EFFECT OF HIGH TEMPERATURE ON OVULE DEVELOPMENT IN FIELD PEA

(*Pisum sativum* L.)

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Abstract

Field pea (*Pisum sativum* L.) is a cool-season crop that is highly vulnerable to high temperature especially during flowering. Temperatures exceeding 28℃ cause abortion of flowers and young fruits in the field, leading to severe yield loss of the crop. In this research, I aimed to investigate the impact of high temperature on ovule development during the reproductive development of pea. Assessments of gynoecium, ovule development, ovule viability, seed set, and ovule abortion from several cultivars exhibiting a wide range in heat tolerance revealed that high temperature altered the normal progression of ovule development under both growth chamber and field conditions. Plants with open flowers at the first reproductive node, but with closed mature buds at the second reproductive node, were exposed to high temperature (35℃/18℃ day/night) for 4 days under growth chamber conditions. The gynoecium evaluated at the first four reproductive nodes of these plants showed contrasting effects of high temperature among nodes and cultivars. A larger size of gynoecium components, such as ovary, style, and stigma, was identified at the youngest reproductive nodes (Node 3) on some heat-treated cultivars compared to the controls, which was consistent with older flower stages found at those nodes. Assessments of embryo sac and ovule size on these nodes revealed that greater size and advanced ovule development were the main effects of high temperature on heat-tolerant cultivars. In turn, less advanced ovule development on diverse nodes of the plants appears to be the factor that separates medium and low heat-tolerant cultivars under heat stress, where medium heat-tolerant cultivars showed poor development at one node, and a low heat-tolerant cultivar at two nodes. Importantly, the occurrence of embryo development at its early stages (zygote to globular-stage embryo) was detected in > 90% of these ovules. A different level of embryo development suggested that high temperature compromised early embryo growth at affected nodes. Ovule viability, analyzed by the presence of callose deposition and reactive oxygen species (ROS), revealed that high temperature could disrupt ovule development in more than one way. An increase of callose accumulation found around the vascular bundle region of ovules suggested that high temperature could disrupt assimilate transport to the embryo sac. Moreover, a heavy presence of ROS was detected in the embryo sac, indicating possible oxidative damage of the embryo sac contents in young ovules, specifically in pods at the raceme's distal position at young nodes (Node 4). Evaluation of abortion in mature pods confirmed a consistent failure of ovules right after fertilization and ovules containing embryos at early stages of embryo development in heat-treated plants. In the field, the

assessment of young ovules and mature pods of 18 cultivars showed a more severe effect of high temperature on ovule development. Ovules collected at 4 days after flowering and a few days (2-3) of high temperature (>28°C) in the field displayed poor embryo sac development, embryo sac decline, and endosperm and embryo growth disruption. Similar to growth chamber conditions, > 90% of these young ovules showed embryos at early development (pro-embryo to globular stage). Finally, seed number reduction in the field occurred mainly because of high ovule abortion (20-57% per pod) at various stages of embryo growth (pro-embryo to late cotyledon stage). Cultivars that showed the least ovule abortion were 40-10, Naparnyk, and CDC Golden, whereas cultivars with the greatest ovule abortion were Carneval, CDC Centennial, and MFR043. Overall, these findings demonstrated that high temperature disrupted normal ovule development, specifically when embryo formation was taking place. Although a certain level of accelerated development was observed on some nodes, poor ovule development on other nodes could be related to a conflict of assimilate availability for an embryo in development. The outcomes from this research provide valuable insights that enlighten how high temperatures hinder the success of reproductive development in field pea. These findings can also be used to select and assess more proficient varieties with high yield performance under warmer environments.

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ABAF	Aborted ovules right before or after fertilization
AEC	Aborted ovules with embryo at early cotyledon stage
AEE	Aborted ovules with early embryo growth
AELC	Aborted ovules with embryo at early or late cotyledon stages
AGH	Aborted ovules with embryo between globular to heart stage
ALC	Aborted ovules with embryo at late cotyledon stage
APE	Aborted ovules with various levels of embryo growth
CDC	Crop Development Centre
DAF	Days after flowering
ESP	Early seeded pea
HBSS	Hank's balanced salt solution buffer
LSD	Least Significant Difference
LSP	Late seeded pea
PAM	Pea Association Mapping panel
PBS	Phosphate-buffered saline
POS	Potential ovules to become seeds
ROS	Reactive oxygen species
SOR	Seed-to-Ovule Ratio
TKW	Thousand kernel weight

Chapter 1. Introduction

1.1 Background and motivation

Climate Change has become one of the greatest concerns in agricultural production around the world. Indeed, the increase of temperatures in the environment is one of the prominent effects of global warming leading to stress conditions in agricultural systems (Rose, 2015; Schlenker and Roberts, 2009; Hedhly et al., 2009). Research has already shown that high temperatures in the environment cause severe yield reduction in crops, such as chickpea (*Cicer arietinum* L.; Wang et al., 2006), soybean (*Glycine max* [L.] Merr.; Djanaguiraman et al., 2013), cotton (*Gossypium hirsutum* L.; Singh et al., 2007), corn (*Zea mays* L.; Schlenker and Roberts, 2009), pea (*Pisum sativum* L.; Sadras et al., 2013), wheat (*Triticum aestivum* L.; Gibson and Paulsen, 1999), and rice (*Oryza sativa* L.; Barnabás et al., 2008). According to Demming-Adams et al. (2008), plants can withstand stress conditions by developing morphological, anatomical, and physiological changes; however, their productivity is the main aspect compromised in the process, particularly when stress is caused by a rise of temperature (Hedhly et al., 2009). Hence, understanding how changes in the environment are affecting the biological processes in plants is crucial to seek new alternatives to allow agricultural production to overcome limitations caused by extreme temperatures.

In field pea (*Pisum sativum* L.), elevated temperatures detrimentally affect its development. Prior research shows that temperatures above 25ºC can cause a negative impact on early and late physiological development of pea (Guilioni et al., 2003; Bueckert et al., 2015; Huang et al., 2017; Tafesse et al., 2019). For example, growth rates of pea plants decreased 22% on average under temperature cycles over 30ºC for four days in greenhouse conditions (Guilioni et al., 2003). Certainly, this effect is important in early physiological processes of plants; however, a major effect is noted in late physiological development, where the reproductive phase of plants is most important and sensitive to high temperature (Prasad et al., 2008; Sánchez et al., 2014; Sage et al., 2015). For instance, temperatures of 31ºC for four days in plants at reproductive development caused reduction of seeds at different reproductive nodes in pea cultivar Solara (Jeuffroy et al., 1990). In parallel, temperatures above 30°C in both growth chamber and field experiments affected pea yield by causing abortion of buds, flowers, and fruits (Guilioni et al., 1997). Overall, stress conditions caused by extreme temperatures can reduce productivity of crops by half (Macedo,

2012). Clearly, heat-stress conditions disturb normal development of pea plants and particularly on the reproductive phase of plants leading to undesired yield loss.

In flowering plants, where successful reproduction involves male (pollen) and female (ovule development) counterparts, any damage or disruption in either reproductive organ can highly compromise the reproductive process leading to a severe yield loss of plants (Goldberg et al., 1994; Herrero, 2003). Although heat stress effects on plant reproduction has been widely investigated, most studies have focused on the effect of high temperature on male counterparts of the process (Prasad et al., 2006; Farooq et al., 2011; Djanaguiraman et al., 2018). In fact, the androecium, being exposed to the environment in various species, has been thought to be more sensitive to high temperatures (Wahid et al., 2007; Barnabás et al., 2008; Giorno et al., 2013). However, the female counterpart of the process (ovule) can also be compromised by high temperatures, as demonstrated in crops such as bean (*Phaseolus vulgaris* L.; Ormrod et al., 1967), wheat (*Triticum aestivum* L.; Saini et al., 1983), and peach (*Prunus persica* [L.] Batch.; Kozai et al., 2004). For example, ovules from heat-treated (30°C) plants of wheat showed absences of embryo sac and abnormal embryo and nucellus development that compromised the reproductive development of plants (Saini et al., 1983). In pea, where both male and female parts are enclosed within a keel petal (Maurer et al., 1966), the high temperature may affect both structures. The male counterpart of the reproductive process in pea has already been investigated (Jiang et al., 2015), leaving the female counterpart (the ovule and its internal components) remaining to be explored.

1.2 Objectives and scope

The overall goal of this research was to investigate the effect of heat stress on ovule development in a range of pea cultivars from different levels of tolerance to heat stress. In this way, a range of 6 to 18 cultivars was tested under growth chamber and field conditions in different experiments. These cultivars were selected from the Pea Association Mapping Panel (PAM) previously studied at the Crop Development Centre (CDC) of the University of Saskatchewan (Diapari et al., 2015; Jiang et al., 2017b).

I proposed the following hypotheses. First, high temperature would constrain (smaller, or more swollen) ovule and gynoecium development (stigma, style, ovary). Second, ovule viability would decrease in flowers exposed to heat stress during or right after fertilization, so that embryos would not develop. Third, there would be cultivars whose ovules would be less sensitive to high

temperature, and therefore less affected. To achieve the overall goal and test the mentioned hypotheses, some specific objectives were proposed, as follows:

1. To investigate the influence of high temperature on female reproductive flower parts (gynoecium) and its ovules in flowers at different stages of development in the first four reproductive nodes of six field pea cultivars under growth chamber conditions (Chapter 3).
2. To assess the influence of high temperature on ovule viability by means of callose and reactive oxygen species (ROS) presence in young ovules on three field pea cultivars selected randomly from Chapter 3. This evaluation was made on different reproductive nodes of plants following heat treatment under growth chamber conditions (Chapter 4).
3. To investigate seed set and ovule abortion at the first four reproductive nodes of plants at the stage of physiological maturity in six field pea cultivars under growth chamber conditions (Chapter 4).
4. To evaluate the effect of the high temperature in young ovules (4 days after anthesis) and seed set in various field pea cultivars under field (early- and late-seeded plots) and growth chamber conditions (Chapter 5).
5. To determine possible relationships among plant traits (pod number, reproductive nodes, canopy temperature, among others) versus ovule and seed-set performance under field conditions (Chapter 5).

Chapter 2. Literature Review

2.1 Pea (*Pisum sativum* L.)

2.1.1 Origin and importance of pea

Pea plants belong to the family Fabaceae, whose cultivation dates from primitive times to the present. According to Smýkal et al. (2012), domestication of this crop has been tracked back to the 9th to 10th millennium B.C, suggesting that its use could predate cereals. Indeed, archeological evidence has demonstrated that pea was cultivated in regions like the Near East and Greece at the Neolithic time (Marx, 1977). Thus, the origin of this crop is localized in Southwest Asia and Northwest Asia, where it was distributed to Europe and the world (Makasheva, 1984). Specifically, it is suggested that pea is native to countries such as Syria, Iraq, Iran, Turkey, Israel, Jordan, and Lebanon (Agriculture and Agri-Food, 2015). Currently, pea is grown in temperate zones in five continents: Africa, America, Asia, Europe, and Oceania. The area registered to this crop in 2018 was 7.9 million hectares around the world, where leaders of its production were countries such as Canada, France, Russia, China, and Inidia (FAOSTAT, 2018). Therefore, being one of the oldest crops cultivated in the world, pea cultivation has spread worldwide.

Here in Canada, field pea is one of the largest pulse crops cultivated by area and yield volume. In 2019, the seeded area reported of this crop was 4.3 million acres, which makes it one of the largest pulse crops produced in the country (Statistics Canada, 2020). The crop is an important source of income, and its role in agriculture is increasing over time in the country. Pea production was estimated to be 4,237 kilotonnes in 2019, which was approximately 18.3% higher than 2018 due to an increase of 19.5% of harvested area. It is mainly produced in the western provinces of Alberta, Saskatchewan, Manitoba, and British Columbia. Remarkably, Saskatchewan accounts for 55% of Canadian pea production (Agriculture and Agri-Food Canada, 2020). Thus, field pea is one of the major crops cultivated in the western part of Canada.

Pea is considered one of the most important grain legumes with a high content of nutrients for human and animal nutrition. In fact, this vegetable is an economical source of protein, carbohydrates, vitamins, and minerals (Taherian et al., 2012). Its remarkable content of protein (23%) is of good quality compared to other legumes, because of its high lysine content (Anderson et al., 2002). In general, this leguminous plant can contribute a source of globulin and proteins of

vital importance for metabolic processes, such as cellular division and protein storage in humans (Taherian et al., 2012). Additionally, pea can supply minerals such as potassium, phosphorus, calcium, copper, iron, and zinc in the human diet (Iqbal et al., 2006). On the other hand, pea is used as a complementary source of protein next to cereals in animal diets. Anderson et al. (2002) mention that pea can be used as a forage crop planted in a mixture with cereals to increase digestibility, protein, and energy content in hay and silage for livestock. In related studies, pea is a source of nutrients for animals such as sheep, bison, swine, and poultry (Anderson et al., 2002). Hence, pea can contribute as a source of energy and digestible amino acids to the diet of monogastrics and ruminants (Pulse Canada, 2017; Castell et al., 1996). In conclusion, pea plants and seeds are an excellent source of nutrients for human and animal consumption.

2.1.2 Vegetative description of pea

Plants of Pea (*Pisum sativum* L.), a species of the Fabaceae family, present a characteristic vegetative morphology including compound leaves and tendrils (Lecoeur, 2010; Makasheva, 1984). These plants display acropetal development with an indeterminate growth habit, where the older structures are found on the bottom, and the youngest on the top of a developed plant (Lecoeur, 2010). The stem cross section is round with little rectangular shape, whose length can be as small as 50 cm for dwarf varieties and can reach 300 cm for tall varieties. On every stem, plants exhibit nodes, which are the points of leaf and stipule attachment (Makasheva, 1984). Two types of nodes are distinguishable. The first ones are vegetative nodes, which give rise to vegetative structures like branches, stipules, and leaves. The second ones are reproductive nodes where flowers and pods appear. The initial two vegetative nodes produce vestigial leaves and are found under the soil (Cousin, 1997; Maurer et al., 1966). The rest of the vegetative nodes display stipules which are leaflike structures that are not considered leaves by plant anatomists but represent important transpiring and photosynthetic areas on the plant (Makasheva, 1984). The real leaves of these plants are compound, whose structure is made up of a petiole and two or three pairs of leaflets terminated by one or two tendrils (Lecoeur, 2010). Depending on the structure of the plants, there are varieties call “leafy”, which have stipules, leaflets, and tendrils, and varieties called “afila” or “leafless” and “semi-leafless”, where the leaflets are replaced by tendrils (McComb, 1977; Lecoeur, 2010). Therefore, the incredible vegetative structure of this plant makes it capable of growing long and tall stems, while climbing and holding onto neighbouring plants.

2.1.3 Flower description of pea

Pea flowers present the typical characteristics of the papilonaceous type within family Fabaceae. They display a set of pentamerous petals and sepals, ten stamens and a carpel (Makasheva, 1984). The five petals are made up of a standard petal, two wing petals, and two keel petals (Fig. 2.1; Ferrándiz et al., 1999). The standard petal is broader than the wing petals, which surround the keel petals that are fused together through a suture along with their abaxial margins (Fig. 2.1; Cousin, 1997; Makasheva, 1984). As a self-pollinated flower, the keel petals enclose the 10 stamens, one freestanding and nine fused at half of length, forming a semi-tube around of the carpel of the flower (Fig. 2.1; Ferrándiz et al., 1999; Tucker, 1989). The pistil exhibits one ovary with one style and one stigma; the ovary is semi sessile containing 4-12 ovules depending on genotype, and the style is bent almost at a right angle to the ovary (Makasheva, 1984; Cousin, 1997). The ovules are campylotropous (curved ovule) and are attached in an alternative way to a suture inside the ovary (Cooper, 1938). The style has a pubescent area followed by a wet stigma, which is localized on the tip of the style above the pubescent area. Thus, the stigma is considered the membranous area on the rim of the elliptical part of the style, the only area where the pollen has been demonstrated to germinate (Warnock and Hagedorn, 1954). Therefore, the complex structure of the pea flower causes the nearness of the anther's pollen and the stigma to make it a self-pollinated species, even though floral nectar produced from nectary tissue located around the base of the gynoecium may attract bees (Razem and Davis, 1999).

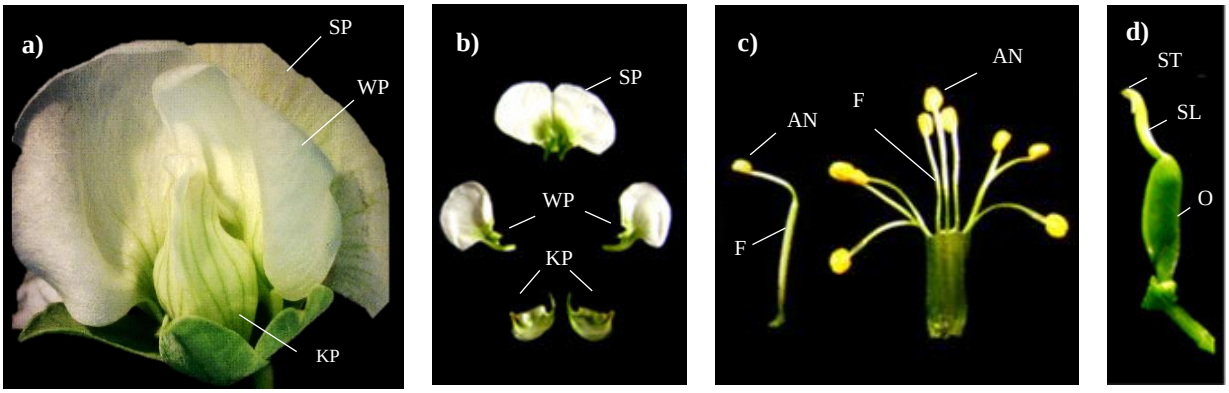


Fig. 2.1 Scheme of an open pea flower and its parts. a) Flower without dissection, displaying standard petal – SP; wing petal – WP; and keel petal – KP. b) Petals dissected from an open flower. c) Stamens displaying anthers – AN; and filaments – F. d) Pistil showing ovary – O; style – SL; and stigma – S. Modified from Kumar et al. (2011).

2.1.4 Reproductive biology of pea

Reproduction in flowering plants is a complex process which involves the development of reproductive structures, pollination, fertilization, and embryo development. In pea, the beginning of the reproductive phase is identified when a floral primordium appears in the axil of a new differentiated leaf (Lejeune-Hénaut and Biarnès, 2010). Flowering stages can be described quite precisely by the development of individual floral organs. The internal development of the flower initiates by the formation of the sepals of the calyx, followed by the petals of the corolla, and finishes with the formation of the stamens on the flower apex (Ferrándiz et al., 1999; Tucker, 1989). By the time the staminal structures are initiated, flower buds are 0.4 to 0.5 mm in size, and differentiation of anther cells has occurred when the bud is 1 mm in size (Makasheva, 1984). After this point, the flower also displays a sequence of stages visually different to each other. An initial stage starts when the tip of the flower petals is visible above the sepals, the intermediate stages begin when petals emerge around 12 mm from the sepals, then the anthesis stage when the flower is completely open, and a final stage when the petals of the flower become withered and part of the pod (ovary) is visible (Maurer et al., 1966). Therefore, each flower goes through a sequence of complex changes to ensure sexual reproduction of the plant.

2.1.4.1 Gamete development in pea

The development of male and female gametes takes place through a series of cell divisions inside the anthers and the ovules, respectively. The development of male gametes starts in the pollen grains (male gametophytes) within the anthers, where many microspore mother cells in structures called anther lobes will first go through a series of meiotic divisions and later through mitotic divisions to give rise to pollen grains (Shivanna, 2003; Lersten, 2008). Simultaneously, the female gametophyte is formed inside an ovule of the ovary, where the apex will divide to form the first sporogenous cells, which through meiotic divisions will generate four megaspores (Cooper, 1938). Whereas the three haploid cells closest to the micropyle will degenerate, one haploid cell nearest the chalaza survives and its nucleus will divide through mitosis to form eight nuclei in the mother cell (Cooper, 1938; Lersten, 2008). At that point, the three nuclei will remain at one side of the ovule to form the three antipodal cells, whereas the three other nuclei will remain at the micropyle side of the ovule to form the egg and two synergid cells. Finally, the two remaining

nuclei will take the middle (central cell) position of the megagametophyte to be the polar nuclei that will fuse during fertilization to form the endosperm to nourish the embryo (Cooper, 1938; Makasheva, 1984).

2.1.4.2 Progamic phase in pea

The onset of reproductive development happens when pollination and fertilization take place. According to the stages of flower development, pollination in pea has been identified to occur 24 to 36 h before its flower opens, when the wing petals are still tightly closed, and the standard petal shows a greenish white colour (Pate and Flinn, 1977; Cooper, 1938). Thus, self-pollination in pea can be ensured by a unique synchrony of development among male gametophytes (pollen grains, which conatin the sperm cells) and the female gametophytes (mature embryo sac) within ovules of the ovary, during suppression of the petal elongation (Tucker, 1989; Wojciechowska, 1978). Thus, pollination happens when the anthers dehisce around 24 h before the flower actually opens, the keel petals becomes loaded with pollen from the anthers, and the stigma gets contacted with the pollen around itself (Tucker, 1989).

Double fertilization can occur between 3 to 10 hours after the pollen gets attached to the stigma (Makasheva, 1984). Pollen tubes grow along the ventral suture of the ovary to reach the micropyle of the ovule, then one pollen tube enters into the embryo sac passing between synergids and the egg, and releases the two male gamete nuclei into it (Cooper, 1938). One sperm nucleus fuses with the two polar nuclei giving rise to triploid endosperm, and another one fuses with the egg cell to yield the diploid zygote. The first triploid nucleus of the endosperm can be observed after 12 h of the fusion between the sperm cell nucleus with polar nuclei, and by that time, the fusion of the other male nucleus with the egg will be completed (Makasheva, 1984; Cooper, 1938). Therefore, the success of the reproductive development in pea plants depends on a sequence of synchronized processes that involve self-pollination and double fertilization.

2.1.4.3 Embryo and seed development of pea

Once fertilization has occurred, a chain of coordinated events takes place inside the ovular tissue to form the future embryo. That is, after fusion of the male nuclei with the two polar cells and the egg, the endosperm and zygote are formed following an exact sequence, where the

endosperm starts to develop first and multiply faster than the zygote (Lersten, 2008; Pate and Flinn, 1977; Cooper, 1938). The sequence of developmental stages is illustrated in Fig.2.2. While the first division of the zygote can be expected 24 h after fusion, the endosperm has already gone through three to four divisions by that time (Lersten, 2008; Makasheva, 1984). Initially, free nuclear divisions take place in the endosperm, and later cell wall formation starts in the micropylar end, while the endosperm continues to stay multinucleate at the chalaza region of the ovule (Cooper, 1938). Meanwhile, the diploid zygote cell divides horizontally to form a basal and an apical cell, both equal in size, where the basal cell will divide longitudinally to form two suspensor cells and the apical cell will divide transversely to form an apical embryo mother cell and a middle cell (Lersten, 2008; Cooper, 1938). The apical embryo mother cells will go through a successive cellular division to form a mass of cells in a globular shape (globular stage). Later, cells at the centre and at the basal side of the embryo will become meristematic and initiate the development of two cotyledons (heart stage), epicotyl, and hypocotyl while the suspensor will start to disintegrate (Cooper, 1938). Finally, the cotyledons will grow (early and late cotyledon stage) to act as storage organs of the seed and the endosperm will be completely absorbed by the embryo (Lersten, 2008; Marinos, 1970).

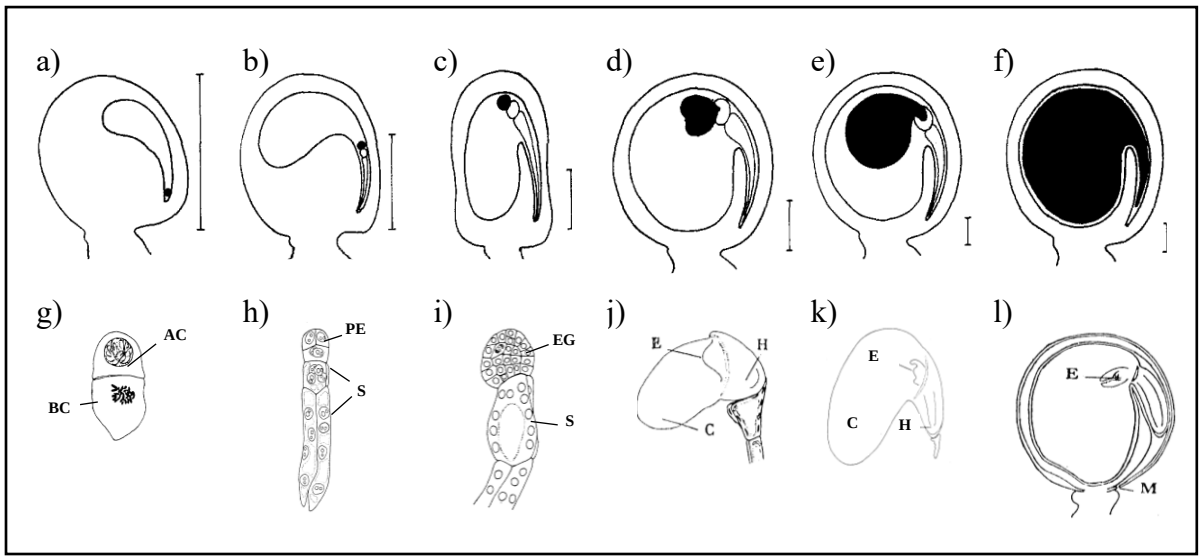


Fig. 2.2 Scheme of post-fertilization development of pea ovules in medial longitudinal sections. a) to f) represent ovule with embryo sac, and embryo (shown in black) at various stages of development. a) Ovule containing zygote, b) ovule containing pro-embryo, c) ovule containing embryo at globular stage, d) ovule containing embryo at heart stage, e) ovule containing embryo at early cotyledon stage, and f) ovule containing embryo at late cotyledon stage. g) to l) represent the cytological progression of embryo development at higher magnification. g) zygote, h) pro-embryo, i) embryo at globular stage, j) embryo at heart stage, k) embryo at early cotyledon stage, and l) embryo at late cotyledon stage. Abbreviations: AC - apical cell, BC - basal cell, C - cotyledon, E - epicotyl, EG - embryo at globular stage, H - hypocotyl, M – micropyle, PE - pro-embryo, S – suspensor. Scale bars for a) to f) = 1mm. Modified from Marinos, (1970) and Cooper (1938).

Some stages of zygote-embryo development have been identified according to the stages of flower development. Firstly, when the flower is fully expanded, with the standard petal fully erect (anthesis), fertilization has been accomplished and the zygote has started to divide (Pate and Flinn, 1977; Cooper, 1938). By the time the corolla is withered and abscinded, the embryo will be at the globular stage at the dome of the embryo sac (Marinos, 1970). Then, when the ovule gets a round shape, the embryo will start to become heart-shaped and the suspensor will reach its maximum stage of elongation (Marinos, 1970). Later, 4 to 5 days after flowering (DAF), histogenesis is identified to start, denoting also the beginning of suspensor disintegration (Reeve, 1948). Although these stages can be a guide to follow embryo development, some variation in the embryo development can still exist among cultivars. For instance, Cooper (1938) and Reeve (1948) reported that embryos of varieties Alderman, Little Marvel, and Asgrow at 3 DAF were spherical or globular prior to histogenesis. Also, King and Heyes (1986) reported that embryos of Alaska cultivar at the same stage were flattened, globose and undifferentiated. Therefore, while stages of

embryo development can be identified along with the flower development, slight differences can still be detected between genotypes.

In general, the development of embryo-seed growth can be divided into three main stages by crop physiologists. An initial stage that starts from fertilization to the beginning of seed filling, a second stage at the beginning of seed filling, and a final stage after physiological maturity (Munier-Jolain et al., 2010). The first stage in pea coincides with plant flowering when cell divisions occur in the embryos. The second stage happens when cell division stops, and dry matter accumulation proceeds within the cotyledons. Finally, the third stage takes place when assimilate is no longer delivered to the seed and the final seed weight is reached (Munier-Jolain et al., 2010; Ney et al., 1993; Duthion and Pigeaire, 1991). A useful indicator of these stages is the water content of seeds, where the beginning of seed filling of pea is identified when each seed has around 85% water, then water content will start to decrease slowly following a rapid reduction after the seed reaches its maturity phase (Munier-Jolain et al., 2010). Hence, embryo and seed development can be described in a sequence of steps depending on the interest of the study to follow.

2.2 Mechanism of plant adaptation to heat stress

Environmental stressors like high temperature, can seriously compromise plants' normal growth and development (Lobell and Asner 2003). To survive under harsh environments, plants experience morphological, anatomical, and physiological changes that affect their healthy development (Altman, 2003). Many of those changes respond to multiple strategies that plants generate to overcome any undesirable stress conditions to maintain homeostasis (Macedo, 2012). In fact, plants have developed a series of tactics that can be classified as avoidance and tolerance mechanisms to adapt to adverse environmental stresses, such as heat stress (Hasanuzzaman et al., 2013).

Heat avoidance, or the plant’s ability to avoid heat stress, involves mechanisms that allow them to maintain growth and development, by reducing the inner temperature, reflecting heat, and protecting sensitive tissue under high temperature (Madlung and Comai, 2004; Bueckert and Clarke, 2013). Some heat avoidance mechanisms are: changing leaf orientation, reduction of absorption of solar irradiation, transpiration cooling, altering of membrane composition, and adjusting leaf morphology, among others (Hall 1992; Wahid et al., 2007; Bueckert and Clarke,

2013). For example, shifting leaf orientation from horizontal to vertical reduces leaf heat and light stress under a lack of water supply (Tozzi et al., 2013). The presence of a thick coat of small hairs on the surface of leaves (hirsuteness) contributes to reduce absorption of solar radiation and protects cuticles under hot environments (Holmes and Keiller, 2002; Hasanuzzaman et al., 2013). Transpirational cooling occurs in plants under well-watered conditions, where evaporation of water through stomata reduces the temperature of the leaves under warm conditions (Crawford et al., 2012; Porch and Hall, 2013).

In contrast to heat avoidance, heat tolerance is described as the plant's ability to maintain its functions and render adequate but lower yield under high temperature (Hall 1992; Hemantaranjan et al., 2014). According to Hasanuzzaman et al. (2013) and Wahid et al. (2007), some remarkable tolerant mechanisms include ion transport, osmoprotectants, free radical scavengers, and factors involved in signaling cascades and transcriptional control. Among these mechanisms, the production of ROS and antioxidants in a control pathway has been identified as part of adaptation to heat stress and acquired thermotolerance (Maestri et al., 2002; Wahid et al., 2007). As such, heat tolerance is a highly specific trait that entails a specialized metabolism and structural organization of the plants (Bueckert and Clarke, 2013).

Also, escape accounts for another essential mechanism that plants used to minimize the effect of heat stress (Prasad et al., 2017). According to Barnabás et al. (2008), plants' escape strategies encompass a short life cycle, a higher rate of growth, and using reserves for production before the onset of severe stress. In fact, early maturation in many crops can reduce yield losses under high temperature (Levitt 1972; Bueckert and Clarke, 2013). A hastened life cycle under high temperature has been observed in faba bean, field pea, lentil, wheat, and durum, among others (Bhandari et al., 2016; Dias and Lidon, 2009; Guilioni et al., 2003). In fact, under warmer temperatures, leaf and crop development rate can be increased leading to a rapid senescence of plants (Prasad et al., 2008). Alternatively, early morning flowering is another strategy to escape high temperatures during the day (Satake and Yoshida 1978; Sheehy et al., 2005). For example, in rice, where flowering time varied during the day (1000 to 1200 h), cultivars with an early morning trait showed reduced spikelet sterility and higher yield (Ishimaru et al., 2010).

2.3 Plant photosynthesis and reproductive development under heat stress

Temperatures above plant optimal conditions (heat stress) can disturb many physiological and metabolic processes that constrain plant development and growth (Bhandari et al., 2016). Among the plants' different processes, photosynthesis is the most sensitive to high temperatures (Wahid et al., 2007). Plants under high temperatures can experience damage to essential components for the photosynthetic process, such as chloroplasts, RUBISCO (ribulose 1,5-bisphosphate carboxylase/oxygenase) enzymes, photosynthetic pigments, photosynthetic apparatus, among others. Under extreme temperatures, the structure of the chloroplast changes and disorganization of thylakoids, stroma and grana swelling are observed (Gounaris et al., 1984; Karim et al., 1997; Zhang et al., 2005). Consequently, chloroplast structure is damaged and linked to an increase in ion-leakage in leaf cells of heat-stressed plants (Wahid and Shabbir, 2005; Bita and Gerats, 2013).

Ribulose pathway enzymes, highly sensitive to high temperatures, can show alterations in their functions and lead to severe reduction of photosynthesis (Maestri et al., 2002). In some cases, although the catalytic activity of RUBISCO enzyme can increase, its affinity for CO_2 change and photosynthesis is still compromised (Salvucci and Crafts-Brandner, 2004). Similarly, the photosynthetic apparatus is highly compromised in plants exposed to heat stress because extreme temperatures can disrupt the photosystem I and II activity (Al-Khatib and Paulsen 1999; Sharkey, 2005). For example, temperature above 40°C is reported to damage the functioning of the photosystem II (Sharkey, 2005). Although moderate heat stress (<40°C) does not cause direct damage to the photosystems, an excessive production of reactive oxygen species is reported to inhibit photosystem II repairing (Allakhverdiev et al., 2008). In addition to these effects, high temperature can impair protein degradation, membrane fluidity, hormone homeostasis, protein folding, among other metabolic processes (Bhandari et al., 2017; Sita et al., 2017). Taken together, the increase in temperature above optimal plant conditions drives critical metabolic changes that can reduce photosynthetic activity in plants.

Several studies have shown that carbohydrate synthesis and carbohydrate transport from sources (mature leaves) to sinks (flowers and fruits) can be disrupted under high temperatures (Aloni et al., 1991; Ruan et al., 2010; Suwa et al., 2010). According to Liu et al. (2019), high

sensitivity during reproductive development can be attributed to sugar depletion in many plants. For example, high temperature (35℃) in pepper caused a reduction of sucrose export from leaf to flower, buds, and roots (Aloni et al., 1991). Similarly, studies in cotton showed that high temperature (38℃) decreased soluble carbohydrates in pistils and constrained reproductive organ development (Snider et al., 2009). Specifically, the reduction of carbohydrate synthesis in plants under heat stress reduces sucrose content translocated to reproductive organs (Sita et al., 2017). Once sucrose is transported to the sinks in development, it is degraded to derivates through enzymes such as invertase (Ruan et al., 2010). However, under high temperatures, the activity of invertase enzymes can be highly inhibited (Frank et al., 2009). In maize, increased biomass and a reduction of yield grain suggested that high temperature affected sink but not source activity (Cheikh and Jones, 1995). Later studies showed that high temperature disrupted invertase activity and constrained sucrose degradation and starch biosynthesis in the grains (Suwa et al., 2010). Besides, plants under high temperature divert resources to produce metabolites, such as heat shock proteins, antioxidants, and osmolytes that allow them to cope with heat stress (Farooq et al., 2011; Mittler et al., 2012). All of those molecules are of high cost and reduce even more sucrose availability leading to starvation of reproductive organs (Wahid et al., 2007). It is worth noticing that although elevated temperature decreased sucrose content in reproductive organs in various crops, studies on the effect of high temperature on sucrose content are still scarce in crops such as legumes (Liu et al., 2019).

2.4 Heat stress and female reproductive organs of flowering plants

High temperatures are a limiting factor for optimal production of many crops. In general, plant development and growth are compromised under heat stress conditions, and sexual reproduction is one of the most stress-sensitive stages (Wang et al., 2006; Zinn et al., 2010). When elevated temperatures coincide with the plant's reproductive phase, male and female organs of the flowers can be negatively affected during the process (Sage et al., 2015). According to Herrero (2003), high temperatures can affect the development of parent tissue (male and female), causing an asynchrony that jeopardizes normal reproductive processes. Although the flower's male organs are believed to be the most sensitive tissue to high temperatures, the additive negative effect of heat stress on female tissue can increase the reduction of fruit set, as well (Hedhly, 2011). This effect has been observed in canola (*Brassica napus* L.), where reciprocal crosses between male

and female parts of the flower showed high yield reduction especially when both structures were exposed to heat stress (Young et al., 2004). Some effects of the elevated temperature on the flowers' female reproductive organs are the disturbance of the growth of gynoecium, the viability of the gametophyte, and embryo development.

The development of the flower's female organs can be altered by an increase of temperature in the environment (Rodrigo and Herrero, 2002; Iwahori, 1966). Indeed, high temperatures during the plant's flowering stage can cause abnormal growth of pistils and female gametophytes, leading to a lack of synchrony in the reproductive development (Hedhly, 2011; Herrero, 2003). For example, when apricot flowers were exposed to an increase of temperature between 6 to 7℃, blooming was accelerated, resulting in flowers with undeveloped pistils that affected the pollination and consecutively fruit set (Rodrigo and Herrero, 2002). In related research in tomato, temperatures of 40℃ for four days caused malformation in internal ovule development displaying nuclear membrane damage, an empty nucellus, and degenerated endosperm after fertilization (Iwahori, 1966). Additionally, elevated temperatures have led to abnormal development or absence of the embryo sac in crops, such as peach and wheat (Kozai et al., 2004; Hedhly, 2011). Hence, heat stress may disturb the healthy growth of the ovule affecting its performance during the reproductive process.

High temperatures during reproductive development can also reduce ovule longevity. In some studies, callose accumulation reduced ovule fertilization and caused starvation of the embryo in fertilized ovules in various crops (Cerović and Ružić, 1992; Hedhly, 2011). The identification of callose deposition in ovule tissue has been widely employed to identify ovule senescence (Dumas and Knox, 1983; Sun et al., 2004). For example, in a study carried out by Cerović and Ružić (1992), flowers of sour cherry (*Prunus cerasus* L.) exposed to temperatures of 25℃ during 10 DAF, presented high intensities of callose deposition in senescent ovules, and pollen tubes displayed abnormal behavior without entering the micropyle of those ovules. Similarly, high intensity callose deposition has been identified in the ovule's chalaza after exposure to high temperatures during senescence in ovules of sweet cherry (Postweiler et al., 1985). According to Piršelová and Matušíková (2013), callose presence in cells is related to stress conditions because the polysaccharide is synthesized as a mechanism of defense in plant tissue. It is also suggested that this mechanism can limit the regular transport of sugar from ovule tissue towards the embryo

(Lersten, 2008; Sun et al., 2004). In pea, although there is not enough evidence to prove whether high temperatures affect ovule viability, Briggs et al. (1987) described that callose deposition in different stages of the ovules is, in fact, a signal of early ovule abortion imposed by maternal tissue. Therefore, ovule viability, fertilization, and embryo development can be disrupted under heat stress.

As a summary, Table 2.1 outlines numerous studies that have investigated the effects of heat treatments on various components of the female reproductive structures of flowers, selected from a diverse assemblage of agricultural and horticultural crop species.

Table 2.1 Some relevant studies of the effect of high temperature on female reproductive structures in flowers from various crops.

Crop	Heat Treatment	Female Structure Affected	High Temperature Effect	References
Arabidopsis thaliana	30°C and 33°C at bolting stage.	Ovules	Reduction of ovule number and increasing ovule abortion.	Whittle et al., 2009
Canola	32/26°C day/night.	Ovules and stigmas	Protruding stigma and aberrant ovule development.	Polowick and Sawhney, 1988
Cherry	20°C during anthesis for 5 d.	Ovules	Reduced ovule viability.	Postweiler et al., 1985
	25°C after anthesis for 10 d.	Ovules	Increased ovule senescence.	Cerović and Mićić, 1999
	30°C at anthesis stage for 4 d.	Stigma	Reduction of stigma receptivity	Hedhly et al., 2003
Common bean	35/26.5°C - day/night.	Ovules	Increased rate of ovule development.	Ormrod et al., 1967
	32/27°C - day/night before anthesis for 2 d.	Embryo sac	Embryo sac failure after anthesis	Gross and Kigel, 1994
Cucumber	30°C constant temperature and 27.5°C - during anthesis for 4 d.	Ovary	Hastening of flower anthesis and fruit growth rate.	Marcelis and Hofman-Eijer, 1993
Peach	20 and 30°C at anthesis stage.	Stigma	Reduction of stigma receptivity.	Hedhly et al., 2005
	25°C and 30°C constant temperature starting before blooming.	Ovules	Hastening flower development but suppressed ovule and embryo sac growth.	Kozai et al., 2004
Rice	45/30°C day/night during vegetative and flowering stage.	Pistil	Pistil hyperplasia.	Takeoka et al., 1991
Tomato	40°C after hand pollination for 4d.	Ovules and pro-embryos	Endosperm collapse, embryo development retarded, and pro-embryo aborted.	Iwahori, 1966
	26.7°C to 33°C constants at flowering bud stage.	Style and stigma	Increased stigma exertion and reduced stigma receptivity.	Charles and Harris, 1972

	33/23℃ day/night.	Ovules and styles	Reduced ovule viability and increased style exertion.	Fernandez-Muñoz and Cuartero, 1991; Levy et al., 1978
	35/30℃ day/night.	Style	Increased stigma exertion.	Lohar and Peat, 1998
	25 ℃ constant temperature after flower opening.	Ovary	Parthenocarpic fruits, reduction of ovary size, and fast fruit growth.	Adams et al., 2001
	35/30℃ - day/night after anthesis for 12 d.	Style	Reduced cell numbers on styles.	Pan et al., 2019
Wheat	30℃ - at onset of meiosis of anthers for 3 d.	Ovary and Ovules	Abnormal and absent embryo sacs in ovules.	Saini et al., 1983
	38℃ during early kernel development.	Embryos	Increased ethylene production in embryonic tissue.	Hays et al., 2007

Chapter 3. Gynoecium and Ovule Development of Field Pea under Heat Stress

3.1 Introduction

High temperature in the environment is a major factor threatening crop productivity around the world (Cross et al., 2003; Waraich et al., 2012; Bueckert et al., 2015; Fahad et al., 2017; Traub et al., 2018). Heat stress or temperatures exceeding the threshold of crop tolerance impairs plant development and leads to seed yield loss in many cereal and legume crops (Wahid et al., 2007; Kaushal et al., 2013; Bueckert et al., 2015). According to several studies, an increased temperature during the reproductive phase of the plants causes damage of reproductive structures, such as flowers, fruits, and early seeds (Snider and Oosterhuis, 2011; Bhandari et al., 2016; Sita et al., 2017). For example, temperature over 30℃ during reproductive development reduced number of flowers and pods in legume crops, such as chickpea (*Cicer arietinum* L.; Wang et al., 2006), cowpea (*Vigna unguiculata* L.; Ahmed et al., 1992), common bean (*Phaseolus vulgaris* L.; Ofir et al.,1993), lentil (*Lens culinaris* Medic.; Bhandari et al., 2016), soybean [*Glycine max* (L.) Merr.; Djanaguiraman et al., 2013], and field pea (*Pisum sativum* L.; Jeuffroy et al., 1990). Understanding the influence of high temperature during the reproductive process is a critical step to improve the selection of robust breeding lines for more efficient reproductive performance under this abiotic stress.

Specifically, in field pea, heat stress reduces the number of seeds by increasing abortion of reproductive organs, such as flowers, seeds, and young fruits (Karr et al., 1959; Guilioni et al., 2003; Bueckert et al., 2015). Jeuffroy et al. (1990), found that pea plants exposed to 31℃ for two to four days during their flowering stage displayed yield reduction even though their number of nodes was not affected, which means that abortion of seeds and pods occurred. In further studies in glasshouse, growth chamber, and field conditions, temperatures ≥ 30℃ for four days during reproduction caused abortion of buds, flowers, and young pods on plants depending on their position on the stem. (Guilioni et al., 1997; Guilioni et al., 2003). In this way, abortion of seeds, flowers, and young fruits has been identified as the foremost effect of heat stress linked to yield reduction; however, the specific causes behind their abortion are still unclear (Bueckert et al., 2015). Recent research of the male contributor (androecium) of the process of reproduction showed that 36℃ imposed on field pea plants for four and seven days during their flowering stage reduced pollen germination and composition, pollen tube length, and seed set (Jiang et al., 2015, 2017a).

Whereas adverse effects of the high temperature on pollen may be part of the cause of reproductive abortion in field pea, the response of the female component, specifically whether high temperature intensifies ovule abortion or not, remains to be elucidated.

Most studies on the effect of heat stress during reproductive development in flowering plants are concentrated on the male component because it has been thought to be the most sensitive and pollen is relatively easy to access (Prasad et al., 2006; Djanaguiraman et al., 2013; Devasirvatham et al., 2012). However, several reports (Gross and Kigel, 1994; Cross et al., 2003; Giorno et al., 2013) indicated that the female contributor of reproduction can be sensitive to high temperature too. In particular, the ovule and its embryo sac can be susceptible to temperature stress and can consequently be aborted in some plants (Erickson and Markhart, 2002; Young et al., 2004). Studies on common bean (*Phaseolus vulgaris* L.), wheat (*Triticum aestivum* L.), peach (*Prunus persica* Batch.), and *Arabidopsis thaliana* (L.) Heynh. all revealed that plants exposed to temperatures over 25°C displayed ovules with poor growth, reduced viability, cellular disorganization of the embryo sac, and absence or retarded embryo sac growth that lead to unsuccessful reproduction (Ormrod et al., 1967; Saini et al., 1983; Kozai et al., 2004; Whittle et al., 2009). Whereas the effects of temperature on both pollen and ovule are mainly related to gamete development and the programic phase (pollination and fertilization), a third phase involving post-zygote and embryo development (after fertilization) can also be susceptible to high temperature (Hedhly, 2011; Ozga et al., 2016). For example, in chickpea, soybean, and pea, high temperature caused abortion of fruits after fertilization and during seed filling (Jeuffroy et al., 1990; Wang et al., 2006; Siebers et al., 2015). In general, the female counterpart of the reproductive process under heat stress has received less research, so its assessment will likely provide critical insights about the reproductive process and consequently how yield of field pea is affected under this abiotic stress.

In this chapter, I aimed to investigate the influence of high temperature on female reproductive flower parts (gynoecium) and its ovules in flowers at different stages of development on the first four reproductive nodes of six field pea cultivars. I hypothesized that if plants were exposed to heat stress, then they would display poor (small) ovaries, styles, and stigma growth, and the ovules would show disrupted embryo sacs relative to node position and the heat tolerance of each pea cultivar.

