties. My results could be confounded by time of sampling / development stage. Flower samples from several plots were not sampled at the optimal flower bud stage. Flower samples were collected by myself with the help from one summer student, which introduced additional sampling variation. The optimal stage of flower sampling was at Stage III (Figure 5.1H, after anther dehiscence and before flowers were fully open), but some samples may have been collected from Stage IV-V (Figure 5.1), because after flowers had already been fully open and the standard petal was closed again, they could appear similar to Stage III. Pollen viability from Stage IV-V was lower compared to Stage III under control conditions (Chapter 5). Attention to improved experimental design and careful flower sampling (anthers at the exact and same stage) are needed for successful mapping pollen vigor when there are more than 24 genotypes.

10.11 Future perspectives

Although this project provides substantial information about the effects of heat stress on pollen development and seed set in field pea, a number of questions remain unanswered:

(1) How does heat stress affect stigmatic receptivity in field pea? The results from the present study indicate that pollen load on the stigma surface decreased after 4 days of heat exposure, although anther dehiscence had occurred. One possibility was that stigmatic receptivity decreased under heat stress. In the future, it is recommended to conduct reciprocal crosses between control pollen/stigma and heat-stress stigma/pollen, as well as to examine the compositions (lipid, protein, and carbohydrates) in control and heat-stressed stigma surfaces using FTIR-ATR spectroscopy.

(2) Does heat stress increase the accumulation of ROS in ovules? Increased synthesis of ROS in pollen grains due to heat stress was observed in the present study. We also found that heat stress reduced pollen viability, pollen germination, and the proportion of ovules that receive a pollen tube, but heat stress had a less pronounced effect on ovule viability. In the future, it is recommended to test the effect of heat stress on ROS accumulation in ovules, and to further explore how genes control ROS synthesis under stressed conditions.

(3) Does pollen grain size differ among pea cultivars? How does pollen grain size affect crowding / the physical aspect on the stigma? Does the overcrowding reduce pollen germinability on stigma surface?

(4) How does pod shape (rounded ends, versus tapered ends) influence survivorship of fertilized ovules? Or does pod shape only have an indirect effect, by the pod's final shape taking form simply by accommodating around those ovules that successfully reached maturity?

(5) What are the confounding effects of drought stress and heat stress on pollen development and seed set in field pea? The present study focused on the effects of heat stress alone on reproductive development in field pea. Under field conditions, however, heat stress and drought stress occur simultaneously. In the future, it is recommended to investigate the interactive effects of heat and drought on reproduction in field pea.

(6) How can we gain more confidence in those significant SNP markers associated with reproductive development in Chapter 9? In the future, it is recommended to utilize more pea varieties, a larger number of SNP markers, and/or data obtained from more site-years to identify and validate those significant SNP markers.

Answering the above questions is essential for a deeper understanding of reproductive development under heat stress.

10.12 Overall assessment

Heat stress reduced percentage pollen germination, pollen-tube length, pollen viability, proportion of ovules evidently fertilized, seed number per pod, and the seed-ovule ratio. Heat stress also tended to decrease ovule viability. Pea appears to have fairly robust pollen, and even after 4 days of heat, some pollen grains survive and can fertilize ovules. However, heat stress increased pollen wall (intine) thickness, modified chemical compositions of the pollen coat, and induced anther indehiscence. Successful seed development is controlled by multiple factors such as ovule position, seed size, and pollen availability. Ovules at the basal ends of pods are most likely to abort. Small-seed pea cultivars are most likely to develop into mature seeds. Genomic regions where genes governing flowering time and duration of flowering are located in pea overlapped with the genomic regions where genes control the number of reproductive nodes. The results obtained from this research will benefit plant physiologists and plant breeders and allow for better understanding of successful reproductive development in field pea and other legume crops under temperature increases projected to result from global climate change.

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