3.2 Materials and Methods

3.2.1 Plant material and growth chamber conditions

The experiment was carried out with six cultivars of field pea (*Pisum sativum*): 40-10, Naparnyk, CDC Meadow, CDC Sage, Carneval, and MFR043. These cultivars were selected for their range of heat tolerance based on their seed-to-ovule ratio exhibited in previous heat stress trials of the Pea Association Mapping (PAM) panel at the University of Saskatchewan (Table 3.1; Jiang et al., 2017a). These plants were grown under control conditions in growth chambers, where the light was supplied by banks of cool fluorescent tubes providing an irradiance of ~ 450±5 µmol photons m^{-2} s^{-1}. In the chambers, photoperiod was set up at 16h light/8h dark, and the temperature was kept at 24℃ during light (day) time and 18 ℃ during dark (night) time. For each pot or experimental unit, four seeds of each cultivar were sown in cylindrical pots of 7.6 L filled with peat base mix (Sunshine®, RR. Horticulture Canada Ltd., Edmonton, AB, Canada), and 20 g of a slow-release fertilizer 14-14-14 (Nutricote®, Brampton, ON, Canada). Watering was provided, so plants avoided conditions of drought stress. After germination, plants were thinned from four to two plants per pot at the 3 to 4-leaf stage (Jeuffroy et al., 1990) and the experimental unit was considered one plant per pot. Half-strength Hoagland nutrient solution (Hewitt, 1952) was provided every other day starting from three weeks until six weeks after sowing.

Table 3.1 List of cultivars with their respective characteristics of leaf type, origin, and level of heat tolerance based on seed-to-ovule ratio from previous trials of the Pea Association Mapping (PAM) panel.

Cultivar	Leaf Type	Origin	Heat Tolerance
40-10	Normal	Germany	High
Naparnyk	Normal	Eastern Europe	
CDC Meadow	Semi-leafless	CDC, Canada	Medium
CDC Sage	Semi-leafless	CDC, Canada	
Carneval	Semi-leafless	Western Europe	Low
MFR043	Normal	CDC, Canada	

3.2.2 Experiment design and treatment

The experiment was set up in a randomized complete block design (RCBD) with 48 plants corresponding to six cultivars, two temperatures, and four replications. In all cases, when plants growing at 24℃ day/18℃ night presented opened flowers (stage 0.5 according to Maurer et al.,

1996) on reproductive Node 1, flower buds with petals tightly closed on reproductive Node 2 (stage 0.2 according to Maurer et al., 1966), and flower buds with sepals covering the flower structure on reproductive Node 3, half of the plants were transferred to a chamber where temperature was set up at 35℃ day/18℃ night in cycles for four days. The cycles of temperature were established according to a photoperiod of 16 h light and 8 h dark, where 18℃ were kept during dark time (night), and temperature was increased 3℃ every hour during light time (day) until achieving 35℃. Then, the temperature was kept for 6 h and dropped by 3℃ every hour until the chamber returned to 18℃. The remaining plants from each subset were maintained under 24℃ day/18 ℃ night, thereby representing the controls.

3.2.3 Sample collection and measurements

When the four days of treatment finished, flowers and young fruits were collected from reproductive Node 1 to Node 4 on plants from control and heat stress conditions (Fig. A2.2). Flower stage was recorded on these samples before and after treatment according to the decimal system of blossom opening described by Maurer et al. (1966). The stage of the flowers was rated from 1 to 10 points based on the most common stages found at the time of collection on the six cultivars, e.g., 1 was assigned to the youngest stage and 10 to the oldest stage of the flower. The gynoecium of these flowers was evaluated by measuring length of style, and length and width of ovaries from the four reproductive nodes of the plants. Since floral organ development took place on reproductive Node 3 and Node 4 during treatment, stigma length was evaluated on flowers localized on those nodes. The measurement was performed by mounting the tissue on slides and evaluating it under light microscopy and software Image J.

Pistils from Node 1 to Node 4 were then dissected under a stereomicroscope by carefully removing one of the ovary walls to keep the ovules attached to the suture of the pod on the other wall. These samples were fixed in a solution made of 3.7% formaldehyde fixative and 5% glacial acetic acid in 1x phosphate-buffered saline (PBS) for at least 24 h (Enugutti et al., 2013). Afterward, the internal development of the ovules was visualized by applying a clearing-staining procedure by using Mayer's Hemalum stain with some modifications (Schneitz et al., 1997). Briefly, the tissue was washed twice in PBS, then stained with Mayer's Hemalum for 30 minutes. Later, the tissue was washed with distilled water, destained with 1% acetic acid for 45 min., and dehydrated in an ethanol series. Finally, the tissue was placed in methyl benzoate (≥98%) overnight

at room temperature and mounted on slides to be evaluated under a light microscope the following day (Enugutti et al., 2013; Fig. A2.3). In these samples, ovule length and embryo sac area were measured by using a Zeiss Axioplan microscope.

Later, ovule fertilization and embryo sac stage were assessed mainly on flowers taken from reproductive Node 1, Node 2, and Node 3 of the plants, because in those flowers, the process of fertilization had already been accomplished. An ovule was considered fertilized when the embryo sac showed signs of expansion and contained an embryo at different stages of development, such as zygote, pro-embryo, or globular stage. In contrast, an unfertilized ovule was recognized when the embryo sac showed lack of growth (small size) or expansion, and absence of an embryo growing in its cavity. In parallel, embryo sac stage was evaluated within ovules from these same nodes on cultivars 40-10, Naparnyk, CDC Meadow, CDC Sage, and Carneval by rating their embryo sacs according to their degree of development (Table 3.2). Cultivar MFR043 was excluded from these assessments because it displayed different flower morphology that compromised its normal process of fertilization and, therefore, its general ovule development status (Appendix 1). Specifically, embryo sac development was rated from 0 to 5 for the five cultivars, as described in Table 3.2.

3.2.4 Data analysis

The analytical procedure was performed by employing a Linear Mixed Model to consider the nested structure of the experimental design. This method was executed using SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). Treatment, cultivar, reproductive node, pod position, ovule position, and their interactions, were treated as fixed effects with their respective nested structure, whereas replication and its interaction terms were considered as random effects. The Kenwardroger option was used to approximate the degrees of freedom for unbalanced data, e.g., plants with one or two pod positions per node. In cases where significant differences were found, a post hoc test was applied to determine the difference of levels from the response variables using the Least Significant Difference (LSD) test. Since field pea cultivars used in this research varied in seed number and seed size genetically, they contained a varied number of ovules aligned on the suture within the ovary/pod. As a result, some of the ovules were positioned closer or further from the maternal supply, so this ovule position effect was standardized across cultivars. Three positions were considered: stylar, ovules localized closest to the style; medial, ovules at the medial

area within the ovary/pod; and basal, ovules closest to the pedicel end of the ovary/pod (Gutiérrez et al., 1996; Jiang et al., 2017a; Fig. 3.1). That is, the total number of ovules within each ovary/pod was divided into three regions, and when the number of ovules could not be divided evenly by three, the maximum difference in the number of ovules between categories was one (Gutiérrez et al. 1996; Table A2.1). Because ovule length and embryo sac area were evaluated on ovules from nodes at different ages in the same plant, data were transformed with the natural logarithm to meet the assumptions of the parametrical statistical analysis, i.e., normality and homoscedasticity.

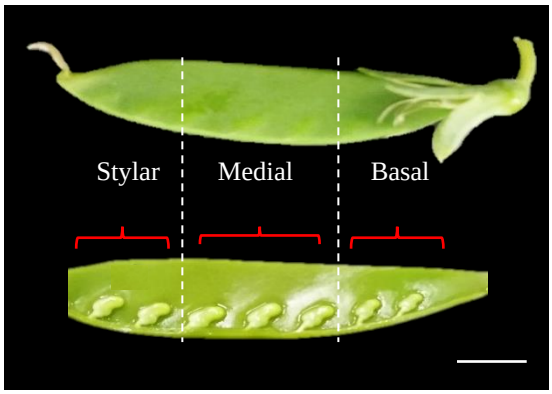
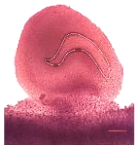
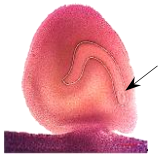
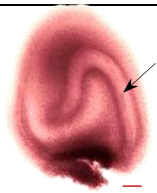
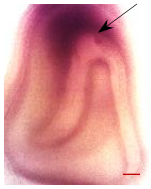
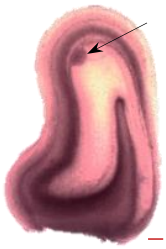


Fig. 3.1 Standardized ovule positions within each pod considered in the evaluation of six field pea cultivars, where ovules closest to the style end were identified as the stylar position, ovules closest to the pedicel end were identified as the basal position, and ovules in the middle were identified as the medial position within the pod. Scale bar = 5 mm.

Table 3.2 Rating and description of embryo sac stage in ovules collected immediately after four days of treatment (36℃/18℃ or 24 ℃/18℃) from flowers at reproductive Node 1, Node 2, and Node 3 of five field pea cultivars. Ovules cleared and stained with Mayer's Hemalum (see section 3.2.2). Scale bar = 100 µm

Description of Embryo Sac Stage	Image of Embryo Sac	Rating
• Embryo sac of small size without presence of the zygote or embryo in its cavity, equivalent to an unfertilized embryo sac.		0
• Embryo sac displaying enlargement of its cavity and presence of the zygote or pro-embryo stage growth (arrow).		1
• Embryo sac displaying enlargement of its cavity and presence of the embryo at early globular stage growth (arrow).		2
• Embryo sac displaying enlargement of its cavity and presence of the embryo at globular stage approaching the dome of the ovule (arrow).		3
• Embryo sac at high expansion, identified by widening of one side of its cavity containing an embryo at globular stage already at the dome of the ovule (arrow).		4
• Embryo sac at maximum expansion, showing a wide cavity containing an embryo in transition from globular to heart stage (arrow).		5

3.3 Results

3.3.1 Flower development

Evaluation of the flower stage on reproductive Node 1 to Node 4 of the six cultivars after treatment revealed that high temperature caused a significant effect on flower development at the level of reproductive node across all cultivars (Table 3.3). Specifically, flowers at reproductive Node 3 and Node 4 tended to show a more advanced stage of the flowers on plants subjected to heat stress, but the effect was mainly significant at reproductive Node 3 on all plants compared with their controls (Fig. 3.2). Independently of the treatment, cultivars Naparnyk, CDC Meadow, and Carneval exhibited the oldest flower stages whereas MFR043 exhibited the youngest flower stages (Table 3.4). The acropetal development of the plant showed a normal trend where reproductive Node 1 had the oldest flower stages, followed by Node 2, Node 3, and Node 4. Similarly, flowers at the proximal positions in the raceme at each node had more advanced stages compared with their respective flower at distal positions (Table 3.4).

3.3.2 Stigma and style length

The effect of high temperature evaluated on stigma and style length at reproductive Node 3 and Node 4 was significant depending on cultivar (Table 3.3). Stigma length on cultivar CDC Sage and style length on cultivar 40-10 were larger on plants exposed to heat stress (Table 3.5). Contrary to this trend, flowers of CDC Meadow displayed significantly shorter stigmas and styles, and flowers of cultivar Carneval showed shorter styles on flowers of plants exposed to heat stress compared to their controls (Table 3.5). Interestingly, although stigmas of flowers at proximal positions were slightly larger than those at distal positions of a flowering raceme (Node 3 and Node 4) under control conditions, the trend changed on plants exposed to high temperature. That is, high temperature treated plants displayed significantly larger stigmas on flowers at distal positions compared with flowers at proximal positions in the raceme of these nodes (Fig. 3.3). Regardless of the treatment, the largest stigmas in the group of cultivars were identified on CDC Sage and Carnival showing average size of 0.60 and 0.57 mm, respectively (Table 3.4). The largest styles were identified on cultivars Naparnyk and MFR043 displaying average size of 7.38 and 7.14 mm, respectively (Table 3.4). Conversely, the shortest stigmas were exhibited by flowers of cultivar 40-

10 with an average size of 0.53 mm, and the shortest styles were observed in flowers of cultivars CDC Meadow and Carneval displaying average size of 6.1 and 5.8 mm, respectively (Table 3.4).

3.3.3 Ovary width and length

Evaluation of ovary width and length from reproductive Node 1 to Node 4 showed a significant effect of temperature depending on cultivar and reproductive node location (Table 3.3). At reproductive Node 1, where flowers were fully open at the beginning of treatment, high temperature reduced ovary width on CDC Meadow and Carneval, and length on CDC Meadow and 40-10, compared with the respective node in plants under control conditions (Table 3.6). At reproductive Node 2, where flowers were still closed (stage 0.2, according to Maurer et al., 1966) when the treatment started, two contrasting effects of high temperature were observed. First, a significantly wider ovary on Naparnyk, and a wider and longer ovary on CDC Meadow; second, a significantly narrower and shorter ovary on Carneval and CDC Sage on plants exposed to heat treatment compared with their respective controls (Table 3.6). At reproductive Node 3, where little buds were present (buds still covered by sepals) when the treatment started, significantly larger ovaries were noted on plants exposed to high temperature. Specifically, flowers at this reproductive node displayed wider and larger ovaries on cultivar CDC Sage and wider ovaries on cultivars 40-10, Carneval, and Naparnyk exposed to high temperature compared with plants under control conditions (Table 3.6). Finally, at reproductive Node 4, where buds were still developing when heat treatment started, cultivar Naparnyk had wider ovaries on plants exposed to heat stress compared with the control plants, whereas heat treatment did not significantly affect the other cultivars (Table 3.6).

In general, most plants exposed to high temperature had reduced ovary size at reproductive Node 1, and increased ovary size at reproductive Node 3 (Table 3.6). Particularly, wider and longer ovaries were evident on plants of cultivar Naparnyk whereas smaller ovaries were obvious on cultivar Carneval after heat treatment (Table 3.5) compared with their controls. In addition, regardless of temperature treatment, ovary width and length were always greater on pods at proximal positions on nodal racemes compared to the distal positions (Table 3.4). Plants of Naparnyk displayed the largest ovaries ($\bar{x}$= 24.1±1.9 mm), whereas MFR043 had the smallest ovaries ($\bar{x}$=10.0 ±0.2 mm) compared with the rest of the cultivars (Table 3.4).

3.3.4 Number of ovules and ovaries (young fruits)

The number of ovules within an ovary (young pod) for the six cultivars was not affected by high-temperature treatment (Table 3.3). Instead, normal characteristics for each cultivar, as grown under chamber conditions, were observed for this trait. Naparnyk and CDC Sage produced more ovules per ovary showing an average of approximately 8 ovules per ovary, whereas cultivars 40-10 and MFR043 produced fewer ovules per ovary displaying an average of approximately 7 ovules per ovary (Table 3.4). Additionally, ovaries at the proximal position (older, dominant) in the raceme of all six cultivars always had a greater number of ovules than in ovaries at distal positions (Table 3.4).

Finally, high temperature did not affect the number of ovaries (young fruits) from reproductive Node 1 to Node 4 on the six cultivars evaluated right after 4 d of treatment (Table 3.3). Instead, significant differences of ovary numbers were detected among cultivars and nodes, regardless of treatment. Thus, plants from Naparnyk, CDC Meadow, and MFR043 were more likely to present two pods per node, while the tendency was reduced on Carneval and CDC Sage. As a general characteristic for all cultivars, reproductive Node 1 tended to produce two pods, and the probability of two pods was reduced as the position of the reproductive node on the main stem of the plant increased (Table 3.4).

Table 3.3 Analysis of variance for the effect of cultivar, treatment, node, pod position, and their interactions on floral stage, stigma and style length, ovary width and length, number of ovules per ovary, and number of ovaries per node after four days of high temperature treatment on six field pea cultivars (*Pisum sativum* L.) grown under growth chamber conditions. The three-way interactions that were non-significant are not shown.

Source of Variation	Floral Stage		Stigma Length (mm)		Style Length (mm)		Ovary Width (mm)		Ovary Length (mm)		No. of Ovule /Ovary		No. of Ovary /Rep. Node	
	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)	13.64	**<.0001**	12.03	**<.0001**	12.96	**<.0001**	42.49	**<.0001**	57.69	**<.0001**	21.39	**<.0001**	8.70	**<.0001**
Treatment (T)	1.08	0.3004	1.16	0.2843	0.04	0.8511	4.04	**0.0456**	1.94	0.1657	0.23	0.6302	0.29	0.5962
Node (N)	706.77	**<.0001**	0.03	0.8704	0.05	0.8273	454.88	**<.0001**	524.11	**<.0001**	0.75	0.5221	9.05	**<.0001**
Pod position (PP)	81.98	**<.0001**	5.99	**0.0168**	0.78	0.3789	118.31	**<.0001**	43.89	**<.0001**	33.74	**<.0001**	-	-
C*T	0.60	0.6989	4.52	**0.0013**	3.82	**0.0029**	3.91	**0.0020**	2.41	**0.0384**	1.47	0.2019	0.51	0.7694
C*N	5.43	**<.0001**	0.45	0.8112	1.02	0.4109	8.56	**<.0001**	14.07	**<.0001**	1.01	0.4499	0.89	0.6009
C*PP	1.41	0.2235	1.54	0.1888	1.39	0.2339	3.33	**0.0064**	1.49	0.1976	3.22	**0.0088**	-	-
T*N	3.05	**0.0305**	2.94	0.0911	2.47	0.1186	11.93	**<.0001**	5.46	**0.0013**	1.53	0.2103	0.62	0.6454
T*PP	3.64	0.0582	10.6	**0.0017**	0.01	0.9178	3.43	0.0655	3.07	0.0816	1.88	0.1719	-	-
N*PP	7.87	**<.0001**	0.01	0.9043	0.48	0.4894	3.69	**0.0127**	4.35	**0.0056**	1.96	0.1231	-	-
C*T*N	0.72	0.7651	0.51	0.7643	0.46	0.805	2.82	**0.0005**	2.33	**0.0048**	0.80	0.6763	0.77	0.7398

Significance levels at P ≤0.05 and P≤0.001 are shown in bold.

Table 3.4 Means (± SE) of flower stage (n=42 to 192), stigma and style length (n=19 to 96), ovary width and length (n=42 to 192), number of ovules per ovary (n=42 to 192), and number of ovaries per reproductive node (n=42 to 84) according to the source of variation: cultivar, reproductive node, and pod position of field pea plants (*Pisum sativum* L.) grown under growth chamber conditions. For more details of sample size, see Table A2.3.

Source of Variation	Floral Stage	Stigma Length (mm)	Style Length (mm)	Ovary Width (mm)	Ovary Length (mm)	No. of Ovules per Ovary	No. of Ovaries per Rep. Node
Cultivar							
40-10	6.53±0.32 b	0.53±0.005 d†	6.69±0.10 bc	4.50±0.40 b	20.35±1.75 b	6.78±0.06 c	1.50±0.08 bcd
CDC Meadow	6.74±0.31 ab	0.54±0.007 cd	6.11±0.10 de	4.85±0.38 b	18.42±1.48 b	7.27±0.09 b	1.88±0.05 ba
CDC Sage	6.48±0.34 b	0.60±0.008 a	6.43±0.08 cd	5.39±0.47 b	21.27±2.01 b	7.72±0.07 a	1.28±0.07 d
Carneval	6.73±0.31ab	0.57±0.007 b	5.79±0.07 e	5.36±0.45 b	20.26±1.87 b	7.29±0.09 b	1.38±0.08 dc
MFR043	5.69±0.19 c	0.55±0.006 bc	7.14±0.11 ab	2.55±0.08 c	10.00±0.22 c	6.95±0.08 bc	1.73±0.07 abc
Naparnyk	6.89±0.30 a	0.54±0.006 cd	7.38±0.12 a	5.59±0.41 a	24.06±1.92 a	7.89±0.09 a	1.98±0.03 a
Reproductive Node							
Node 1	9.34±0.12 a	-	-	8.80±0.34 a	36.14±1.58 a	7.39±0.08 a	1.77±0.06 a
Node 2	7.74±0.09 b	-	-	5.33±0.19 b	20.10±0.80 b	7.36±0.09 a	1.75±0.06 a
Node 3	5.32±0.12 c	0.55±0.005 a	6.59±0.079 a	3.01±0.10 c	11.22±0.32 c	7.25±0.08 a	1.65±0.06 ba
Node 4	3.64±0.07 d	0.55±0.005 a	6.58±0.080 a	2.03±0.03 d	8.79±0.14 d	7.28±0.07 a	1.50±0.07 b
Pod Position							
Proximal	6.77±0.17 a	0.55±0.005 b	6.56±0.056 a	5.29±0.25 a	21.05±1.12 a	7.46±0.06 a	-
Distal	6.25±0.17 b	0.56±0.005 a	6.61±0.055 a	4.29±0.20 b	17.07±0.86 b	7.18±0.05 b	-

†Values within a column and source of variation followed by the same letter are not significantly different at P<0.05.

Table 3.5 Effect of high temperature (35℃) on stigma and style length, ovary width and length on six cultivars of field pea (*Pisum sativum* L.) grown under growth chamber conditions. Means of four replications (stigma and style length n= 9 to 16; ovary width and length n=20 to 32, Table A2.3) and their respective standard error are shown.

Cultivar	Stigma Length (mm)		Style Length (mm)		Ovary Width (mm)		Ovary Length (mm)	
	Control (24°C)	Heat (35°C)	Control (24°C)	Heat (35°C)	Control (24°C)	Heat (35°C)	Control (24°C)	Heat (35°C)
40-10	0.53±0.004 de†	0.53±0.009 de	6.51±0.10 d	6.87±0.16 bc	5.02±0.615 b	4.97±0.530 b	21.41±2.72 c	19.29±2.22 cde
CDC Meadow	0.56±0.009 bc	0.52±0.007 e	6.26±0.18 df	5.96±0.07 eg	4.83±0.55 b	4.88±0.53 b	18.98±2.14 cde	17.87±2.08 e
CDC Sage	0.57± 0.012 b	0.63±0.012 a	6.35±0.16 cde	6.50±0.07 cd	5.53±0.68 b	5.25±0.67 b	21.53±2.86 cd	21.02±2.88cd
Carneval	0.56±0.006 bc	0.58±0.013 b	5.94±0.06 ef	5.63±0.12 g	5.62±0.70 b	5.11±0.59 b	21.44±2.67 c	19.08±2.64 de
MFR043	0.55±0.012 bcd	0.56±0.007 bcd	7.19±0.19 ab	7.10±0.10 ab	2.54±0.15 c	2.56±0.08 c	10.22±0.41 f	9.79±0.19 f
Naparnyk	0.54±0.010 cde	0.54±0.009 cde	7.24±0.21 ab	7.52±0.10 a	5.16±0.56 b	6.03±0.61 a	23.05±2.74 b	25.06±2.73 a

†Values within columns and variable followed by the same letter are not significantly different at P<0.05.

Table 3.6 Effect of high temperature on ovary width and length at the first four reproductive nodes of six field pea (*Pisum sativum* L.) cultivars right after four days of treatment under growth chamber conditions. Data means ± SE of four replications (n= 4 to 8, Table A2.3).

Cultivar	Ovary Width (mm)							
	Node 1		Node 2		Node 3		Node 4	
	Control	Heat	Control	Heat	Control	Heat	Control	Heat
40-10	9.7±0.8 a-d†	8.7±0.9 cd	6.0±0.8 f-i	5.6±0.7 f-i	2.5±0.2 p-t	3.4±0.4 l-n	2.0±0.08 t-v	2.1±0.12 r-v
CDC Meadow	9.4±0.4 a-c	8.2±0.9 de	4.9±0.5 ik	6.3±0.6 e-h	3.0±0.5 m-q	3.0±0.2 m-p	2.0±0.09 t-v	1.9±0.10 t-v
CDC Sage	11.0±0.7 ab	10.8±0.9 a-c	6.5±0.3 e-h	5.0±0.7 ik	2.6±0.2 p-s	3.3±0.4 l-o	2.0±0.05 t-v	1.8± 0.09 v
Carneval	11.4±0.7 a	9.5±1.1 b-d	6.3±0.4 e-g	5.5±0.6 h-j	2.9±0.3 n-q	3.4±0.2 lm	1.9±0.08 uv	2.0±0.13 t-v
MFR043	3.3±0.4 m-o	3.0±0.1 m-p	2.8±0.1 m-q	2.9±0.1 m-p	2.1±0.1 r-v	2.3±0.1 q-u	1.9±0.03 uv	2.0±0.07 t-v
Naparnyk	9.7±0.7 a-d	10.8±0.7 ab	5.5±0.4 gij	6.6±0.6 ef	3.4±0.4 mn	4.2±0.5 kl	2.1±0.09 s-v	2.6±0.10 o-r
	Ovary Length (mm)							
40-10	42.1±3.8 a	34.8±4.4 bc	24.5±3.6 def	21.6±2.9 efg	10.2±0.5 l-r	12.2±1.2 k-m	8.9±0.1 n-t	8.6±0.3 o-t
CDC Meadow	37.5±1.1 ab	31.6±4.2 cd	18.5±2.1 fh	22.2±2.2 eg	11.2±1.4 k-q	10.0±0.5 l-s	8.7±0.5 o-t	7.7±0.3 st
CDC Sage	43.8±4.7 ab	45.3±4.6 a	24.6±1.9 de	17.9±2.3 ghi	9.3±0.8 n-t	12.4±1.2 j-l	8.4±0.5 q-t	8.5±0.2 p-t
Carneval	43.1±3.3 ab	39.4±5.3 a-c	24.1±1.9 de	18.4±2.7 gh	10.3±0.8 l-r	11.0±0.3 k-o	8.2±0. 2r-t	7.5±0.3 t
MFR043	12.4±1.3 j-l	10.6±0.2 k-p	10.3±0.1 k-r	10.9±0.1 k-r	9.2±0.2 m-t	9.4±0.1 l-t	9.0±0.3 n-t	8.3±0.1 q-t
Naparnyk	45.8±4.0 a	47.3±3.4 a	22.6±2.4 efg	25.7±2.9 de	13.6±1.2 i-k	15.8±1.5 h-j	10.3±0.6 l-r	11.4±0.2 k-n

†Values within column and node followed by the same letter are not significantly different at P<0.05.

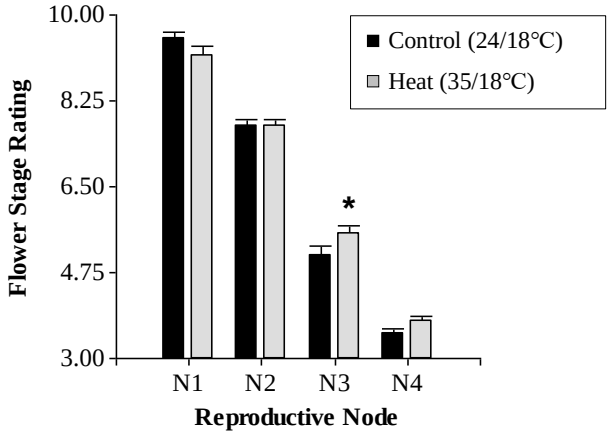


Fig. 3.2 Effect of high temperature on flower stage at the first four reproductive nodes of six field pea cultivars right after four days of treatments on plants grown under growth chamber conditions. Flower stage was rated from 1 to 10 according to decimal system of blossom opening described by Maurer et al. (1966). Means of four replications (n=34 to 42; for more details of sample size, see Table A2.3) with their respective error bars are shown. * Indicates a significant temperature treatment effect at P<0.05.

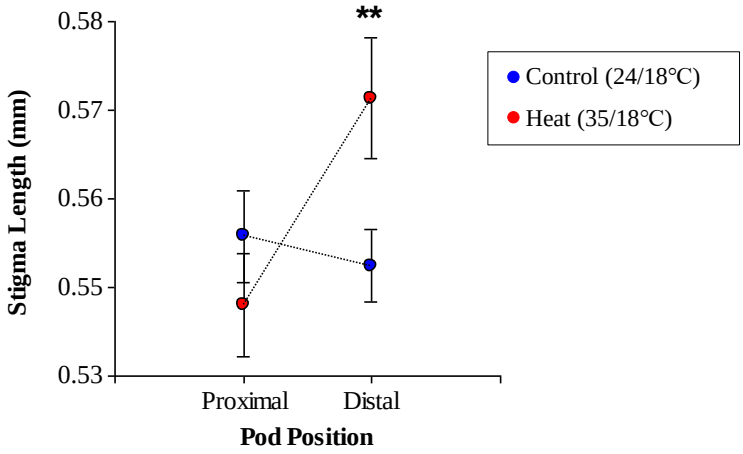


Fig. 3.3 Overall effect of high temperature on stigma length according to pod position evaluated on flowers of reproductive Nodes 3 and Node 4 on six field pea (*Pisum sativum* L.) cultivars grown under growth chamber conditions. Means of four replications (n=21 to 48; for more details of sample size, see Table A2.3) with their respective error bars are shown. ** Indicates a significant temperature treatment effect at P<0.01.

3.3.5 Ovule length and embryo sac area

Ovule length and embryo sac area were highly associated through a nonlinear relationship. The increase of ovule length is explained by the increase of embryo sac area with an efficiency of 0.97 and a bias equivalent to $-5.97e^{-11}$. In fact, the three parameters of the nonlinear regression, alpha, beta, and gamma, were highly significant (P<0.001; Table A2.2). In other words, an increase in embryo sac area meant an increase of ovule length under

control and heat stress conditions (Fig. 3.4). Interestingly, the curve between both variables also reflected a rapid increase of ovule length at initial ovule stages that later slows down specially when the ovules reached approximately 1 mm in length (Fig. 3.4).

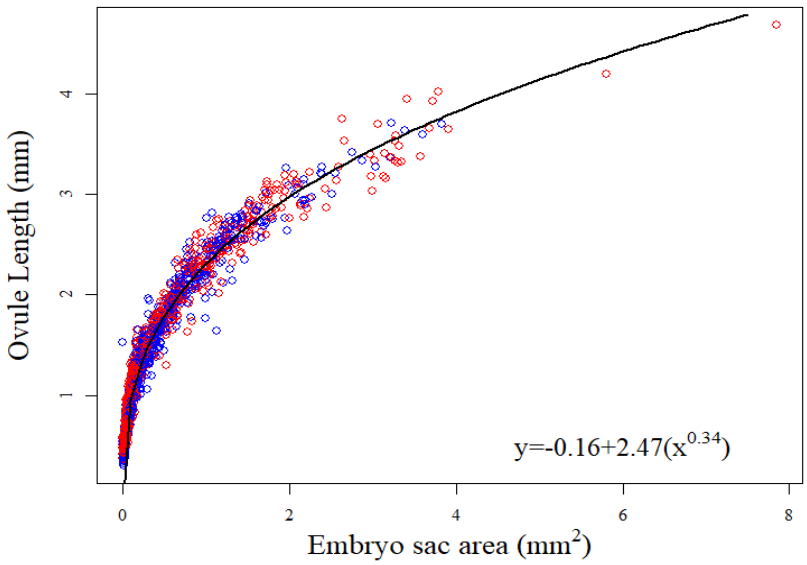


Fig. 3.4 Relationship between embryo sac area (mm^2) and ovule length (mm) of ovules in pods from reproductive Node 1 to Node 4 of five field pea cultivars (40-10, Naparnyk, CDC Meadow, CDC Sage, and Carneval) grown under growth chamber conditions. Bottom right of the plot displays the fitted equation from the nonlinear regression (power model) with efficiency =0.97 and bias =-5.97e^{-11}, P<0.001. Blue circles indicate ovules from plants that experienced 24℃ light /18℃ dark (control) conditions, whereas red circles indicate ovules from plants that experienced 35℃ light /18℃ dark (heat) conditions.

In terms of treatment, the effect of high temperature on ovule length and embryo sac area of the ovules was significant depending on cultivar and reproductive node (Table 3.7). Plants of cultivars Naparnyk and 40-10 exposed to heat displayed greater ovule lengths and embryo sacs. This effect was significant at reproductive Node 1 on Naparnyk and at reproductive Node 3 on 40-10 compared to the same reproductive node on plants under control conditions (Figs. 3.5 and 3.6). On plants from cultivars CDC Meadow and CDC Sage, two effects of the high-temperature treatment were identified. First, significantly smaller ovule lengths and embryo sacs area were detected at reproductive Node 1 on both cultivars compared to their controls; and second, significantly greater ovule lengths and embryo sacs were identified at reproductive Node 2 and Node 4 on CDC Meadow, and at reproductive Node 3 on CDC Sage compared to the same nodes on plants under control conditions (Figs. 3.5 and 3.6).

In contrast, although heat-treated Carneval plants had greater ovule lengths and embryo sacs areas on reproductive Node 1, an adverse effect was noticed at reproductive Node 2 and Node 3. Here, the ovule lengths and embryo sacs at these nodes on heat-treated plants were smaller than nodes at a similar position on plants under control conditions (Figs. 3.5 and 3.6). Finally, heat treated MFR043 had similar sized ovules and embryo sacs on all reproductive nodes but had smaller sizes on reproductive Node 1 compared to the respective reproductive node of plants under control conditions (Figs. 3.5 and 3.6). Overall, when the six cultivars were considered together, ovule length and embryo sac were both larger in ovaries of plants exposed to heat treatment (Table 3.8).

Independently from temperature treatment, Carneval, Naparnyk, and 40-10 had the largest ovules and embryo sacs, whereas MFR043 had the smallest ovules and embryo sacs in the group of cultivars. As expected, the first (and oldest) reproductive node had the largest ovules and embryo sacs, and reproductive Node 4 (the youngest) had the smallest ovules and embryo sacs in the plants (Table 3.8). Within a pod, ovules and embryo sacs at the medial position were significantly larger than those at stylar and basal positions regardless of cultivar, reproductive node, and temperature treatment (Table 3.8).

Table 3.7 Analysis of variance for the effect of cultivar, treatment, node, ovule position, and their interactions on ovule length, embryo sac area, and rating of the embryo sac stage after four days of treatment on six field pea (*Pisum sativum* L.) cultivars grown under growth chamber conditions.

Source of Variation	Ovule Length (mm)		Embryo Sac Area (mm^2)		Rating of Embryo Sac Stage					
					Node 1		Node 2		Node 3	
	F Value	P Value	*F* Value	*P* Value	*F* Value	*P* Value	*F* Value	*P* Value	*F* Value	*P* Value
Cultivar (C)	18.54	**<.0001**	14.31	**<.0001**	3.75	**0.0073**	3.69	**0.0077**	9.49	**<.0001**
Treatment (T)	19.58	**<.0001**	5.93	**0.0153**	0.86	0.3531	16.68	**<.0001**	1.87	0.1728
Node (N)	255.50	**<.0001**	237.56	**<.0001**	-	-	-	-	-	-
Ovule position (OP)	54.32	**<.0001**	35.82	**<.0001**	4.66	**0.012**	3.79	**0.0261**	3.2	**0.0457**
C*T	4.96	**0.0002**	7.72	**<.0001**	8.04	**<.0001**	9.79	**<.0001**	12.74	**<.0001**
T*N	7.68	**<.0001**	3.35	**0.0189**	-	-	-	-	-	-
C*N	5.22	**<.0001**	5.75	**<.0001**	-	-	-	-	-	-
C*OP	3.04	**0.0010**	2.00	**0.0322**	0.33	0.9506	0.31	0.9605	0.49	0.859
N*OP	1.65	0.1314	0.98	0.4398	-	-	-	-	-	-
T*OP	0.03	0.9714	0.02	0.9828	0.83	0.4356	0.01	0.9923	1.96	0.1422
C*T*N	6.68	**<.0001**	7.66	**<.0001**	-	-	-	-	-	-
C*T*OP	1.05	0.3963	0.71	0.7199	1.33	0.2277	0.59	0.7848	2.55	**0.0102**

Significance levels at P <0.05 and P<0.001 are shown in bold

Table 3.8 Means (± SE) of ovule length (mm) and embryo sac area (mm^2) according to source of variation: treatment (n=1132 to 1166), cultivar (n=324 to 498), reproductive node (n=502 to 620) and ovule position (n=748 to 800) on six field pea (*Pisum sativum* L.) cultivars grown under growth chamber conditions. For more details, see Table A2.4.

Source of Variation	Ovule Length (mm)	Embryo Sac Area (mm^2)
Cultivar		
40-10	1.21±0.077 ab†	0.421±0.070 ab
CDC Meadow	1.05±0.058 b	0.248±0.033 b
CDC Sage	1.25±0.074 a	0.364±0.056 ab
Carneval	1.36±0.090 a	0.549±0.088 a
MFR043	0.73±0.014 c	0.058±0.002 c
Naparnyk	1.30±0.073 a	0.366±0.051 ab
Treatment		
Control (24°C)	1.13±0.040 b	0.310±0.030 b
Heat (35°C)	1.17±0.042 a	0.358±0.037a
Reproductive Node		
Node 1	2.03±0.063 a	1.009±0.068 a
Node 2	1.22±0.028 b	0.231±0.013 b
Node 3	0.79±0.015 c	0.069±0.003 c
Node 4	0.56±0.006 d	0.029±0.000 d
Ovule Position		
Stylar	1.14±0.049 b	0.327±0.041 b
Medial	1.24±0.055 a	0.382±0.044 a
Basal	1.07±0.047 c	0.294±0.039 b

†Values within column and variable followed by the same letter are not significantly different at P<0.05.

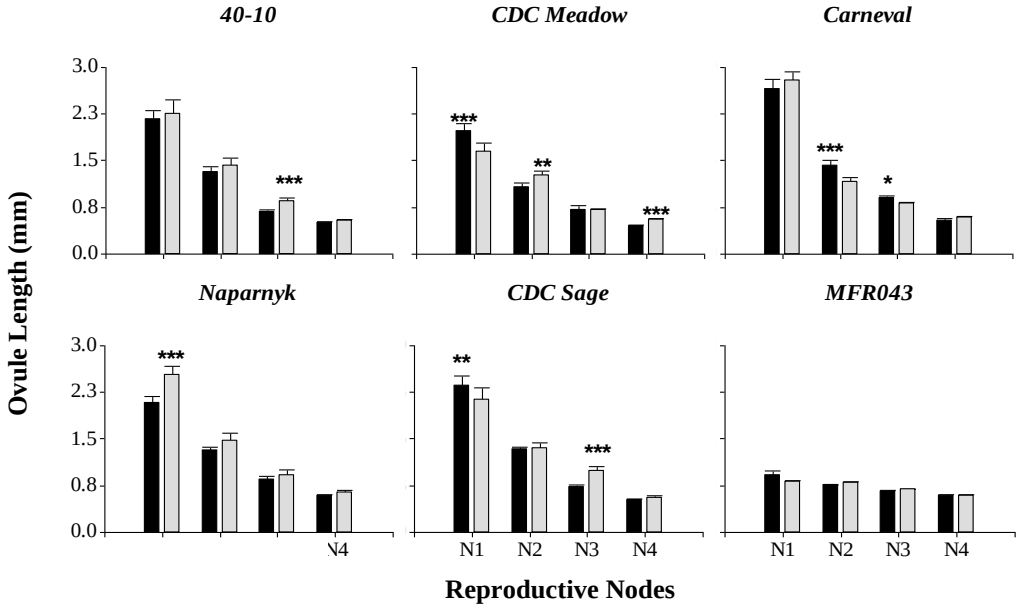


Fig. 3.5 Effect of high temperature (35/18°C) on ovule length (mm) on the first four reproductive nodes of six field pea (*Pisum sativum* L.) cultivars grown under growth chamber conditions. Means of four replications (n=27 to 68, Table A2.4) with their respective standard error bars are shown. Black bars represent the control; Grey bars represent heat treatment. *Indicates a significant temperature treatment effect at P<0.05; **Indicates a significant temperature treatment effect at P<0.01; ***Indicates a significant temperature treatment effect at P<0.001.

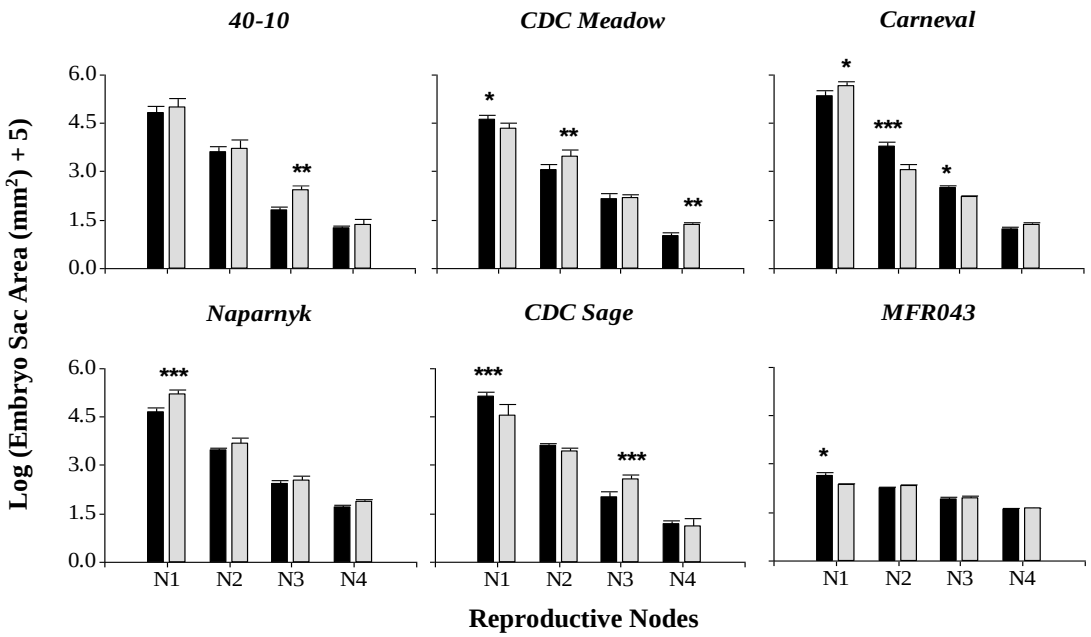


Fig. 3.6 Effect of high temperature (35/18°C) on embryo sac area (mm^2) in ovules of the first four reproductive nodes on six field pea (*Pisum sativum* L.) cultivars grown under growth chamber conditions. Means of four replications (n=27 to 68; for more details of sample size see, Table A2.4) with their respective standard error bars are shown. Black bars represent the control; Grey bars represent heat treatment. *Indicates a significant temperature treatment effect at P<0.05; **Indicates a significant temperature treatment effect at P<0.01; ***Indicates a significant temperature treatment effect at P<0.001.

3.3.6 Fertilization and embryo sac stage

Fertilization and embryo sac stage were assessed on ovules from reproductive Node 1, Node 2, and Node 3 of the plants. The evaluation of approximately 1500 ovules from five cultivars Naparnyk, 40-10, CDC Meadow, CDC Sage, and Carneval revealed the existence of two categories of fertilized ovules and one type of unfertilized ovule (Fig. 3.7). Specifically, ovules with signs of fertilization by displaying an embryo between the pro-embryo to globular stages corresponded to 90.0% and 86.8 % of the ovules under control and heat stress conditions, respectively (Category A; Fig. 3.7). Ovules with signs of fertilization by displaying an embryo between the zygote to early pro-embryo stages corresponded to 9.0% and 12.1% of the ovules under control and heat stress conditions, respectively (Category B; Fig. 3.7). Finally, unfertilized ovules displaying an undamaged but small embryo sac without any signs of embryo growth corresponded to 0.92% and 1.09% of the ovules under control and heat stress conditions, respectively (Category C; Fig. 3.7).

Since the flower morphology of MFR043 differed from the other cultivars, and therefore influenced the degree of ovule fertilization (Appendix 1), the proportion of fertilized ovules on this cultivar was evaluated separately. Thus, the evaluation of 390 ovules from this cultivar revealed that 96% and 99% of these ovules had no signs of fertilization under control and heat stress conditions, respectively.

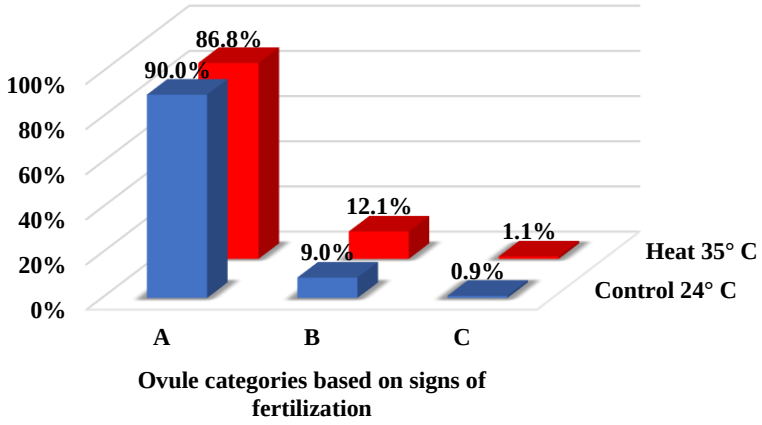


Fig. 3.7 Proportion of ovules (n=1500) at different degrees of development according to treatment on five cultivars of field pea (*Pisum sativum* L.) right after four days of temperature treatment. Category A: ovules showing embryo sac containing pro-embryo or embryo at globular stage; Category B: ovules showing embryo sac growth right after fertilization with presence of zygote or early pro-embryo; and Category C: Ovules without signs of fertilization.

The frequency of these three categories of the embryo sac development revealed that the proportion of ovules displaying a zygote or early pro-embryo stage was increased under high temperature in most of the cultivars. Hence, heat-treated CDC Meadow had more ovules at this embryo sac stage at reproductive Node 2 and 3, Carneval and 40-10 at Node 2, and Naparnyk at Node 3 compared with their respective controls (Table 3.9).

Table 3.9 Proportion of ovules displaying embryo and fertilization occurrence on five field pea cultivars and their three first reproductive nodes according to treatment. Category A: ovules showing embryo sac containing pro-embryo or embryo at globular stage; Category B: ovules showing embryo sac growth right after fertilization with presence of zygote or early pro-embryo; and Category C: Ovules without signs of fertilization.

Cultivar	Reproductive Nodes	Embryo Sac Categories According to Treatment					
		Control (24/18°C)			Heat (35/18°C)		
		A	B	C	A	B	C
40 -10	1	0.33	0.00	0.00	0.37	0.00	0.00
	2	0.36	0.00	0.00	0.32	0.05	0.00
	3	0.11	0.20	0.00	0.21	0.05	0.00
	Total	**0.80**	**0.20**	0.00	**0.90**	**0.10**	0.00
Naparnyk	1	0.33	0.00	0.00	0.32	0.00	0.01
	2	0.35	0.00	0.00	0.33	0.00	0.00
	3	0.30	0.02	0.00	0.25	0.07	0.02
	Total	**0.98**	**0.02**	0.00	**0.90**	**0.07**	**0.03**
CDC Meadow	1	0.33	0.02	0.01	0.34	0.01	0.00
	2	0.29	0.02	0.01	0.27	0.05	0.005
	3	0.21	0.08	0.02	0.13	0.19	0.005
	Total	**0.84**	**0.12**	**0.04**	**0.74**	**0.25**	**0.01**
CDC Sage	1	0.32	0.01	0.00	0.33	0.00	0.01
	2	0.38	0.01	0.00	0.31	0.02	0.00
	3	0.18	0.10	0.00	0.25	0.08	0.00
	Total	**0.88**	**0.12**	0.00	**0.89**	**0.10**	**0.01**
Carneval	1	0.38	0.00	0.00	0.32	0.00	0.00
	2	0.36	0.01	0.00	0.33	0.05	0.00
	3	0.27	0.01	0.00	0.28	0.01	0.00
	Total	**0.98**	**0.02**	0.00	**0.93**	**0.07**	0.00

Rating of the embryo sac stage considering degree of embryo development on the five cultivars showed a significant interaction of treatment by cultivar at the three reproductive nodes (Table 3.7). Plants of Naparnyk and 40-10 exposed to high temperature had advanced ovule stages at reproductive Node 1 and Node 3 (respectively) compared to those on nodes at similar positions under control conditions (Fig. 3.8). CDC Meadow exposed to high temperature had lower stages of ovules at reproductive Node 1, but advanced stages on reproductive Node 2 compared to nodes at similar positions under control conditions. Following a similar trend but at different nodes, CDC Sage exposed to high temperature had lower stages of the ovules at reproductive Node 2 but advanced stages on reproductive Node 3 compared to the respective nodes of plants under control conditions (Fig. 3.8). In contrast, Carneval exposed to high temperature had more advanced stages at reproductive Node 1 but lower ovule stages at reproductive Node 2 and Node 3 compared to nodes at similar positions of plants under control conditions. In general, the high temperature negatively tended to affect most of the cultivars at reproductive Node 2, where some cultivars showed significant lower ovule stages compared to the node at same location on plants under control conditions (Fig. 3.8).

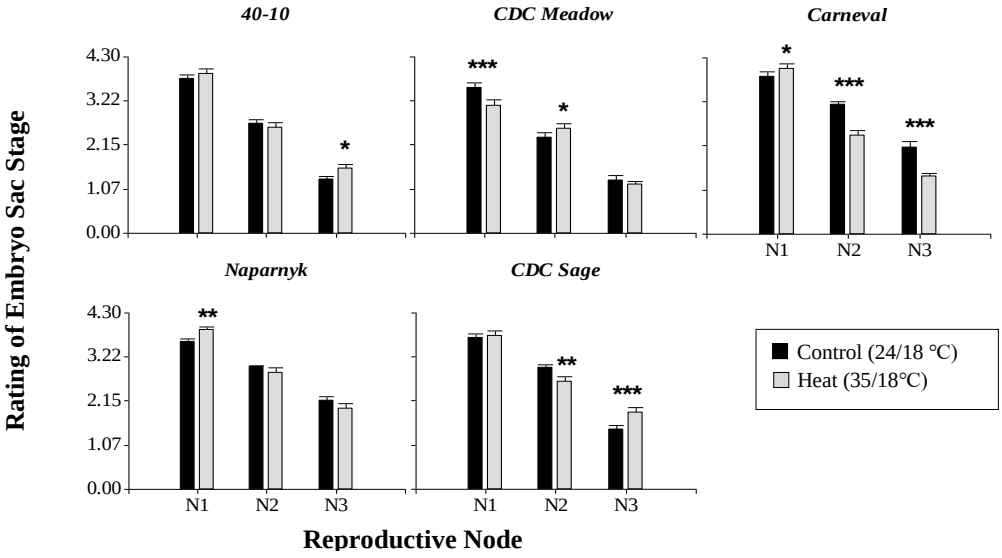


Fig. 3.8 Effect of high temperature (35°C) on embryo sac stage in ovules at reproductive Node 1, Node 2, and Node 3 of five field pea (*Pisum sativum* L.) cultivars right after four days of treatment. Means of four replications (n=34 to 68; for more details of sample size, see Table A2.4) with their respective standard error bars are shown. *Indicates a significant temperature treatment effect at P<0.05; **Indicates a significant temperature treatment effect at P<0.01; ***Indicates a significant temperature treatment effect at P<0.001.

Additionally, the analysis of the rating scale for embryo sac stage revealed a significant three-way interaction of cultivar by treatment by ovule position at reproductive Node 3 (Table 3.7). Ovules at this reproductive node of cultivar 40-10 and CDC Sage exposed to heat treatment had advanced stages at the three ovule positions compared with their controls (Fig. 3.9). However, the effect was only significant on ovules at the basal position within pod on both cultivars (Fig. 3.9). Moreover, ovules at this node (Node 3) on Naparnyk exposed to high temperature had slightly advanced stages at a medial position within the pod compared with their controls, whereas lower ovule stages were identified at basal positions within pod (Fig. 3.9). In contrast, ovules at the referred node (Node 3) on CDC Meadow and Carneval had lower ovule stages on plants exposed to heat stress compared to the controls. Specifically, ovules at the medial position within pods on cultivar CDC Meadow, and ovules at stylar and medial positions on Carneval had significantly lower ovule stages on plants exposed to heat treatment compared to similar ovule positions under control conditions (Fig. 3.9).

Finally, regardless of treatment, CDC Meadow showed the lowest ovule stages compared to other cultivars. In terms of ovule position, ovules at the medial position were always more advanced, followed by ovules at stylar and basal positions within the pod (Table 3.10).

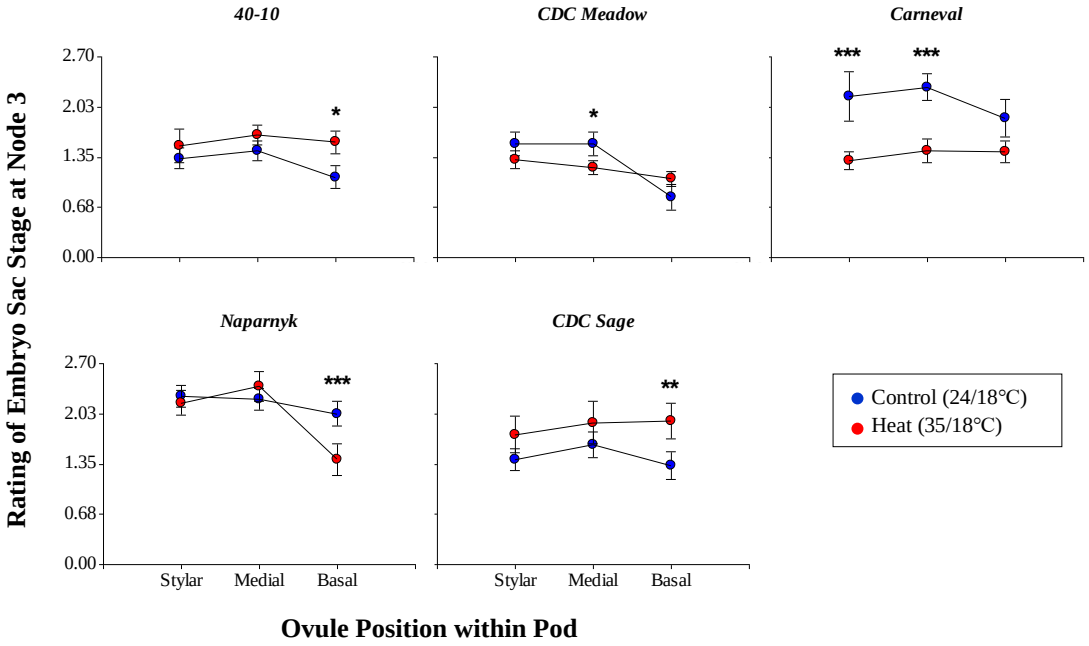


Fig. 3.9 Effect of high temperature (35/18°C) on embryo sac stage in ovules at three positions within pods on reproductive Node 3 of five field pea (*Pisum sativum* L.) cultivars after four days of treatment. Means of four replications (n=46 to 90; Tables A2.1 and A2.4) with their respective standard error bars are shown. *Indicates a significant temperature treatment effect at P<0.05; **Indicates a significant temperature treatment effect at P<0.01; ***Indicates a significant temperature treatment effect at P<0.001.

Table 3.10 Means (± SE) of the embryo sac stage rated in ovules at reproductive Node 1, Node 2, and Node 3 according to source of variation: cultivar (n=76 to 129), treatment (n=877 to 919), and ovule position (n=586 to 623) within pods on field pea plants (*Pisum sativum* L.) grown under growth chamber conditions.

Source of Variation	Embryo Sac Stage Rating by Reproductive Node		
	Node 1	Node 2	Node 3
Cultivar			
40-10	3.84±0.13 a†	2.65±0.20 ab	1.38±0.18 bc
Carneval	3.89±0.13 a	2.78±0.20 a	1.70±0.18 b
CDC Meadow	3.33±0.12 b	2.43±0.20 b	1.22±0.17 c
CDC Sage	3.68±0.14 a	2.83±0.20 a	1.71±0.19 b
Naparnyk	3.75±0.12 a	2.92±0.19 a	2.07±0.17 a
Treatment			
Control	3.67±0.07 a	2.82±0.18 a	1.65±0.15 a
Heat	3.72±0.07 a	2.62±0.18 b	1.58±0.15 a
Ovule Position			
Stylar	3.74±0.10 ab	2.76±0.19 ab	1.66±0.16 ab
Medial	3.87±0.09 a	2.85±0.19 a	1.75±0.16 a
Basal	3.48±0.10 b	2.56±0.19 b	1.44±0.16 b

†Values within a column and within source of variation followed by the same letter are not significantly different at P<0.05.

3.4 Discussion

The increase of temperature in the environment disturbs growth and productivity of legume crops (Sadras et al., 2013; Bhandari et al., 2016; Sita et al., 2017). Particularly, the reproductive stage of the plants is broadly identified as the most vulnerable to heat stress (Lambert and Linck, 1958; Guilioni et al., 2003; Bueckert et al., 2015). Traditionally, studies on high temperature during reproductive stages of various crops have mainly concentrated on the male reproductive structures (pollen and anthers) of the flowers, as they are thought to be the most sensitive to high temperature and pollen is relatively easy to collect (Ahmed et al., 1992; Porch and Jahn, 2001; Djanaguiraman et al., 2013; Mesihovic et al., 2016). Nevertheless, my study revealed that high temperature can influence the female reproductive structures and that the effect can vary depending on the reproductive node of the plant. In general, high temperature (35℃ day/18℃ night) imposed for four days on field pea plants during the early flowering stage affected gynoecium and ovule development in flowers at the first four reproductive nodes. This is a first approach to determine any effect of high temperature focused on young ovules of field pea, considering its complex reproductive nodal structure where young and advanced flower stages are found simultaneously on the same plant.

3.4.1 Effect of high temperature on gynoecium morphology

Plants exposed to high temperature displayed varied responses with respect to the size of female reproductive structures depending on their reproductive node positions on plants. The variation in response of the reproductive nodes to heat stress has been normally attributed to an adverse and a compensatory effect when they return to optimal conditions; however, those observations are mainly based on seed yield evaluated at physiological maturity of the plants (Jeuffroy et al., 1990). Here, an advanced flower development right after heat stress suggests that a mechanism of acceleration may be taking place during development of these structures. For instance, although cultivars of diverse level of tolerance were evaluated, most of the heat-treated plants displayed flowers at advanced stages particularly on younger reproductive nodes (Node 3 and Node 4) compared with nodes at similar location on plants under control conditions. This observation may be in close association to a mechanism of early aging of these annual plants, because the acceleration on the program of development is one of the effects of heat stress identified on field pea (Guilioni et al., 1997). Several authors explain that, as sessile organisms,

plants go through morphological, anatomical, and physiological changes to adapt to stress conditions (Wahid et al., 2007; Macedo, 2012; Hasanuzzaman et al., 2013). As such, acceleration of the life cycle is considered a strategy of the plants to avoid or escape potential dangerous environmental conditions (Adams et al., 2001; Macedo, 2012). This phenomenon has been also observed in other legume crops such as soybean, chickpea, mung bean, and lentils (Bhandari et al., 2016; Kaushal et al., 2013; Malaviarachchi et al., 2016; Ruiz-Vera et al., 2018). Thus, hastening development of the flower may well be part of the escape strategy identified for various plant species under high temperature.

Under heat stress conditions, most of the plants displayed wider and larger ovaries at Node 3, in congruence to the advanced flower development observed at that node; however, these ovary traits displayed varied responses on other reproductive nodes of the same plants exposed to heat stress. For example, heat-treated cultivars 40-10, CDC Meadow, CDC Sage, and Carneval displayed either narrower or shorter ovaries at Node 1 and Node 2 compared to their controls. Since flowers on these nodes correspond to the oldest on the plants, and that they were already pollinated and fertilized before or during treatment, this finding suggests that high temperature may have constrained the early fruit growth. Accordingly, under heat stress conditions, photosynthesis of the plants decline and resources are diverted to cope to the unfavored conditions; as a result, assimilate availability for the sinks in development can be limited (Georgieva et al., 2000; Wahid et al., 2007; Snider et al., 2009). Studies by Jahnke et al. (1989) showed that although ovaries initially have a steady development, later after fertilization they become a strong sink of assimilates that compete against other structures of the plant. In parallel, histological studies on pea demonstrated that maximal elongation of the ovary occurs specifically from two to five days after anthesis (Ozga et al., 2016). Thus, ovaries at reproductive Node 1 and Node 2 may have been at a stage of high demand of assimilates that may not have been entirely satisfied by the plants. In related studies of pea, Karr et al. (1959) found that flowers (at the first reproductive node) after several (~6 d) days from anthesis were highly sensitive to heat stress in terms of seed yield; unfortunately, they do not report any evaluation of the ovaries right after heat exposure. Finally, in my study, ovary size at reproductive Node 4 (youngest), where male and female reproductive flower organs were developing during heat stress, the heat treatment did not show a significant effect, suggesting that ovary development may not have been disturbed on those young flowers. Perhaps, flowers on that node being the youngest on the plant, initially enclosed within the sepals

and petals, were probably more protected during the whole heat treatment, but this hypothesis needs further investigation.

Curiously, stigma and style length evaluated on flowers at reproductive Node 3 and Node 4 (youngest), also revealed a diverse effect of high temperature depending on the cultivar. For example, heat-treated cultivars CDC Sage and 40-10 displayed longer stigmas and/or styles, whereas these structures in heat-treated cultivars CDC Meadow and Carneval were shorter, compared to plants under control conditions. Although studies on female flower morphology under heat stress in legume crops are scarce, related findings on other crops, such as tomato (*Lycopersicon esculentum* Mill.) and rice (*Oryza sativa* L.) showed that high temperature caused an asynchronous growth of female floral structures, like the style (Rudich et al., 1977; Lohar and Peat, 1998; Saeed et al., 2007; Giorno et al., 2013). In those studies, it was assumed that high temperature caused stigma protrusion due to increased growth of the style of the flowers; however, Pan et al. (2019) found that high temperature actually reduced style and stamen length, and a protrusion of the stigma was observed due to shorter stamens. In my study, although size of styles and stigmas of plants exposed to heat stress varied among pea cultivars compared to their controls, the affected cultivars (CDC Sage, 40-10, CDC Meadow, and Carneval) never displayed stigmas protruding from their flower arrangement. Indeed, pea flowers possess a keel petal that helps to maintain contact between stigma and pollen from the anthers (Tucker, 1989; Etcheverry et al., 2012). In this sense, the keel petal was always observed to keep the style bended and therefore the stigma at the same level as anthers on the affected cultivars in my study. The larger structures observed on flowers of CDC Sage and 40-10 may be related instead to the advanced flower stage detected on Node 3 and Node 4 of the plants exposed to heat stress, as they also exhibited larger ovaries compared to plants growing under optimal conditions (Fig. 3.1; Table 3.6).

On the other hand, a smaller size of style and stigma observed on cultivars CDC Meadow and Carneval may suggest the existence of a poor development of the whole flower structure, since these cultivars also presented smaller ovaries under heat stress conditions (Table 3.6), as partially seen in tomato plants (Pan et al., 2019). Furthermore, as the reduction in size of style and stigma was just detected on plants that correspond to medium and low seed yield tolerant cultivars (Table 3.1), the smaller styles and stigmas may indicate increased susceptibility of these cultivars to heat stress at early flower development. Similarly, in chickpea, less heat tolerant cultivars had shorter

styles on plants exposed to high temperature for several days (7-10 d) in field and growth room conditions (Devasirvatham et al., 2012). An additional explanation of the reduced size of these structures may be due to a hormonal unbalance and reduced plant assimilate availability on plants exposed to heat stress. Indeed, high temperature can influence phytohormone signals such as indole-3-acetic acid (IAA), jasmonic acid (JA), abscisic acid (ABA), and ethylene (Larkindale and Huang, 2005; Sakata et al., 2010; Teplova et al., 2000), where the first two have been demonstrated to influence processes such as cell division and expansion of stamens and pistils (Pan et al., 2019). Also, a lack of assimilate availability to the pistils has been clearly demonstrated in studies of cotton (*Gossypium hirsutum)* where high temperature 38/20℃ day/night caused up 16.8% reduction in the net photosynthesis in leaves of subtended pistils that displayed lower carbohydrate content (Snider et al., 2009). Therefore, the multiple effect of high temperature on style and stigma length from different pea cultivars may be due to an advanced or lowered development that was influenced by a superior or reduced maternal supply of the cultivars under stress conditions.

3.4.2 Effect of high temperature on ovule and embryo sac

The assessment of the internal embryo sac in ovules at the first four reproductive nodes did not reveal any sign of deformation or injury on either treated or control plants. However, high temperature influenced ovule length and embryo sac area. Gross and Kigel (1994) found that although heat-treated ovules and embryo sacs of common bean were apparently normal under microscopic examination, their gynoecium performance was impaired. In my study, although not ovule damage was observed, variation of ovule and embryo sacs size was detected on heat treated cultivars. At Node 1 some heat-treated cultivars (Naparnyk, 40-10, and Carneval) had larger ovules and embryo sacs, whereas other heat-treated cultivars (CDC Meadow and CDC Sage) had smaller ovule and embryo sacs compared to their controls. At Node 2 and Node 3 some cultivars (CDC Meadow, CDC Sage, and 40-10) had larger ovules and embryo sacs whereas one cultivar (Carneval) had smaller ovule and embryo sacs compared to their controls. The larger ovule and embryo sac in some of the cases (CDC Sage and 40-10) may be associated to the advanced development observed also on ovary length and width. Interestingly, embryo sac area from both control and high temperature conditions was positively correlated with embryo sac stage (Fig. 3.10), implying that larger ovules corresponded to an advanced embryo sac stage, whereas smaller ovules were related to a younger embryo sac stage (Fig. 3.5 and Fig. 3.7). In the case of small

ovules on heat treated plants, it could be argued that lack of fertilization occurred; however, embryo presence in over 90% of ovules on plants from heat stress (similar to the controls) suggested that the process of embryo growth, rather than fertilization, was affected in those ovules (Fig. 3.6). In this case, an adjustment of the maternal expenditure to maximize fitness of the plant under unfavorable conditions should be considered (Dinar and Rudich, 1985; Barnabás et al., 2008; Aloni et al., 1991). Furthermore, since maturation of the reproductive structures of these plants starts from the base to the top of the plant (acropetally), similar to an inflorescence raceme, spatial and temporal advantages associated with resource (assimilate, water) availability under stress may have influenced their development (Lloyd, 1980; Diggle, 1995).

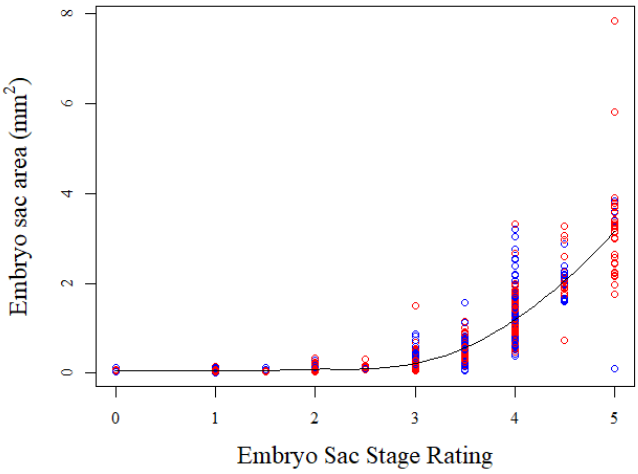


Fig. 3.10 Relationship between embryo sac stage and embryo sac area (mm^2) of ovules within pods from reproductive Node 1 to Node 3 of five field pea cultivars grown under growth chamber conditions. $r = 0.80$; $P \leq 0.001$. Blue circles indicate ovules from plants that experienced 24°C light /18°C dark (control) conditions, whereas red circles indicate ovules from plants that experienced 35°C light /18°C dark (heat) conditions.

According to Guilioni et al. (2003), depending on the severity of the stress, pea plants may prioritize assimilate availability to some nodes in the plants given that leaf size is reduced in plants under heat stress. In my study, while priority of ovule development at some nodes may be observed, the effect seems to vary among cultivars and their reproductive nodes probably depending on its level of efficiency or strategy to overcome heat stress. For example, heat-treated CDC Meadow, identified as a medium heat tolerant cultivar, displayed less advanced ovule development at Node 1 (oldest), and yet, advanced or larger ovules on the rest of reproductive nodes. From the other end of the heat tolerance spectrum, the low heat tolerant cultivar Carneval displayed advanced ovule development only on Node 1, with poor or less advanced embryo sacs

on the rest of reproductive nodes compared to their controls. In this sense, some authors state that although plants can adapt to adverse environmental conditions, their level of tolerance can vary among species and cultivars depending on their hormonal response (Ozga et al., 2016; Wahid et al., 2007). For instance, Savada et al. (2017), studying cultivar Carneval, found that ovaries from young flowers (-2 days from anthesis) displayed up to 48% reduction of ovary growth that was associated with increase ethylene evolution in flowers at young age under heat stress conditions.

In addition, the cultivar leaf type may have influenced the effect of high temperature on ovule and embryo sac size identified at the different reproductive nodes of the plants. Heat-treated cultivars with normal leaf type (40-10 and Naparnyk) displayed either advanced ovule stages or no difference in ovule size on their reproductive nodes compared with the controls. In contrast, heat-treated semi-leafless cultivars (e.g. CDC Meadow and Carneval) always presented a varied effect of advanced and less advanced ovule stages along the reproductive nodes of the plants compared to their controls. A normal leaf type cultivar consists commonly of leaflets, stipules, and tendrils, whereas a semi-leafless cultivar has stipules and tendrils instead leaflets (Mihailović and Mikić, 2004; Mikić et al., 2011). Heath and Hebblethwaite (1985), studying different leaf type cultivars (leafless, semi-leafless, and leafed) found that despite the different leaf components (leaflets, tendrils), the cultivars did not show differences in the conversion of the assimilates into dry matter at the field level. But different effects seem to exist under heat stress and sufficient water supply. In my research, the normal leaf type cultivars tended to maintain better performance in terms of ovule stage and size compared with the semi-leafless cultivars. Indeed, some studies on normal leaf type cultivars reveal that they can possess a high yield potential; however, their characteristics of lodging and yield reduction due to drought stress made them less desirable (Alvino and Leone, 1993; Stelling, 1994). In my study, as water supply was not restricted, the conventional leaf cultivars may use their higher photosynthetic ability as an advantage to display its own potential to overcome heat stress conditions. Interestingly, Wilson et al. (1981) studying water use on conventional and semi-leafless cultivars found that although a conventional cultivar showed slightly fewer seeds than the semi-leafless cultivar under irrigated conditions, the conventional cultivar had greater 1000-seed weight. In contrast, Baigorri et al. (1999) found that under irrigated conditions, the normal leaf cultivar produced higher shoot and pod dry matter, but lower seed dry matter. Clearly, there are various criteria about the efficiency of normal leaf type cultivars; however, the normal leaf cultivars evaluated in this study showed an interesting outcome

right after high temperature that may involve valuable physiological traits to investigate in the future, such as the role of leaf size and their components.

Signs of embryo formation were identified within most of the ovules (>90%) at reproductive Node 1, Node 2, and Node 3, on five heat-treated cultivars (Naparnyk, 40-10, CDC Meadow, CDC Sage, and Carneval). The existence of embryos in these ovules indicates that ovule fertilization was probably not strongly disrupted in the experiment, since flower stages (open flower and buds) at these nodes corresponded to ovules where fertilization and early embryo growth was taking place during heat treatment. In many studies, high temperature is reported to disrupt pollen performance, and therefore, fertilization (Monterroso and Wien, 1990; Porch and Jahn, 2001; Devasirvatham et al., 2012), but the great amount of pollen produced by plants may compensate this effect in some species (Hedhly et al., 2003; Hinojosa et al., 2019). For example, in quinoa (*Chenopodium quinoa* Willd.) whereas high temperature (40/24°C) reduced up to 70% of pollen viability, seed set of the treated plants did not reduce under this abiotic stress (Hinojosa et al., 2019). In field pea, pollen composition and in-vitro pollen germination has been detected to be perturbed after treatment with temperatures of 36°C/18°C day/night for 4 d on cultivars CDC Sage and CDC Golden (Jiang et al., 2015). However, in later studies it was also observed that high temperature did not affect the area of the in-vivo pollen tubes in the styles nor the proportion of fertilized ovules on the same two cultivars exposed to similar heat treatment (Jiang et al., 2019). Therefore, these findings support the idea that although pollen performance can be altered under heat stress, it may not necessarily lead to a fertilization failure. Possibly, the amount of pollen produced by plants under high temperature may be enough for self-fertilization (Hedley and Ambrose, 1981) of 6-10 ovules per ovary in these pea plants, even if part of the pollen was affected (Hedhly et al., 2003; Hinojosa et al., 2019). In fact, annual legume species with similar pollen release mechanism (brush style) to pea can produce around 4758 to 10442 pollen grains per flower (Galloni et al., 2007).

In addition, the existence of fertilized ovules despite pollen disruption in self-pollinated flowers has been associated to occurrence of cross-pollination in some species under adverse conditions (Weerakoon et al., 2009; Bishop et al., 2017; Van Ginkel and Flipphi, 2020). According to Solbrig (1976) a progeny from cross-pollination may be advantageous under varying environments since the new offspring can acquire new genes to adapt to extreme conditions.

Correspondingly, the Diversity Assurance theory indicates that under adverse conditions such as heat stress, male sterility arises but female organs maintain their functionality to ensure pollination from wind-borne pollen from stress-tolerant plants (Van Ginkel and Flipphi, 2020). For example, research in rice (*Oryza sativa*) has shown that although high temperature (31-32°C) affected pollen sterility, a reduction of spikelet (grain) fertility is not constrained because pollen from adjacent plants or other panicles could be deposited on stigmas of the affected flowers (Weerakoon et al., 2009). Similarly, when plants of faba bean (*Vicia faba*) were exposed to elevated temperature and then moved to either flight cages with bumblebees or to field conditions, the proportions of outcrossed seed increased from 17% and 31% to 33% and 80% in flight cages and field conditions, respectively (Bishop et al., 2017). In field pea, while pollen performance can be restricted under heat stress (Jiang et al., 2015), the high proportion of fertilized ovules found on plants exposed to high temperature (35°C) may involve some degree of cross-pollination as an adaptative mechanism to unfavorable conditions as well.

Alternatively, asexual seed reproduction (apomixis) may also explain the observed presence of embryos in ovules of field pea under heat stress. Here, it can be argued the possibility of embryos developed from an unreduced and unfertilized egg cell (Hojsgaard et al., 2014; Rodrigo et al., 2017), since high temperature damages pollen effectiveness in pea (Jiang et al., 2015). Some studies suggest that the frequency of sexual and apomictic embryo sac incidence in angiosperm species can be triggered by environmental stress factors, such as light, drought, temperature, among others (Carman et al., 2011; Knox, 1967; Marshall and Brown, 1981; Rodrigo et al., 2017). For instance, Knox (1969), studying *Dichanthium aristatum* under various environmental conditions, found that the prevalence of apomictic embryo sacs in this species was associated to the photoperiods during flowering stage of these plants. Comparably, in a study carried out with five apomictic *Boechera* species, it was found that plants exposed to drought and continuous heat (32°C) showed reduced frequency of apomictic ovules. Given that these studies prove the influence of the environment on facultative apomictic species to shift to a sexual stage, it is hypothesized that all angiosperms may have inherited a shift to apomixis (Carman et al., 2011; Hojsgaard and Hörandl, 2019). In legume species, although there are no reports of facultative apomictic species, Smartt (1979) proposed that the lack of consistency on hybrid traits detected in interspecific hybridization studies would suggest a low occurrence of spontaneous apomixis on these plants. However, there is a lack of studies on this topic to confirm it at present. Specifically, in pea, the

closest reported form of fruits without fertilization are parthenocarpic (or seedless) fruits, where emasculated young ovaries need the application of hormones such as gibberellins to promote pod growth (Vercher and Carbonell, 1991). In my study, since the presence of embryo growth was identified in ovules of flowers where pollen performance may have been disturbed under heat stress, apomixis reproduction (an aspect beyond this research) should not be ruled out but it will need future investigation. In addition, the existence of fertilized ovules on self- pollinated flowers where pollen is disrupted under adverse conditions has been associated to an increase of cross-pollination in some species.

3.5 Conclusions

Whereas the study of high temperature on the female component of the reproductive process in legumes and other crops has been scarce, my results elucidate interesting insights about the influence of heat stress on development of the female reproductive structures. In this part of the study, measurements of the gynoecium components and inspection of embryo sacs revealed that heat stress can influence gynoecium and ovule growth in field pea. Plants exposed to high temperature exhibited advanced flower development accompanied with growth of their gynoecium components (stigma, style, and ovary) specifically noticed on upper reproductive nodes, such as Node 3 on most cultivars. Likewise, the assessment of the ovule and its embryo sac revealed that advanced development took place also on the bottom reproductive nodes (Node 1 or Node 2) on some cultivars. In contrast, less advanced ovule development on diverse nodes appears to be the factor that separates medium and low tolerant cultivars under heat stress, where medium tolerant cultivars showed poor development at one node, and low tolerant cultivars at two nodes. Based on this outcome, the proposed hypothesis that high temperature causes poor development of gynoecium and ovules can be partially accepted, since an opposite effect (advanced development) was also observed. Here, although flower stage could account for the advanced development observed on Node 3, variation of ovule development on the different nodes may be related to hormonal and maternal expenditure of each cultivar affected by high temperature, aspects that need further investigation.

Transition section between Chapter 3 and Chapter 4

In Chapter 3, gynoecium components with particular attention to the ovule development at the first four reproductive nodes of six field pea cultivar were evaluated right after heat stress. This study showed that high temperature influenced the gynoecium and ovule of the plants. Although an advanced development of the ovule was observed in some nodes, a poor or reduced development was found in other nodes of the same plants. This effect was consistent with the heat tolerance of each cultivar, where high heat tolerant cultivars tended to show advanced or no effect of high temperature whereas the low tolerant cultivars displayed a poor development in some nodes and advanced development in other nodes. In Chapter 4, ovule viability was evaluated in three cultivars, whereas seed set and abortion were evaluated in five cultivars under growth chamber conditions.

Chapter 4. Ovule Viability, Seed Set, and Ovule Abortion of Field Pea under Heat Stress

4.1 Introduction

High air temperatures negatively impact productivity and yield of many crops including legumes (Teixeira et al., 2013; Sita et al., 2017; Barnabás et al., 2008). Plants have a range in their heat stress sensitivity within their life cycles, with the reproductive phase being one of the most stress-susceptible (Gross and Kigel, 1994; Porch and Jahn, 2001; Porter and Semenov, 2005). Indeed, when plants experience heat over their threshold tolerance, they can suffer alterations in their reproductive organs (Giorno et al., 2013; Sage et al., 2015). Various studies in pea (*Pisum sativum* L.) have shown that temperature over 27°C leads to abortion of reproductive structures such as buds, flowers, young pods and seeds and therefore yield reduction (Lambert and Linck, 1958; Nonnecke et al., 1971; Jeuffroy et al., 1990; Guilioni et al., 2003). While abortion of buds and flowers can be attributed to damage of the gametophytes (male and female) at early development (Monterroso and Wien, 1990; Kokubun et al., 2001; Abernethy et al., 1977), the abortion of young pods and seeds indicate a failure during embryo formation in the female gametophyte (Warrag and Hall, 1983; Jeuffroy et al., 1990; Ozga et al., 2017). Therefore, an assessment of seed set and ovule stage abortion at physiological maturity of the plants can provide valuable information about the susceptibility, or robustness, of the reproductive process of field pea under heat stress.

In flowering plants, reproduction encompasses a complex sequence of steps where any disturbance to the male and the female gametophytes can compromise seed formation. Under ideal conditions, pollen germinates on the stigma and style, and thereafter, two sperms cells from the pollen (male gametophyte) fertilize the egg and the central cell in the embryo sac (female gametophyte). This process gives rise to the embryo and its endosperm, that later will become the seed (Goldberg et al., 1994; Herrero, 2003; Yadegari, 2004). Unfortunately, adverse conditions during reproduction can cause failure of one or both gametophytes and leads to disruption of the reproductive process at any stage (Warrag and Hall, 1983; Briggs et al., 1987; Cerović and Mićić, 1999). Studies involving abiotic stresses such as lack of water, high salinity, and metal toxicity demonstrate that the female gametophyte can be impaired and aborted in soybean, common bean, and *Arabidopsis thaliana* (Kokubun et al., 2001; Hauser et al., 2006; Chehregani and Kavianpour, 2007). For example, salt-stressed plants of *Arabidopsis* displayed a loss of ovule viability prior or

after fertilization accompanied by heavy callose deposition in cells of endothelium, suspensor, and embryos (Sun et al., 2004). In the case of high temperature, numerous studies suggest a detrimental effect on male gametophyte viability (Halterlein et al., 1980; Nikolova et al., 2012; Sakata and Higashitani, 2008). Specifically, in field pea, pollen viability under high temperature has been already studied (Jiang et al., 2015). But the female gametophyte has been less explored under heat stress in most plants and crops (Barnabás et al., 2008; Sage et al., 2015). Therefore, the investigation of ovule viability, and specifically the female gametophyte contained within the ovule, may provide complementary insights of how high temperature causes seed yield reduction in pea.

While ovule abortion can be assessed through seed set in plants at physiological maturity, the evaluation of callose deposition and reactive oxygen species (ROS) allow the identification of early signs of damage to young tissue (Dumas and Knox, 1983; Kristiansen et al., 2009; Piršelová and Matušíková, 2013). Callose is a polysacharide normally synthesized during reproduction, cell cytokinesis, molecule movement regulation, and in response to biotic and abiotic stress (Chen and Kim, 2009; Xie and Hong, 2011; Shi et al., 2016). Under temperature stress, callose accumulates on sieve plates and plasmodesmata of injured tissue, and consequently causes a reduction of solute translocation in affected tissue (Bilska and Sowiński, 2010; Furch et al., 2007). In pea, callose deposition has been identified as a first sign of ovule abortion since callose was found on ovules displaying nuclear and cytoplasm damage (Briggs et al., 1987). In contrast, ROS are molecular derivates of oxygen that are normally produced in cell organelles at low levels under optimal conditions (Mittler et al., 2004; Dietz et al., 2016). However, under stress conditions such as severe drought, high salinity, and extreme temperatures, ROS are often overproduced and become toxic for cells (Luna et al., 2011; Sun et al., 2004; Suzuki and Mittler, 2006). Sharma et al. (2012) explain that an increased production of ROS during stress conditions can lead to oxidative damage of the cell components entailing the activation of programmed cell death in the tissue. Therefore, assessment of callose and ROS in young ovules can provide valuable information of the mechanism of abortion occurring in this tissue under abiotic stress.

During sexual reproduction, male (pollen) and female gametophytes (embryo sac in the ovule) play unquestionable key roles for seed formation. Although the study of the effect of high temperature in the male gametophyte has made considerable progress, the influence of this

environmental stress on the ovule and its embryo sac remain unknown in field pea. Within this context, investigating the impact of high temperature on the ovule is critical to obtain a complete panorama of how this abiotic stress constrains seed yield on field pea. Here, I aimed to investigate the influence of high temperature on ovule viability by means of callose and ROS assessment of young ovules. This evaluation was made on different reproductive nodes of plants following heat treatment. I also assessed seed set and ovule abortion at different reproductive nodes of plants at physiological maturity stage. For that, I hypothesized that if plants were exposed to heat stress, ovule viability would be reduced on all reproductive nodes of the plants evaluated. Also, I expected that plants exposed to heat stress would display low seed set and high abortion relative to node position of the plants depending on degree of heat tolerance of pea cultivars.

4.2 Materials and Methods

4.2.1 Plant material and growth chamber conditions

Evaluation of seed set and abortion was carried out with six cultivars of field pea (*P. sativum*): 40-10, Naparnyk, CDC Meadow, CDC Sage, Carneval, and MFR043. As described in Chapter 3, they were selected based on their range of seed-to-ovule ratio exhibited in previous heat stress trials of the Pea Association Mapping Panel (PAM) at the University of Saskatchewan (Table 4.1; Jiang et al., 2017a). The evaluation of ovule viability was performed on three cultivars (Naparnyk, CDC Sage, and Carneval) that represented the main trend of ovule development identified previously (Chapter 3). These plants were grown under controlled conditions in growth chambers, where the light was supplied by banks of cool fluorescent tubes providing an irradiance of ~ 450±5 μmol photons m^{-2} s^{-1}. In the chambers, photoperiod was set up at 16h light/8h dark, and the temperature was kept at 24℃ during light (day) time and 18 ℃ during dark (night) time. For each pot or experimental unit, four seeds of each cultivar were sown in cylindrical pots of 7.6 L filled with peat base mix (Sunshine®, RR. Horticulture Canada Ltd., AB., Canada), and 20 g of a slow-release fertilizer 14-14-14 (Nutricote®, Brampton, ON, Canada). Watering was provided so plants avoided any drought stress. After germination, plants were thinned from four to two plants per pot at the 3 to 4-leaf stage (Jeuffroy et al., 1990), and half-strength Hoagland nutrient solution (Hewitt, 1952) was provided every other day starting from three weeks until six weeks after sowing.

Table 4.1. List of cultivars with their respective characteristics of leaf type, origin, and seed-to-ovule ratio used in the study of ovule development and seed set.

Cultivar	Leaf Type	Origin	Seed-to-Ovule Ratio
40-10	Normal	Germany	High
Naparnyk	Normal	Eastern Europe	
CDC Meadow	Semi-leafless	CDC, Canada	Medium
CDC Sage	Semi-leafless	CDC, Canada	
Carneval	Semi-leafless	Western Europe	Low
MFR043	Normal	CDC, Canada	

4.2.2 Experiment design and treatment

For the analysis of ovule viability, two sets of plants were used. One of these sets was employed for evaluation of callose depositions whereas the other for ROS. In this part of the study, I evaluated a total of 48 plants that corresponded to two sets of plants, three cultivars (Naparnyk, CDC Sage, and Carneval), two temperatures (control and heat), and four replications. Additionally, for analysis of seed set at the physiological maturity stage of the plants, a set of 48 plants corresponding to six cultivars (40-10, Naparnyk, CDC Meadow, CDC Sage, and Carneval), two temperatures, and four replications was used. In each case (sets of plants), the experiments were set up in a randomized complete block design (RCBD).

To impose the heat treatment, half of the plants from each set that was growing at 24℃ day/18℃ night and reached early flowering stage were transferred to a chamber where temperature was set up at 35℃ day/18℃ night in cycles for four days. Plants were considered to be at early flowering stage when they displayed opened flowers on reproductive Node 1 (stage 0.5, Maurer et al., 1966), flower buds with petals tightly closed on reproductive Node 2 (stage 0.2, Maurer et al., 1966), and flower buds covered by their sepals on reproductive Node 3. Cycles of temperature treatment were established according to a photoperiod of 16 h light and 8 h dark, where 18℃ was maintained during dark time (night), and temperature was increased in steps of 3℃ every hour during light time (day) until achieving 35℃. Then, the temperature was kept for 6 h and dropped by 3℃ every hour until the chamber returned to 18℃. Plants that remained under 24℃ day/18 ℃ (half of each group) were considered the controls.

4.2.3 Sample collection and measurements

Callose deposition and reactive oxygen species (ROS)

Since the nature of both tests were suitable for analysis of very young tissue, callose deposition was evaluated on ovules from pistils at reproductive Node 2 to Node 5, whereas ROS was assessed on ovules from pistils at reproductive Node 4 and Node 5 (youngest) of plants right after treatment. Pea plants can bear one or two flowers in a single inflorescence on each reproductive node, where the flower at lower position (proximal) tends to be approximately 12 to 24 h older than the flower at upper position (distal) in the raceme (Makasheva, 1984; Savada et al., 2017). When two flowers were produced on a node, pistils from both flowers were collected. The pistils were dissected under a stereomicroscope by carefully removing one of the ovary walls to keep the ovules attached to the suture on the other ovary wall. For analysis of callose deposition, samples were fixed in formalin-acetic acid-alcohol solution (FAA50) and processed according to the protocol of Martin (1959) by using 0.1% Aniline Blue solution. Visualization of these samples was performed using an epifluorescence microscope and UV filter (excitation 365 nm and emission 420 nm). The proportion of ovules with signs of callose accumulation was determined by dividing the number of ovules with signs of callose by the total number of ovules per ovary. Also, fertilization of these samples was assessed by the presence of pollen tubes entering the micropyle area of the ovule and the remains of callose deposits found in the micropyle side of the ovule as a product of successful fertilization.

In the case of ROS evaluation, only ovules from pistils at Node 4 were considered in the analysis, since those at Node 5 were extremely delicate and whenever dissecting tools were in contact with the ovules, they caused external damage and triggered false-positive ROS. The assessment was performed using an Image-iTTM Live Green ROS Detection Kit (Molecular Probes Inc., Eugene, OR, USA). Briefly, ovules attached to one wall of the ovary were incubated in 5-(-6)-carboxyl-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) for 30 minutes, then they were gently washed three times with Hank's balanced salt solution buffer (HBSS), and immediately mounted on slides to be evaluated under an epifluorescence microscope. Visualization of ROS was achieved by using a filter allowing excitation from 450 to 490 nm and emission detection of 520 nm. The presence of ROS was identified by a green fluorescence dye on the affected tissue (Hauser et al., 2006; Kristiansen et al., 2009).

Pod and seed assessment at physiological maturity stage of the plants.

When plants from the six cultivars finished their heat exposure treatment (35℃ day/18 ℃ night for 4 d), they were returned to control conditions (24℃ day/18 ℃ night). In these plants, pods (mature fruits) from reproductive Node 1 to Node 4 were collected from plants exposed to heat and control conditions when the crop canopy was approaching physiological maturity. This stage refers to the point when plants turned green-yellow, and seeds at the fourth reproductive node reached at least 6 to 8 mm in diameter for small seed size and large seed size cultivars, respectively (Ney and Turc, 1993). In these samples, pod length was measured with a digital caliper. The number of seeds was recorded from each pod considering pod position on the raceme of each node. Seed-to-ovule ratio was obtained by dividing the number of seeds to the number of ovules in each pod. Seed diameter was measured from the hilum to the opposite side of the seed by using a digital caliper. Ovules and early seeds that failed to reach a mature seed stage, i.e. the embryo did not fill the seed coat, were considered aborted, and proportion of abortion was determined by dividing the number of aborted ovules by the number of total ovules within the pod. The aborted stage of the ovules was defined under a dissecting microscope according to morphological characteristics of the embryo sac and embryo stage displayed by their aborted structures (Briggs et al., 1987; Cooper, 1938; Marinos, 1970).

4.2.4 Data Analysis

The statistical analysis was performed as a Linear Mixed Model for the nested structure of the experimental design, with SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). Treatment, cultivar, reproductive node, pod position, ovule position, and their interactions, were treated as fixed effects with their respective nested structure, whereas replication and its interaction terms were considered as random effects. The Kenwardroger option was used to approximate the degrees of freedom for unbalanced data, e.g., plants with one or two pod positions per node. In cases where significant differences were found, a post hoc test was applied to determine the difference of levels from the response variables using the Least Significant Difference (LSD) test. Since field pea cultivars used in this research varied in seed number and seed size genetically, they contained a varied number of ovules aligned on the suture within the ovary/pod. As a result, some of the ovules were positioned closer or further from the maternal supply, so ovule position effect was standardized across cultivars. Three positions were considered: stylar, ovules localized closest

to the style; medial, ovules at the medial area within the ovary/pod; and basal, ovules closest to the pedicel end of the ovary/pod (Gutiérrez et al., 1996; Jiang et al., 2017a). That is, the total number of ovules within an ovary/pod was divided into three, and when the number of ovules could not be divided by three, the maximum difference in the number of ovules between categories was one (Gutiérrez et al., 1996; Table A2.1).

4.3 Results

4.3.1 Callose depositions and reactive oxygen species (ROS) right after treatment

In cultivars Naparnyk, CDC Sage, and Carneval, callose deposits were detected close to, and around, the vascular bundle area of ovules (Fig. 4.1). Heat stress affected the proportion of ovules with callose depending on the reproductive node position within these cultivars (Table 4.2). In general, the number of ovules with callose increased at reproductive Node 3 on the plants exposed to heat stress (Table 4.3). Flowers on this node were around 48 hours from the opened flower stage, and their ovules contained a zygote or early pro-embryo growth. In addition, an analysis performed individually on each reproductive node revealed that besides the effect of high temperature on reproductive Node 3 on all plants, high temperature increased the number of ovules with callose at reproductive Node 2, specifically in Naparnyk (Table 4.4 and 4.5).

Table 4.2 Analysis of variance for the effect of treatment, cultivar, node, and their interaction on proportion of ovules with presence of callose and proportion of fertilized ovules per pod of three field pea cultivars grown under growth chamber conditions.

Source of Variation	Prop. of Ovules with Callose per Ovary		Prop. of Fertilized Ovules per Ovary	
	F Value	P Value	F Value	P Value
Treatment (T)	2.12	0.148	2.07	0.153
Cultivar (C)	1.57	0.234	3.01	0.053
Node (N)	1.31	0.320	66.08	**<.0001**
C*T	0.84	0.434	0.98	0.377
C*N	0.74	0.626	2.03	0.066
T*N	5.05	0.003	0.21	0.889
C*T*N	1.77	0.113	0.81	0.560

Significance levels at P <0.05 and P<0.001 are shown in bold.

Table 4.3 Means (± SE) of the proportion of ovules with callose (n=12 to 24 pods) and proportion of fertilized ovules per ovary (n= 24 to 48 pods) according to reproductive nodes on the main stem of three field pea cultivars grown under growth chamber conditions.

Reproductive Node	Prop. of Ovules with Callose per Ovary		Prop. of Fertilized Ovules per Ovary
	Control	Heat	
Node 2	0.12±0.04 b†	0.17±0.04 b	0.98±0.01 a
Node 3	0.08±0.05 b	0.37±0.09 a	0.94±0.03 a
Node 4	0.09±0.05 b	0.05±0.04 b	0.76±0.06 b
Node 5	0.22±0.08 ab	0.13±0.06 b	0.17±0.06 c

†Values within a column and within variable followed by the same letter are not significantly different at P<0.005.

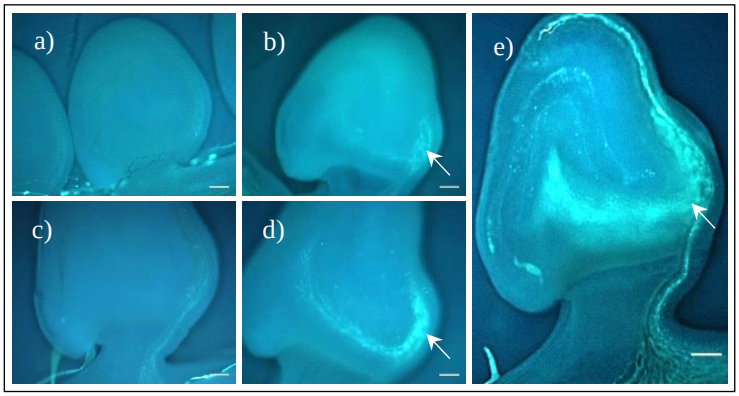


Fig. 4.1 Ovules of field pea with and without presence of callose deposition in the area of the vascular bundle and chalaza of the ovules. a) and c) Ovules from Node 3 and Node 2 without signs of callose deposition, respectively. b) and d) Ovules from Node 3 and Node 2 with signs of callose deposition (arrows), respectively. e) Ovule at an advanced stage of abortion displaying heavy callose accumulation around the vascular bundle and bottom region (arrow) of ovule. Scale bars 100 µm.

Table 4.4 Analysis of variance for the effect of treatment, cultivar, and their interaction on the proportion of ovules with presence of callose at four reproductive nodes collected from the main stem of field pea cultivars grown under growth chamber conditions.

Source of Variation	Node 2		Node 3		Node 4		Node 5	
	F Value	P Value	F Value	P Value	F Value	P Value	F Value	P Value
Treatment (T)	0.66	0.4199	10.56	**0.0027**	0.45	0.5074	0.57	0.4562
Cultivar (C)	0.58	0.5674	2.00	0.1515	0.61	0.5480	0.66	0.5271
C*T	5.74	**0.0065**	1.53	0.2312	0.61	0.5493	0.40	0.6759

Significance levels at P <0.05 and P<0.001 are shown in bold.

Table 4.5 Means (± SE) of the proportion of ovules with callose per ovary (n = 4 to 8 pods) at reproductive Node 2 of three field pea cultivars grown under growth chamber conditions.

Cultivar	Prop. of Ovules with Callose at Node 2	
	Control	**Heat**
Naparnyk	0.03±0.06 b†	0.30±0.06 a
CDC Sage	0.18±0.07 ab	0.05±0.06 b
Carneval	0.17±0.07 ab	0.16±0.07 ab

†Values followed by the same letter are not significantly different at P<0.05

The test for callose deposition also allowed visualization of the presence of pollen tubes entering the micropylar area of ovules; therefore, the number of ovules with this sign of fertilization was evaluated on ovules of the three cultivars (Naparnyk, CDC Sage, and Carneval). Here, high temperature did not cause an effect on the proportion of fertilized ovules (Table 4.2). Instead, fertilized ovule proportion per ovary was affected by reproductive node position of the plants (Table 4.2). Thus, fertilized ovule number per pod was greater on reproductive Node 2 and Node 3 compared to Node 4 and Node 5 (Table 4.3). In Node 2 and Node 3, where flowers were around 72 and 48 hours from the opened flower stage, the average proportion of fertilized ovules was 0.96 per ovary. In contrast, in Node 4 and 5, where flowers were just opened or still closed at the time of collection, the number of fertilized ovules was 0.76 and 0.17 per pod, respectively (Table 4.3).

Finally, the analysis of ROS performed on ovules from reproductive Node 4 of the three cultivars showed a significant effect of temperature on ovaries at the distal position, depending on cultivar (Table 4.6). Specifically, a heat stress effect was observed on ovules from distal ovaries for CDC Sage (Table 4.7 and Fig. 4.2). In this cultivar, whereas 0.32 ovules per ovary on the distal position of an inflorescence displayed ROS under heat stress, the proportion was 0 on pods at a similar position under control conditions. There was a slight but insignificant increase in number of ovules displaying ROS in pods at proximal and distal position in Naparnyk exposed to heat stress (Table 4.7).

Table 4.6 Analysis of variance for the effect of treatment, cultivar, and their interactions on proportion of ovules with presence of Reactive Oxygen Species (ROS) per ovary position at reproductive Node 4.

Source of Variation	Ovary Position			
	Proximal		Distal	
	F Value	P Value	F Value	P Value
Treatment (T)	0.52	0.4813	1.88	0.1922
Cultivar (C)	0.46	0.6416	3.46	0.0607
C*T	1.83	0.1856	7.29	**0.0080**

Significance levels at P <0.05 and P<0.001 are shown in bold.

Table 4.7 Effect of high temperature on proportion of ovules with presence of ROS per ovary position at the reproductive Node 4 on three cultivars of field pea. Means ± SE (n= 3 to 4 pods).

Cultivar	Ovary Position			
	Proximal		Distal	
	Control	Heat	Control	Heat
Carneval	0.14±0.09 a†	0.04±0.04 a	0.16±0.0 ab	0.05±0.1 bc
Naparnyk	0.00±0.00 a	0.08±0.06 a	0.00±0.0 c	0.06±0.1 bc
CDC Sage	0.16±0.08 a	0.08±0.08 a	0.00±0.0 bc	0.32±0.1 a

†Values within a column and within variable followed by the same letter are not significantly different at P<0.005.

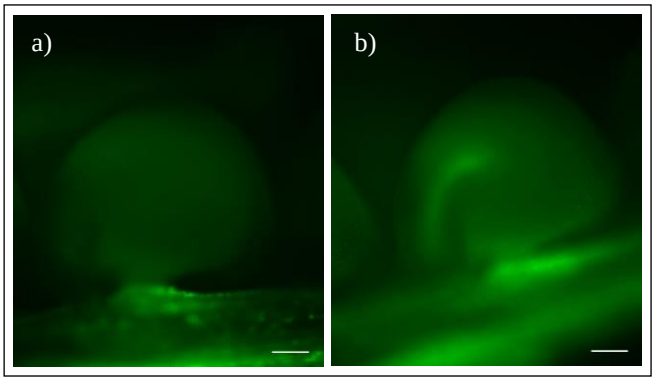


Fig. 4.2 Ovules within ovaries at reproductive Node 4 of field pea with and without presence of Reactive Oxygen Species (ROS) in the embryo sac region. a) Ovule without ROS and b) Ovule with signs of ROS over the embryo sac. Scale bars 100 µm.

4.3.2 Pod and seed assessment at plant physiological maturity

Pod length, number of seeds, and seed-to-ovule ratio

High temperature applied at the early flowering stage significantly affected pod length of mature pods on plants depending on cultivar and reproductive node (Table 4.8). Specifically, heat-treated CDC Sage had smaller pods at reproductive Node 1. Heat-treated Carneval had smaller pods at reproductive Node 3 but longer pods at reproductive Node 4 compared to nodes at a similar

position on plants from control conditions (Fig. 4.3). Across cultivars, pods from reproductive Node 1 and Node 2 tended to be smaller on plants under high temperature compared to plants under control conditions through the experiment (Fig. 4.3).

Importantly, temperature increase also significantly affected the number of seeds and seed-to-ovule ratio on some cultivars at the level of reproductive node (Table 4.8). In particular, heat treatment reduced the number of seeds and seed-to-ovule ratio of cultivar Carneval at reproductive Nodes 1-3, of CDC Sage at reproductive Node 1 and Node 2, and of CDC Meadow at reproductive Node 2 and Node 3 (Fig. 4.4). In contrast, heat treatment on 40-10 increased seeds and seed-to-ovule ratio at reproductive Node 3 and Node 4 compared with their respective controls (Fig. 4.4). Cultivar Naparnyk did not show any significant effect of temperature on any of these variables. Overall, the negative effect of high temperature on seed-to-ovule ratio was mainly identified at reproductive Node 1 and 2 across cultivars (Fig. 4.4).

Table 4.8 Analysis of variance for the effect of cultivar, treatment, reproductive node, and pod position on pod length, number of seeds, and seed-to-ovule ratio of five field pea (*Pisum sativum* L.) cultivars grown under growth chamber conditions. Three-way interactions that were not significant are not shown.

Source of Variation	Pod Length (mm)		Number of Seeds		Seed-to-Ovule Ratio	
	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
Cultivar (P)	23.53	**<.0001**	23.64	**<.0001**	34.76	**<.0001**
Treatment (T)	2.97	0.0961	17.39	**<.0001**	21.93	**<.0001**
Reproductive Node (N)	23.90	**<.0001**	6.75	**0.0006**	6.47	**0.0008**
Pod Position (PP)	26.37	**<.0001**	16.84	**<.0001**	10.01	**0.0018**
C*T	0.09	0.9840	7.54	**<.0001**	10.77	**<.0001**
C*N	1.41	0.1608	1.29	0.2495	1.34	0.2240
C*PP	0.33	0.8608	0.68	0.6061	0.42	0.7945
T*N	4.50	**0.0044**	7.48	**<.0001**	6.30	**0.0004**
T*PP	0.65	0.4223	0.27	0.6072	0.69	0.4078
N*PP	1.02	0.3833	2.35	0.0736	3.93	**0.0096**
C*T*N	6.24	**<.0001**	3.91	**<.0001**	3.84	**<.0001**

Significance levels at P <0.05 and P<0.001 are shown in bold

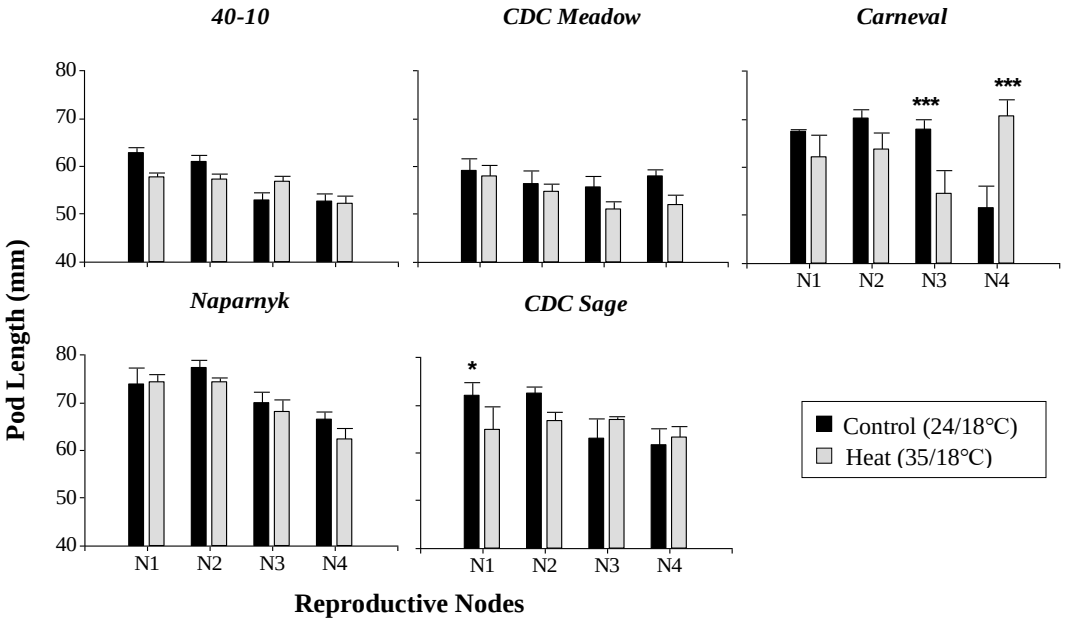


Fig. 4.3 Effect of high temperature (35/18°C) on pod length of the first four reproductive nodes on five field pea (*Pisum sativum* L) cultivars grown under growth chamber conditions. Means of four replications (n=4 to 8 pods) with their respective standard error bars are shown. *Indicates a significant temperature treatment effect at P<0.05; ***Indicates a significant temperature treatment effect at P<0.001.

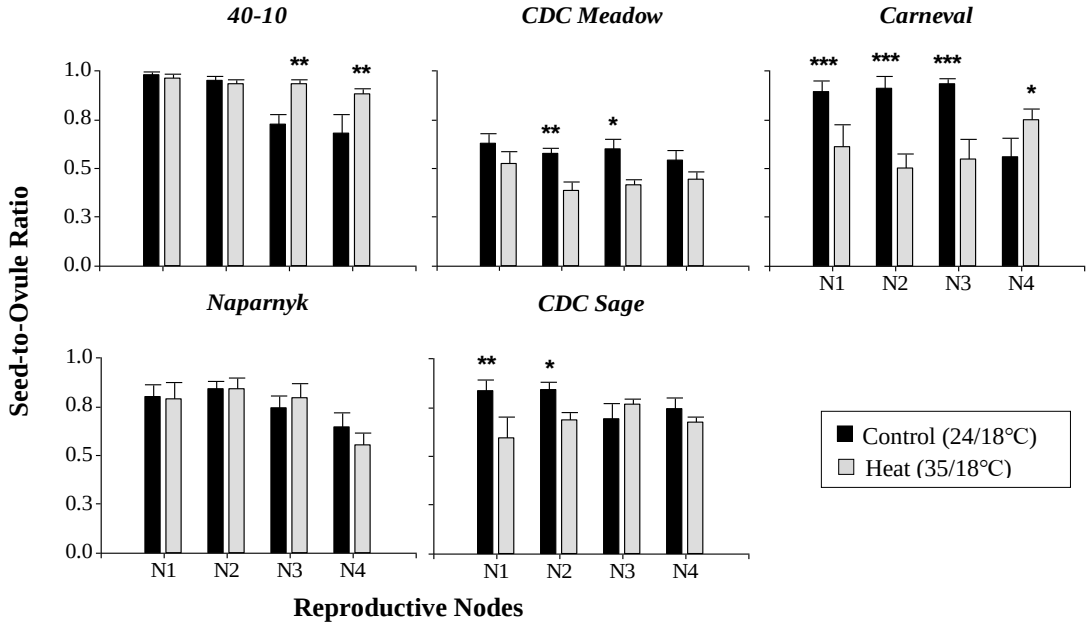


Fig. 4.4 Effect of high temperature (35/18°C) on seed-to-ovule ratio of the first four reproductive nodes on five field pea (*Pisum sativum* L) cultivars grown under growth chamber conditions. Means of four replications (n=4 to 8 pods) with their respective standard error bars are shown. *Indicates a significant temperature treatment effect at P<0.05; **Indicates a significant temperature treatment effect at P<0.01; ***Indicates a significant temperature treatment effect at P<0.001.

Independent of the treatments, a longer pod was displayed on Naparnyk and CDC Sage, and greater number of seeds and seed-to-ovule ratios on 40-10, Naparnyk, and CDC Sage (Table 4.9). Pod length, seed number, and seed-to-ovule ratio were always greater at reproductive Node 1 and 2 compared to Node 4 on plants, and in pods at proximal positions compared to distal ones for all cultivars (Table 4.9).

Table 4.9 Means (± SE) of pod length (mm), number of seeds per pod, and seed-to-ovule ratio according to source of variation: treatment (n=127 to 130), cultivar (n=44 to 63), reproductive node (n=59 to 71), and pod position (n=100 to 157) of field pea (*Pisum sativum* L.) plants grown under growth chamber conditions.

Source of Variation	Pod Length (mm)	Number of Seeds per Pod	Seed-to-Ovule Ratio
Cultivar			
40-10	56.65±0.642 c†	6.02±0.151 a	0.878±0.020 a
CDC Meadow	55.58±0.775 c	3.81±0.138 c	0.512±0.019 c
CDC Sage	66.34±1.103 b	5.70±0.180 a	0.728±0.022 b
Carneval	63.48±1.424 b	5.27±0.249 b	0.712±0.034 b
Naparnyk	70.77±0.928 a	5.68±0.195 b	0.749±0.025 b
Treatment			
Control (24°C)	63.59±0.780 a	5.56±0.126 a	0.755±0.016 a
Heat (35°C)	61.54±0.779 a	5.03±0.136 b	0.677±0.019 b
Reproductive Node			
Node 1	65.17±1.078 a	5.60±0.205 a	0.760±0.027 a
Node 2	65.39±1.023 a	5.62±0.191 a	0.745±0.025 a
Node 3	60.64±1.103 b	5.23±0.179 a	0.713±0.025 a
Node 4	59.06±1.065 b	4.73±0.157 b	0.646±0.023 b
Pod Position			
Distal	60.88±0.779 b	5.04±0.134 b	0.690±0.018 b
Proximal	64.25±0.766 a	5.55±0.128 a	0.742±0.017 a

†Values within a column and within source of variation followed by the same letter are not significantly different at P<0.005.

Seed diameter

The increase of temperature at early flowering stage influenced seed diameter and the effect varied among cultivars (Table 4.10). Specifically, seeds of 40-10, Naparnyk, CDC Meadow, and CDC Sage displayed between 1 to 2.7 % smaller diameter on plants exposed to heat stress compared to controls. Contrastingly, seeds of Carneval exhibited 2.7% larger diameter on heat-treated plants compared to the controls (Fig. 4.5). The highest reduction (2.7%) in seed diameter was observed on Naparnyk and lowest reduction (1%) on CDC Sage (Fig. 4.5). Regardless of treatment, 40-10 showed the smallest seed size in the group. In terms of reproductive nodes, Node

1 and Node 2 tended to show the largest seed size compared to Node 3 and Node 4 on all plants (Table 4.11).

Table 4.10 Analysis of variance for the effect of cultivar, treatment, reproductive node, and their interactions on seed diameter (mm) of five field pea (*Pisum sativum* L.) cultivars grown under growth chamber conditions.

Source of Variation	Seed Diameter (mm)	
	***F* Value**	***P* Value**
Cultivar (C)	40.56	**<.0001**
Treatment (T)	24.76	**<.0001**
Reproductive Node (N)	21.51	**<.0001**
C*T	12.9	**<.0001**
C*N	4.94	**<.0001**
T*N	0.96	0.4126
C*T*N	0.98	0.4636

Significance levels at P <0.05 and P<0.001 are shown in bold

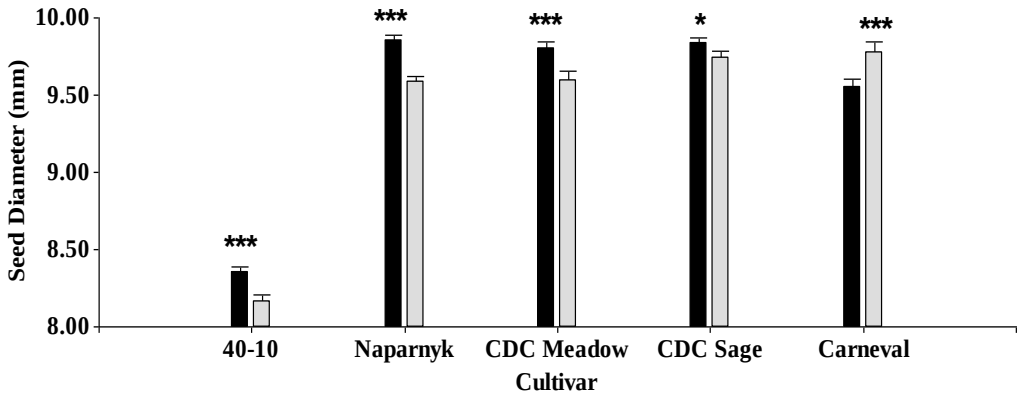


Fig. 4.5 Effect of high temperature (35/18°C) on seed diameter from first four reproductive nodes on field pea (*Pisum sativum* L.) cultivars grown under growth chamber conditions. Means of four replications with their respective standard error bars are shown.

Table 4.11 Means (± SE) of seed diameter (mm) according to cultivar, treatment, and reproductive node on field pea (*Pisum sativum* L.) plants grown under growth chamber conditions.

Cultivar	Seed Diameter (mm)
40 -10	8.27±0.03 b
Carneval	9.71±0.03 a
CDC Meadow	9.73±0.03 a
CDC Sage	9.79±0.03 a
Naparnyk	9.73±0.03 a
Treatment	
Control	9.47±0.03 a
Heat	9.34±0.03 b
Reproductive Node	
Node 1	9.53±0.04 a
Node 2	9.46±0.04 a
Node 3	9.41±0.04 b
Node 4	9.16±0.05 c

†Values within a column followed by the same letter are not significantly different at P<0.005.

Ovule and early seed abortion

Evaluation of ovule and early seed abortion at plant maturity revealed the existence of three main categories according to their degree of development. The first category had ovules less than 1 mm that failed right before or after fertilization (ABAF); the second category had ovules between 1 to less than 3 mm that failed after fertilization, and they contained embryos either globular or heart stages (AEE); and the third category had ovules between 3 to 5 mm that failed when their embryos were at early or late cotyledon stages (AELC). In particular, the proportion of aborted ovules of less than 1 mm or ABAF per pod was significantly increased under high temperature depending on the cultivar (Table 4.12). Carneval and CDC Meadow exhibited an increased proportion of ABAF on plants exposed to high temperature compared to plants under control conditions (Fig. 4.6). The proportion of aborted ovules from 1 to less than 3 mm or AEE per pod were significantly affected at level of interaction cultivar by treatment by node and interaction cultivar by treatment by ovule position (Table 4.12). High temperature increased significantly the proportion of aborted ovules AEE per pod on Carneval at Node 2 and Node 3 and on CDC Sage at Node 1, compared to similar node locations on control plants (Fig. 4.7). In addition, heat treatment increased the presence of AEE specifically at medial positions within pods of Carneval, at basal positions within pods of CDC Sage, and at stylar positions within pods of CDC Meadow

compared with control plants (Fig. 4.8). In contrast to this trend, high temperature did not increase abortion of ovules between 3 to 5 mm, corresponding to category AELC (Table 4.12). Regardless of the temperature treatment, Carneval had more aborted ovules of category ABAF, whereas CDC Meadow had more aborted ovules of category AEE and AELC. In general, ovules at medial positions tended to show the fewest aborted ovules (Table 4.13).

Table 4.12 Analysis of variance for the effect of cultivar, treatment, reproductive node, and ovule positions on three categories of ovule abortion found in mature pods of field pea (*Pisum sativum* L.) plants grown under growth chamber conditions. ABAF: ovules of less than 1 mm that failed right before or after fertilization; AEE: fertilized ovules between 1 to less than 3 mm that failed at early embryo growth (globular to heart stages); AELC: early seeds between 3 to 5 mm that did not fill the seed coat and presented embryos between early and late cotyledon stages.

Source of Variation	Ovule and Seed Failure Categories					
	ABAF (<1mm)		AEE (1 < 3 mm)		AELC (3 to 5 mm)	
	***F* Value**	***P* Value**	***F* Value**	***P* Value**	***F* Value**	***P* Value**
Treatment (T)	21.57	**<.0001**	3.30	0.0700	0.00	0.9701
Cultivar (C)	9.30	**<.0001**	7.03	**0.0037**	3.24	**0.0500**
Reproductive Node (N)	2.50	0.0595	1.94	0.1223	1.56	0.1980
Ovule Position (OP)	8.23	**0.0003**	57.38	**<.0001**	1.45	0.2368
C*T	8.79	**<.0001**	2.82	**0.0251**	0.55	0.6971
C*N	1.88	**0.0361**	2.54	**0.0031**	0.67	0.7808
C*OP	1.91	0.0572	4.55	**<.0001**	1.90	0.0595
T*N	1.04	0.3740	1.74	0.1575	1.02	0.3854
T*OP	0.37	0.6913	4.80	**0.0087**	0.53	0.5864
N*OP	0.64	0.6987	1.90	0.0804	0.56	0.7660
C*T*N	1.50	0.1232	1.98	**0.0249**	1.67	0.0715
C*T*OP	0.67	0.7182	2.25	**0.0234**	0.82	0.5816

Significance levels at P <0.05 and P<0.001 are shown in bold

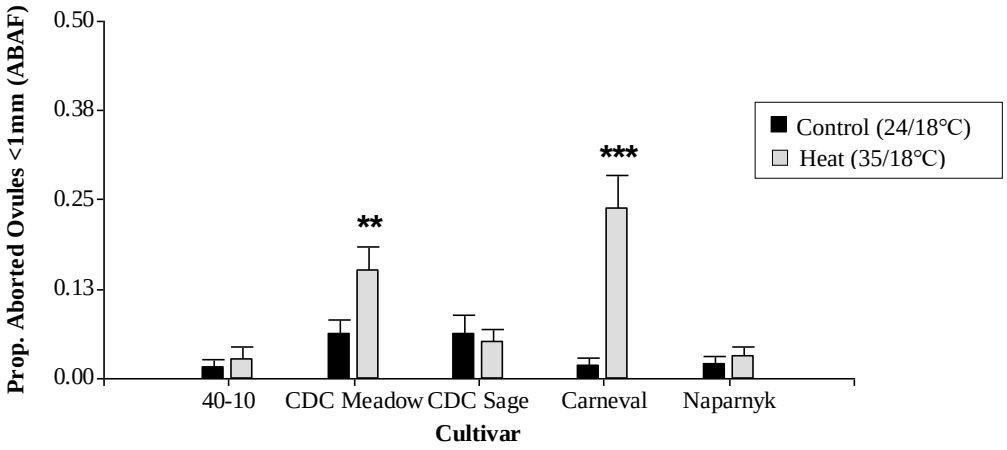


Fig. 4.6 Effect of high temperature (35/18°C) on proportion of aborted ovules at category ABAF (ovules of less than 1 mm) per pod from four reproductive nodes per plant in five cultivars of field pea (*Pisum sativum* L.) grown under growth chamber conditions. Means of four replications (n= 19 to 32 pods) with their respective error bars are shown. **Indicates a significant temperature treatment effect at P<0.01; ***Indicates a significant temperature treatment effect at P<0.001.

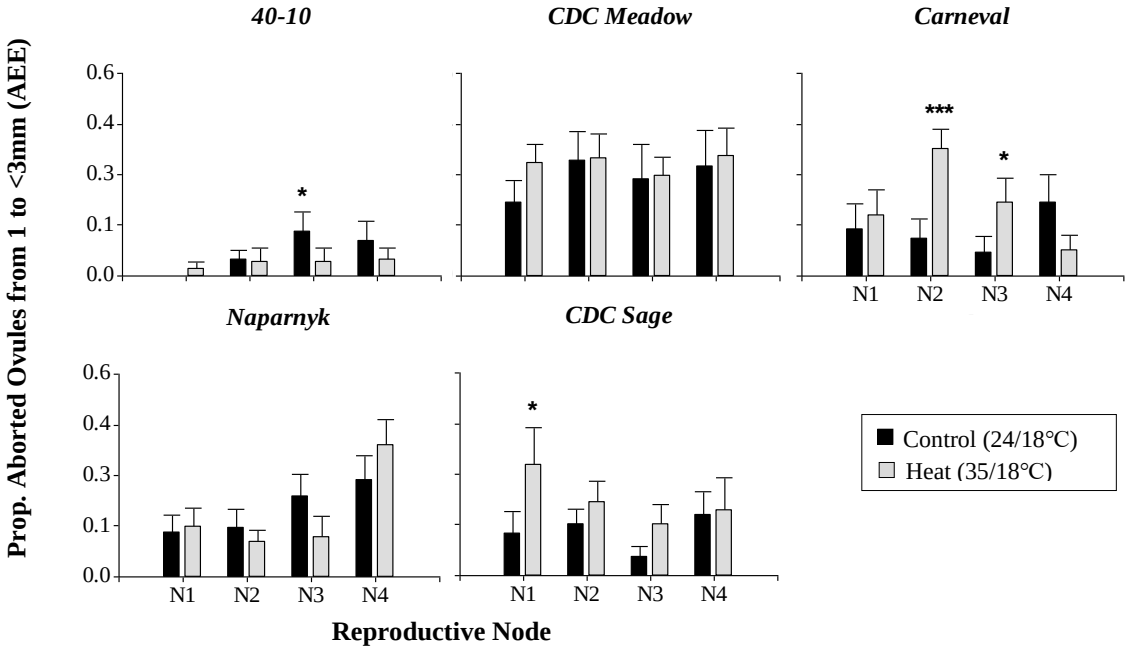


Fig. 4.7 Effect of high temperature (35/18°C) on proportion of aborted ovules at category AEE (fertilized ovules between 1 to less than 3 mm) per pod of the first four reproductive nodes on five field pea (*Pisum sativum* L.) cultivars grown under growth chamber conditions. Means of four replications (n = 4 to 8 pods) with their respective error bars are shown. *Indicates a significant temperature treatment effect at P<0.05; ***Indicates a significant temperature treatment effect at P<0.001.

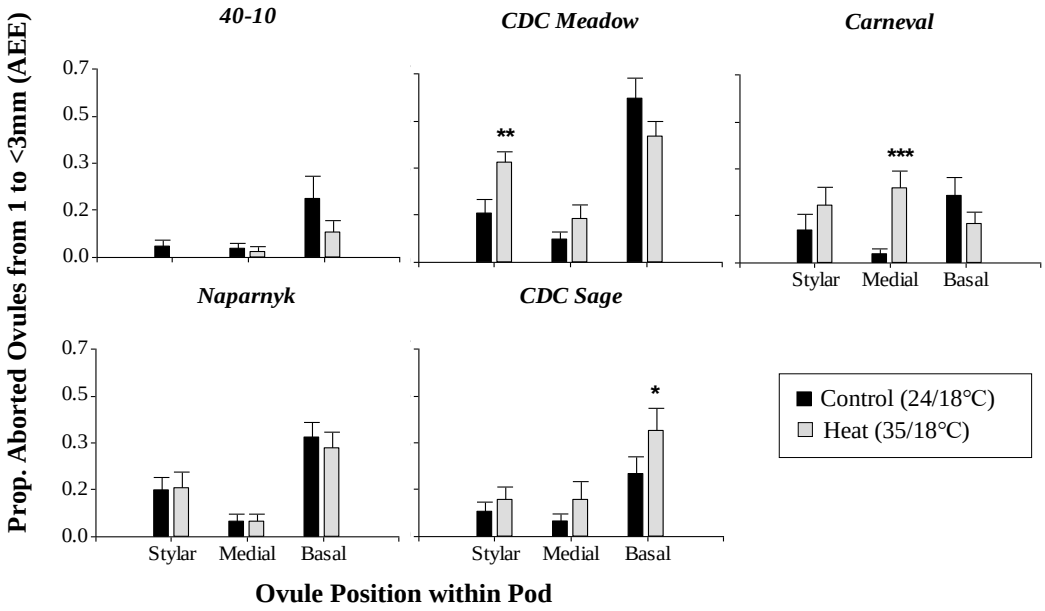


Fig. 4.8 Effect of high temperature (35°C) on proportion of aborted ovules at category AEE (fertilized ovules between 1 to less than 3 mm) per pod, according to three ovule positions within pods on five field pea (*Pisum sativum* L.) cultivars grown under growth chamber conditions. Means of four replications (n = 4 to 8 pods) with their respective error bars are shown.

Table 4.13 Means (± SE) of ovule and early seed abortion at three categories according to cultivar (n = 44 to 63 pods), treatment (n = 127 to 130 pods), and ovule position within the pod (n =257 pods) of field pea (*Pisum sativum* L.) plants grown under growth chamber conditions. ABAF: ovules of less than 1 mm that failed right before or after fertilization; AEE: fertilized ovules between 1 to less than 3 mm that failed at early embryo growth (globular to heart stages); AELC: early seeds between 3 to 5 mm that did not fill the seed coat and presented embryos between early and late cotyledon stages.

Source of Variation	Aborted Ovules Category		
	ABAF (<1mm)	AEE (1 to 3 mm)	AELC (>3 to 5 mm)
Cultivar (C)			
40-10	0.02±0.010 b†	0.07±0.018 c	0.01±0.004 b
CDC Meadow	0.11±0.019 a	0.31±0.028 a	0.06±0.011 a
CDC Sage	0.06±0.016 b	0.18±0.025 b	0.02±0.009 b
Carneval	0.13±0.026 a	0.17±0.024 b	0.03±0.009 b
Naparnyk	0.03±0.008 b	0.20±0.023 b	0.03±0.009 b
Treatment (T)			
Control (24°C)	0.04±0.008 b	0.17±0.016 a	0.03±0.006 a
Heat (35°C)	0.10±0.013 a	0.20±0.016 a	0.03±0.005 a
Ovule Position (OP)			
Stylar	0.10±0.015 a	0.15±0.016 b	0.03±0.008 a
Medial	0.03±0.011 b	0.09±0.014 c	0.02±0.005 a
Basal	0.07±0.015 a	0.31±0.023 a	0.04±0.008 a

†Values within a column and within source of variation followed by the same letter are not significantly different at P<0.005.

Correlation of variables from pods at mature stage

Variables evaluated at pod maturity showed both positive and negative associations. For positive associations, the number of seeds, seed-to-ovule ratio, and pod length were correlated with each other in plants exposed to both high temperature and control conditions (Table 4.14). Similarly, in plants from control conditions, ovule number was positively associated with pod length and number of seeds; however, in plants exposed to high temperature conditions, this relationship did not exist. Furthermore, the number of ovules per pod was positively associated with the proportion of aborted ovules of category ABAF and AEE per pod, specifically in plants under heat stress conditions (Table 4.14).

In contrast, in control plants, pod length was inversely associated with the proportion of the three types of aborted ovules per pod, namely ABAF, AEE, and AELC. In heat-treated plants, pod length was inversely associated with two of the aborted categories, namely ABAF and AEE (Table 4.14). As expected, the number of seeds and seed-to-ovule ratio per pod were also negatively associated with the proportion of the three types of aborted ovules per pod (ABAF, AEE, AELC) in plants from both control and high temperature conditions. In these plants, aborted ovules AEE showed the strongest negative association with the number of seeds and seed-to-ovule ratio per pod (Table 4.14). Also, aborted ovules of category ABAF showed a stronger negative association with the number of seeds and seed-to-ovule ratio in plants exposed to heat stress conditions (r= -0.68 and -0.67 respectively) compared to plants under control conditions (r= -0.33 and -0.36 respectively). Finally, the number of ovules per pod was inversely associated with seed-to-ovule ratio in plants under heat stress conditions (Table 4.14).

Table 4.14 Correlation matrix showing main associations between variables evaluated at mature stage of pods on five field pea cultivars according to treatment, high temperature (up right side) and control conditions (down left side). ABAF: ovules of less than 1 mm that failed right before or after fertilization; AEE: fertilized ovules between 1 to less than 3 mm that failed at early embryo growth (globular to heart stages); AELC: early seeds between 3 to 5 mm that did not fill the seed coat and presented embryos between early and late cotyledon stages.

Heat / Control	PL	NO	NS	SOR	A1	A2	A3
Pod Length (PL)		0.21	0.61***	0.48***	-0.48***	-0.33**	-0.03
Number of Ovules (NO)	0.54***		-0.20	-0.43***	0.25*	0.50***	0.14
Number of Seeds (NS)	0.77***	0.33**		0.97***	-0.68***	-0.76***	-0.38***
Seed-to-Ovule Ratio (SOR)	0.64***	0.02	0.95***		-0.67***	-0.82***	-0.39***
Prop. of ABAF per Pod (A1)	-0.27*	-0.03	-0.33**	-0.36**		0.23	0.13
Prop. of AEE per Pod (A2)	-0.47***	0.14	-0.79***	-0.87***	-0.02		0.12
Prop. of AELC per Pod (A3)	-0.32**	-0.03	-0.49***	-0.50***	-0.02	0.27*	

*Indicates a significant temperature treatment effect at P<0.05; **Indicates a significant temperature treatment effect at P<0.01; ***Indicates a significant temperature treatment effect at P<0.001.

4.4 Discussion

High temperature during reproductive development in field pea plants is a major factor associated with yield reduction (Guilioni et al., 2003; Prasad et al., 2008; Bueckert et al., 2015). As a flowering plant, seed yield of this crop relies on a successful sexual reproduction. Therefore, any environmental disturbance can seriously harm the male and female gametophytes during the reproductive process and cause severe seed loss (Warrag and Hall, 1983; Tischner et al., 2003; Sita et al., 2017). In this sense, the investigation of the ovule is critical to uncover the weakness of the reproductive development under high temperature; however, its study has been neglected since this reproductive structure has been considered to be less sensitive (Barnabás et al., 2008; Sage et al., 2015). Interestingly, some studies evaluating seed set of reciprocal crosses between male and female heat-stressed structures in various species have suggested a possible failure of the female gametophyte under this abiotic stress, but this phenomenon has not been clearly confirmed (Gross and Kigel, 1994; Young et al., 2004; Djanaguiraman et al., 2018). Here, in field pea, the analysis of viability of young ovules and seed abortion at physiological maturity stage of the plants revealed that high temperature negatively affected ovule from fertilization to early embryo formation.

4.4.1 Effect of high temperature on callose deposition

In response to heat, field pea plants of the three estudied cultivars exhibited an increase in callose deposition in ovules at one out of four reproductive nodes evaluated. The accumulation of this polysaccharide was found on the vascular bundle area (chalaza) and nucellus of these ovules. These signs of ovule senescence are consistent with those reported in various species, such as almond (*Prunus dulcis* [Mill.] D.A. Webb), peach (*Prunus persica* [L.] Batsch), alfalfa (*Medicago sativa* L.), and *Arabidopsis* (Dumas and Knox, 1983; Arbeloa and Herrero, 1991; Rosellini et al., 1998; Sun et al., 2004). The presence of callose in some of these studies has been associated with tissue degenerated in unfertilized ovules (sterile). Curiously, most of those studies have a common theme, that callose accumulation on the chalaza area of the ovule could be attributed to obstruction of assimilates imported to the embryo sac. In my study, since 94% of the ovules at the affected node displayed signs of fertilization (Table 4.2), confirmed by the presence of pollen tubes in the micropylar area of the ovules, a sterility of the ovules may be very low. Instead, the presence of callose deposits around the vascular bundle and nucellus area could be more related to an assimilate disruption in these ovules. In fact, earlier studies in pea showed that presence of callose at similar locations in ovules was accompanied by lignin accumulation that caused a lack of permeability of the tissue, leading to starvation of early embryo and endosperm in the ovules (Briggs et al., 1987).

According to Chamberlin et al. (1993), ovule ultrastructure and autoradiographic studies for carbon flow in soybean revealed that photo-assimilates in ovules are continuously regulated spatially and temporarily at two specific spots around the vascular bundle of the ovules, the chalaza and micropylar area of the ovule. Moreover, research in *Arabidopsis* has shown that cells at the chalaza tend to display a high frequency of plasmodesmata activity implying the existence of a symplastic control of nutrient flow in the chalaza toward the embryo sac (Thijssen, 2003; Sager and Lee, 2014). Thus, it is plausible that callose accumulation in the chalaza area of field pea ovules under heat stress was associated with a disruption of plasmodesmata activity and symplastic transport of assimilates into embryo sac. This may agree with numerous studies where abiotic stress such as wounding, temperature, and mineral toxicity caused an increase of callose deposition on plasmodesmata and a subsequent inhibition of assimilate transport in leaves, shoots, petioles, and roots of the affected plants (Webster and Currier, 1968; Smith and McCully, 1977; Bilska and

Sowiński, 2010; Zavaliev et al., 2011; Cui and Lee, 2016). Similarly, the increase of callose deposits observed in ovules at a specific reproductive node of heat-treated plants in my study, may support the fact that a change in the dynamics of assimilate transport occurred also at the plant structural level. Hence, accumulation of this polysaccharide detected in ovules under heat stress may demonstrate disruption of ovule development by means of blockage of assimilate transport into the tissue.

4.4.2 Effect of high temperature on presence of reactive oxygen species

The analysis for Reactive Oxygen Species (ROS) in ovules at reproductive Node 4 of the plants revealed that one out of the three cultivars exhibited accumulation of ROS in the gametophyte area of the ovules. ROS (O_2^-, H_2O_2, OH, $^1O_2$), as a normal by-product of the metabolism of the cells, can lead to a harmful oxidation in the cells (Apel and Hirt, 2004; Burton and Jauniaux, 2011; Sharma et al., 2012). According to Dat et al. (2000) and Choudhury et al. (2017), ROS can normally act as a powerful signal to regulate growth and activate defense mechanisms in the plants. Several reports has demonstrated that when ROS is produced to control homeostasis in plant tissue, ROS work in a signal pathway to activate cellular processes, such as defense against pathogens and acclimation to environmental conditions (Neill et al., 2002; Ren et al., 2002). However, if abiotic stress e.g. severe drought, metal toxicity, temperature stress, among others, appear the fragile balance of the cell metabolism can be disrupted. As a result, an overproduction of ROS occurs in the tissue causing oxidative stress and damage to the cell contents (Dat et al., 2000; Maheshwari and Dubey, 2009; Sharma et al., 2012). In my study, given that the accumulation of ROS was observed specially in ovules within pods at the distal position in the inflorescence raceme, their presence may be associated with a harmful effect in the cells of those ovules. Indeed, according to various studies in legumes, pods and its ovules at distal positions in a raceme tend to be highly compromised under adverse conditions, especially since they are localized farther from the maternal supply (Brun and Betts, 1984; Diggle, 1995; Guilioni, 1997). Furthermore, the presence of the reactive species specifically in the embryo sac may indicate damaged cells at the ultrastructure level as an initial stage of ovule abortion. Sun et al. (2005), evaluating the effect of salt stress in *Arabidopsis*, found that ROS accumulated initially in the embryo sac and later spread out into the chalaza and integuments. In those studies, cellular and ultrastructure analysis of these ovules revealed that cell components such as cytoplasm, vacuoles

(embryos), and mitochondria membranes were disrupted, which was associated with possible programmed cell death activated within the affected ovules (Sun et al., 2004, 2005; Hauser et al., 2006).

The increase of ROS in tissue may be part of a process that accompanies callose deposition in tissue affected by biotic and abiotic stress (Wu et al., 2018). Studies in *Arabidopsis* mutants have shown that callose in cellular tissue appears to control plasmodesmata activity in response to ROS signal in the cells (Benitez-Alfonso et al., 2009; Cui and Lee, 2016). For example, endogenous application of H_2O_2 on *Arabidopsis* leaves decreased plasmodesmata permeability in consistency with an increase of callose deposits around the plasmodesmata in the leaf tissue (Cui and Lee, 2016). In my study, although the accumulation of ROS was observed in ovules that also presented some level of increased callose deposition on one cultivar (CDC Sage) under heat stress (Table A2.3), their different localization within the ovule, callose in the chalaza and ROS in the embryo sac, may imply the existence of various effects of heat stress. Whereas the presence of ROS in the embryo sac could be related to toxicity and programmed cell death, callose deposition around the vascular area could be associated to a posterior effect in a signal mechanism to control or limit maternal supply toward the embryo sac. Various authors explain that ROS can act as signal molecules for cellular processes e.g. callose deposition, but this function is coordinated in a delicate balance where, right after the signal, the reactive species need to be removed efficiently from the tissue by enzymes and antioxidant scavengers (Mittler et al., 2004; Suzuki and Mittler, 2006; Sharma et al., 2012). Overall, the presence of ROS in embryo sacs may indicate disruption of the metabolic activity of the cells in the gametophyte in ovules about to be aborted during heat stress. Although ROS detected in the affected ovules did not follow the same pattern observed on callose deposition, both molecules may be involved in some degree of disruption in the ovular tissue in plants exposed to heat stress.

4.4.3 Effect of high temperature on seed number and pod length

Pod length, seed number, and seed-to-ovule ratio exhibited analogous effects of high temperature at plant physiological maturity, indicating the close relationship between successful seeds and pod length (Jeuffroy and Chabanet, 1994; Ozga et al., 2017). These three variables were positively correlated, meaning that reduction of one variable resulted in reduction of the other and vice versa (Table 4.13). Importantly, although the trend was similar for the three variables, seed

number and seed-to-ovule ratio exhibited more pronounced negative responses to the high temperature. This is consistent with other studies where the major effect of high temperature was identified through the reduction of seed yield on legume and cereal crops (Prasad et al., 2008; Sadras et al., 2013; Kaur et al., 2015).

As expected, cultivars previously categorized as having medium or low tolerance to high temperature consistently exhibited reduced seed number and seed-to-ovule ratio under heat stress. This reduction was observed at the first two to three reproductive nodes on the plants, where opened flowers (Node 1) and buds (Node 2 and Node 3) were present during treatment. Comparably, Jeuffroy et al. (1990), evaluating various reproductive nodes on the pea cultivar Solara, identified that high temperature (31°C) reduced seed number at the first two nodes. They attributed the effect to a critical stage of the flower (>six days after open flower) localized at the affected nodes, where a low concentration of plant assimilates under heat stress could affect early embryo development. In related studies, Guilioni et al. (2003) identified that cultivar Messire exposed to high temperature before and after flowering caused seed reduction by changing the pattern of seed production along the main stems of the plants regardless of the age of the flower. In the same study, the effect was ascribed to the competition of assimilates between the different sinks in development. As such, seeds in pods on the first nodes (older) were prioritized to maintain growth and maturity in plants under heat stress. Whereas Jeuffroy et al. (1990) and Guilioni et al. (2003) point out various responses of the plants to heat stress, perhaps due to the different cultivars evaluated, their findings coincide in that assimilate availability may drive the fate of the seeds at specific plant nodes.

In my study, although the reduction of seed yield on the first reproductive nodes may be attributed to young sensitive flower stages (open flower and buds) to heat stress, the variation of node response among cultivars may also indicate dependence on resource availability of each cultivar. Indeed, the low heat-tolerant cultivar Carneval displayed reduction of seed-to-ovule ratio at the three first reproductive nodes of the plants, whereas the medium heat-tolerant cultivars CDC Meadow and CDC Sage showed reduction of seed-to-ovule ratio at two of these reproductive nodes. This effect could be related to a physiological mechanism in the plants where sinks (seeds) in development were adjusted according to the availability of photo-assimilates of the plant under stress (Aloni et al., 1991; Guilioni et al., 2003). Moreover, evaluation of diverse tolerant cultivars

on legume crops such as chickpea and lentil have shown that yield and reproductive failure on them was associated with proper physiological response of the plants e.g chlorophyll content alteration, sucrose availability, and photosynthesis efficiency (Kumar et al., 2013; Bhandari et al., 2016; Chand et al., 2018). Therefore, the reduction on seed number and seed-to-ovule ratio in medium and low heat-tolerant cultivars may indicate a physiological adjustment of the resource availability in each cultivar.

4.4.4 Effect of high temperature on ovule and early seed abortion

Under high temperature, abortion of young ovules (<1mm and 1 to 3 mm in length) was increased. This small size of the aborted ovules suggests that failure during fertilization and early embryo growth was enhanced in plants exposed to heat. On one side, while aborted ovules of less than 1mm in length could be associated to some level of fertilization malfunction, this idea may apply to only a small portion of these aborted ovules. Indeed, signs of fertilization or embryo growth (e.g., a zygote, embryo) found in more than 90% of the ovules on reproductive Node 1 to Node 3 right after similar heat treatment (Chapter 3; Fig. 3.7) suggests that abortion of ovules of less than 1mm could also involve those ovules containing a zygote or pro-embryo growth. Jahnke et al. (1989), studying assimilate partitioning in pea, explained that ovaries (containing ovules) right after fertilization start a stage of high activity and therefore, they become a sink for assimilates that compete against other organs in development on the plant. Under these conditions, it is possible that embryo starvation occurred as a consequence of maternal expenditure adjustment (Dinar and Rudich, 1985; Aloni et al., 1991), where development of offspring that had received the least investment may have been arrested (Nakamura, 1988; Diggle, 1995).

In contrast, the increase of aborted ovules between 1 to <3 mm (ovules with embryos between globular to heart stages) observed at different reproductive nodes of the plants may indicate vulnerability to heat at young embryo development (early seed). In fact, following fertilization, the consecutive embryo, suspensor, and endosperm formation requires an active cell division in the ovule (King and Heyes, 1986; Ruan et al., 2010) that is under maternal control (Weber et al., 2005). In the present study, it is possible that high temperature enhanced this abortion by disrupting metabolic and hormonal activity of the plants, and therefore, compromised young seed development (Ozga et al., 2017; Liu et al., 2019). For example, in common bean, young pod abortion on heat sensitive cultivars was associated with a low level of indol-3-acetic acid (IAA)

transport that accumulated at pedicels of aborted reproductive structures (Ofir et al., 1993). In similar context, research in wheat showed that high temperature induced high levels of ethylene in structures such as developing embryos and kernels on a low heat-tolerant cultivar displaying low seed set. They hypothesized that ethylene was excessively produced in response to heat stress to regulate senescence and seed abortion on the plants (Hays et al., 2007). Thus, in this study, ovule abortion being related to early seed growth may follow disruption of cell division and changes in metabolic activity in plants under heat stress, possibilities that will need further research.

Additionally, the increased abortion of ovules at an embryo stage detected at specified ovule positions (stylar, medial, basal) within pods may be related to an asynchronous development of the young seeds in the pod (Linck, 1961). Hedley and Ambrose (1981) proposed that ovules within the same pod can exhibit different growth rates where seeds with higher growth rates in the middle of the pod would be less aborted. Alternatively, O'Donnell and Bawa (1993), following the pattern of ovule and seed abortion in *Sophora japonica*, suggested that abortion at specific ovule positions within a pod may be attributed to the sequence of ovule fertilization, where ovule positions that are fertilized first, varying among species, would have lower probabilities of abortion. In my study, although early seed abortion tended to occur at stylar and basal positions in the pod on some cultivars, an occurrence of ovule abortion at middle positions on others cultivars may perhaps be more closely related to the sequence of ovule fertilization in those pods.

4.4.5 Effect of high temperature on seed diameter

The increase of temperature reduced seed diameter on high and medium heat-tolerant cultivars. In congruency to this finding, seed size reduction has been also observed in legume crops such as common bean, cowpea, lentil, faba bean, chickpea, and soybean exposed to temperature stress (Egli and Wardlaw, 1980; Sadras et al., 2013; Awasthi et al., 2014). In lentil, for example, evaluation of several accessions showed that high temperature in the field caused 5.7% to 28.3% reduction in seed size. In the present study, although the effect was not as high as in lentil, heat-treated plants of high and medium heat-tolerant cultivars displayed 1% to 2.7% seed size reduction. Various studies revealed that the effect of high temperature on seed size can be attributed to disturbance of reproductive development of the plants (McDonald and Paulsen, 1997; Wang et al., 2006; Tacarindua et al., 2012). Work on legumes and cereal crops showed that the increase of temperature at stages of pre-anthesis, full bloom, and seed filling on the plants affected final seed

size mainly due to a reduction of seed filling duration (Savin et al., 1999; Wang et al., 2006; Prasad et al., 2008). In fact, under a short period of seed filling, seeds can not reach a satisfactory development and an insufficient accumulation of photosynthates caused a reduced seed size (Kumar et al., 2013; Wang et al., 2006). In addition, high temperature can increase senescence of lower leaves in pea (McDonald and Paulsen, 1997) leading to assimilate limitations for seeds in development (Farooq et al., 2011; Fahad et al., 2017). On the other hand, related research also has revealed that high temperature induced acceleration of the seed-filling rate as a compensatory effect for reduction of seed-filling duration (Prasad et al., 2008); however, in many cases the increase in seed-filling rate can not compensate the reduction in seed-filling duration (Marcelis and Hofman-Eijer, 1993; Farooq et al., 2011). In this sense, it is possible that the advanced ovule stage observed on plants right after similar treatment (Chapter 3) corresponded to the accelerated rate that accompanied a reduction of seed-filling duration in the plants. Complementarily, it has been demonstrated that high temperature disrupted metabolic activity of cytokinin and invertase enzyme on various crops (Banowetz et al., 1999; Bhandari et al., 2016) which could lead to reduced cell division and limited assimilate partitioning for embryos and endosperm in development (Wang et al., 2006; Tacarindua et al., 2012).

In contrast to the reduced seed size identified on high and medium heat-tolerant cultivars, the low heat-tolerant cultivar Carneval displayed an increase of seed size under high temperature. Although this increase in size was low (2.7%), it can be ascribed to a compensatory effect for the high seed loss observed on this heat-treated plants, as observed on related studies under field and control conditions in pea and chickpea, respectively (Poggio et al., 2005; Wang et al., 2006). In the present study, since the variation in seed size (1 to 2.7%) was relatively low compared to the reduction in seed number (4 to 43%), it appears that seed number was the most affected trait by high temperature. This assertion agrees with observations of Sadras (2007) when reviewing the trade-off effect between seed size and number in various crops, concluded that seed number can be the most plastic trait related with allocation of plant resources under adverse environment conditions. In parallel, research on cereal and legume crops adaptation has shown that seed number could explain most of the variation of seed yield in response to adverse thermal conditions (Sadras and Dreccer, 2015). In general, in my study, it is apparent that high heat-tolerant cultivars managed to maintain seed number at the expense of a slight reduction of seed size, whereas the low heat-

tolerant cultivar exhibiting high reduction of seed number exhibited a slight increase in seed size under heat stress conditions.

4.5 Conclusions

As a critical element of the reproductive process in field pea, ovules at young stages and at physiological plant maturity were evaluated after high temperature exposure. Ovule viability, abortion, and seed set provided evidence of how high temperature may be influencing ovule and seed development on reproductive nodes of pea plants.

In terms of viability, callose accumulation increased around the vascular bundle area of the ovules at Node 3, suggesting a conflict of assimilate transport to the embryo sac in the three cultivars evaluated. The presence of ROS evaluated on Node 4 also revealed that ovules of one of the cultivars (CDC Sage) may be susceptible to high temperature, especially in pods at the distal position of the inflorescence (raceme) of the node. In this context, the hypothesis that ovule viability would be affected on all the nodes evaluated on plants exposed to heat stress may be partially accepted since callose accumulation was observed on just one of the reproductive nodes of the plants.

In contrast, pod length, seed number, and seed-to-ovule ratio showed consistent effects of high temperature with medium and low heat-tolerant cultivars displaying a reduction of these variables on the first three reproductive nodes evaluated. Interestingly, seed diameter revealed that low heat-tolerant plants displayed the lowest seed-to-ovule ratio and exhibited a slightly larger seed diameter, perhaps as part of a compensatory effect for the loss of seeds. In contrast, high heat-tolerant cultivars maintained seed number with a slight reduction of seed size.

In addition, ovule abortion findings demonstrated that ovules right after fertilization and ovules containing embryos at early development were those most affected by high temperature treatment. It is worth noting that aborted ovules right after fertilization (<1mm) were not related to a particular node of the plant. Instead, this abortion may result from a mechanism for assimilate adjustment on the affected plants. Here, the hypothesis that high temperature would reduce seed set while ovule abortion would be increased according to the cultivar's level of heat tolerance is accepted.

Overall, whereas high temperature reduced seed-to-ovule ratio as expected in cultivars CDC Meadow, CDC Sage, and Carneval, ovule abortion found at plant physiological maturity and ovule viability right after heat treatment both suggest that susceptibility of ovules at early development (after fertilization) occurs, because aborted ovules contained some level of early embryo growth.

Transition section between Chapter 4 and Chapter 5

In Chapter 4, the assessment of ovule viability was achieved by evaluating callose deposition at Node 2 to Node 5 and ROS at Node 4 on three cultivars. Also, seed set and abortion at the first four reproductive nodes were evaluated on six cultivars at plant physiological maturity. The high temperature increased the proportion of ovules that had callose deposition on the chalaza area, especially on reproductive Node 3 of the plants. Increased accumulation of ROS was observed in the embryo sac of ovules at distal pods on the plants' reproductive Node 4. Although both tests revealed some level of ovule disruption under high temperature, callose suggested a conflict of assimilate adjustment, and ROS suggested damage of the embryo sac components in ovules of heat-treated plants. Evaluation of abortion showed that ovules containing early embryo growth tended to be aborted under high temperatures in growth chamber conditions, specifically in medium and low tolerant cultivars. In Chapter 5, the effect of high temperature on 6 cultivars under field conditions and 18 cultivars under growth chamber and field conditions was evaluated on young ovules, seed set, and ovule abortion.

Chapter 5. Effect of High Temperature on Ovule Development and Seed Set of Field Pea Cultivars under Field and Growth Chamber Conditions

5.1 Introduction

Ambient rising temperatures, a product of climate change, threaten the potential seed yield for many crops worldwide (Barnabás et al., 2008; Macedo, 2012; Teixeira et al., 2013; Sita et al., 2017). Under adverse heat conditions, plants experience morphological, anatomical, and physiological changes that constrain their normal development and productivity (Bhattacharya and Vijaylaxmi, 2010; Macedo, 2012; Teixeira et al., 2013). According to several studies, above-optimum temperatures negatively affect a plant's reproductive stages, ultimately leading to yield loss in cereal, oilseed, and legume crops (Young et al., 2004; Prasad et al., 2017; Kaushal et al., 2013). Specifically, in cool-season pulses, 25℃ is considered the threshold temperature where plant yield reduction is observed (Guilioni et al., 2003; Sadras and Dreccer, 2015). In fact, legumes, such as chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medic.), and pea (*Pisum sativum* L.) are reported to exhibit flower and pod losses when ambient air temperature exceeds 27℃ (Lambert and Linck, 1958; Guilioni et al., 1997; Bhandari et al., 2016; Kaushal et al., 2013). With climate change, episodes of high environmental temperature are expected to become increasingly frequent, and further, the global annual air temperature is predicted to increase by 0.2 ℃ per decade (IPCC, 2014). In this scenario, understanding the main weakness of the sexual reproductive process under high temperature is vital to generate and select robust heat-tolerant pea varieties for the future.

During the plant's reproductive phase, important processes, such as organ formation, fertilization, and fruit development, can all be disturbed by high temperature leading to failure of seed formation (Gross and Kigel 1994; Wahid et al., 2007; Wang et al., 2006; Barnabás et al., 2008). Studies on field pea have revealed that even 2 to 4 days of high temperature exposure trigger significant yield loss by causing abortion of flowers, early seeds, and young fruits (Bueckert et al., 2015; Latef and Ahmad, 2014; Guilioni et al., 2003). Early reports in this crop have suggested that early seed growth (~ 6 days after open flower) was a highly sensitive stage and embryonic development failed between 27℃ to 31℃ (Lambert and Linck, 1958; Jeuffroy et al., 1990). A different study by Guilioni et al. (1997) evaluated various levels of temperature stress, and found that young buds were aborted after severe stress (33/30℃), but abortion after moderately high

temperature (31/20°C) was not related to any specific stage of reproductive organ. As such, Guilioni et al. (1997) proposed that the abortion of reproductive organs depended on their position on the plant stem. The variable response observed in pea studies can be attributed to different cultivars used in those investigations, but it is still unclear how high temperature affects the reproductive process in field pea. Considering that reproduction in legumes involves male and female reproductive organs of the flower (Leppik, 1966; Tucker, 1989), various authors have indicated that seed reduction due to high temperature is associated with pollen damage, fertilization failure, and ovule impairment (Gross and Kigel, 1994; Ormrod et al., 1967; Prasad et al., 2006). While screening various cultivars in pea, Jiang et al. (2017) and Petkova et al. (2008), found that pollen viability and in-vitro pollen growth were highly affected in plants exposed to temperatures between 36°C and 45 °C. The ovule, as the female contributor of the reproductive process, has been suggested to be also affected under heat stress in various plant species, such as common bean (*Phaseolus vulgaris* L.), wheat (*Triticum aestivum* L.), and *Arabidopsis thaliana* (L.) Heynh. (Saini et al., 1983; Whittle et al., 2009; Gross and Kigel, 1994). However, in field pea, the influence of high temperature on ovule development remains to be explored.

Since diverse cultivars have varied mechanisms to overcome stress, studying a range of genetically different cultivars may provide a more thorough understanding of how plants are affected under stress. According to Wahid et al. (2007), cultivars belonging to the same species are capable of coping to heat-stress conditions by reacting in a dissimilar manner. Indeed, the reaction of plants to heat-stress conditions is related to their genetic capability of sensing the stimulus and transducing it to physiological changes, where some genotypes are more proficient at producing high yield (Farooq et al., 2017; Wahid et al., 2007). Therefore, screening of field pea cultivars in terms of ovule response to high temperature may provide a more complete prospect of failure of the reproductive process in these plants. Furthermore, ovule assessment in various cultivars under field conditions may provide a realistic view of how these structures are being affected during hot days. In the field, a common and practical technique to test cultivar performance under high temperatures is to use a normal and a delayed seeding date, where plants at late seeding have their flowering phase displaced later in the season, and are expected to be exposed to more frequent waves of high temperature (French, 1990; Kaushal et al., 2013; Bhandari et al., 2016).

In this study, I aimed to evaluate the effect of high temperature in young ovules (4 days after anthesis) and seed set in various field pea cultivars under field (early- and late-seeded plots) and growth chamber conditions. The cultivars represented a range of genetically inherited seed size. Complementarily to this, correlations between plant traits (pod number, reproductive nodes, canopy temperature, among others) with ovule and seed-set performance were carried out to identify key associations. Here, I hypothesized that if diverse field pea cultivars were exposed to high temperature there would be cultivars that display high tolerance by exhibiting less ovule damage and abortion, and high seed set. I also hypothesized that important associations would exist between plant traits and ovule failure, where identification of these 'bottlenecks' could be used to improve seed retention and seed yield in heat in future cultivars.

5.2 Materials and Methods

5.2.1 Plant material and growing conditions

Field experiments were carried out during spring-summer 2017 and 2018 in Sutherland (52º10' N, 106º30'W), Saskatoon, Saskatchewan. In 2017, six field pea cultivars: Naparnyk, 40-10, CDC Sage, CDC Meadow, MFR043, and Carneval examined in previous experiments (Chapter 3 and 4) were assessed in field plots. In 2018, 18 cultivars including the six mentioned above from the Pea Association Mapping (PAM) panel were also screened. Overall, these cultivars corresponded to 14 semi-leafless and four normal leaf type cultivars (Table 5.1). The plot size was 1.37 m in width by 3.66 m in length. Prior to seeding, weed control was accomplished by application of herbicides Edge (ethalflurafin) plus Pursuit (imazethapyr) in the fall. During the growing season, weed control was achieved by spraying Viper (imazamox and benzon) around four weeks after seeding and Axial (pinoxaden) plus Centurion (clethodim) around six weeks after seeding. Although fertilization was not applied in any of the years, the seeds were inoculated with commercial rhizobia (*Rhizobium sp.*) to ensure nitrogen fixation by the experimental plants.

Table 5.1 Description of origin and leaf type of 18 field pea cultivars from the Pea Association Mapping (PAM) panel assessed under field and growth chamber conditions.

Cultivar	Leaf Type	Origin
40-10	Normal	Germany
Aggasiz	Semi-leafless	AAFC, Canada
Argus	Semi-leafless	AAFC, Canada
Carneval	Semi-leafless	Sweden
CDC Bronco	Semi-leafless	CDC, Canada
CDC Centennial	Semi-leafless	CDC, Canada
CDC Golden	Semi-leafless	CDC, Canada
CDC Meadow	Semi-leafless	CDC, Canada
CDC Mozart	Semi-leafless	CDC, Canada
CDC Patrick	Semi-leafless	CDC, Canada
CDC Sage	Semi-leafless	CDC, Canada
CDC Striker	Semi-leafless	CDC, Canada
CDC Treasure	Semi-leafless	CDC, Canada
Cutlass	Semi-leafless	CDC, Canada
MFR043	Normal	CDC, Canada
Naparnyk	Normal	Eastern Europe
Nitouche	Semi-leafless	Denmark
TMP15213	Normal/Semi-leafless	Eastern Europe

AAFC, Agriculture and Agri-Food Canada.
CDC, Crop Development Centre, University of Saskatchewan.

In terms of weather conditions, the mean maximum temperatures during the growing seasons (May to August) in 2017 and 2018 were similar but the years differed in cumulative precipitation and number of days with temperatures above 28^{o}C (Table 5.2). Cumulative precipitation in the growing season was 127.9 mm in 2017 and 103.2 mm in 2018. The number of days with temperature above 28 ^{o}C was 29 d in 2017 and 34 d in 2018 (Table 5.2). In particular, the flowering phase occurred between June to July, where the number of days with temperature above 28 ^{o}C was 20 d in 2017 and 18 d in 2018 (Table 5.2).

The same 18 cultivars of pea were also evaluated under growth chamber conditions. In the chamber, light was supplied by banks of cool fluorescent tubes providing an irradiance of 450±5 μmol photons m^{-2} s^{-1}. The photoperiod was established at 16h light/8h dark, and the temperature was kept at 24^{o}C during light (day) time and 18^{o}C during dark (night) time. Five seeds of each cultivar were sown in cylindrical pots of 7.6 L filled with peat base mix (Sunshine®, RR. Horticulture Canada Ltd., Edmonton, AB, Canada), and 20 g of a slow-release fertilizer 14-14-14 (Nutricote®, Brampton, ON, Canada). Watering was provided so that plants avoided conditions of drought stress. Plants were thinned from five to two plants per pot at the 3 to 4-leaf stage

(Jeuffroy et al., 1990), and half-strength Hoagland nutrient solution (Hewitt, 1952) was provided every other day starting from two weeks until six weeks after sowing.

Table 5.2 Monthly mean maximum and mean temperature, monthly precipitation, and number of days with temperature over 28°C during growing season 2017 and 2018.

Season	Month	Mean Max Temperature (°C)	Mean Temperature (°C)	Total Precipitation (mm)	Number of days with Temperature >28 °C
2017	May	20.0	12.1	46.3	3
	June	23.6	16.2	30.9	5
	July	27.6	19.6	25.5	15
	August	25.9	17.8	25.2	6
	Season	24.3	16.4	127.9	29
2018	May	23.0	14.3	35.0	5
	June	25.0	17.3	19.9	6
	July	26.2	18.6	31.1	12
	August	25.1	17.2	17.2	11
	Season	24.8	16.8	103.2	34

Weather data obtained from Environment Canada (http://climate.weather.gc.ca)

5.2.2 Experiment design and treatment

For field experiments in 2017 and 2018, plants were sown at two seeding dates, namely early (normal) and late seeded plots in the study. Early seeded pea (ESP) were sown between April to May whereas late seeded pea (LSP) were sown two to four weeks later. In LSP, plant flowering phases were expected to be under greater heat stress since they occurred in mid-July where environment temperatures tended to exceed 30℃. For each seeding date, plots were set up in a randomized complete block design with four replications.

In the growth chamber, half of the plants that were growing at 24℃ day/18℃ night and reached the early flowering stage were transferred to chambers where heat treatment cycles (35℃/18℃) were provided for four consecutive days. The remaining plants were kept under 24℃ day/18℃ to serve as the control. Specifically, plants were identified to be at the early flowering stage when they developed fully opened flowers at the first reproductive node (stage 0.5; Maurer et al., 1965) but with flowers still closed (stage 0.2; Maurer et al., 1965) at the second reproductive node. The cycles of heat treatment were established in a similar manner to previous experiments (Chapter 3; Section 3.2.2), so plants were exposed to six hours of 35℃ during daytime while 18℃ was maintained during nighttime. After heat treatment, heat-stressed plants were returned to

control conditions, where they remained until physiological maturity stage. The trial was set up in a randomized complete block design (RCBD) with four replications.

5.2.3 Sample collection, processing, and measurements

Young ovule assessment

In the field, once flowering started and air temperature was >28°C, two to three flowers (subsamples) at the open flower stage were tagged, and pistils were collected four days later (4 days after flowering or 4DAF) from each plot. In 2017, the pistils were collected from both ESP and LSP. Given that ovules from LSP in 2017 were observed to be more affected, the screening of the ovules from 18 cultivars in 2018 was performed only in the LSP. After collection, ovaries of the flowers were immediately fixed in formalin–acetic acid–alcohol (FAA50). In the laboratory, the length and width of these ovaries were determined with a caliper. The ovaries were carefully dissected by removing one of the ovary walls to keep the ovules attached to the suture of the pod on the other wall. The internal structure of the ovules was assessed by applying a clearing-staining procedure using Mayer's Hemalum stain (Enugutti et al., 2013; Schneitz et al., 1997) as described previously (Chapter 3; Section 3.2.3). In these samples, embryo sac area, status (normal or aborted), and ovule length were evaluated by using a Zeiss Axioplan microscope and the resulting digital images were analyzed with Image J software. Aborted ovules were identified when their embryo sacs displayed signs of damage such as embryo sac wall and endosperm collapse. Finally, the proportion of aborted ovules per ovary was determined by dividing the number of aborted ovules by the total number of ovules within ovary.

Mature pod, seed, and ovule abortion

In 2018, pods of 18 cultivars from ESP and LSP in the field were collected and measured at the the stage of the plants' physiological maturity, when the crop canopy turned yellow. Two plants were randomly selected per plot and two representative pods from the middle reproductive nodes of the plants were collected, e.g., if a plant had 10 reproductive nodes, pods from Node 4 and Node 5 in the stem were collected. In parallel, under growth chamber conditions, pods from the same 18 cultivars in plants at physiological maturity stage were also collected. In these plants, pods from reproductive Node 2, Node 3, and Node 4 were collected as they were estimated to be

in the course of development during the heat treatment and therefore more affected according to previous experiments (Chapter 4).

After the pods were collected, pod length was measured with a caliper. Ovule number, seed number per pod, and aborted ovules or early seeds were then recorded. The abortion stages of ovules and early seeds were determined under a stereomicroscope. In these aborted structures, the stage was determined by the degree of embryo sac development, presence of an embryo at different stages of development, and the length of the ovules according to earlier experiments (Chapter 4; Section 4.3.2). Seed-to-ovule ratio was calculated by dividing the total number of seeds by the total number of ovules per pod. Similarly, the proportion of aborted ovules was calculated by dividing the number of ovules at the specific stage of abortion by the total number of ovules per pod.

Plant performance under field conditions

During the field seasons in 2017 and 2018, two plants per plot were randomly selected and marked. They were used to record the number of vegetative nodes, reproductive nodes, reproductive node with fruit, aborted fruit nodes, pod number, and pod-to-node ratio at physiological maturity. Pod-to-node ratio was obtained by dividing the number of pods by the number of reproductive nodes in the main stem of each plant. Temperature of the plant canopy was measured twice in ESP and LSP during flowering stage of the plants when temperature of the environment exceeded 25°C. This measurement was performed with a handheld infrared thermometer (Model 6110.4 ZL, Everest Interscience Inc, Tucson, AZ, USA.). Canopy Temperature Depression (CTD) was obtained by subtracting canopy temperature of the plant (T_c) from the temperature of the air (T_a) which is CTD= T_a -T_c (Balota et al., 2008; Hatfield, 1983). Flower duration (days) was estimated from when 50% of plants in a plot showed at least one open flower to when 50% of plants in a plot reached terminal flowering. Seed size, referred to as Thousand Kernel Weight, was assessed by weighing around 100 seeds per plot, and the average weight of a seed was multiplied by 1000. Additionally, in plots from 2017, greenness of the plants was measured twice during flowering by using a SPAD chlorophyll meter (Konica Minolta Sensing Americas Inc., USA). Greenness was measured on fully expanded stipules on the second or third node counted down from the tip of the plant.

5.2.4 Data Analysis

The mixed model procedure from SAS statistical software (PROC MIXED) was employed to analyze variables evaluated for both field and growth chamber data sets, with some variations. For variables evaluated in the field, the model included seeding dates and cultivar as fixed effects, whereas replications and their interactions with treatment factors (seeding dates, cultivar) were considered as random effects. For variables evaluated in growth chambers, the model involved temperature treatment, cultivar, and node as fixed effects and replications and interactions with treatment factors as random effects. Additionally, ovule position was also accounted as a fixed effect for certain variables such as seed-to-ovule ratio, proportion of ovules and early seed abortion, embryo sac area, ovule length, proportion of fertilized and aborted ovules in young ovaries. The effect of ovule position within a pod was standardized to three positions across cultivars, as described previously (Chapter 3, Section 3.2.4). The three positions were: stylar, ovules localized closest to the style; medial, ovules at the medial area within the ovary/pod; and basal, ovules closest to the pedicel end of the ovary/pod (Gutiérrez et al., 1996). The DDFM = Kenwardroger option in the model was used to account for degrees of freedom for unbalanced data. The differences between mean values were obtained by using the least significant difference (LSD) at $P \leq 0.05$ significance. Also, correlation analyses among the variables was performed by the Pearson Correlation procedure (PROC CORR) in SAS statistical software. Finally, the relationship between seeding date, early ovary assessment, variables, and plant performance in the field 2017 was analyzed by using principal component analysis (PCA). This technique establishes multiple associations of highly correlated variables as different orthogonal components. Given that the variables have varied units of measurements, they were first scaled using the function *scale* in R statistical software.

5.3 Results

5.3.1 Ovary assessment at 4 days after flowering (4DAF) of six field pea cultivars collected under field conditions in 2017

Ovary length and width

Ovaries (young pods) at 4DAF had differing lengths and widths depending on the seeding date of the plots, cultivars, and interaction seeding date by cultivar (Table 5.3). Both variables

showed the same consistent pattern, where ovaries from early seeded pea (ESP) were significantly larger compared to ovaries of the same age from late seeded pea (LSP) (Table 5.3). Ovaries of Carneval, CDC Meadow, and CDC Sage were significantly larger in ESP compared to LSP (Fig. 5.1). Although ovaries of 40-10 and MFR043 were slightly longer and wider ovaries in ESP compared to LSP, the difference was not significant. Regardless of seeding date, 40-10 and Naparnyk had the largest ovaries in the group with length by width of 40.5 by 9.7 mm and 34.2 by 8.8 mm, respectively, whereas CDC Meadow and Carneval produced the smallest ovaries with length by width of 23.5 by 5.8 mm and 20.8 by 5.4 mm, respectively (Table 5.3).

Table 5.3 Effect of seeding date, cultivar, and their interaction on ovary length and width, and fertilization of pods collected at 4 days after flowering (DAF) from plants under field conditions. The values of each effect and column followed by different letters differ significantly at P <0.05.

Source of Variation	Ovary Length (mm)	Ovary Width (mm)	Fertilization
Seeding date			
Early	35.0±1.68 a†	8.4±0.36 a	0.95±0.025 a
Late	22.3±1.86 b	5.9±0.40 b	0.93±0.026 a
Cultivar			
40-10	40.5±2.91 a	9.7±0.63 a	0.99±0.035 a
Naparnyk	34.2±3.57 ab	8.8±0.77 ab	0.99±0.042 a
CDC Sage	28.3±2.91 bc	7.0±0.63 bc	0.97±0.035 a
MFR 043	24.5±3.15 c	6.4±0.68 c	0.79±0.035 b
CDC Meadow	23.5±2.91 c	5.8±0.63 c	0.95±0.035 a
Carneval	20.8±2.91 c	5.4±0.63 c	0.94±0.037 a
***P* Values**			
Seeding Date (SD)	**<.0001**	**<.0001**	0.5993
Cultivar (C)	**0.0004**	**0.0002**	**0.0053**
C*SD	**0.0475**	**0.0181**	0.9360

Significance levels at P <0.05 and P<0.001 are shown in bold. †Values within a column and within variable followed by the same letter are not significantly different at P<0.005.

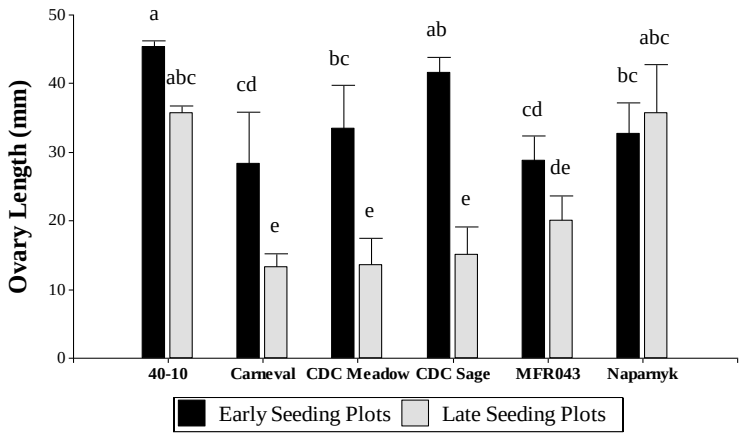


Fig. 5.1 Effect of seeding date on ovary length (mm) of flowers at 4 DAF from six field pea cultivars grown under field conditions. Means of 4 replications (n=12 ovaries) with their respective error bars are shown. Means with similar smaller-case letters are not significantly different at $P<0.05$.

Ovule length, embryo sac area, and fertilization

Both ovule length and embryo sac area characteristics were consistent under field conditions, similar to previous experiments in growth chambers (Chapter 3). Both variables displayed marked differences by seeding date of pea, cultivar, and ovule position within ovary. A two-way interaction between seeding date and ovule position was also significant (Table 5.4). Interestingly, whereas the ovule length from ESP was 27% larger than those from LSP, the average area of the embryo sac was over twice as large in ESP compared with LSP (Table 5.5). Ovule length and embryo sac area of ovules at stylar, medial, and basal positions within an ovary were larger in ESP compared to LSP (Fig. 5.2). Ovules from medial and stylar positions within an ovary did not differ significantly from each other and only differed from ovules at the basal position in LSP (Fig. 5.2). Regardless of seeding date, 40-10 had significantly larger ovule length and embryo sac area compared to the other cultivars. In general, ovules from the medial position within an ovary were significantly larger, followed by ovules at stylar and basal positions (Table 5.5).

Table 5.4. Analysis of variance of the effect of seeding date, cultivar, ovule position, and their interactions on ovule length (mm), embryo sac area (mm^2), and proportion of aborted ovules of ovaries collected at 4 DAF from plants in field conditions.

Source of Variation	Ovule Length (mm)		Embryo Sac Area (mm^2)		Proportion of Aborted Ovules per Ovary	
	***F* Value**	***P* Value**	***F* Value**	***P* Value**	***F* Value**	***P* Value**
Seeding Date (SD)	26.72	**<.0001**	30.09	**<.0001**	17.42	**0.0002**
Cultivar (C)	4.72	**0.0023**	3.68	**0.0093**	5.45	**0.0011**
Ovule Position (OP)	125.42	**<.0001**	80.82	**<.0001**	11.52	**<.0001**
SD*C	1.52	0.2119	1.40	0.2496	3.71	**0.0098**
SD*OP	4.48	**0.0149**	16.93	**<.0001**	0.87	0.4255
C*OP	1.86	0.0670	0.43	0.9290	1.26	0.2701
SD*C*OP	0.93	0.5087	1.09	0.3828	1.06	0.4029

Significance levels at P <0.05 and P<0.001 are shown in bold.

Table 5.5 Means (± SE) of ovule length (mm), embryo sac area (mm^2), and proportion of aborted ovules according to seeding date, cultivar, and ovule position within ovaries at 4DAF from six field pea cultivars grown under field conditions.

Effect	Ovule Length (mm)	Embryo Sac Area (mm^2)	Proportion of Aborted Ovules per Pod
Seeding Date			
Early	2.03±0.08 a†	0.79±0.05 a	0.20±0.06 b
Late	1.43±0.09 b	0.36±0.06 b	0.47±0.06 a
Cultivar			
40-10	2.25±0.13 a	0.91±0.09 a	0.04±0.09 c
Naparnyk	1.87±0.16 ab	0.59±0.11 b	0.26±0.10 bc
CDC Sage	1.65±0.13 b	0.59±0.09 b	0.34±0.09 ab
MFR043	1.61±0.15 b	0.42±0.10 b	0.33±0.09 b
CDC Meadow	1.53±0.13 b	0.52±0.09 b	0.56±0.09 a
Carneval	1.45±0.13 b	0.43±0.09 b	0.49±0.09 ab
Ovule Position			
Stylar	1.79±0.06 b	0.60±0.04 b	0.31±0.06 b
Medial	1.91±0.06 a	0.71±0.04 a	0.28±0.06 b
Basal	1.47±0.06 c	0.42±0.04 c	0.41±0.06 a

†Values within column and variable followed by the same letter are not significantly different at P<0.05.

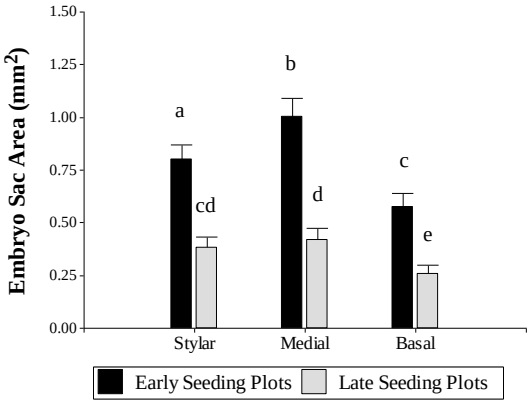


Fig. 5.2 Effect of seeding date on embryo sac area (mm^2) of ovules at stylar, medial, and basal positions within ovaries collected at 4DAF from six field pea cultivar grown under field conditions. Means of 4 replications (n=72 ovaries) with their respective error bars are shown. Means with similar smaller-case letters are not significantly different at $P<0.05$.

In addition, embryo sac morphology and the presence of a pro-embryo or embryo at the globular stage revealed that fertilization failure was low in most cultivars tested. Interestingly, the proportion of ovules per pod that contained embryos varied only among cultivars and not by seeding date or their interactions (Table 5.3). Specifically, the proportion of ovules displaying embryos went from 0.94 to 0.99 in five out of six cultivars. Only MFR043 had the smallest proportion with an 0.79 ovules per pod (Table 5.3).

Proportion of aborted ovules per ovary

Early ovule abortion in ovaries 4DAF was determined by signs of internal embryo sac disruption such as breakdown of the embryo sac lining, lack of embryo sac expansion, endosperm shrinkage, and complete collapse of the embryo sac lining in extreme cases (Fig. 5.3). The proportion of ovules with these signs of abortion per ovary varied significantly by seeding date, cultivar, ovule position, and the interaction of seeding date by cultivar (Table 5.4). The proportion of ovules with signs of abortion was significantly greater at LSP, where the average proportion was 0.47 ovules per ovary whereas the average in ESP was 0.20 ovules per ovary (Table 5.5). Cultivars such as Carneval, CDC Meadow, and CDC Sage had greater proportions of ovules with signs of abortion at late compared to early seeding (Fig. 5.4). Independently of seeding date, Carneval, CDC Meadow, and CDC Sage also showed greater proportions of aborted ovules compared to the rest of the cultivars. In contrast, 40-10, and Naparnyk had the smallest proportion of aborted ovules

per ovary (Table 5.5). At the ovule position level, ovules with signs of abortion were always more likely at basal positions within the ovary (Table 5.5).

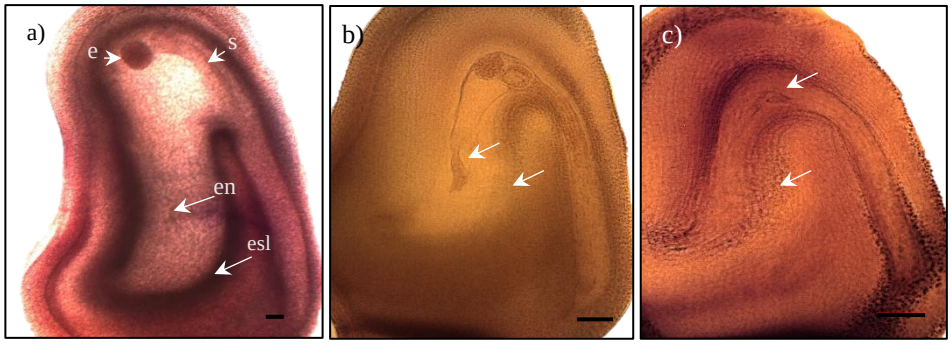


Fig. 5.3 Ovules of field pea at 4 DAF displaying healthy and disrupted embryo sacs. Cleared ovules according to Schneitz et al. (1997). A) Healthy ovule with normal globular-stage embryo (e), suspensor (s), endosperm (en), and embryo sac lining (esl) growth. B) Aborted ovule displaying embryo sac wall and endosperm breakdown (arrows). C) Aborted ovule displaying lack of embryo sac expansion and complete collapse of the embryo sac content (arrows). Scale bar= 100 µm.

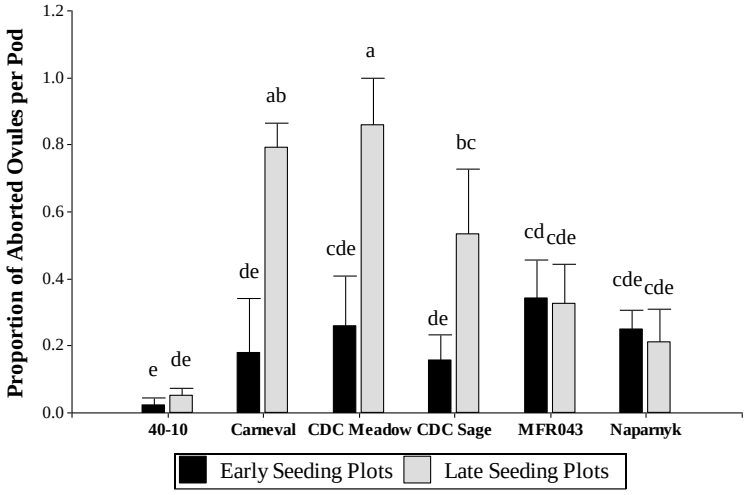


Fig. 5.4 Effect of seeding date on the proportion of aborted ovules per young pods (4DAF) of 6 field pea cultivars collected from plants under field conditions. Means of 4 replications (12 ovaries) with their respective error bars are shown. Means with similar smaller-case letters are not significantly different at $P<0.05$.

Correlation matrix among variables of young ovaries and plant performance

Variables evaluated in young ovaries (4DAF) collected at the middle of flowering showed inverse and positive associations with plant performance characteristics in the field. Ovary length and width were inversely associated with the number of reproductive nodes and pods of plants in LSP (Table 5.6). This means that plants with high numbers of reproductive nodes and pods are

associated with small ovaries in LSP. Furthermore, ovary length and width, embryo sac area, and ovule length were inversely associated with number of aborted fruit nodes on plants in ESP and LSP (Table 5.6). In other words, small-sized ovaries, ovules, and embryo sacs at 4DAF were related with a high number of aborted fruit nodes on plants at ESP and LSP. In contrast, the proportion of aborted ovules within ovaries was positively associated with the number of the pods produced by ESP. This means that ovaries with more aborted ovules were related to plants producing a high number of pods in ESP. Additionally, ovary length and width, embryo sac area, and ovule length were positively associated with canopy temperature in LSP. This means that the largest ovaries (pods) and ovules were found in plants with high canopy temperature in LSP (Table 5.6). Finally, flower duration and TKW of the plants did not show any association with ovary variables (Table 5.6).

Table 5.6 Correlation matrix among variables evaluated in ovaries 4DAF and variables of plant performance under field conditions. Variables of ovaries 4DAF included ovary length and width, embryo sac area, ovule length, and proportion of aborted ovules. Variables of plant performance included reproductive nodes, number of reproductive nodes with fruit, pod number, number of aborted fruit nodes, plant greenness, flower duration, canopy temperature, and thousand kernel weight from six field pea cultivars from early and late seeded pea.

	Early seeded pea					**Late seeded pea**				
	Ovary Length	**Ovary Width**	**Embryo Sac Area**	**Ovule Length**	**Proportion of aborted ovules**	**Ovary Length**	**Ovary Width**	**Embryo Sac Area**	**Ovule Length**	**Proportion of aborted ovules**
Number of Reproductive Nodes (RN)	-0.10	-0.09	-0.29	-0.22	0.33	**-0.44***	**-0.46***	-0.31	-0.33	0.27
Number of Reproductive Nodes with Fruit (RWF)	0.14	0.14	-0.08	0.01	0.18	-0.23	-0.26	-0.16	-0.14	0.08
Pod Number (PN)	-0.29	-0.28	-0.40	-0.33	**0.44***	**-0.48***	**-0.48***	-0.37	-0.36	0.23
Number of Aborted Fruit Nodes (AbFN)	**-0.52****	**-0.51***	**-0.50***	**-0.53****	0.38	**-0.61****	**-0.61****	**-0.43***	**-0.52***	**0.52****
Flower Duration (FD)	-0.20	-0.18	-0.08	-0.18	0.09	0.34	0.34	0.24	0.29	-0.15
Canopy Temperature (CT)	0.30	0.26	0.30	0.33	-0.10	**0.49***	**0.44***	**0.54***	**0.49***	-0.26
Thousand Kernel Weight (TKW)	-0.40	-0.36	-0.32	-0.23	0.16	-0.22	-0.21	-0.32	-0.26	0.23

Significant level of the correlation coefficient at P≤0.05, ≤0.01, and ≤0.0001 were denoted in bold and symbols *, **, ***, respectively.

Relationship of seeding date, ovaries 4DAF, and plant performance under field conditions 2017

The relationships among seeding date, early ovary, and plant performance variables were observed in the ordination analysis (PCA). The diagram displays two clear clusters identifying ESP and LSP (Fig. 5.5). Principal component 1 explains 26% of the separation between the two groups. Variables, such as the proportion of potential seeds (POS) from young pods, days to flower (DTF), and ovary width-to-length ratio (OWL) from young pods were positively associated with ESP. In contrast, variables linked to abortion, e.g., the proportion of aborted ovules per young pod (RA) and proportion of fertilized aborted ovules (RAF), were highly related to LSP (Fig. 5.5). Principal component 2 is the main representative of yield-related variables. In this case, variables such as seed yield (Y), pod number (PN), reproductive nodes (RN), among others, were highly correlated with ESP, whereas canopy temperature at the middle of flowering stage (CT1) was in the opposite direction, being associated with LSP (Fig. 5.5)

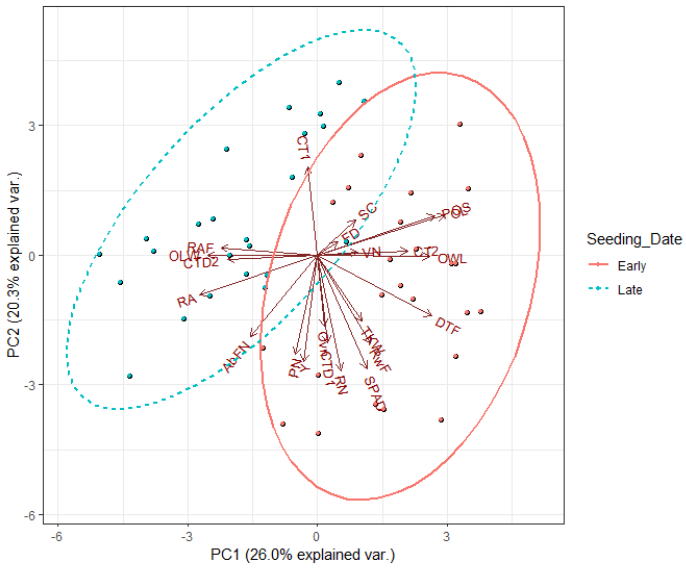


Fig. 5.5 Biplot representing the principal component analysis of variables ovary length (OL), ovary width (OW), potential future seeds (POS), proportion of aborted ovules per young pods (RA), proportion of fertilized aborted ovules (RAF), ovary length to width ratio (OLW), ovary width to length ratio (OWL), ovule number (Ovn)vegetative nodes number (VN), reproductive nodes number (RN), pod number (PN), aborted fruit node number (AbFN), number of reproductive nodes with fruit (RWF), seed yield (Y), plant greenness (SPAD), flower duration (FD), days to flower (DTF), canopy temperature at middle of flowering stage (CT1), canopy temperature at end of flowering stage (CT2), canopy temperature depression at middle of flowering stage (CTD1), canopy temperature depression at end of flowering stage (CTD2), and thousand kernel weight (TKW).

5.3.2 Eighteen field pea cultivars under growth chamber and field conditions in 2018.

5.3.2.1 Seed set under growth chamber conditions

Seed-to-ovule-ratio and ovule number

Seed-to-ovule ratio evaluated on Node 2 to Node 4 of the plants was influenced by high temperature (Table 5.7). An analysis performed by each reproductive node revealed that seed-to-ovule ratio was specifically reduced at particular nodes in some cultivars (Table 5.7). A smaller average seed-to-ovule ratio was identified in CDC Sage, Nitouche, Kahuna-PGRO, CDC Meadow, Aggasiz, and CDC Mozart at reproductive Node 2, Cutlass, Aggasiz, and Nitouche at reproductive Node 3, and CDC Striker and TMP15213 at reproductive Node 4 on plants under heat stress conditions compared to their respective controls (Fig. 5.6 a-c). Furthermore, heat stress also reduced seed-to-ovule ratio at medial positions within pods specifically at Node 4, where the average ratio went from 0.89 to 0.76 at this ovule position in control and heat-stressed plants, respectively (Fig. 5.7).

Regardless of temperature treatment, CDC Patrick, 40-10, CDC Golden, CDC Sage, and Naparnyk showed the greatest seed-to-ovule ratio with averages over 0.75 seeds per pod and CDC Centennial, Nitouche, Cutlass, Carneval, and CDC Meadow showed the smallest average with 0.60 seeds per pod. In general, seed-to-ovule ratio was significantly greater at medial positions, followed by stylar, and basal positions within the pod (Table 5.7).

The number of ovules per pod differed among cultivars only but not among temperature treatments, nodes, or their interactions (Table A2.5). Naparnyk and CDC Striker produced the greatest number of ovules with over 7 ovules per pod, whereas Aggasiz, Argus, Kahuna-PGRO, TMP15213, and CDC Golden the smallest number with less than 6 ovules per pod (Table A2.5).

Table 5.7 Effect of temperature treatment, cultivar, ovule position on seed-to-ovule ratio per pod at reproductive Node 2, Node 3, Node 4, and these three nodes combined together, in 18 field pea cultivars grown under growth chamber conditions.

Source of Variation	Seed-to-Ovule Ratio per Pod			
	Node 2	Node 3	Node 4	Average of Nodes 2-4
Temperature treatment				
Control	0.71±0.02 a†	0.68±0.02 a	0.66±0.02 a	0.69±0.01 a
Heat	0.62±0.02 b	0.64±0.02 a	0.65±0.02 a	0.63±0.01 b
Cultivar				
40-10	0.84±0.04 a	0.83±0.04 a	0.77±0.05 ab	0.81±0.02 a
Aggasiz	0.54±0.06 de	0.62±0.05 c-f	0.85±0.04 a	0.67±0.03 bc
Argus	0.66±0.06 b-d	0.64±0.07 b-e	0.53±0.07 d-h	0.62±0.04 cd
Carneval	0.55±0.04 de	0.50±0.05 d-f	0.46±0.05 h	0.51±0.03 d
CDC Bronco	0.68±0.05 a-d	0.60±0.06 c-f	0.64±0.05 b-g	0.64±0.03 bc
CDC Centennial	0.68±0.07 a-d	0.47±0.08 ef	0.62±0.07 b-h	0.59±0.04 cd
CDC Golden	0.77±0.05 a-c	0.80±0.06 a-c	0.76±0.06 ab	0.77±0.03 ab
CDC Meadow	0.45±0.05 e	0.53±0.05 d-f	0.54±0.05 e-h	0.50±0.03 d
CDC Mozart	0.58±0.06 c-e	0.55±0.08 d-f	0.74±0.06 a-c	0.62±0.04 cd
CDC Patrick	0.79±0.06 a-c	0.86±0.04 a	0.87±0.04 a	0.84±0.03 a
CDC Sage	0.76±0.06 a-c	0.82±0.05 ab	0.70±0.07 a-d	0.76±0.03 ab
CDC Striker	0.66±0.06 a-d	0.64±0.07 b-f	0.57±0.06 c-h	0.62±0.04 cd
CDC Treasure	0.69±0.04 a-d	0.66±0.05 b-d	0.64±0.06 b-f	0.66±0.03 bc
Cutlass	0.66±0.05 a-d	0.58±0.05 d-f	0.49±0.06 f-h	0.58±0.03 cd
Kahuna-PGRO	0.68±0.06 a-d	0.82±0.06 ab	0.67±0.07 b-e	0.71±0.04 a-c
Naparnyk	0.78±0.03 ab	0.77±0.03 a-c	0.73±0.04 a-c	0.76±0.02 ab
Nitouche	0.56±0.06 de	0.45±0.07 f	0.73±0.06 a-c	0.58±0.04 cd
TMP15213	0.66±0.06 a-d	0.67±0.09 a-e	0.46±0.07 gh	0.60±0.04 cd
Ovule Position				
Stylar	0.68±0.02 a	0.68±0.02 b	0.69±0.02 b	0.68±0.01 b
Medial	0.81±0.02 a	0.81±0.02 a	0.83±0.02 a	0.81±0.01 a
Basal	0.51±0.02 b	0.48±0.02 c	0.45±0.02 c	0.48±0.01 c
***P* Value**				
Temperature treatment (T)	**<.0001**	0.1305	0.4425	**<.0001**
Cultivar (C)	**0.0102**	**0.0007**	**<.0001**	**<.0001**
Ovule Position (OP)	**0.0077**	**0.0011**	**0.0001**	**<.0001**
T*C	**0.0028**	**0.0035**	**0.0173**	**0.0479**
C*OP	**0.0111**	**0.0071**	**0.0076**	**<.0001**
T*OP	0.0898	0.2301	**0.0016**	**<.0001**
T*C*OP	0.4570	0.9374	0.4876	0.2865

†Values within a column and within variable followed by the same letter are not significantly different at P<0.05. Significance levels at P <0.05 and P<0.001 are shown in bold.

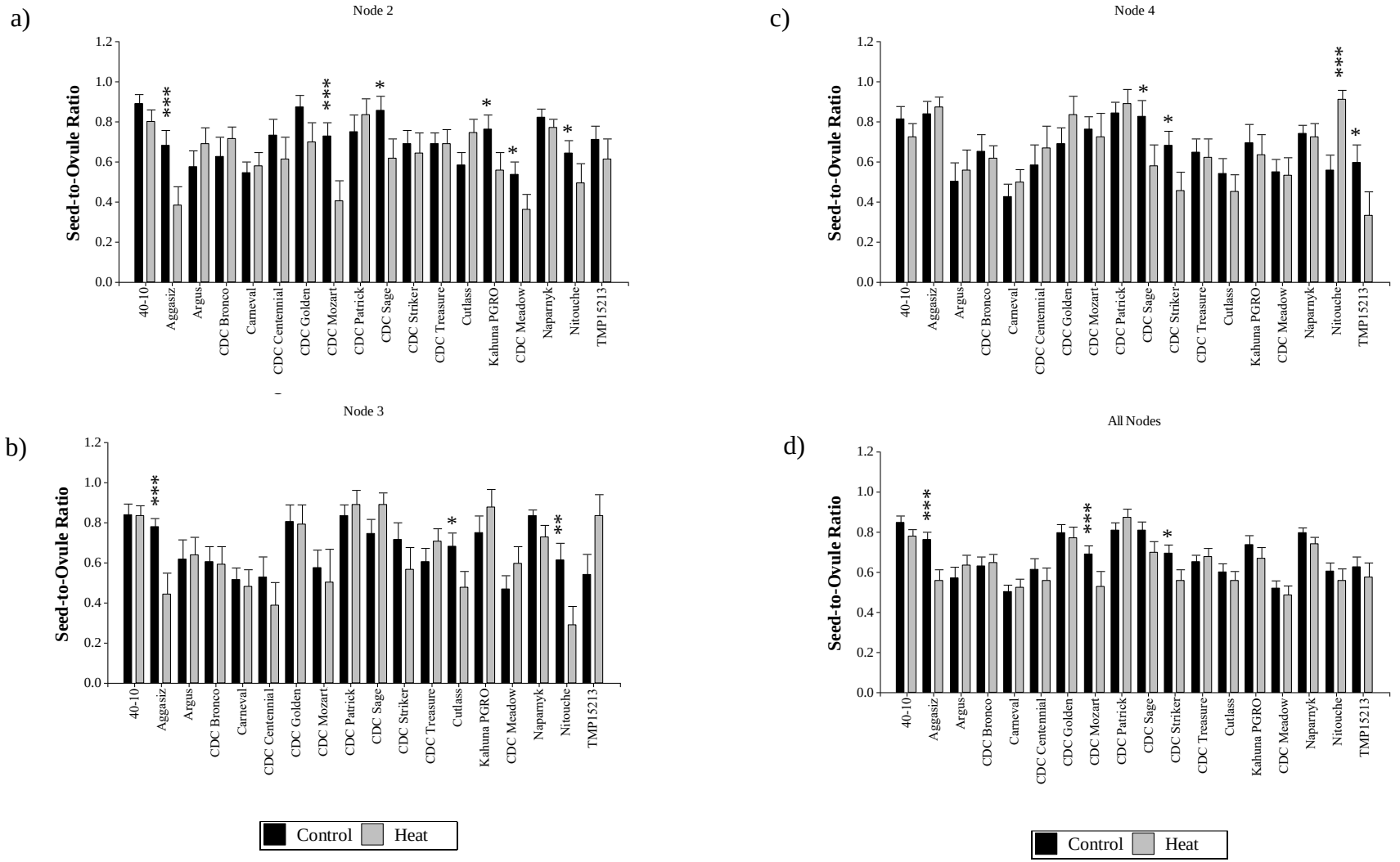


Fig. 5.6 Effect of high temperature (35/18°C) on seed-to-ovule ratio at a) Node 2, b) Node 3, c) Node 4, and d) the average of these nodes in 18 field pea cultivars grown under growth chamber conditions. Means of 4 replications (n=4 to 8 pods per node) with their respective error bars are shown. *, **, *** indicate significant differences between temperature treatment within each cultivar at P<0.05, P<0.01, and P<0.001, respectively.

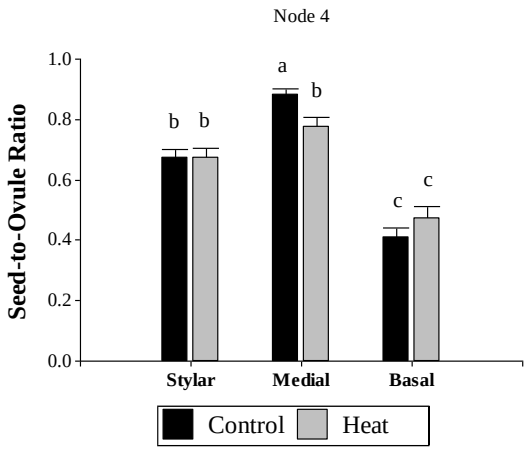


Fig. 5.7 Effect of high temperature (35/18°C) on seed-to-ovule ratio at stylar, medial, and basal position within pods of reproductive Node 4 from 18 cultivars grown under growth chamber conditions. Means of 4 replications with their respective error bars are shown. Means with similar smaller-case letters are not significantly different at $P<0.05$.

Ovule and early seed abortion in mature pods

Ovule abortion occurred at different stages throughout ovule development. Mainly, two categories of aborted ovules were seen: ovules that were aborted right after or before fertilization (ABAF) and aborted ovules with presence of an embryo (APE). Specifically, aborted ovules with a round shape, lack of embryo sac expansion, and less than 1 mm in length were considered as ABAF. Aborted ovules greater than 1 mm length, but with signs of an embryo sac and embryo development were identified as APE. Given that APE ovules had embryo sacs and embryos at various degrees of development, they were subdivided into aborted ovules with an early embryo or pro-embryo (AEE), aborted ovules with an embryo between globular to heart stage (AGH), and aborted ovules with an embryo between the early to late cotyledon stage (AELC) or early seeds that did not complete their development and failed to fill their seed coat.

The proportion of ovules that aborted right before or after fertilization (ABAF) per pod differed significantly by temperature treatment, cultivar, ovule position, and interaction of temperature treatment by cultivar (Table 5.8). The average proportion of these aborted ovules increased in heat -stressed plants that showed a proportion of 0.15 of ABAF per pod compared to the controls that had a proportion of 0.07 per pod (Table 5.8). Particularly 40-10, Aggasiz, CDC Centennial, Cutlass, Kahuna-PGRO, Nitouche, CDC Meadow, CDC Sage, and CDC Striker had a greater proportion of aborted ovules from this category in plants under heat-stressed conditions compared to the controls (Fig. 5.8). Independently from temperature treatment, CDC Meadow,

Aggasiz, and Nitouche had the greatest proportion of ABAF that ranged from 0.18 to 0.19 per pod, whereas CDC Bronco, CDC Patrick and Naparnyk had the smallest proportions with less than 0.04 per pod. The proportion of ABAF was always greater in basal positions (0.12), followed by stylar (0.11) and medial (0.07) positions within the pod (Table 5.8).

Table 5.8 Effect of temperature treatment, cultivars, ovule position and their interaction on the proportion of aborted ovules right after or before fertilization (ABAF), with presence of a pro-embryo or early embryo development (AEE), with an embryo between the globular to heart stage (AGH), with an embryo between the early to late cotyledon stage (AELC) per pod in plants grown under growth chamber conditions.

Source of Variation	Categories of Aborted Ovules Proportion per Pod			
	ABAF	**AEE**	**AGH**	**AELC**
Temperature treatment				
Control	0.07±0.01 b†	0.15±0.01 a	0.03±0.00 a	0.08±0.01 a
Heat	0.15±0.01 a	0.15±0.01 a	0.02±0.00 b	0.05±0.01 b
Cultivar				
40-10	0.04±0.01 de	0.10±0.02 d-f	0.01±0.00 cd	0.04±0.01 e
Aggasiz	0.19±0.03 a	0.09±0.02 d-f	0.02±0.01 b-d	0.03±0.01 e
Argus	0.17±0.03 a-c	0.10±0.02 d-f	0.02±0.01 b-d	0.12±0.02 a-c
Carneval	0.12±0.02 a-d	0.20±0.02 a-c	0.03±0.01 b-d	0.14±0.02 ab
CDC Bronco	0.03±0.01 de	0.18±0.02 a-d	0.06±0.01 a	0.10±0.02 a-d
CDC Centennial	0.10±0.03 a-e	0.25±0.04 a	0.01±0.01 b-d	0.05±0.02 de
CDC Golden	0.05±0.02 c-e	0.10±0.02 d-f	0.04±0.01 a-c	0.03±0.01 e
CDC Meadow	0.18±0.03 ab	0.21±0.02 ab	0.04±0.01 ab	0.07±0.01 c-e
CDC Mozart	0.12±0.03 a-d	0.13±0.03 b-f	0.04±0.01 a-c	0.03±0.01 e
CDC Patrick	0.02±0.01 de	0.07±0.02 ef	0.02±0.01 b-d	0.07±0.02 c-e
CDC Sage	0.10±0.03 a-e	0.11±0.02 c-f	0.02±0.01 b-d	0.02±0.01 e
CDC Striker	0.13±0.03 a-d	0.16±0.03 a-d	0.01±0.01 b-d	0.07±0.02 c-e
CDC Treasure	0.08±0.02 b-e	0.14±0.02 b-f	0.06±0.01 a	0.06±0.01 de
Cutlass	0.07±0.02 b-e	0.21±0.02 ab	0.03±0.01 b-d	0.10±0.02 b-d
Kahuna-PGRO	0.08±0.03 a-e	0.05±0.01 f	0.00±0.00 d	0.16±0.03 a
Naparnyk	0.01±0.00 e	0.15±0.02 a-d	0.02±0.01 b-d	0.03±0.01 e
Nitouche	0.19±0.03 a	0.15±0.03 a-e	0.01±0.01 b-d	0.06±0.01 de
TMP15213	0.12±0.03 a-e	0.17±0.03 ab	0.00±0.00 cd	0.09±0.02 c-e
Ovule position				
Stylar	0.11±0.01 a	0.11±0.01 b	0.02±0.00 b	0.08±0.01 b
Medial	0.07±0.01 b	0.07±0.01 c	0.01±0.00 b	0.03±0.00 c
Basal	0.12±0.01 a	0.26±0.01 a	0.05±0.01 a	0.11±0.01 a
P-Value				
Temperature treatment (T)	**<.0001**	0.2993	**0.0049**	**<.0001**
Cultivar (C)	**0.0219**	**0.0027**	**0.0189**	**0.0001**
Ovule Position (OP)	**0.0011**	**<.0001**	**<.0001**	**<.0001**
T*C	**0.0012**	**0.0003**	**0.1066**	0.1633
C*OP	0.7650	**<.0001**	**0.0051**	**<.0001**
T*OP	0.2405	**<.0001**	0.1185	**0.0057**
T*C*OP	0.9891	0.2933	0.4870	0.1349

†Values within a column and within variable followed by the same letter are not significantly different at P<0.005. Significance levels at P <0.05 and P<0.001 are shown in bold.

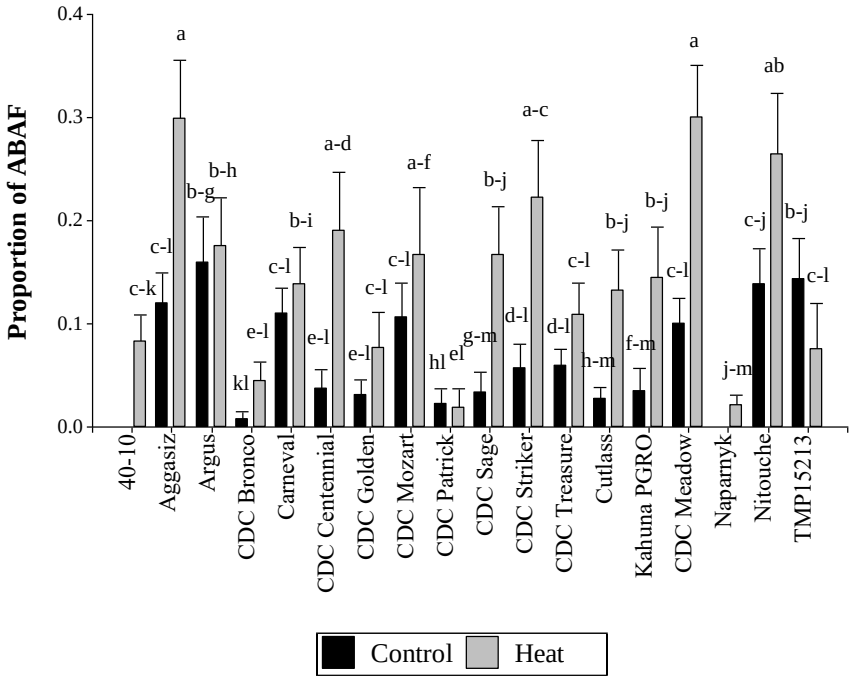


Fig. 5.8 Effect of high temperature (35/18°C) on the proportion of aborted ovules right before or after fertilization (ABAF) in pods collected from reproductive Node 2, Node 3, and Node 4 on plants of 18 cultivars grown under growth chamber conditions. Means of 4 replications with their respective error bars are shown. Means with similar smaller-case letters are not significantly different at P<0.05.

The proportion of aborted ovules AEE varied significantly for cultivar, ovule position, interactions of cultivar by ovule position, temperature treatment by cultivar, and temperature treatment by ovule position (Table 5.8). Cultivars TMP15213 and CDC Bronco had a significantly greater proportion of AEE in plants under heat stress with-proportions of 0.32 and 0.21 per pod compared to 0.10 and 0.13 per pod under control conditions, respectively (Fig. 5.9). The proportion of this aborted category increased greatly at medial positions within the pod under high temperature with 0.12 of AEE per pod compared to the control with 0.04 AEE per pod (Fig. 5.10). Regardless of temperature treatment, CDC Meadow, Cutlass, and CDC Centennial showed the greatest proportion of AEE with proportions ranging from 0.21 to 0.25 per pod, whereas Aggasiz, CDC Patrick, and Kahuna-PGRO had the smallest proportions with less than 0.10 per pod. In general, the proportion of AEE was greater at basal positions, followed by stylar and medial positions within the pod (Table 5.8). In particular, the greatest proportion of AEE was found in CDC Meadow, Cutlass, Carneval, and CDC Centennial at basal positions within the pod (Table A2.8).

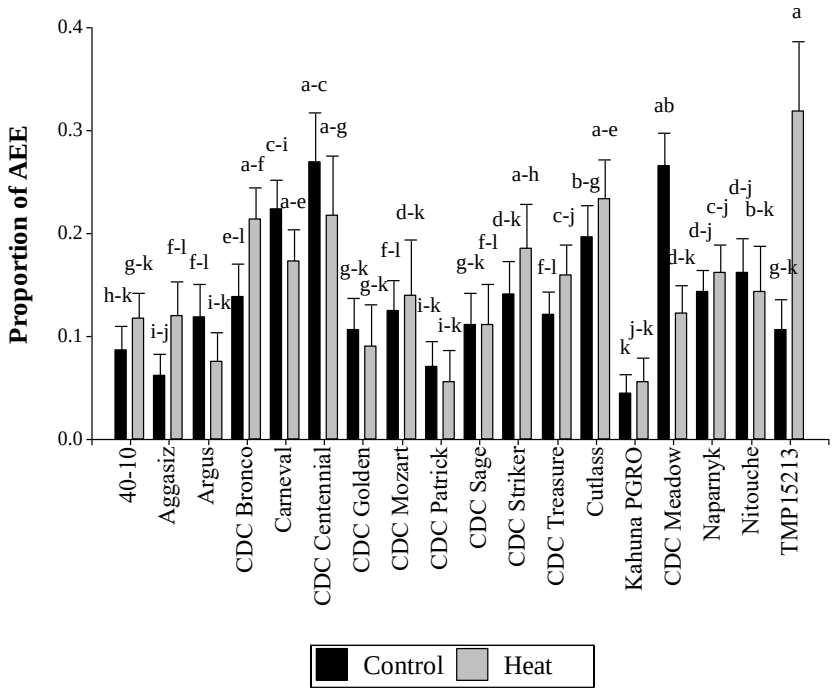


Fig. 5.9 Effect of high temperature (35/18°C) on the proportion of aborted ovules containing pro-embryo or early embryos in development (AEE) in pods collected from reproductive Node 2, Node 3, and Node 4 in plants of 18 cultivars grown under growth chamber conditions. Means of 4 replications with their respective error bars are shown. Means with similar smaller-case letters are not significantly different at P<0.05.

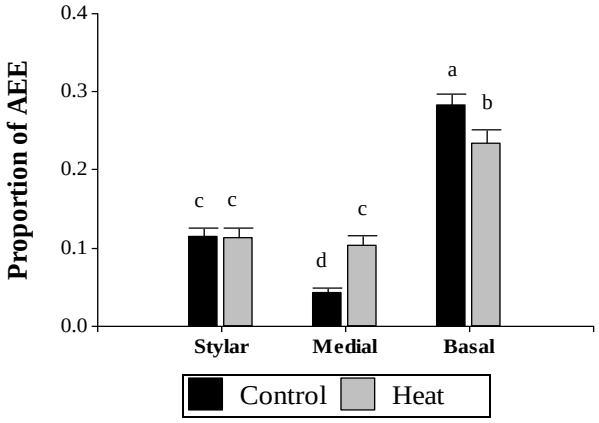


Fig. 5.10 Effect of high temperature (35/18°C) on the proportion of aborted ovules containing pro-embryo or early embryos in development (AEE) at stylar, medial and basal position within pods collected from reproductive Node 2, Node 3, and Node 4 in plants of 18 cultivars grown under growth chamber conditions. Means of 4 replications with their respective error bars are shown. Means with similar smaller-case letters are not significantly different at P<0.05.

Since pods analyzed in this study were collected from reproductive Node 2 to Node 4, where flowers were still closed, and fertilization was taking place when plants were exposed to heat

stress, the proportion of aborted ovules between globular to heart stage (AGH) was relatively smaller at the moment of sample collection. However, the proportion of these aborted ovules still varied throughout temperature treatment, cultivar, ovule position, among cultivars by ovule position (Table 5.8). The proportion of AGH was slightly greater in plants under control conditions with 0.03 per pod compared to the heat stress conditions with 0.02 per pod. Independently from temperature treatment, CDC Bronco and Treasure had the greatest values with over 0.06 AGH per pod. In particular, the proportion was greater at basal positions that exhibited 0.05 AGH per pod (Table 5.8). Cultivars CDC Golden, CDC Bronco, and CDC Treasure showed the greatest abortion of these ovules at basal positions with over 0.09 per pod (Table A2.8).

Similarly, although there was a small proportion of aborted ovules with the presence of an embryo between the early to late cotyledon stage (AELC), it varied according to temperature treatment, cultivar, and ovule position. Furthermore, the interaction of temperature treatment by ovule position was significant for these types of aborted ovules (Table 5.8). The proportion of AELC aborted ovules per pod was smaller in plants under heat stress compared to plants under control conditions (Table 5.8). Specifically, this proportion was smaller at stylar and basal positions within the pod in plants under heat stress compared to plants under control conditions (Fig. 5.11). Regardless of the temperature treatment, CDC Bronco, Cutlass, Argus, Carneval, and Kahuna-PGRO showed the greatest proportion of AELC with a range from 0.10 to 0.16 per pod whereas CDC Mozart, Aggasiz, CDC Sage, Naparnyk and CDC Golden had the smallest with 0.03 or less per pod. As expected, most of these aborted ovules were found at basal positions followed by stylar and medial positions within the pod (Table 5.8). Particularly, Kahuna-PGRO and Argus had the greatest AELC abortion at basal positions (Table A2.8).

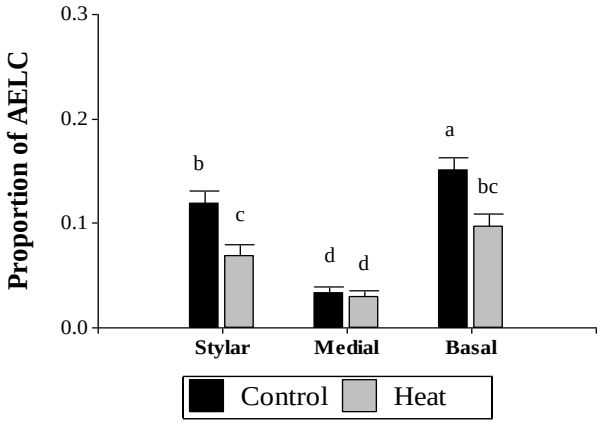


Fig. 5.11 Effect of high temperature (35/18°C) on the proportion of aborted ovules containing embryos between early to late cotyledon stage (AELC) at stylar, medial and basal position within pods collected from reproductive Node 2, Node 3, and Node 4 in plants of 18 cultivars grown under growth chamber conditions. Means of 4 replications with their respective error bars are shown. Means with similar smaller-case letters are not significantly different at $P<0.05$.

Correlation matrix among seed-set variables under growth chamber conditions

In plants exposed to control or heat-stress conditions, pod length was positively correlated with ovule number, seed number, and seed-to-ovule ratio. In contrast, in plants under control or heat stress, pod length was inversely associated with aborted ovules that had presence of early embryo development. Also, pod length was inversely associated with ovule abortion that occurred when an embryo was between the globular to heart stage under control conditions and with abortion that happens right after or before fertilization under heat stress (Table 5.9). Seed-to-ovule ratio was positively correlated with seed number in plants from both temperature treatments. In contrast, seed-to-ovule ratio and seed number were inversely correlated with all stages of abortion in plants from control conditions, and with abortion at early stages of ovule development ABAF and AEE in heat-stressed plants. In other words, seed-set variables were negatively associated with various stages of ovule abortion under control conditions, but they were mainly negatively associated with ovule abortion that occurred at early ovule stages under heat stress (Table 5.9).

Table 5.9 Correlation matrix among variables pod length (PL), ovule number (ON), seed number (SN), seed-to-ovule ratio (SOR) and proportion of aborted ovules right before or after fertilization (ABAF), with presence of a pro-embryo or early embryo development (AEE), with an embryo between globular to heart stage (AGH), with an embryo between the early to late cotyledon stage (AELC) per pod of 18 cultivars grown under growth chamber conditions.

Heat Conditions / Control Conditions	PL	ON	SN	SOR	ABAF	AEE	AGH	AELC
Pod Length (PL) mm		0.37***	0.63***	0.53***	-0.32***	-0.34***	-0.12	0.09
Ovule Number (ON)	0.41***		0.47***	0.06	-0.26**	0.18*	0.005	-0.04
Seed Number (SN)	0.61***	0.46***		0.90***	-0.65***	-0.37***	-0.08	-0.15
Seed-to-Ovule Ratio (SOR)	0.46***	-0.02	0.87***		-0.65***	-0.47***	-0.09	-0.14
Prop. of aborted ovules ABAF	-0.13	0.02	-0.42***	-0.49***		-0.12	-0.08	-0.19*
Prop. of aborted ovules AEE	-0.38***	0.13	-0.45***	-0.56***	-0.15		-0.07	-0.20**
Prop. of aborted ovules AGH	-0.22**	-0.09	-0.27**	-0.27**	-0.07	0.09		0.05
Prop. of aborted ovules AELC	-0.14	-0.20*	-0.45***	-0.41***	-0.09	-0.02	-0.03	

Significance level of the correlation coefficient at P≤0.05, ≤0.01, and ≤0.0001 are denoted by symbols *, **, ***, respectively.

5.3.2.2 Seed set and assessment of ovaries (4DAF) under field conditions during 2018

5.3.2.2.1 Seed set and abortion in representative mature pods

Pod length

The length of the pod varied significantly among seeding dates, cultivars, and their interaction (Table 5.10). Pod length was greater in early seeded pea (ESP) where length was 61.7 mm compared to 59.9 mm in late seeded pea (LSP). Specifically, 40-10 and MFR043 had significantly larger pods in ESP compared to LSP (Fig. 5.12). Regardless of seeding date, CDC Bronco, CDC Striker, and Naparnyk showed the longest pods within the range of 65 to 71 mm, whereas CDC Treasure and 40-10 had the shortest with 53 - 56 mm, respectively (Table 5.10).

Ovule number and seed-to-ovule ratio (SOR)

Ovule number and seed-to-ovule ratio were highly influenced by seeding date and cultivar, but not by their interaction (Table 5.10). Both variables displayed opposite trends to each other. Whereas ESP plants had the least number of ovules, they showed the greatest seed-to-ovule ratio compared to plants in LSP that had the greatest ovule number and the smallest seed-to-ovule ratio (Table 5.10). Irrespective of seeding date, CDC Sage, Carneval, and CDC Striker produced the most ovules with over 8 ovules per pod, and CDC Golden, Argus, TMP15213, and Aggasiz the smallest number with 6.4 to 6.8 ovules per pod. For seed-to-ovule ratio, 40-10, Naparnyk, and CDC Golden had the greatest seed-to-ovule ratio per pod of with 0.70 to 0.78, whereas MFR043, CDC Centennial, and Carneval had the smallest ratios per pod, ranging from 0.36 to 0.46 (Table 5.10).

Table 5.10 Effect of seeding date, cultivar, and their interaction on pod length (mm), ovule number, seed-to-ovule ratio, and proportion of aborted ovules right before or after fertilization (ABAF), with presence of embryo growth (APE), with pro-embryo or embryo at early growth (AEE), with an embryo between globular to heart stage (AGH), with an embryo at early cotyledon stage (AEC), and with embryo at late cotyledon stage (ALC) in representative pods (Section 5.2.3) of plants grown under field conditions.

Effect	Pod Length (mm)	Ovule Number	Seed-to-ovule ratio	Proportion of aborted Ovules		Proportion of aborted Ovules with Presence of Embryo (APE)			
				ABAF	APE	AEE	AGH	AEC	ALC
Seeding Date									
Early	61.7±0.6 a†	7.3±0.1 b	0.60±0.01 a	0.04±0.00 a	0.36±0.01 b	0.13±0.01 a	0.05±0.01 a	0.09±0.01 a	0.08±0.01 a
Late	59.9±0.7 b	7.6±0.1 a	0.54±0.02 b	0.04±0.01 a	0.42±0.02 a	0.16±0.01 a	0.06±0.01 a	0.11±0.01 a	0.09±0.01 a
Cultivar									
40-10	56.3±2.6 gh	7.3±0.15 de	0.78±0.04 a	0.02±0.01 b-d	0.20±0.04 i	0.05±0.02 f	0.02±0.01 c-e	0.09±0.02 b-g	0.04±0.02 f
Aggasiz	61.9±1.6 c-f	6.8±0.09 fg	0.60±0.04 c-f	0.02±0.01 cd	0.38±0.04 d-f	0.11±0.03 d-f	0.08±0.02 b-e	0.08±0.02 e-h	0.11±0.02 bc
Argus	59.9±1.1 d-g	6.5±0.12 gh	0.54±0.04 e-h	0.03±0.01 b-d	0.42±0.04 c-e	0.08±0.02 f	0.06±0.02 b-d	0.20±0.04 a	0.09±0.02 b-f
CDC Bronco	65.9±1.3 bc	7.6±0.16 cd	0.61±0.03 c-e	0.01±0.01 cd	0.38±0.03 d-g	0.09±0.02 ef	0.06±0.02 b-e	0.11±0.02 b-g	0.12±0.03 b
Carneval	58.3±1.7 e-g	8.1±0.14 b	0.46±0.04 hi	0.03±0.01 b-d	0.52±0.04 ab	0.17±0.03 b-e	0.14±0.03 a	0.12±0.02 b-f	0.09±0.02 b-f
CDC Centennial	57.0±0.9 gh	7.3±0.09 de	0.44±0.03 hi	0.04±0.01 a-c	0.52±0.03 a-c	0.28±0.04 a	0.03±0.01 b-e	0.07±0.02 e-h	0.13±0.03 b
Cutlass	63.6±1.6 b-d	7.4±0.14 c-e	0.58±0.05 d-f	0.04±0.01 a-c	0.37±0.05 d-g	0.08±0.02 f	0.04±0.02 b-e	0.15±0.02 b	0.10±0.03 b-e
CDC Golden	58.9±1.2 d-g	6.4±0.14 h	0.70±0.04 a-c	0.04±0.01 a-c	0.25±0.04 g-i	0.14±0.03 c-e	0.02±0.01 e	0.06±0.02 gh	0.04±0.01 ef
MFR043	58.8±2.2 d-g	7.1±0.13 ef	0.36±0.04 i	0.07±0.03 a	0.57±0.04 a	0.17±0.03 c-e	0.03±0.01 b-e	0.13±0.03 b-e	0.24±0.02 a
CDC Meadow	57.9±1.3 f-h	7.8±0.15 bc	0.56±0.04 d-g	0.05±0.02 ab	0.39±0.04 cd	0.18±0.03 b-d	0.05±0.02 b-d	0.11±0.02 b-f	0.04±0.01 c-f
CDC Mozart	57.8±1.3 f-h	7.7±0.11 bc	0.53±0.03 e-h	0.05±0.02 a-c	0.42±0.03 b-d	0.20±0.03 a-c	0.09±0.02 bc	0.06±0.01 gh	0.07±0.02 b-f
Naparnyk	70.9±2.5 a	7.5±0.13 c-e	0.72±0.06 ab	0.04±0.01 a-c	0.23±0.06 hi	0.07±0.03 f	0.03±0.01 b-e	0.08±0.02 c-g	0.04±0.02 f
Nitouche	62.7±1.6 b-e	7.2±0.12 ef	0.49±0.03 f-h	0.01±0.00 d	0.50±0.03 a-c	0.21±0.03 b-d	0.05±0.02 b-e	0.14±0.02 bc	0.10±0.02 b-d
CDC Patrick	60.4±1.6 d-g	7.8±0.14 bc	0.62±0.05 b-e	0.07±0.02 ab	0.31±0.04 e-h	0.13±0.02 d-f	0.05±0.01 b-e	0.07±0.01 e-h	0.05±0.02 c-f
CDC Sage	60.7±1.9 d-g	8.0±0.12 b	0.58±0.04 d-f	0.07±0.01 a	0.35±0.04 d-f	0.16±0.04 c-e	0.08±0.02 b	0.08±0.02 d-g	0.04±0.02 c-f
CDC Striker	67.5±1.6 ab	8.8±0.16 a	0.50±0.03 f-h	0.06±0.02 a-c	0.44±0.02 a-d	0.22±0.03 ab	0.05±0.01 b-e	0.07±0.01 f-h	0.10±0.02 b-f
TMP15213	63.0±0.9 b-e	6.8±0.12 fg	0.67±0.03 b-d	0.05±0.01 a-c	0.28±0.03 f-i	0.11±0.02 d-f	0.03±0.01 de	0.03±0.01 h	0.12±0.03 b
CDC Treasure	53.1±1.7 h	7.7±0.14 bc	0.47±0.03 gh	0.04±0.01 a-d	0.49±0.02 a-c	0.22±0.03 ab	0.09±0.02 bc	0.13±0.02 b-d	0.05±0.02 d-f
***P* Value**									
Seeding Date (SD)	**0.0113**	**<.0001**	**0.0003**	0.3503	**0.0006**	0.1724	0.2048	0.2853	0.9133
Cultivar (C)	**<.0001**	**<.0001**	**<.0001**	**0.0212**	**<.0001**	**<.0001**	**0.0209**	**<.0001**	**0.0002**
SD*C	**0.0140**	0.5873	0.1740	0.2849	0.2616	0.9287	0.4303	0.2789	**0.0162**

†Values within a column and within variable followed by the same letter are not significantly different at P<0.05. Significance levels at P <0.05 and P<0.001 are shown in bold.

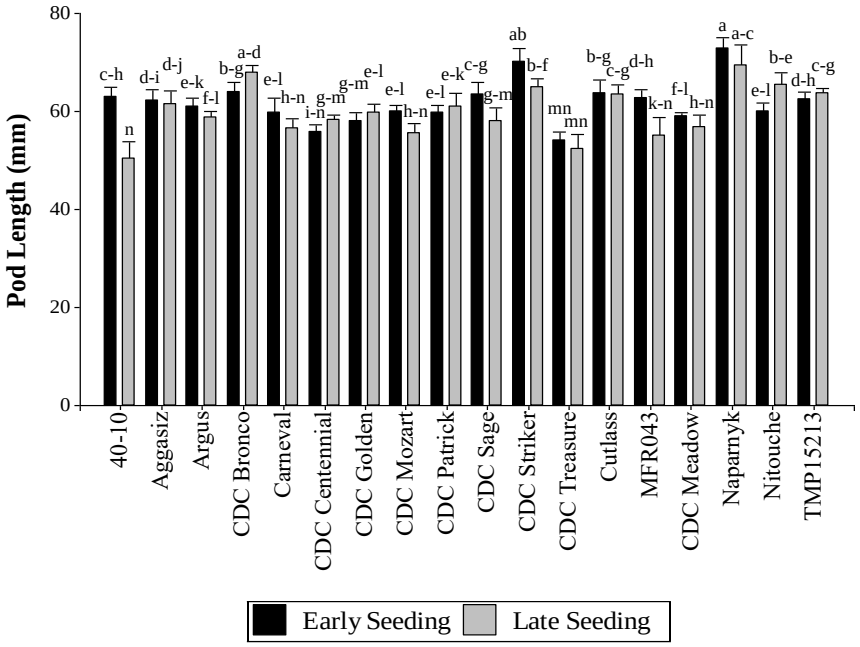


Fig. 5.12 Effect of seeding date on length of representative pods (Section 5.2.3) of 18 cultivars grown under field conditions. Means of 4 replications (n=16 pods) with their respective error bars are shown. Means with similar smaller-case letters are not significantly different at $P<0.05$.

Ovule and early seed abortion in mature pods

According to the characteristics described in earlier experiments of ovule development and abortion, two main types of aborted ovules were identified in this experiment, too. A first type was ovules that aborted right before or after fertilization (ABAF), and a second type of aborted ovules with the presence of embryo development (APE). Evaluation of these types of aborted ovules showed trends that varied by cultivar and seeding dates in some cases.

The proportion of the first type of aborted ovules, ABAF, differed significantly just at the level of cultivar but not seeding date or their interaction. In contrast, the proportion of the second type of aborted ovules (APE) per pod was highly influenced by seeding date and cultivars (Table 5.10). In the first category of aborted ovules (ABAF), MFR043, CDC Patrick, and CDC Sage had more of these aborted ovules with proportions of 0.07 within a pod and 40-10, Aggasiz, CDC Bronco, and Nitouche had the smallest proportions (0.01-0.02) within a pod (Table 5.10; Fig. 5.14). In contrast, the second type of aborted ovules, APE, increased in LSP that showed 0.42 aborted ovules per pod compared to ESP that showed 0.36 aborted ovules per pod. Regardless of temperature treatment, this type of aborted ovule was more prolific in MFR043, Carneval, CDC

Centennial and Nitouche with proportions of over 0.50 per pod, whereas the least was seen in Naparnyk and 40-10, with 0.23 and 0.20 per pod, respectively (Table 5.10; Fig. 5.13).

Aborted ovules with the presence of embryo growth (APE) was composed of four main subcategories that were classified according to the embryo stage that they displayed. In this way, these subcategories were identified as aborted ovules with presence of an early embryo or pro-embryo (AEE), an embryo between globular to heart stage (AGH), an embryo at early cotyledon stage (AEC), and an embryo at late cotyledon stage (ALC). Specifically, the three first subcategories AEE, AGH, and AEC of aborted ovules were highly influenced just by the type of cultivar but not by seeding date. In contrast, the fourth subcategory (ALC) showed the influence of cultivar plus the interaction of seeding date by cultivar (Table 5.10). The average proportion of aborted ovules from ALC increased significantly up to two and three-fold in Cutlass and CDC Mozart at LSP and in Argus and CDC Striker at ESP (Fig. 5.14). Regardless of seeding date, most aborted ovules from subcategory AEE were found in CDC Centennial, CDC Striker, and CDC Treasure, CDC Mozart, and Nitouche, subcategory AGH in CDC Mozart, CDC Treasure, and Carneval, subcategory AEC in Cutlass and Argus, and subcategory ALC in Aggasiz, CDC Bronco, CDC Centennial, TMP15213 and MFR043 (Table 5.10).

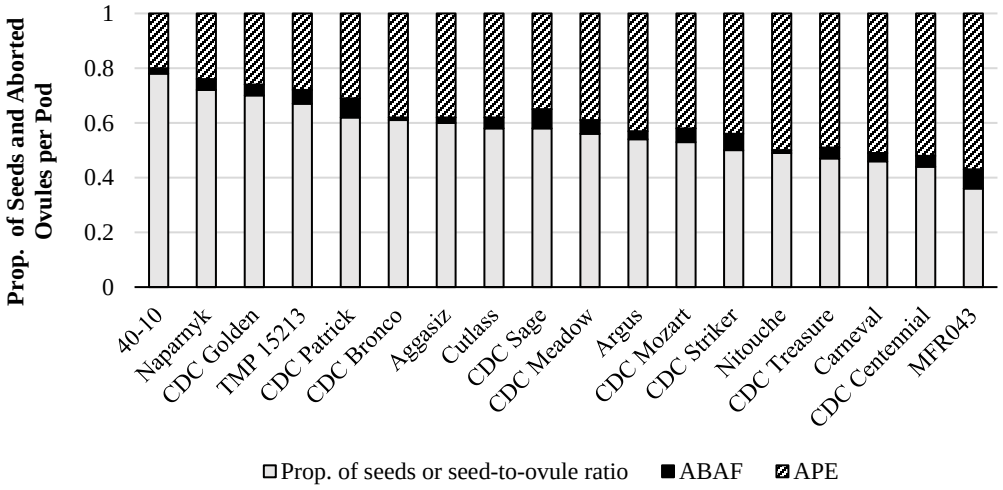


Fig. 5.13 Proportion of seeds or seed-to-ovule ratio, aborted ovules right before or after fertilization (ABAF), and aborted ovules with presence of embryo at young development (APE) within pods of 18 field pea cultivars grown under field conditions in both early and late seeded pea during 2018.

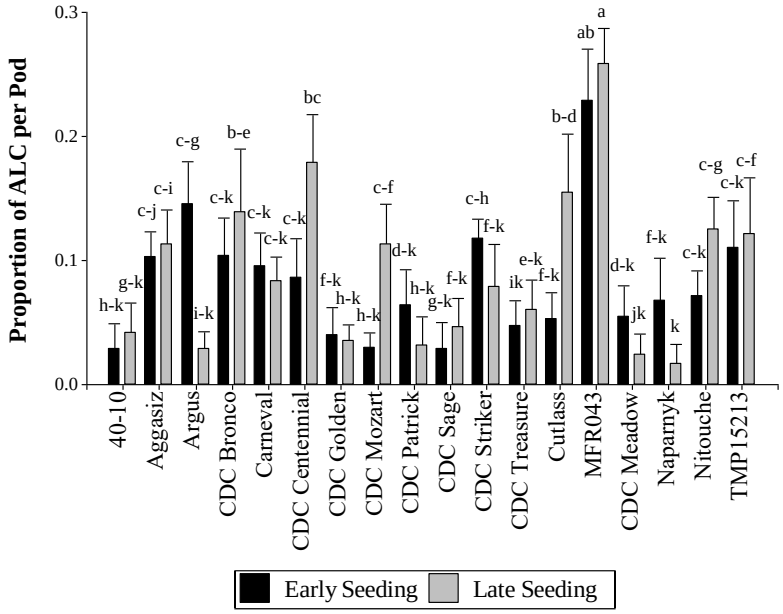


Fig. 5.14 Effect of seeding date on the proportion of aborted ovules at late cotyledon stage (ALC) in representative pods (Section 5.2.3) of 18 cultivars grown under field conditions. Means of 4 replications plots (n=16 pods) with their respective error bars are shown. Means with similar smaller-case letters are not significantly different at P<0.05.

Correlation between seed set and ovule abortion in mature pods

In ESP and LSP, pod length was positively correlated with seed-to-ovule ratio. Specifically, pod length from both seeded plots was inversely associated with aborted ovules that contained early embryo or pro-embryo development (AEE). Moreover, pod length from LSP was inversely correlated with ovules that were aborted right before or after fertilization (ABAF) and ovules that were aborted when their embryos were between globular and heart stage (AGH). The number of ovules per pod was positively correlated with aborted ovules AEE and AGH in LSP. In terms of seed-to-ovule ratio, the variable was inversely associated with all categories of aborted ovules in LSP and with three categories of the aborted ovules with presence of embryo growth AEE, AEC, and ALC in ESP. Interestingly, seed-to-ovule ratio was strongly inversely correlated (R= -0.96 and -0.94) to aborted ovules with presence of embryo growth (APE) in both ESP and LSP (Table 5.11).

Table 5.11 Correlation matrix among variables: pod length (PL), ovule number (ON), seed-to-ovule ratio (SOR), and proportion of aborted ovules right before or after fertilization (ABAF), with presence of embryo growth (APE), pro-embryo or embryo at early growth (AEE), embryo between globular to heart stage (AGH), embryo at early cotyledon stage (AEC), and embryo at late cotyledon stage (ALC) in representative pods (Section 5.2.3) of 18 field pea cultivars grown under field conditions.

Early seeded pea \ Late seeded pea	PL	ON	SOR	ABAF	AEE	AGH	AEC	ALC	APE
Pod Length (PL)		0.12	0.48***	-0.44***	-0.30**	-0.27*	-0.12	0.01	-0.35**
Ovule Number (ON)	0.20		-0.18	0.18	0.35**	0.25*	-0.23*	-0.07	0.15
Seed-to-ovule ratio (SOR)	0.49***	-0.19		-0.36**	-0.53***	-0.37**	-0.28*	-0.59***	-0.94***
Prop. of aborted ovules ABAF	-0.02	0.11	-0.17		0.07	0.01	-0.14	0.05	0.01
Prop. of aborted ovules AEE	-0.50***	0.13	-0.64***	-0.03		0.03	-0.30*	0.15	0.56***
Prop. of aborted ovules AGH	-0.15	0.14	-0.14	-0.05	-0.25*		0.09	-0.04	0.40**
Prop. of aborted ovules AEC	-0.16	0.11	-0.41**	-0.04	-0.05	-0.03		-0.03	0.34**
Prop. of aborted ovules ALC	0.07	-0.08	-0.45***	-0.08	0.05	-0.12	-0.01		0.61***
Prop. of aborted ovules APE	-0.48***	0.18	-0.96***	-0.10	0.66***	0.15	0.42**	0.47***	

Significant level of the correlation coefficient at P≤0.05, ≤0.01, and ≤0.0001 are denotated by symbols *, **, ***, respectively.

5.3.2.2.2 Ovule assessment at 4DAF in late seeded pea

Embryo sac area and ovule length

Evaluation of embryo sac and ovule length in young ovaries from LSP showed congruent information, similar to previous analysis of ovule development under growth chamber conditions (Section 5.3.1). Both variables differed significantly among the three ovule positions within the pod and cultivar but not at the level of interaction of ovule position by cultivar (Table 5.12). Ovules at medial positions had the greatest embryo sac area and ovule length, followed by ovules at stylar and basal positions within the pod. Cultivars CDC Centennial, 40-10, CDC Bronco, Carneval, and CDC Patrick had the greatest embryo sac area and ovule length whereas CDC Meadow, CDC Treasure, CDC Mozart, MFR043, and CDC Golden had the smallest dimensions in the group of cultivars examined (Table 5.12).

Table 5.12 Effect of cultivar, ovule position, and their interaction on embryo sac area (mm^2), ovule length (mm), and proportion of fertilized ovules, potential ovules to become seed, and aborted ovules per ovary at 4 DAF collected from plants grown under field conditions in late seeded pea.

Cultivar	Embryo Sac Area (mm^2)	Ovule Length (mm)	Prop. Fertilized Ovules	Prop. Potential Seeds per Ovary	Prop. Aborted Ovules per Ovary
40-10	0.91±0.08 a†	2.10±0.08 a-d	1.00±0.00 a	0.92±0.05 a	0.08±0.05 g
Aggasiz	0.78±0.15 a-c	1.89±0.13 a-f	0.93±0.03 a	0.65±0.07 a-d	0.35±0.07 d-g
Argus	0.50±0.06 b-g	1.70±0.11 d-h	0.94±0.04 a	0.48±0.09 c-f	0.52±0.09 b-e
Carneval	0.84±0.05 ab	2.16±0.06 a-c	1.00±0.00 a	0.72±0.07 a-c	0.28±0.07 e-g
CDC Bronco	0.88±0.06 a	2.20±0.07 ab	1.00±0.00 a	0.85±0.04 ab	0.15±0.04 fg
CDC Centennial	0.92±0.12 a	2.27±0.13 a	0.98±0.02 a	0.57±0.09 b-e	0.43±0.09 c-f
CDC Golden	0.24±0.02 g	1.21±0.07 ij	0.90±0.06 a	0.40±0.08 d-g	0.60±0.08 a-d
CDC Meadow	0.43±0.05 d-g	1.53±0.09 f-i	1.00±0.00 a	0.56±0.08 b-f	0.44±0.08 b-f
CDC Mozart	0.34±0.12 e-g	1.13±0.12 j	0.91±0.04 a	0.21±0.08 g	0.79±0.08 a
CDC Patrick	0.80±0.10 a-c	2.07±0.11 a-d	0.98±0.02 a	0.71±0.08 a-c	0.29±0.08 e-g
CDC Sage	0.70±0.08 a-d	1.87±0.11 b-g	0.96±0.02 a	0.72±0.08 a-c	0.28±0.08 e-g
CDC Striker	0.64±0.10 a-e	1.71±0.13 d-h	0.94±0.05 a	0.34±0.08 e-g	0.66±0.08 a-c
CDC Treasure	0.42±0.06 d-g	1.49±0.11 g-j	0.95±0.03 a	0.27±0.08 fg	0.73±0.08 ab
Cutlass	0.60±0.09 a-f	1.79±0.12 c-h	1.00±0.00 a	0.58±0.09 b-e	0.42±0.09 c-f
MFR043	0.29±0.02 fg	1.47±0.04 h-j	0.97±0.02 a	0.47±0.08 c-g	0.53±0.08 a-e
Naparnyk	0.62±0.06 a-e	1.97±0.09 a-e	1.00±0.00 a	0.74±0.07 a-c	0.26±0.07 e-g
Nitouche	0.63±0.05 a-e	1.93±0.08 a-e	0.95±0.03 a	0.53±0.08 c-f	0.47±0.08 b-e
TMP15213	0.50±0.04 c-g	1.67±0.06 e-h	1.00±0.00 a	0.62±0.08 b-e	0.38±0.08 c-f
Ovule Position					
Stylar	0.63±0.03 b	1.83±0.04 b	0.97±0.01 a	0.61±0.03 b	0.39±0.03 b
Medial	0.76±0.04 a	1.97±0.05 a	0.98±0.01 a	0.73±0.03 a	0.27±0.03 c
Basal	0.47±0.03 c	1.57±0.05 c	0.94±0.01 b	0.40±0.03 c	0.60±0.03 a
***P* Value**					
Cultivar (C)	**<.0001**	**<.0001**	0.5484	**0.0001**	**0.0001**
Ovule Position (OP)	**<.0001**	**<.0001**	**0.0152**	**<.0001**	**<.0001**
C*OP	0.8962	0.9002	0.7023	0.9130	0.9008

†Values within a column and within variable followed by the same letter are not significantly different at P<0.05. Significance levels at P <0.05 and P<0.001 are shown in bold.

Ovule Status

Congruently to previous field evaluations (Section 5.3.1), some ovules showed signs of abortion, such as loss of embryo sac lining, endosperm disintegration, and a thin embryo sac cavity. Ovules with these signs of disruption were considered aborted, whereas ovules without signs of damage were considered as ovules in good condition or ovules still with promise to become viable seeds (POS). Specifically, the proportion of aborted ovules and ovules in good condition (POS) per ovary varied significantly at the level of ovule position and cultivar (Table 5.12). Consistently, the greatest proportion of aborted ovules and the smallest proportion of potential seeds was identified at the basal position within the ovary (Table 5.12). Cultivars 40-10, CDC Bronco, and Naparnyk had the greatest proportion of potential seed and the smallest proportion of aborted ovules at the three ovule positions. In contrast, CDC Mozart, CDC Treasure, and CDC Striker had the smallest proportion of potential ovules and the greatest proportion of aborted ovules (Table 5.12).

Furthermore, fertilization was determined in these ovules by characteristics such as degree of embryo sac development and presence of an embryo at early growth (pro-embryo or globular embryo) stage. Interestingly, the proportion of fertilized ovules per ovary differed by ovule position within the pod, but not among cultivars (Table 5.12). In this sense, they were significantly greater in stylar and medial positions with proportions of 0.97 and 0.98, respectively, whereas the proportion was smaller in the basal position with 0.94 per pod (Table 5.12).

Correlations among variables from ovaries 4DAF, seed set, and plant performance in late seeded pea

The embryo sac area and ovule length from ovaries 4DAF were positively associated with seed diameter and seed-to-ovule ratio of mature pods (Table 5.13). In contrast, the embryo sac area and ovule length were inversely associated with the proportion of ovules that aborted right before or after fertilization (ABAF) and aborted ovules that had signs of embryo growth (APE) in mature pods. As expected, the proportion of potential ovules to become seeds (POS) from ovaries 4DAF was positively associated with seed diameter and seed-to-ovule ratio from mature pods (Table 5.13). In turn, the proportion of POS from ovaries 4 DAF was inversely associated with aborted ovules that displayed embryo growth (APE) of mature pods. Congruently, the proportion of aborted ovules of ovaries 4DAF correlated positively with aborted ovules at various levels of

development in mature pods. Overall, the proportion of POS and aborted ovules from ovaries 4DAF was associated with the proportion of seeds and aborted ovules, respectively, in mature pods (Table 5.13).

In contrast, variables such as ovule length, ovule number per ovary, and proportion of potential ovules to become seeds (POS) of ovaries at 4DAF were inversely correlated with canopy temperature (CT) obtained in the middle of flowering (Table 5.14), meaning that ovaries with ovules of great length, high ovule number, and high proportion of potential seed were related with plants of lower canopy temperature. Interestingly, the number of reproductive nodes with fruit (RWF) was positively associated with POS and inversely correlated with the proportion of aborted ovules 4DAF (Table 5.14). In other words, plants with high number of reproductive nodes with fruits were related to ovaries at 4DAF that had high proportion of potential ovules to become seeds. Finally, the proportion of aborted ovules in ovaries 4DAF was positively correlated with canopy temperature (CT) and negatively correlated with canopy temperature depression (CDT) at the middle of flowering (Table 5.14). This means that the high proportion of aborted ovules per ovary was related to plants with high canopy temperature in the middle of flowering.

Table 5.13 Correlation matrix among variables evaluated in young ovaries (4DAF) such as embryo sac area (mm^2), ovule length (mm), ovule number per ovary, proportion of potential ovules to become seed (POS), proportion of aborted ovules and variables from mature pods such as seed-to-ovule ratio, and proportion of aborted ovules right before or after fertilization (ABAF), with presence of a growing embryo (APE), with a pro-embryo or embryo at early growth (AEE), with an embryo between globular to heart stage (AGH), with an embryo at early cotyledon stage (AEC), and with an embryo at late cotyledon stage (ALC) per pod collected from 18 cultivars in late seeded pea.

Mat. Pod / Ovary 4DAF	Seed Diameter (mm)	Seed-to-ovule ratio	Proportion of ABAF	Proportion of APE	Proportion of AEE	Proportion of AGH	Proportion of AEC	Proportion of ALC
Embryo Sac Area (mm^2)	0.34***	0.35***	-0.22**	-0.30***	-0.33***	-0.10	-0.15*	0.03
Ovule Length (mm)	0.35***	0.33***	-0.26***	-0.28***	-0.36***	-0.11	-0.10	0.08
Ovule Number per Ovary	-0.15*	-0.13*	0.08	0.12	0.11	0.16*	-0.04	0.06
Proportion of POS	0.25**	0.20**	-0.17*	-0.16*	-0.23**	-0.12	-0.02	0.09
Proportion of Aborted Ovules	-0.25**	-0.20**	0.17*	0.16*	0.23**	0.12	0.02	-0.09

Significant level of the correlation coefficient at P≤0.05, ≤0.01, and ≤0.0001 are denoted by symbols *, **, ***, respectively.

Table 5.14 Correlation matrix among variables evaluated in young ovaries (4DAF) such as embryo sac area (mm^2), ovule length (mm), ovule number per ovary, proportion of potential ovules to become seed (POS), proportion of aborted ovules and plant performance variables such as number of reproductive nodes (RN), number of reproductive nodes with fruit (RWF), aborted fruit nodes (AbFN), flower duration (FD), canopy temperature at middle of flowering stage (CT), and canopy temperature depression at middle of flowering (CTD), pod number (PN), pod-node-ratio (PRN) in 18 cultivars from late seeded pea.

Plant Traits / Ovary 4DAF	RN	RWF	AbFN	FD	CT	CTD	PN	PRN
Embryo Sac Area (mm^2)	0.06	0.12	-0.09	0.20	-0.19	0.19	0.04	0.04
Ovule Length (mm)	0.13	0.18	-0.09	0.23*	-0.27*	0.27*	0.04	0.06
Ovule Number per Ovary	0.01	0.03	-0.02	0.03	-0.33**	0.33**	-0.18	-0.27*
Prop. of POS	0.21	0.32**	-0.18	0.21	-0.26*	0.26*	0.21	0.22
Prop. of Aborted Ovules	-0.21	-0.32**	0.18	-0.21	0.26*	-0.26*	-0.21	-0.23

Significant level of the correlation coefficient at P≤0.05, ≤0.01, and ≤0.0001 are denoted by symbols *, **, ***, respectively.

5.4 Discussion

High temperature in the environment is one of the main factors that constrains seed yield in field pea (Karr et al., 1959; Guilioni et al., 2003; Bueckert et al., 2015). During the plant life cycle, the reproductive stage is the most sensitive phase to the increase of temperature leading to seed reduction (Prasad et al., 1999; Tacarindua et al., 2012; Wang et al., 2006). There are several studies in pea that have focused on the reproductive stage and yield response under heat stress (Lambert and Linck, 1958; Jeuffroy et al., 1990; Sadras, 2007); however, relatively low attention has been put to the ovule (future seed) and its development under heat stress. In my study, the internal structure (embryo sac) of ovules at four days after open flower stage (4DAF) and seed set at the maturity stage of pea plants were evaluated in six cultivars during 2017 and in 18 cultivars during 2018 under field and growth chamber conditions. In general, ovules containing embryos at early and late growth were disturbed, and therefore, they were aborted in young ovaries and mature pods under high temperature in field (>28℃) and growth chamber conditions (35℃).

5.4.1 High temperature on six cultivars under field conditions during 2017

Seeding at a later date than the recommended time in a season is a useful technique to expose and evaluate cultivars under adverse environmental conditions, such as high temperature (Kaur et al., 2015; Bhandari et al., 2016; Huang et al., 2017). In my study, by using early (normal) and late seeding time (four weeks later than the early), it was possible to detect differences in ovary and ovule development at 4 DAF on flowers from those plots. Ovaries of low and medium heat-tolerant cultivars (Carneval, CDC Meadow, and CDC Sage) on late seeded pea (LSP) showed poorer development compared with ovaries from early seeded pea (ESP). Consistently, ovaries from LSP displayed smaller ovules and embryo sacs than the ovaries at similar age from ESP. Furthermore, the proportion of ovules with signs of early abortion, such as damage to the embryo sac perimeter, small embryos, and disrupted endosperm were higher on ovaries from LSP. Although these early signs of abortion were not detected previously under heat stress conditions (35℃, 4 d) in the growth chamber (Chapter 3), it is apparent that disruption of ovule development was exacerbated by more adverse conditions in the field. In fact, whereas plants from both ESP and LSP experienced temperatures above 28℃ (8-10 d) during their flowering stage, plants from LSP also faced about three extra days of temperatures that exceeded 30℃ during flowering (Fig. 5.15). Thus, the

intermittent heat waves in the field caused a more harmful effect on the ovules compared to growth chamber conditions.

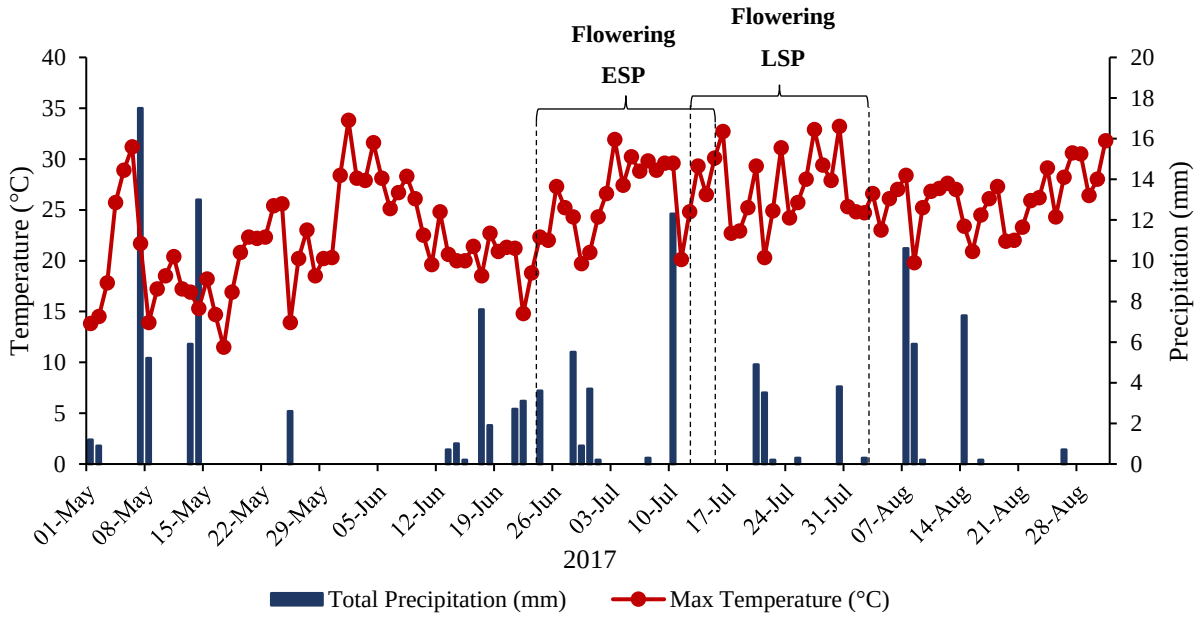


Fig. 5.15 Daily precipitation and maximum temperature during the growing season in 2017 at Saskatoon. Brackets show flowering time in early seeded pea (ESP) and late seeded pea (LSP). Weather data obtained from Environment Canada (http://climate.weather.gc.ca)

In addition, cumulative precipitation differing during flowering stage between ESP and LSP may have influenced the development of the ovaries in the field. While the total rainfall during flowering was 26 mm in ESP, it was only 13.5 mm in LSP. Thus, plants from LSP could be affected not only by high temperatures but also by low precipitation during flowering. When both high temperature and water limitations appear in the field, the effect can be more destructive for the plants than when they appear individually (Craufurd and Peacock, 1993; Prasad et al., 2008). For example, Awasthi et al. (2014), studying heat stress and drought in chickpea, found that rubisco activity on leaves decreased slightly under drought but in a combined stress of heat and drought, the rubisco activity decreased severely specially on sensitive cultivars. Chen et al. (2006) investigating the optimal seeding date for pea and lentil, identified that 10 to 14 mm of rain during summer (July) 2003 in the USA was not sufficient for pea plant development; as a result, a great reduction in yield was observed. Additionally, higher precipitation on ESL may have mitigated the effect of high temperatures (28 ℃) on the plants and therefore reduced abortion was observed. Kutcher et al. (2010) explained that under abundant precipitation, high temperature stress can be alleviated on plants specially via transpiration cooling due to water availability. Overall, conditions

of high temperature accompanied with lack of precipitation (drought) could be factors that influenced development and abortion of ovules during flowering at ESP and LSP.

Although the reduction in seed yield under heat stress has been attributed to lack of fertilization due to pollen damage in various crops (Devasirvatham et al., 2013; Porch and Jahn, 2001), here, ovules of pea collected at 4 DAF after heat waves (>28°C) in the field showed low or no signs of fertilization failure. Indeed, more than 90% of the ovules displayed embryos at various stages of growth (pro-embryo to globular stage) on both ESP and LSP. Interestingly, the inverse correlation detected between ovary size, reproductive nodes and pod number on plants at LSP point to a mechanism of resource availability adjustment on these plants. In fact, poor ovule development accompanied by signs of embryo sac damage observed at LSP could be a consequence of reduced resource availability (e.g. photo-assimilates). For example, similar studies in mungbean, lentils, and chickpea have demonstrated that plants seeded at a later time in the season exhibited lower chlorophyll content, stomatal conductance, water content, and sucrose concentration compared to plants seeded at the normal time (Kaushal et al., 2013; Kaur et al., 2015; Bhandari et al., 2016). Similarly, studies on tomato (*Lycopersicon esculentum* Mill.) have shown that high temperature can change assimilate movement in plants resulting in lower translocation towards young fruits (Dinar and Rudich, 1985). Therefore, it is possible that plants of LSP being exposed to high temperature (>28°C) and low precipitation (13 mm) experienced severe lack of assimilate availability and consequently, higher embryo abortion.

Finally, a positive association between plant canopy temperature, ovary, and ovule size found only on LSP may suggest a mechanism of accelerated ovule development on stressed plants. As described previously in Chapter 3, this condition may be attributed to the accelerated phenology in the plant observed also in other legumes, such as lentil, chickpea, and mungbean (Kaushal et al., 2013; Sharma et al., 2016; Bhandari et al., 2016). In my study, while flowering lasted approximately 22 d in ESP, the stage was shorter in LSP and lasted 17 d. Altogether, my study confirmed previous findings (under growth chamber conditions) where the ovules showed signs of embryo growth constrained under high temperature, especially in low and medium tolerant cultivars. In addition, since the principal component analysis revealed that higher yield and greenness of the plants was associated in ESP, it may be suggested that the higher precipitation received by these plots mitigated effects of adverse high temperature (>28°C, 9 d). Therefore, these

findings also agreed with studies where precipitation ameliorated high temperature effects (Machado and Paulsen, 2001; Gupta et al., 2001; Bueckert et al., 2015).

5.4.2 High temperature on 18 cultivars under growth chamber and field conditions

Under growth chamber conditions, the average seed-to-ovule ratio (SOR) revealed that high temperature affected mainly cultivars Aggasiz, CDC Mozart, and CDC Striker. However, when the analysis was performed per individual node (Node 2, Node 3, and Node 4), it was detected that SOR was also diminished on heat-treated CDC Sage, Nitouche, Kahuna-PGRO, CDC Meadow, Cutlass, and TMP15213 in at least one node. Given that most of the affected cultivars exhibited reductions of SOR at Node 2, where flowers were still closed (stage 0.3; Maurer et al., 1966) at the moment of the heat-treatment, these findings imply that fertilization and embryo formation were susceptible to heat stress, and therefore, early embryo failure occurred. Correspondingly, the existence of an inverse association between SOR, aborted ovules right after or before fertilization (ABAF), and aborted ovules with presence of embryos at the pro-embryo stage (AEE) on heat-treated plants supported the above finding (Table 5.9). Interestingly, since the effect of high temperature on certain cultivars was only detected when individual nodes were analysed, the existence of a compensatory effect may suggest some level of resilience in some cultivars. In this sense, cultivars CDC Sage, Nitouche, Kahuna-PGRO, CDC Meadow, Cutlass, and TMP15213 may be more resilient than cultivars Aggasiz, CDC Mozart, and CDC Striker under heat conditions and unlimited water supply.

When the same 18 cultivars were evaluated under field conditions, the effect of high temperature on SOR was more severe in LSP than that observed on heat-treated plants under growth chamber conditions. According to Suzuki et al. (2014), the more detrimental effect on plants under field conditions is normally attributed to the existence of additional factors such light irradiance, precipitation, vapor pressure, wind, plant density, and others that make the influence of high temperature more harmful on the plants. In my study, the most evident difference between field (2018) and growth chamber experiments points to water supply conditions. Whereas plants were supplied with plenty of water to isolate the effect of high temperature in the growth chamber, plants in the field depended on the seasonal precipitation in 2018. As such, rainfall contributed only 21 mm in a short lapse of 3 d during the flowering stage (Fig. 5.16). Since heat is usually

concomitant with drought conditions in the field (Barnabás et al., 2008; Kutcher et al., 2010; Prasad et al., 2008), a more realistic effect of high temperature on cultivar may have been observed under field conditions. In this way, beside cultivars identified under growth chamber conditions, cultivars CDC Centennial, Carneval, MFR043, and CDC Treasure can be considered as susceptible since they showed the lowest SOR and highest abortion per pod in the field. In the particular case of MFR043, its slightly different floral morphology (Appendix 1) may have also affected its normal reproductive development.

A contrasting response found on ovule number and seed-to-ovule ratio (SOR) in ESP and LSP, where high ovule number but low SOR was detected in LSP and low ovule number but high SOR was detected in ESP, may be related to the overlapped flowering stage of these plots. Indeed, although ESP were seeded on the middle of May and LSP two weeks later, their flowering phase took place at a similar time in July with only a few days (4 d) of difference (Fig. 5.16). Flowering phases in ESP and LSP experienced an average temperature of 27.17 ℃ and 26.31℃, respectively. Interestingly, although plants from both seeding treatments had the exact same cumulative precipitation (21 mm for 3 d) during flowering (July), the rain may have benefitted them in a different manner. On the one side, the 3 d of precipitation could have influenced organ formation (ovules) on plants in LSP since it coincided with the beginning of flowering stages of those plots. On the other side, the 3 d of precipitation could have stimulated development of fertilized ovules (young seeds) in ESP since it occurred 4 d after flowering initiation on those plants. The reproductive development of the plants is highly vulnerable to environmental conditions where not only temperature but also water supply can influence its success (Barnabás et al., 2008; Flohr et al., 2017; Suzuki et al., 2014). Furthermore, plant reproduction, as a highly phasic (complex) process, can showed high responsiveness to water availability at all stages of development including organ formation and embryo development (Prasad et al., 2008). Studies evaluating irrigation on chickpea have identified that watering at flower initiation and/or pod filling stage can improve the number of grains per plant up to two-fold compared with plants without water supply (Dahiya et al, 1993; Shamsi et al., 2010). Thus, in my study, precipitation during flowering (July) could have benefitted ovule formation and seed development in a contrasting manner in ESP and LSP.

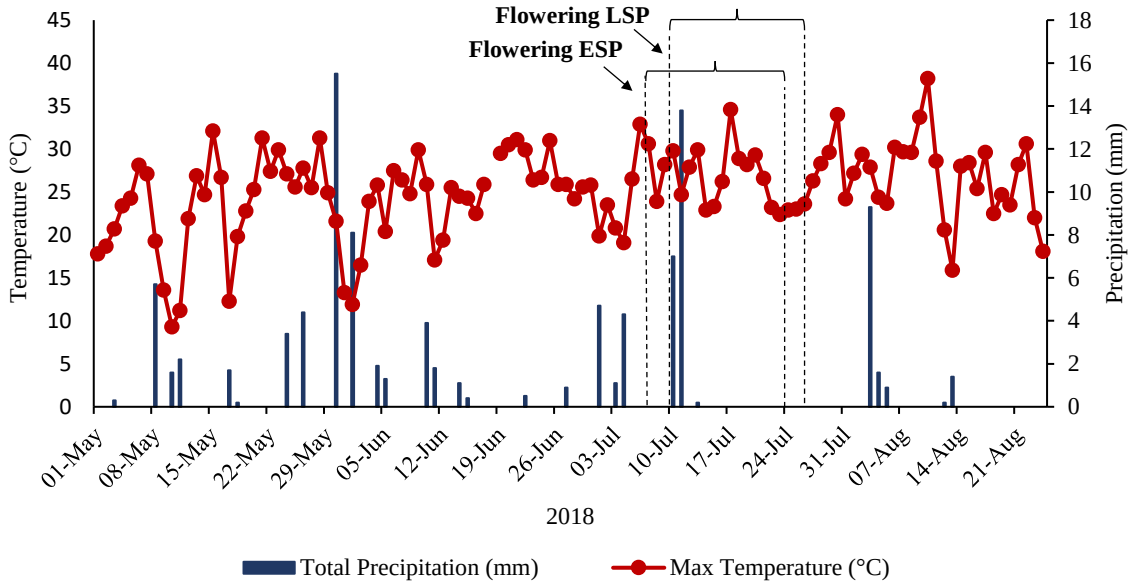


Fig. 5.16 Daily precipitation and maximum temperature during the growing season at Saskatoon in 2018. Brackets show flowering time in early seeded pea (ESP) and late seeded pea (LSP). Weather data obtained from Environment Canada (http://climate.weather.gc.ca).

The analysis of ovule abortion in plants from growth chamber conditions revealed an increased abortion of ovules with early embryo growth (AEE), especially at the pod's medial position on heat-treated plants. The probability of either seed success or failure related to ovule position within a pod has already been observed in many legume species (Cooper, 1938; Hossaert and Valero, 1988; Nakamura, 1988). Based on the linear arrangement of the pod's ovules, the non-random ovule abortion has been associated with two essential gradients in the pod. In the first case, a gradient of fertilization, where ovules closest to the style would be fertilized first, and their probability of being aborted would be lower (Bawa and Webb, 1984; Hossaert and Valero, 1988; Gutiérrez et al., 1996). In the second case, a gradient related to maternal resources proximity, where ovules closest to pedicel end would be benefited by the maternal resources, and thus, those ovules would be less prone to abortion (Watson and Casper, 1984; Harper and Wallace, 1987). In my study, the increased abortion AEE at the pod's medial position on heat-treated plants could be partially explained by the above theories. However, the increase in temperature could also affect the normal pattern of ovule abortion within the pod by restricting assimilate availability in heat-treated plants. In fact, a strategy of assimilate adjustment characteristics in plants under adverse conditions could have particularly affected the development of young ovules containing early embryo growth (Lloyd, 1980; Lee and Bazzaz, 1986). Furthermore, although the abortion of ovules

at a pod's medial position was increased under high temperature, the pattern of high abortion at the stylar and basal end of the pods was always maintained. This outcome is consistent with other findings in pea, where a common abortion at both ends of the pod has been attributed to spatial restrictions at the pod ends on tapered pod shapes (Linck, 1961; Hedley and Ambrose, 1981). Overall, the increased temperature augmented ovule abortion at the medial position, likely due to reduced assimilate availability, and the pattern of high abortion at the pod ends was always kept.

The inverse association detected between seed-to-ovule ratio (SOR) and ovule abortion showed slightly different patterns of seed failure under growth chamber and field conditions. Whereas in growth chambers, SOR was inversely associated with aborted ovules at young stages (ABAF and AEE), in the field it was inversely associated to aborted ovules that contain embryo growth at various levels of development (APE). The effect can be explained by the fact that growth chamber plants were subjected to heat at young flowering stages where processes such as fertilization and early embryo growth could have been compromised. In contrast, field plants faced several heat waves (>28°C, 6 to 9 d) during their entire flowering phase where all stages of embryo development could have been compromised. Additionally, the short precipitation lapse during flowering (21 mm, 3d) could have also influenced the increased embryo abortion on these plants. Water deficit during flowering can constrain embryo growth by diminishing cell division and causing endosperm and embryo desiccation (Westgate and Boyer, 1986; Westgate, 1994; Setter and Flannigan, 2001). Hence, in my study, ovules containing young and late embryo development were affected by high temperature in growth chamber and field conditions, the latter in combination with water availability.

In the field, the assessment of ovules at 4 days after flowering (4DAF) provided analogous information to that from mature pods. The proportion of ovules without damage or potential of ovules to become seeds (POS) at 4DAF was positively associated to SOR in mature pods; meaning that a high proportion of 4DAF ovules in good condition was related to a high proportion of seeds in mature pods. Furthermore, embryo sac area and ovule length were positively associated with seed diameter and SOR, implying that large ovules and embryo sacs (4DAF) were related to high seed diameter and seed-to-ovule ratio in mature pods. Nevertheless, a low strength of these associations (0.20 to 0.35) may indicate the existence of other factors that could have influenced the further seed development (after 4DAF) in the field. In this sense, besides high temperature

(>28°C, 6 to 9 d) during flowering in the field, an apparent terminal drought at this stage could have also disturbed normal development of the seeds and increased embryo abortion. In fact, although at the beginning of July (2018) flowering benefitted from a few days of rainfall (21mm), the rest of the month was characterized by lack of precipitation (Fig. 5.16). Correspondingly, Vadez et al. (2012) and Kashiwagi et al. (2013) explained that a terminal drought stress in the plants can easily occur after a post-rainy season as a consequence of severe reduction of rainfall during key plant stages, such as pod set and seed filling. For example, in chickpea, terminal drought caused high seed yield reduction from 42 to 52%, mainly by reductions of pod number and seed number per pod (Leport et al., 1999). Moreover, studies in soybean have shown that a reduced water potential and an increase of abscisic acid (ABA) in flowers and pods at 3 to 5 d after anthesis under drought conditions contributed to pod abortion on the plants (Liu et al., 2003). In my study, although evaluation of the ovules 4 DAF provided similar information as the evaluation of mature pods, further environmental conditions such as drought can also constrain seed development.

5.5 Conclusions

The adverse effect of high temperature on the reproductive development of plants has been largely identified to reduce seed yield in many crops including pea. In my study, the evaluation of 6 and 18 field pea cultivars under field and growth chamber conditions revealed that high temperature constrained ovule and embryo development in various cultivars. The inspection of the embryo sacs within ovules 4 DAF, and aborted ovules in mature pods, both revealed that failure of these ovules occurred during early embryo development, implying failure of fertilized ovules. Cultivars that always exhibited a high proportion of seeds per pod and low ovule abortion were 40-10, Naparnyk, CDC Bronco, CDC Golden, and CDC Patrick, whereas cultivars that tended to show less seeds per pod and high ovule damage were Nitouche, CDC Mozart, CDC Treasure, Carneval, CDC Centennial and MFR043. Given that among cultivars that showed a high seed-to-ovule ratio, only 40-10 was a small seed-size cultivar, therefore, seed size should not be considered as the only criterion that influences successful seed development. The hypothesis that there would be cultivars displaying high tolerance in terms of ovule performance is accepted. Nevertheless, the hypothesis that important associations between plant performance and ovule failure would exist is partially accepted. Indeed, although some associations between plant performance and young ovules (4DAF) were detected in 2017, the relationship in 2018 was not as strong as expected. More

consistency in results could be improved by recording plant traits at the same time as flower (4DAF) collection in future research. Finally, although high temperature influenced the fate of fertilized ovules, additional factors, such as drought conditions likely exacerbated effects in the field. However, future research considering the combination of both adverse conditions (heat and drought) would be needed to confirm this aspect.

Chapter 6. General Discussion and Conclusions

In field pea, temperatures above 25℃ during flowering cause abortion of reproductive structures, such as flowers and fruits, ultimately leading to seed yield loss (Guilioni, 1997; Sadras et al., 2013; Bueckert et al., 2015). As a flowering plant, field pea relies on the successful performance of the male and female gametophytes to produce seeds. In this sense, any environmental disturbance during reproductive development can harm both male and female gametophytes and constrain seed formation at any stage of development (Polowick and Sawhney, 1988; Gross and Kigel, 1994; Barnabás et al., 2008). Although studies on effects of high temperature on the female component during reproduction in pea and other crops are scarce, my findings elucidate exciting insights of the influence of heat stress on ovule development. Here, the evaluation of the gynoecium (ovary, style, and stigma), ovule development, ovule viability, seed set, and seed abortion in various cultivars revealed that high temperature constrained normal development of fertilized ovules in growth chamber (35℃) and field (>28℃) conditions. These findings contribute to a better understanding of how high-temperature influences and limits seed formation in field pea. Outcomes from this research will contribute to the future selection of robust cultivars with more efficient performance in terms of seed yield under warmer environments.

6.1 Flower and gynoecium development under high temperature

In this study, the evaluation of flowers from Node 1 (oldest) to Node 4 (youngest) on plants right after heat treatment (35℃, 4 d) revealed multiple effects of high temperature that depended on reproductive node position and cultivar. Advanced development of the flowers at reproductive Node 3 and 4 (youngest) was commonly accompanied by the increased size of gynoecium components, such as ovary, style, and stigma, especially on medium and high heat-tolerant cultivars. This finding implies a close association between heat stress and accelerated termination of the life cycle observed in various crops, including field pea (Guilioni, 1997; Malaviarachchi et al., 2016; Ruiz-Vera et al., 2018). The hastening of development is considered a strategy of the plants to escape dangerous environmental conditions, such as drought, that typically follow to heat stress in the field (Macedo, 2012; Bueckert and Clarke, 2013). In contrast, reduced ovary size on the oldest reproductive nodes (Node 1 and Node 2) in some cultivars (40-10, CDC Meadow, CDC Sage, and Carneval) implied that high temperature could constrain early ovary growth in young fruits. Indeed, in flowers at those nodes, early embryo formation was taking place at sampling time.

Jahnke et al. (1989) and Ozga et al. (2016) explain that an ovary after fertilization starts a phase of rapid elongation and becomes a strong assimilate sink. Thus, the poor ovary growth under heat stress may indicate that plants' assimilate partitioning was altered in those floral structures.

Moreover, various researchers have revealed that photosynthesis declines and plant resources are diverted to cope with the unfavorable conditions; as a result, assimilate availability for the sinks in development is limited on heat-treated plants (Georgieva et al., 2000; Wahid et al., 2007; Snider et al., 2009). Additionally, since a fertilized ovule requires a complex sequence of molecular, biochemical, and structural changes to become a fruit (Ozga et al., 2016), hormonal disturbance under heat stress should also be considered. In fact, a large body of research have revealed that high temperature can influence fluctuations of phytohormone signals, such as indole-3-acetic acid (IAA), jasmonic acid (JA), abscisic acid (ABA), and ethylene (Larkindale and Huang, 2005; Sakata et al., 2010; Teplova et al., 2000). Overall, multiple responses of the gynoecium components observed at different reproductive nodes could be related to early aging of the plant (Node 3 and Node 4) and assimilate and hormonal disturbances (Node 1 and Node 2) in plants under heat stress.

6.2 Ovule and embryo sac response to high temperature

A careful inspection of cleared ovules and embryo sacs of both heat-treated and control plants did not reveal any deformation or direct damage of the ovule and embryo sac under growth chamber conditions. However, high temperatures caused a high variation in ovule length and embryo sac area that depended on reproductive node and cultivar. In this way, both larger and smaller ovule and embryo sacs were detected on some nodes of heat-treated cultivars compared to the controls. Given that embryo sac area and embryo sac stage were highly correlated ($r=0.80$; $P\leq0.001$), the results implied that large embryo sacs were linked to an advanced embryo sac stage, whereas small embryo sacs corresponded to young embryo sac stage. In this sense, larger ovules and embryo sacs were related to more advanced flower development identified on some nodes and cultivars. However, smaller ovules and embryo sacs on other nodes and cultivars reflected poor ovule development. Embryo sac evaluation in these samples revealed that >90% of the ovules contained embryos at different degrees of development. Thus, fertilization failure could not be the leading cause of reduced ovule size. In turn, a conflict in resource availability could have driven the difference in the development of these structures. Thus, the variation in sizes of ovules and embryo sacs were most likely consistent with an adjustment of the maternal expenditure to maximize the

fitness of the plants under unfavorable conditions (Dinar and Rudich, 1985; Aloni et al., 1991). Furthermore, a prioritization of assimilate availability to some nodes (Guilioni et al., 2003) could also take place in some cultivars where advanced ovule development was observed under high temperatures. Importantly, whereas different strategies of the cultivars to cope heat stress appear to occur (Wahid et al., 2007; Bhandari et al., 2016; Ozga et al., 2016), a less advanced (poor) ovule development was common in medium and low heat-tolerant cultivars.

Also, a cultivar's leaf type could have played an essential role in ovule development under heat stress. Normal leaf-type cultivars tended to have either advanced ovule stages or no difference in ovule size compared to the controls. In contrast, semileafless cultivars tend to show less advanced ovule stages. Although some normal leafed cultivars can have high seed potential, their characteristics of lodging and drought sensitivity make them less competitive in the field and disliked by growers who use mechanical harvesters (Alvino and Leone, 1993; Stelling, 1994). Here, it is possible that normal leafed cultivars displayed their full potential under high temperatures, because of favorable water supply. Although there is some discrepancy about the efficiency of the normal leaf-type cultivars (Baigorri et al., 1999; Wilson et al., 2011), my findings suggest that normal leafed cultivars may have physiological characteristics that allow them to maintain optimum ovule size under heat stress. Overall, the variation in ovules and embryo sacs on different reproductive nodes of plants under heat stress may be from a conflict between accelerated development and resource availability, and the outcome depended on each cultivar's strategy to overcome heat stress conditions.

The assessment of gynoecium and ovule development of six cultivars from various levels of tolerance to heat stress provided a deeper understanding of their reproductive development during high temperature. Although development variation of female flower traits was observed among the reproductive nodes of these cultivars, the main response to high temperature in most of the cultivars was consistent with the categorization of tolerance used in this study (Table 3.1). As such, heat-treated cultivars 40-10 and Naparnyk tended to maintain healthy and vigorous development of the gynoecium components such as style, ovary, ovules, and embryo sacs accordingly to a high level of heat tolerance. Heat-treated CDC Meadow and CDC Sage tended to show a wide range in development response of their gynoecium components such as ovary, ovules, and embryo-sac sizes, among their reproductive nodes, consistent with a medium level of heat tolerance. Heat-

treated cultivar Carneval was prone to show poor development of gynoecium traits such as style, ovary, ovules, and embryo sacs in most of its reproductive nodes in correspondence to a low level of tolerance to heat stress. In contrast, MFR043 was the only cultivar that could not fit within the categorization used for this study since its particular flower morphology affecting its pollination led it to low seed set under control and heat stress conditions. Hence, the rank of heat tolerance of the cultivars in terms of seed-to-ovule ratio was consistent with their gynoecium performance under high temperature for five out of six cultivars evaluated.

6.3 Reduced ovule viability under high temperature

The presence of callose and reactive oxygen species (ROS) in the ovule tissue revealed more than one aspect where high temperature could constrain normal ovule development. On one side, the increased accumulation of callose on the chalaza and nucellus area of ovules from heat-treated plants may indicate a conflict of assimilate imported to the embryo sac. In fact, this is a common sign of abortion observed in various species, where its presence has been associated with assimilate obstruction in tissue (Arbeloa and Herrero, 1991; Rosellini et al., 1998; Sun et al., 2004). In pea, a histological study of ovule abortion showed that the accumulation of callose in the chalaza zone was commonly accompanied by a high accumulation of lignin in the tissue, indicating a lack of tissue permeability that led to starvation of the young embryos (Briggs et al., 1987). Complementarily, vanishing of the embryo sac lining detected in ovules at an advanced stage of abortion in the field may confirm a damage of transfer cells around the embryo sac as part of callose deposition accumulation. Furthermore, studies in *Arabidopsis* showed that cells in the ovule's chalaza zone had a high frequency of plasmodesmata activity that indicates a control of assimilate flow toward the embryo sac via symplastic transport (Sager and Lee, 2014). Therefore, the accumulation of callose in the chalaza of ovules from heat-treated plants was likely related to assimilate regulation and possible blockage of nutrient transport in ovule tissue. Indeed, multiple studies have demonstrated that abiotic stresses, such as wounding, temperature, and mineral toxicity increase accumulation of callose deposition around plasmodesmata and inhibit assimilate transport to leaves, shoots, petioles, roots, and other plant tissues (Bilska and Sowiński, 2010; Cui and Lee, 2016; Smith and McCully, 1977).

In contrast, an accumulation of ROS in ovules from heat-treated plants could indicate cellular damage in the affected ovules' embryo sac. ROS was consistently detected in the embryo sac of

ovules within pods at distal positions (Node 4) in one out of three studied cultivars. Although ROS is a normal by-product of the metabolism of the cells, its accumulation is related to a high toxicity of the cellular components (Burton and Jauniaux, 2011; Sharma et al., 2012). In various studies, abiotic stressors such as drought, high light intensity, metal toxicity, and temperature, have led to a disruption of the metabolism of the cells and overproduction of ROS As a result, high ROS caused oxidative stress and damage to the cell contents in leaves and seedlings (Dat et al., 2000; Sharma and Dubey, 2005; Maheshwari and Dubey, 2009). Sun et al. (2004) studied the ovules of *Arabidopsi*s under salt stress and found that an accumulation of ROS in the embryo sacs of ovules occurred in an initial stage of abortion that later spread out to the integuments of the ovule. When cellular and ultrastructure study of those ovules was performed, it was determined that damage of cytoplasm, vacuoles, and mitochondria was linked to signs of programmed cell death in those cells (Sun et al., 2005; Hauser et al., 2006). In my study, a similar accumulation ROS detected in embryo sac of young ovules may suggest the existence of oxidative damage in cells of the embryo sac from heat-treated plants.

6.4 Ovule abortion and early seed failure in response to high temperature

Whereas high temperature under growth chamber (35°C) and field conditions (>28°C) caused abortion of ovules containing various levels of embryo growth, the intensity of the effect differed between both conditions. In the field, failure of embryo growth was always more pronounced than in the growth chamber. For example, in growth chambers, the high temperature increased abortion of ovules right after fertilization and ovules containing early embryo growth (zygote, pro-embryo, embryos globular stage). In contrast, in the field, a high amount of abortion occurred in ovules containing a wide range of embryo growth (pre-embryo to late cotyledon stage). The abortion observed under growth chamber conditions (6 and 18 cultivars) was consistent with exposure of the plant's early flowering stage to heat treatment. In fact, flowers on those plants were at young stages (buds to open flowers) of development, where early embryo growth was taking place. In this sense, a reduction in assimilate availability in heat-treated plants (Dinar and Rudich, 1985; Hasanuzzaman et al., 2013) could constrain early embryo growth. In addition, abortion of young ovules could occur as part of a maternal expenditure adjustment in plants where the organs that received the least investment were terminated (Lloyd, 1980; Rudich et al., 1977; Aloni et al., 1991). The abortion at various stages of embryo growth in the field can be explained by plants facing various heatwaves (>28°C) accompanied by low precipitation (13 - 21 ml) during flowering.

According to various authors, water reduction during the flowering phase restricts embryo growth by reducing cell division and causing embryo and endosperm desiccation (Westgate and Peterson, 1993; Westgate, 1994; Liu et al., 2006). Overall, it is apparent that high temperature can affect embryo growth at any stage, depending on plant proficiency to maintain consistent and undisrupted assimilate partitioning under heat stress.

6.5 Seed size under high temperature

Seed size reduction is a common effect of high temperature detected in crops in general, as well as in legume crops, such as common bean, cowpea, lentil, fava bean, chickpea, and soybean (Egli and Wardlaw, 1980; Sadras et al., 2013; Awasthi et al., 2014). Findings from my research revealed that among the evaluated cultivars, seed size showed two particular patterns under heat stress. In medium and high heat-tolerant cultivars displaying a high seed-to-ovule ratio, a slight reduction of seed size was observed. Contrastingly, in low heat-tolerant cultivars with low seed-to-ovule ratio, a slight increase of seed size was identified. On one side, the reduction in seed size could be attributed to the plants' accelerated life cycle under heat stress. Studies in legumes and cereal crops indicate that exposure of plants to high temperature during flowering causes reduction in seed size in response to a short seed filling duration (Savin et al., 1999; Wang et al., 2006; Prasad et al., 2008). In fact, since the life cycle of the plant is accelerated under high temperature, a shortened seed-filling duration constrains seed development, and an insufficient accumulation of assimilate in the seeds occur (Kumar et al., 2013; Prasad et al., 2008). Also, studies in legume crops have shown that high temperature disrupts cytokinin and invertase metabolic activity in the plants (Banowetz et al., 1999; Bhandari et al., 2016). This disruption has been related to reduced cell division and limited assimilated partitioning for the seed in development (Tacarindua et al., 2012; Wang et al., 2006). Smaller seeds are associated with less cell division in early developing seeds and with less assimilate supply.

For a second explanation, the slight increase in seed size found in low heat-tolerant cultivar could obey a compensatory effect for seed loss, as identified in other studies of pea and chickpea (Poggio et al., 2005; Wang et al., 2006). Interestingly, the variation in seed size was small compared to the variation in seed number in any affected cultivar, which is congruent with the fact that seed number variation is the most plastic plant trait related to the allocation of the plant resources under adverse conditions (Sadras, 2007; Sadras and Dreccer, 2015). Overall, although seed size displayed

some variation in plants under heat stress compared to the controls, this effect was not strong. Specifically, whereas medium and high heat tolerant cultivars displayed a mild reduction in seed size, the low heat-tolerant cultivar showed a small but significant increase in seed size in heat-treated plants.

6.6 Future Research

In my research, the examination of young ovules, abortion, and seed set under growth chamber and field conditions provided consistent information of the effect of high temperature on ovule development in field pea. As a leading approach in the evaluation of the effect of high temperature on seed reduction, the investigation of the ovule opens aspects that will require future exploration in field pea.

1. Since the high temperature affected the growth of ovules containing embryos at various stages, the possibility of embryo starvation should be considered. In this sense, a study of plant physiological traits (plant greenness, sucrose content, invertase activity, among others) on leaves along with their subtended ovaries and ovules will uncover important heat tolerance mechanisms in cultivars of various sensitivities to heat.
2. The internal inspection of young ovules in my study allowed visualization of some variations in ovule and embryo growth among the evaluated cultivars, e.g., ovules of 40-10 tended to show more advanced stages than the rest of cultivars independent of temperature treatment. Evaluation of seed growth in terms of cellular division and expansion on cultivars of varied sensitivity to heat will be of interest.
3. The constraining of embryo and endosperm development in various cultivars under heat stress could have occurred from disruption of hormonal homeostasis. An investigation of the hormonal profile in ovules and leaves under heat stress will provide a deeper insight into the variation in ovule growth detected under high temperatures.
4. Since the presence of callose deposition and ROS were detected in different zones of ovules, a possible disruption of plasmodesmata activity and cellular components of the ovules is suggested. An ultrastructural study of ovules on at least one sensitive and one tolerant cultivar will be needed to identify the mechanism of tolerance taking place at the cellular level in the ovules.

5. A common pattern of high seed-to-ovule ratio and low ovule abortion detected on normal leaf type cultivars indicate some advantages of photosynthetic activity on these cultivars. Investigation of the role of leaf size and cellular characteristics of this structure accompanied by seed number assessment at different reproductive nodes of the plants will be of interest.
6. A detrimental effect of high temperature observed on ovule abortion under field conditions was probably exacerbated by other factors, such as lack of precipitation. The evaluation of heat, drought, and their combination under growth chamber conditions will be importance to assess the contribution of each factor to failure of early seeds.
7. Inhibited embryo development and increased callose deposition around the ovules' vascular bundle implied a conflict of assimilate availability. In this sense, a study of plant assimilate partitioning between the source (leaf) and sink (flower) in cultivars with various sensitivities to heat could help to elucidate possible plant mechanisms to heat tolerance.
8. Finally, since a contrasting relationship between canopy temperature and ovule size was identified among years (2017 and 2018), the advantage or disadvantage of that relationship requires further investigation.

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Appendices

Appendix 1: Floral morphology of cultivar MFR043

Flowers of cultivar MFR043 behaved differently compared to other cultivars under growth chamber conditions. Typically, following self-pollination, most cultivars' flowers developed into mature fruits. However, flowers of MFR043 at reproductive Node 1, 2, 3, and 4 failed to produce fruits and they abscised approximately 2 to 3 days after they were open (stage 0.5 of Maurer et al., 1966). When the size of the flower was analyzed, this cultivar had small-sized flowers like CDC Meadow, but differed by a longer style. Thus, the average length of an open flower was 17.37 mm in MFR043 and 18.70 mm in CDC Meadow; however, the style length of MFR043 was 7.25 mm and 6.23 mm for CDC Meadow.

Furthermore, the presence of pollen on stigmas from 10 opened flowers of CDC Meadow and 20 opened flowers of MFR043 (5 plants) under control conditions was evaluated under the micro-stereoscope. I found 100% of the flowers from CDC Meadow displayed stigmas fully covered with pollen, whereas only 30% of flowers from MFR043 had some pollen on the stigma (2 to 5 pollen grains). Later, the presence of pollen tube growth on the stigma and ventral suture of the ovary of MFR043 confirmed that just a couple of these flowers had signs of pollen tube growth (Fig. A1.1). Hence, these observations infer that flowers of MFR043 had some limitations for self-pollination under growth chamber conditions.

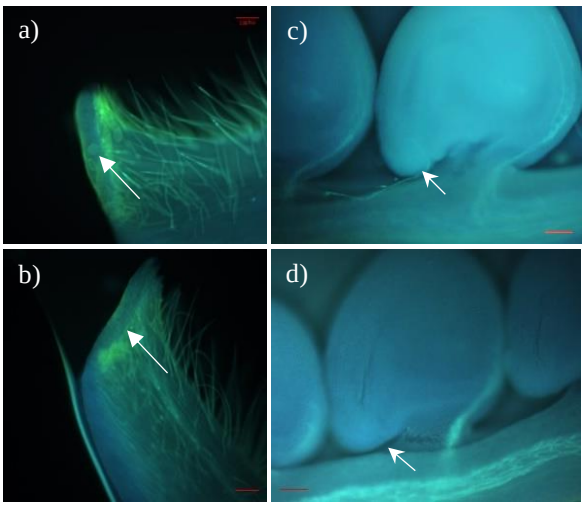

Fig. A1.1 Stigma and ovules of MFR043 with and without signs of pollen grains and pollen tubes on ventral suture of the ovary, a) and b) Stigmas from flowers at pollination stage with some attached and non-attached pollen grains, respectively (arrows). c) and d) Ovules from open flowers, at 0.5 stage (Maurer et al., 1966), with (c) and without (d) signs of pollen tubes in the micropyle (arrows) of the ovule. Cleared and stained stigmas and ovules show callose fluorescence using the aniline blue protocol according to Martin (1959).

A careful inspection of the petals on flowers of MFR043 also revealed that although most of the petals displayed a normal shape common to papilionaceous flowers, the keel petal on these flowers exhibited a different feature compared to the other cultivars. Although this petal presented a normal suture on the ventral side of the petal that gives its shape, the opposite side of the petal, that wraps the anthers and style together, showed a small dentate edge that coincided with the top of the style allowing its' stigma to protrude from this arrangement (Fig. A1.2). This feature of the flower was found on 17 of 22 flower buds evaluated at stage 0.3 (Maurer et al., 1966) when anthers had dehisced, and pollination occurred. According to Tucker (1989), the typical shape of this petal (without dentate edge) may facilitate self-pollination of the flower by allowing pollen to adhere on its walls and contact the stigma at the top of the style. In my observations, the normal shape of this petal may also facilitate the style to bend closer to pollen grains on anthers, encouraging self-pollination of a common pea flower. This impression was confirmed by measurement of the style length, from the base of the style to top of the stigma, which showed that the style of these flowers was 1 to 1.5 mm larger than the rest of cultivars with normal keel petals. Indeed, it was also noticed that the styles of MFR043 tended to be straighter than CDC Meadow (Fig. A1.3). Finally, hand pollination by just pushing pollen close to the stigma of flowers at stage 0.3 on reproductive Nodes 1, 2, 3, and 4 of four plants of MFR043 allowed them to set fruits on all manipulated flowers compared to plants that were not hand pollinated on the same cultivar. Overall, these findings suggest that MFR043 likely requires a unique mechanism performed by either bees or wind (movement) in the field to facilitate self-pollination, and probably support cross-pollination.

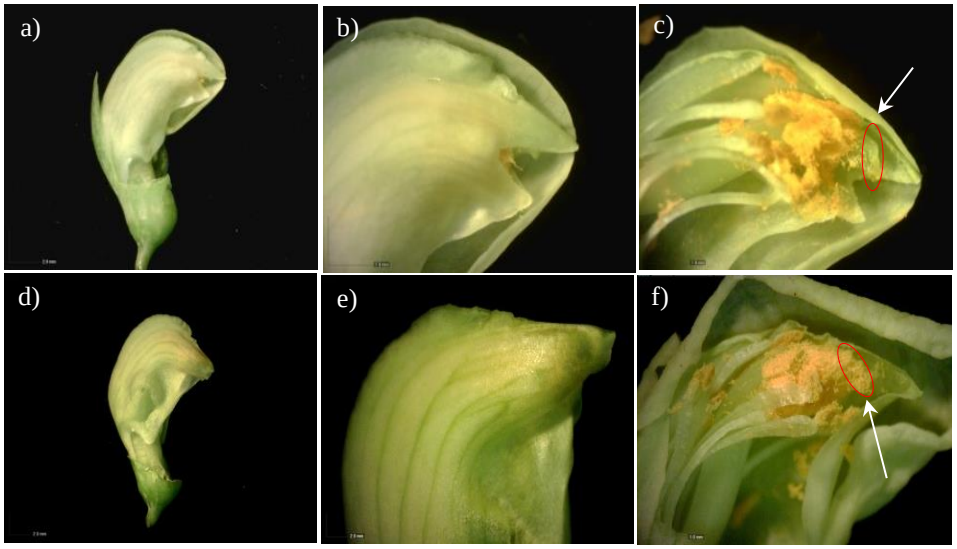

Fig A1.2 Keel petal on cultivars MFR043 and CDC Meadow. a), b) and c) Flower of MFR043 showing keel petal with dentate edge allowing stigma (arrows) to protrude ; d), e), and f) Flower of CDC Meadow displaying normal petal with smooth edge with stigma covered with pollen (arrow).

Fig. A1.3 Styles of cultivar MFR043 and CDC Meadow. a) Style of MFR043 with pollen grains on style hairs but none on stigma (arrow). b) Style of CDC Meadow with pollen grains on style hairs and stigma (arrow).

Appendix 2. Figures and Tables to Support Main Results of Chapter 3, 4, and 5

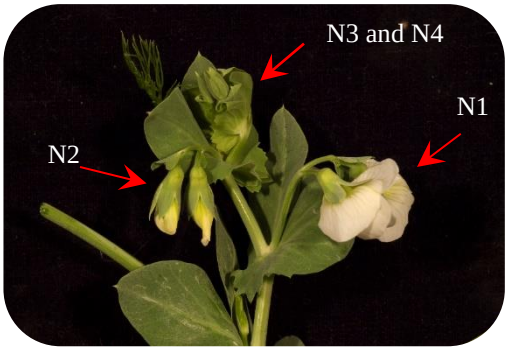

Fig. A2.1 Flowering stage of field pea considered for heat stress exposure (35/18 ºC day/night) under growth chamber conditions where Node 1 (N1) displays open flowers, Node 2 (N2) shows flower buds, and Node 3 (N3) and Node 4 (N4) exhibit flower primordium.

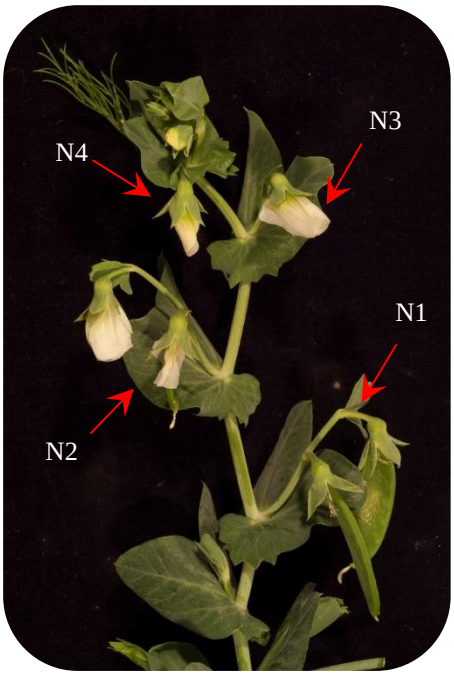

Fig. A2.2 Flowering stage of field pea after four days of heat stress exposure (35/18 ºC day/night) displaying four reproductive nodes (N1, N2, N3, and N4) with flowers at various stages of development.

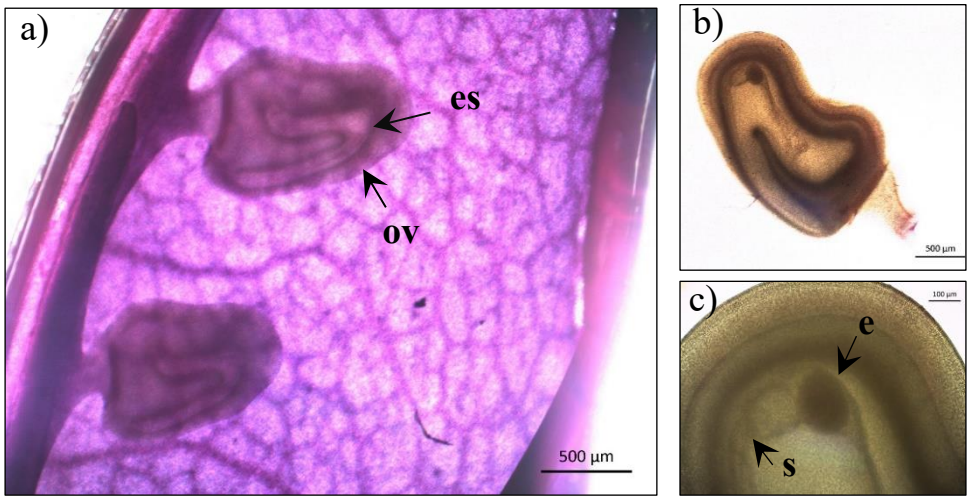

Fig. A2.3 Ovary and ovules of field pea processed by clearing technique. a. Ovary with attached ovules (ov) displaying embryo sac (es). b. and c. Ovules depicting embryo (e) at globular stage and suspensor (s).

Fig. A2.4 Panoramic view of the pea plots from field experiment carried out in Sutherland 2018.

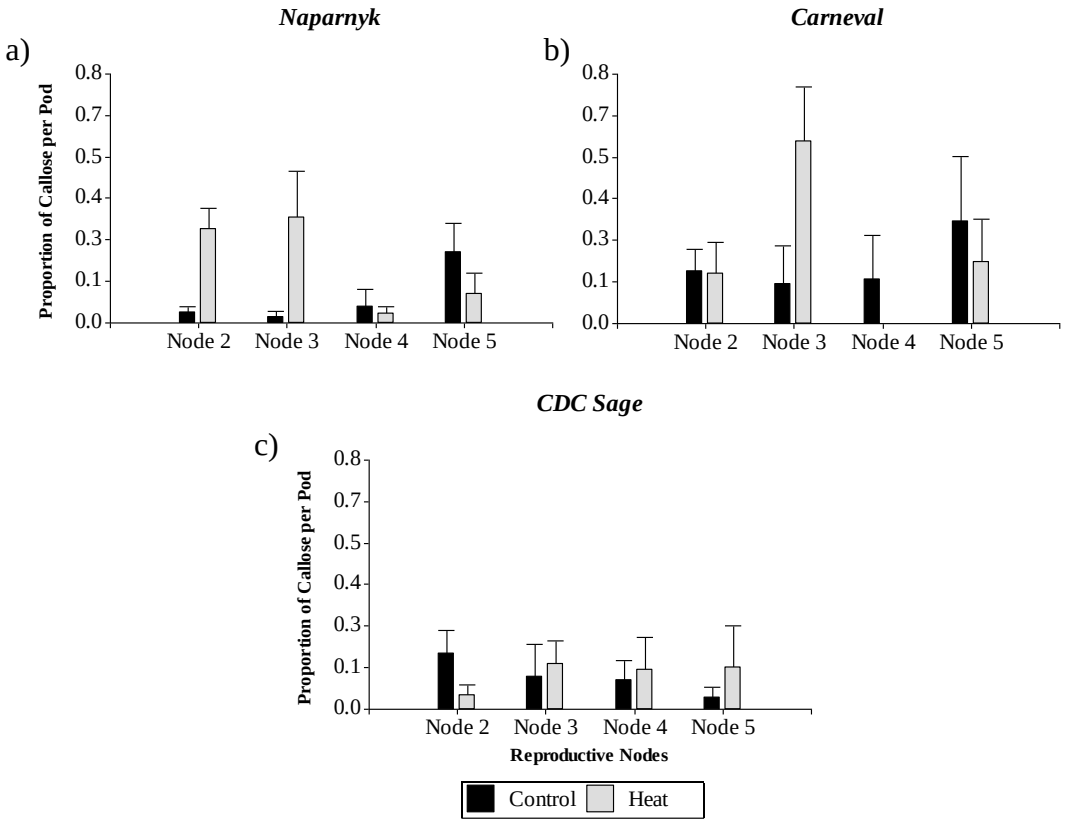

Fig. A2.5 Effect of high temperature (35/18°C) on proportion of ovules with callose deposition per ovary in three cultivars of field pea a) Naparnyk, b) Carneval, and c) CDC Sage and their reproductive nodes (Nodes 2-5) after four days of treatment in plants grown under growth chamber conditions. Means of four replications with their respective standard error bars are shown.

Table A2.1 Variability in number of ovules per pod of six field pea cultivars, and their distribution at three standardized positions within the pod.

Number of Ovules per Pod	Number of Ovules per Standardized Position within Pod		
	Stylar	Medial	Basal
6	2	2	2
7	2	3	2
8	3	2	3
9	3	3	3
10	3	4	3

Table A.2.2 Equation Parameters of the nonlinear regression ($y = a + b(x^c)$) found between ovule length and embryo sac area, where y= ovule length and x=embryo sac area. The referred equation was used in Fig. 3.4.

Equation Parameters	Estimated	*t* Value	*P* Value
Alpha (a)	-0.156918	-7.135	**<.0001**
Beta (b)	2.466418	103.381	**<.0001**
Gama (c)	0.345102	69.677	**<.0001**

Significance levels at P<0.001 are shown in bold; efficiency =0.97; bias=-5.97e^{-11}

Table A2.3 Number of proximal (P), distal (D), and total flowers on six field pea (*Pisum sativum* L.) cultivars, collected right after four days of treatment for the first four reproductive nodes (N1 to N4) on four plants from each treatment.

Node	Treat.	Cultivar																		Total P	Total D	Total
		Carneval			Meadow			MFR043			Naparnyk			Sage			40-10					
		P	D	Subt.	P	D	Subt.	P	D	Subt.	P	D	Subt.	P	D	Subt.	P	D	Subt.			
N1	Control	4	2	6	4	4	8	4	4	8	4	4	8	4	2	6	4	2	6	24	18	42
	Heat	4	2	6	4	4	8	4	4	8	4	4	8	4	1	5	4	3	7	24	18	42
N2	Control	4	2	6	4	4	8	4	3	7	4	4	8	4	3	7	4	3	7	24	19	43
	Heat	4	3	7	4	3	7	4	3	7	4	4	8	4	1	5	4	3	7	24	17	41
N3	Control	4	2	6	4	4	8	4	3	7	4	4	8	4	1	5	4	2	6	24	16	40
	Heat	4	2	6	4	3	7	4	2	6	4	4	8	4	1	5	4	1	5	24	13	37
N4	Control	4	1	5	4	3	7	4	2	6	4	4	8	4	0	4	4	0	4	24	10	34
	Heat	4	0	4	4	2	6	4	3	7	4	3	7	4	1	5	4	2	6	24	11	35
Total Control		16	7	23	16	15	31	16	12	28	16	16	32	16	6	22	16	7	23	96	63	159
Total Heat		16	7	23	16	12	28	16	12	28	16	15	31	16	4	20	16	9	25	96	59	155
Total N3 to N4		16	5	21	16	12	28	16	10	26	16	15	31	16	3	19	16	5	21	96	50	146
Total N1 to N4		32	14	46	32	27	59	32	24	56	32	31	63	32	10	42	32	16	48	192	122	314

Table A2.4 Number of ovules in proximal (P) and distal (D) flowers on six field pea (*Pisum sativum* L.) cultivars, collected right after four days of treatment for the first four reproductive nodes (N1 to N4) on four plants from each treatment.

Node	Treat.	Cultivar																		Total P	Total D	Total
		Carneval			Meadow			MFR043			Naparnyk			Sage			40-10					
		P	D	Subt.	P	D	Subt.	P	D	Subt.	P	D	Subt.	P	D	Subt.	P	D	Subt.			
N1	Control	29	15	44	31	30	61	28	27	55	34	32	66	30	15	45	28	14	42	180	133	313
	Heat	28	16	44	29	28	57	29	26	55	31	30	61	32	8	40	28	21	49	177	129	306
N2	Control	32	15	47	28	27	55	28	21	49	34	34	68	32	22	54	28	19	47	182	138	320
	Heat	32	21	53	30	22	52	28	19	47	31	30	61	31	8	39	28	20	48	180	120	300
N3	Control	28	15	43	30	27	57	28	19	47	33	29	62	30	7	37	28	12	40	177	109	286
	Heat	28	13	41	29	22	51	29	13	42	33	31	64	31	8	39	27	7	34	177	94	271
N4	Control	30	7	37	28	19	47	30	13	43	33	29	62	31	0	31	27	0	27	179	68	247
	Heat	28	0	28	29	13	42	30	22	52	32	22	54	31	8	39	27	13	40	177	78	255
Total Control		119	52	171	117	103	220	114	80	194	134	124	258	123	44	167	111	45	156	718	448	1166
Total Heat		116	50	166	117	85	202	116	80	196	127	113	240	125	32	157	110	61	171	711	421	1132
Total N1 to N4		325	102	337	234	188	422	230	160	390	261	237	498	248	76	324	221	106	327	1429	869	2298

Table A2.5 Effect of temperature treatment, cultivar, and ovule position on seed number per pod from 18 field pea cultivars grown under growth chamber conditions.

Source of Variation	Ovule Number per Pod
Treatment	
Control	6.4±0.1 a†
Heat	6.4±0.1 a
Cultivar	
40-10	6.4±0.1 d-f
Aggasiz	5.9±0.2 fg
Argus	5.8±0.1 f-h
Carneval	7.0±0.1 bc
CDC Bronco	7.0±0.1 bc
CDC Centennial	6.2±0.1 d-f
CDC Golden	5.3±0.2 h
CDC Meadow	6.5±0.1 c-e
CDC Mozart	6.2±0.2 ef
CDC Patrick	6.7±0.1 cd
CDC Sage	6.7±0.1 c-e
CDC Striker	7.8±0.2 a
CDC Treasure	6.7±0.1 cd
Cutlass	6.5±0.1 c-e
Kahuna-PGRO	5.5±0.1 gh
Naparnyk	7.5±0.1 ab
Nitouche	6.5±0.1 c-e
TMP15213	5.3±0.1 h
Ovule Position	
Stylar	-
Medial	-
Basal	-
***P* Value**	
Temperature treatment (T)	0.4446
Cultivar (C)	**<.0001**
Node (N)	0.6953
T*C	0.6854
C*N	0.0987
T*N	0.8781
T*C*N	0.7161

†Values within a column and within variable followed by the same letter are not significantly different at P<0.05. Significance levels at P <0.05 and P<0.001 are shown in bold.

Table A 2.6 Means (± SE) of seed-to-ovule ratio at stylar, medial, and basal position within pods on reproductive nodes 2-4 from 18 cultivars grown under growth chamber conditions.

Cultivar	Node 2			Node 3			Node 4		
	Stylar	Medial	Basal	Stylar	Medial	Basal	Stylar	Medial	Basal
40-10	0.84±0.07 a-g†	0.95±0.05 a	0.74±0.08 a-k	0.91±0.05 a-c	0.92±0.05 a-d	0.69±0.08 c-l	0.88±0.06 a-c	0.89±0.06 ab	0.53±0.09 i-s
Aggasiz	0.59±0.11 h-r	0.61±0.11 f-p	0.44±0.11 m-t	0.59±0.09 h-q	0.80±0.09 a-i	0.56±0.09 j-r	0.87±0.06 a-f	0.87±0.08 a-d	0.82±0.08 a-f
Argus	0.68±0.09 a-l	0.85±0.08 a-e	0.35±0.10 p-u	0.77±0.09 a-j	0.88±0.08 a-e	0.23±0.07 st	0.50±0.12 l-u	0.73±0.14 a-n	0.36±0.10 p-u
Carneval	0.60±0.06 i-p	0.75±0.07 a-k	0.32±0.07 st	0.49±0.09 k-r	0.77±0.07 b-j	0.24±0.06 t	0.38±0.05 q-u	0.73±0.07 a-l	0.26±0.07 u
CDC Bronco	0.73±0.07 a-k	0.87±0.05 a-d	0.44±0.10 p-u	0.57±0.11 h-n	0.89±0.07 a-e	0.33±0.08 o-t	0.66±0.06 c-o	0.93±0.05 ab	0.32±0.08 s-u
CDC Centennial	0.80±0.11 a-k	0.80±0.09 a-j	0.45±0.12 l-t	0.50±0.13 i-s	0.57±0.12 i-s	0.35±0.15 m-t	0.75±0.08 a-n	0.85±0.08 a-g	0.25±0.13 s-u
CDC Golden	0.79±0.10 a-k	0.93±0.07 a-e	0.71±0.10 b-o	0.75±0.13 a-l	0.90±0.07 a-f	0. 75±0.11 a-l	0.86±0.10 a-d	0.86±0.07 a-f	0.50±0.12 i-t
CDC Meadow	0.41±0.08 o-u	0.67±0.08 e-p	0.31±0.08 t	0.56±0.08 h-m	0.64±0.09 e-l	0.35±0.09 n-t	0.55±0.08 h-q	0.74±0.08 b-n	0.34±0.09 r-u
CDC Mozart	0.68±0.11 d-q	0.74±0.09 a-l	0.46±0.11 n-t	0.77±0.12 a-l	0.71±0.12 b-l	0.18±0.08 t	0.90±0.07 a-c	0.95±0.05 a-d	0.40±0.07 p-u
CDC Patrick	0.69±0.12 a-p	0.94±0.06 a-d	0.70±0.13 a-l	0.78±0.09 a-k	1.00±0.00 ab	0.78±0.09 a-k	0.78±0.09 a-i	0.94±0.06 ab	0.85±0.08 a-g
CDC Sage	0.64±0.11 b-p	0.75±0.11 a-k	0.82±0.10 a-i	0.78±0.11 a-j	0.92±0.06 a-c	0.73±0.07 a-l	0.77±0.10 a-l	0.88±0.09 a-e	0.48±0.14 m
CDC Striker	0.65±0.10 b-p	0.75±0.10 a-l	0.61±0.10 e-p	0.68±0.12 c-l	0.88±0.09 a-f	0.38±0.09 m-t	0.64±0.09 d-p	0.80±0.10 a-k	0.30±0.06 s-u
CDC Treasure	0.83±0.05 a-g	0.93±0.04 a-c	0.31±0.05 st	0.64±0.06 e-l	1.00±0.00 a	0.31±0.06 q-t	0.78±0.06 a-g	0.86±0.07 a-f	0.28±0.07 tu
Cutlass	0.63±0.09 c-q	0.91±0.04 a-d	0.37±0.08 n-t	0.69±0.08 e-l	0.83±0.07 a-h	0.28±0.07 r-t	0.44±0.09 p-u	0.77±0.09 a-j	0.29±0.09 tu
Kahuna-PGRO	0.50±0.11 k-t	0.88±0.08 a-e	0.62±0.08 d-q	0.75±0.13 a-l	0.90±0.07 a-f	0.75±0.11 a-j	0.59±0.13 e-q	0.82±0.10 a-h	0.59±0.11 f-q
Naparnyk	0.85±0.05 a-h	0.92±0.03 a-d	0.63±0.06 g-r	0.84±0.05 a-f	0.87±0.04 a-g	0.67±0.06 f-p	0.75±0.05 a-j	0.97±0.03 a	0.48±0.06 o-u
Nitouche	0.59±0.10 f-p	0.70±0.10 b-m	0.44±0.09 n-t	0.54±0.11 i-s	0.51±0.13 l-t	0.38±0.11 m-t	0.71±0.11 a-m	0.88±0.05 a-d	0.49±0.11 g-r
TMP15213	0.75±0.08 a-k	0.71±0.11 d-q	0.56±0.10 j-s	0.50±0.14 f-s	0.70±0.15 a-l	0.61±0.16 c-o	0.45±0.14 n-u	0.55±0.14 o-u	0.55±0.12 i-u

†Values within a column and within variable followed by the same letter are not significantly different at P<0.05.

Table A2.7 Means (± SE) of seed-to-ovule ratio at stylar, medial, and basal position within pods of nodes collected (N2 to N4) from 18 field pea cultivars under growth chamber conditions.

Cultivar	Ovule Position (All Nodes)		
	Stylar	Medial	Basal
40-10	0.87±0.03 a-e	0.92±0.03 ab	0.65±0.05 j-q
Aggasiz	0.68±0.06 h-p	0.76±0.06 c-k	0.60±0.06 l-s
Argus	0.66±0.06 h-n	0.83±0.06 a-g	0.32±0.05 v
Carneval	0.50±0.04 p-u	0.75±0.04 d-l	0.28±0.04 v
CDC Bronco	0.65±0.05 i-q	0.89±0.03 a-c	0.37±0.05 uv
CDC Centennial	0.68±0.07 f-o	0.74±0.06 c-n	0.35±0.08 t-v
CDC Golden	0.80±0.06 a-j	0.90±0.04 a-f	0.66±0.06 i-q
CDC Meadow	0.50±0.05 q-u	0.68±0.05 i-n	0.33±0.05 v
CDC Mozart	0.77±0.06 b-l	0.79±0.05 a-k	0.35±0.05 v
CDC Patrick	0.75±0.06 b-m	0.96±0.03 a	0.78±0.06 a-k
CDC Sage	0.73±0.06 c-n	0.85±0.05 a-g	0.69±0.06 e-o
CDC Striker	0.66±0.06 i-q	0.81±0.06 a-k	0.44±0.05 s-v
CDC Treasure	0.75±0.04 c-k	0.94±0.03 ab	0.30±0.03 v
Cutlass	0.60±0.05 m-s	0.85±0.04 a-h	0.32±0.04 v
Kahuna-PGRO	0.60±0.07 k-s	0.87±0.05 a-g	0.65±0.06 h-q
Naparnyk	0.81±0.03 a-i	0.92±0.02 a-c	0.59±0.03 n-s
Nitouche	0.61±0.06 j-q	0.70±0.06 g-o	0.43±0.06 r-v
TMP15213	0.60±0.07 i-q	0.66±0.07 j-r	0.57±0.07 n-t

†Values within a column and within variable followed by the same letter are not significantly different at $P<0.05$.

Table A2.8 Means (± SE) of proportion of aborted ovules with presence of pro-embryo or early embryo development (AEE), embryo between globular to heart stage (AGH), and embryo between early to late cotyledon stage (AELC) at stylar, medial, and basal positions within the pod of 18 cultivars grown under growth chamber conditions.

Cultivar	AEE			AGH			AELC		
	Stylar	Medial	Basal	Stylar	Medial	Basal	Stylar	Medial	Basal
40-10	0.07±0.02 m-p†	0.03±0.02 op	0.20±0.04 c-j	0.00±0.00 i	0.01±0.01 hi	0.01±0.01 g-i	0.02±0.01 mn	0.02±0.01 l-n	0.07±0.03 e-n
Aggasiz	0.09±0.03 i-p	0.07±0.03 l-p	0.10±0.04 i-p	0.00±0.00 i	0.00±0.00 i	0.05±0.02 c-h	0.02±0.02 l-n	0.00±0.00 n	0.05±0.02 f-n
Argus	0.01±0.01 p	0.01±0.01 p	0.27±0.05 b-f	0.01±0.01 g-i	0.00±0.00 i	0.04±0.02 e-i	0.12±0.03 d-h	0.00±0.00 n	0.23±0.04 a
Carneval	0.15±0.03 h-o	0.07±0.02 n-p	0.39±0.04 a	0.02±0.01 f-i	0.03±0.01 f-i	0.03±0.02 e-i	0.18±0.03 a-d	0.06±0.02 f-n	0.18±0.03 a-d
CDC Bronco	0.14±0.03 h-o	0.09±0.03 j-p	0.31±0.05 a-c	0.07±0.03 a-e	0.01±0.01 hi	0.10±0.03 ab	0.08±0.03 e-m	0.01±0.01 n	0.20±0.04 a-c
CDC Centennial	0.13±0.04 g-p	0.19±0.06 c-k	0.42±0.08 ab	0.00±0.00 g-i	0.00±0.00 g-i	0.03±0.02 e-i	0.05±0.03 f-n	0.06±0.03 e-n	0.05±0.03 f-n
CDC Golden	0.11±0.05 h-p	0.06±0.03 l-p	0.13±0.04 g-p	0.01±0.01 f-i	0.00±0.00 g-i	0.10±0.03 a-c	0.00±0.00 n	0.03±0.02 i-n	0.06±0.03 e-n
CDC Meadow	0.15±0.03 f-n	0.09±0.02 i-p	0.39±0.05 ab	0.03±0.02 e-i	0.05±0.02 c-g	0.03±0.02 e-i	0.11±0.03 e-i	0.04±0.02 g-n	0.05±0.02 f-n
CDC Mozart	0.03±0.02 n-p	0.06±0.03 i-p	0.29±0.06 a-g	0.03±0.02 f-i	0.00±0.00 hi	0.09±0.03 a-d	0.03±0.02 k-n	0.02±0.02 k-n	0.04±0.02 f-n
CDC Patrick	0.11±0.04 h-p	0.02±0.02 op	0.07±0.03 k-p	0.00±0.00 g-i	0.00±0.00 g-i	0.06±0.03 c-i	0.14±0.04 b-g	0.02±0.02 k-n	0.04±0.03 f-n
CDC Sage	0.09±0.04 i-p	0.05±0.03 n-p	0.19±0.05 d-m	0.03±0.02 e-i	0.01±0.01 g-i	0.01±0.01 g-i	0.03±0.02 j-n	0.01±0.01 n	0.03±0.02 j-n
CDC Striker	0.14±0.05 e-o	0.03±0.02 n-p	0.30±0.05 a-d	0.01±0.01 f-i	0.00±0.00 hi	0.01±0.01 f-i	0.07±0.03 e-n	0.02±0.01 l-n	0.12±0.03 d-k
CDC Treasure	0.10±0.03 i-p	0.04±0.02 n-p	0.27±0.03 a-d	0.06±0.02 b-f	0.00±0.00 i	0.12±0.03 a	0.05±0.02 i-n	0.01±0.01 n	0.14±0.03 d-f
Cutlass	0.20±0.04 c-i	0.04±0.02 n-p	0.38±0.05 ab	0.03±0.02 f-i	0.01±0.01 f-i	0.05±0.02 d-i	0.10±0.03 e-i	0.05±0.02 h-n	0.15±0.03 c-e
Kahuna-PGRO	0.03±0.02 n-p	0.06±0.03 l-p	0.06±0.03 n-p	0.00±0.00 hi	0.00±0.00 hi	0.00±0.00 hi	0.24±0.06 ab	0.00±0.00 n	0.25±0.05 a
Naparnyk	0.13±0.02 h-o	0.06±0.02 n-p	0.27±0.03 a-d	0.02±0.01 f-i	0.01±0.01 g-i	0.03±0.01 e-i	0.03±0.01 k-n	0.02±0.01 l-n	0.04±0.02 g-n
Nitouche	0.16±0.05 e-o	0.12±0.04 h-p	0.19±0.05 d-l	0.01±0.01 g-i	0.00±0.00 i	0.02±0.02 e-i	0.04±0.02 f-n	0.02±0.01 mn	0.11±0.03 e-l
TMP15213	0.10±0.03 h-p	0.18±0.06 c-h	0.24±0.06 a-e	0.00±0.00 g-i	0.01±0.01 f-i	0.00±0.00 g-i	0.16±0.06 d-j	0.05±0.03 e-n	0.06±0.03 f-n

†Values within a column and within variable followed by the same letter are not significantly different at $P<0.05$.

Appendix 3: Number of nodes per plant at 45, 60, and 75 days after seeding in early and late seeded pea 2018

Total number of nodes per plant

The number of total nodes differed significantly by seeding date, plant age, cultivar, and interactions of seeding date by plant age, cultivar by plant age, and seeding date by cultivar (Table A3.1). Plants from late seeded pea developed more nodes ($\bar{x}$ =20) than early seeded pea ($\bar{x}$ =19.1). Cultivars Aggasiz, CDC Bronco, CDC Meadow, and Naparnyk produced 1 to 2 more nodes at late seeded pea (LSP) compared to their counterparts at early seeded pea (ESP) (Table A3.2). Congruently, plants after 45, 60, and 75 days from seeding time produced a significantly different number of nodes displaying 13.4, 21.6, and 23.7 nodes, respectively (Table A3.1). Interestingly, the average number of total nodes varied according to the seeding dates at 45 and 60 days, but it did not at 75 days. Plants from LSP produced 13.7 and 22.5 nodes at 45 and 60 days, respectively, whereas these numbers were lower in ESP, with 13.0 and 20.7 nodes, respectively, at similar plant ages (Fig. A3.1). In general, CDC Treasure, Carneval, and CDC Golden produced most nodes and CDC Bronco, 40-10, and CDC Centennial, the least regardless of seeding date (Table A3.1). The interaction between cultivar and plant age revealed that CDC Treasure and CDC Golden stood out with 15.1 and 14.5 nodes at 45 days, Carneval and CDC Treasure with 23.0 and 22.6 nodes at 60 days, and MFR043 and Nitouche with 25.3 and 24.9 nodes at 75 days, respectively (Table A3.3).

Table A3.1 Effect of seeding date, cultivar, and plant age on the number of vegetative nodes, number of reproductive nodes, and total number of nodes evaluated from plants of 18 field pea cultivars grown under field conditions.

Source of Variation	Total Number of Nodes	Number of Vegetative Nodes	Number of Reproductive Nodes
Seeding Date			
Early	19.1±0.32 b†	14.5±0.12 b	4.66±0.24 a
Late	20.0±0.33 a	15.1±0.12 a	4.94±0.25 a
Cultivar			
40-10	18.5±0.97 hi	14.4±0.34 c-g	4.0±0.69 e
Aggasiz	19.5±0.98 b-h	14.4±0.27 d-g	5.2±0.80 ab
Argus	19.7±0.95 a-g	14.9±0.32 a-f	4.8±0.72 b-d
Carneval	20.7±0.98 a	15.2±0.25a-e	5.5±0.84 a
CDC Bronco	18.5±1.02 hi	13.9±0.34 fg	4.6±0.73 cd
CDC Centennial	18.2±0.93 i	13.5±0.28g	4.7±0.72 b-d
CDC Golden	20.5±0.93 ab	15.9±0.31a	4.7±0.74 cd
CDC Meadow	19.9±1.10 a-g	15.1±0.33a-e	4.8±0.75 b-d
CDC Mozart	19.1±0.88 d-i	14.1±0.28e-g	5.1±0.73 a-c
CDC Patrick	20.2±1.03 a-d	15.5±0.34a-c	4.7±0.74 b-d
CDC Sage	18.8±0.94 g-i	14.3±0.31e-g	4.5±0.71 de
CDC Striker	19.0±0.95 f-i	14.6±0.34b-f	4.4±0.70 de
CDC Treasure	20.8±0.87 a	15.7±0.21ab	5.0±0.76 a-c
Cutlass	19.1±0.89 e-i	14.3±0.29e-g	4.8±0.72 b-d
MFR043	20.3±0.99 a-c	15.6±0.40ab	4.7±0.75 cd
Naparnyk	19.2±1.16 c-i	14.4±0.75d-g	4.9±0.72 b-d
Nitouche	20.2±1.04 a-e	15.5±0.43a-d	4.7±0.77 b-d
TMP15213	20.0±1.04 a-f	14.6±0.27b-f	5.4±0.82 a
Plant Age			
45 DAS	13.4±0.09 c	13.3±0.09 b	0.06±0.02 c
60 DAS	21.6±0.15 b	15.5±0.15 a	6.10±0.10 b
75 DAS	23.7±0.16 a	15.5±0.15 a	8.23±0.10 a
***P* Value**			
Seeding Date (SD)	0.0088	0.0409	0.0858
Cultivar (C)	<.0001	0.0002	<.0001
Plant Age (PA)	<.0001	<.0001	<.0001
SD*C	0.0333	0.1045	0.0502
SD*PA	<.0001	0.6021	<.0001
C*PA	0.0313	0.1251	<.0001
SD*C*PA	0.6075	0.6902	0.1932

†Values within a column and within variable followed by the same letter are not significantly different at P<0.05.

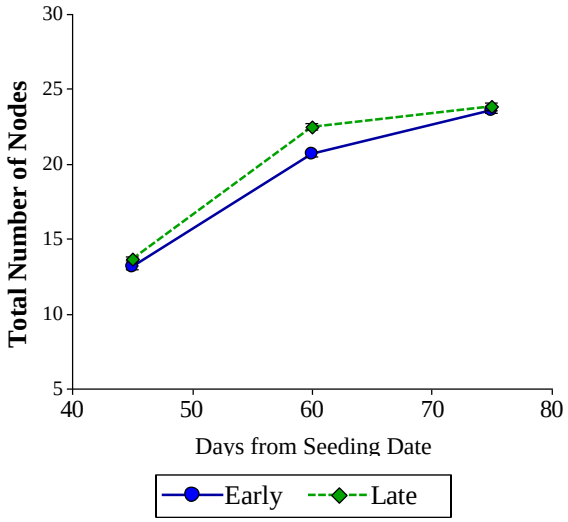

Fig. A3.1 Effect of seeding date on the total number of nodes evaluated at 45, 60, and 75 days after seeding in 18 field pea cultivars under field conditions. Means of 4 replications (n=144 plants) with their respective error bars are shown.

Table A3.2 Means (± SE) of total node number and reproductive nodes from 18 field pea cultivars according to seeding date.

Cultivar	Total Number of Nodes		Number of Reproductive Nodes	
	Early Seeding	Late Seeding	Early Seeding	Late Seeding
40-10	18.4±1.4 i-m†	18.5±1.4 g-m	4.1±1.0 k-m	4.0±1.0 m
Aggasiz	18.5±1.3 h-m	20.6±1.5 a-d	4.8±1.1 b-k	5.5±1.2 a-c
Argus	19.1±1.3 d-m	20.3±1.4 a-e	4.6±1.0 e-m	5.1±1.0 a-h
Carneval	20.1±1.4 a-g	21.2±1.4 a	5.3±1.2 a-d	5.7±1.2 a
CDC Bronco	17.7±1.3l m	19.3±1.6 c-l	4.2±1.0 i-m	5.0±1.1 a-h
CDC Centennial	17.5±1.2 m	18.8±1.5 e-m	4.5±1.0 f-m	4.8±1.1 c-k
CDC Golden	20.1±1.3 a-g	20.9±1.3 ab	4.7±1.1 d-m	4.7±1.0 d-l
CDC Meadow	18.8±1.3 e-m	20.9±1.4 ab	4.5±1.0 g-m	5.0±1.1 a-h
CDC Mozart	18.8±1.3 e-m	19.5±1.2 b-k	5.2±1.1 a-e	4.9±1.0 b-j
CDC Patrick	19.8±1.5 a-i	20.7±1.5 a-d	4.5±1.1 g-m	4.9±1.1 b-j
CDC Sage	18.1±1.4 j-m	19.6±1.3 b-j	4.2±1.0 j-m	4.9±1.1 b-j
CDC Striker	20.1±1.5 a-g	17.9±1.2 k-m	4.8±1.1 b-j	4.0±0.9 lm
CDC Treasure	20.3±1.2 a-e	21.2±1.3 a	4.9±1.0 b-i	5.2±1.2 a-g
Cutlass	18.7±1.3 f-m	19.4±1.3 b-k	4.7±1.0 d-l	4.8±1.0 d-k
MFR043	20.1±1.6 a-h	20.5±1.6 a-d	4.4±1.1 h-m	5.0±1.1 b-h
Naparnyk	18.2±1.4 i-m	20.3±1.9 a-f	4.9±1.1 b-i	4.9±1.0 b-k
Nitouche	20.7±1.5 a-c	19.7±1.5 ab-j	4.2±1.0 i-m	5.3±1.2 a-f
TMP15213	19.5±1.4 b-k	20.6±1.5 a-d	5.3±1.2 a-d	5.5±1.2 ab

†Values within a column and within variable followed by the same letter are not significantly different at P<0.05.

Number of vegetative nodes per plant

The number of vegetative nodes was highly influenced by seeding date, cultivar, and age of plant; however, the interaction of seeding date by plant age was not significant (Table A3.1 and Fig. A3.2). Consistent with total nodes, plants from LSP developed a greater number of vegetative nodes compared to ESP. Whereas the number of vegetative nodes was 15.1 in LSP, the number was 14.5 nodes in ESP (Table A3.1). Regardless of seeding date, CDC Golden, CDC Treasure, and MFR043 had more vegetative nodes exhibiting 15.9, 15.7, and 15.6 nodes, and CDC Centennial and CDC Bronco had the least with 13.5 and 13.9 nodes, respectively (Table A3.1). Although the number of vegetative nodes per plant differed between 45 days and 60 days with 13.3 and 15.5 nodes, the 60-day number did not change when plants were evaluated 75 days after seeding (Table A3.1 and Fig. A3.2).

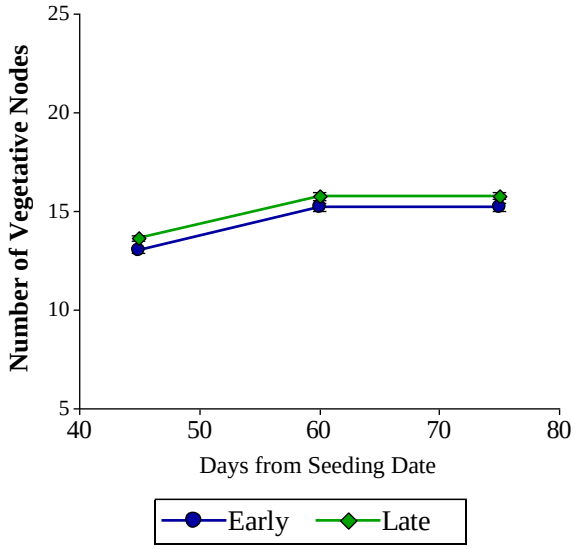

Fig. A3.2 Effect of seeding date on the number of vegetative nodes per plant evaluated at 45, 60, 75 days after seeding, evaluated in 18 field pea cultivars under field conditions. Means of 4 replications (n=144 plants) with their respective error bars are shown.

Number of reproductive nodes per plant

The number of reproductive nodes per plant significantly differed by cultivar, age of the plant, interactions of seeding dates by cultivar, seeding dates by plant age, and cultivar by plant age (Table A3.1). Cultivars such as Carneval and TMP15213 showed the most reproductive nodes with 5.50 and 5.41 nodes, respectively, whereas 40-10 and CDC Striker had the smallest number with 4.04 and 4.41, respectively (Table A3.1). As expected, plants had a significantly different number of reproductive nodes at different stages, starting with 0.06 nodes at 45 days, followed by

6.1 nodes at 60 days and 8.23 at 75 days from seeding (Table A3.1). Interestingly, reproductive node number was similar for LSP and ESP at 45 days from seeding; however, the number was significantly greater in LSP at 60 days and contrastingly smaller at 75 days compared to the respective ESP (Fig. A3.3). Whereas the average number of reproductive nodes was 6.72 in LSP and 5.48 in ESP at 60 days, it was 8.05 in LSP and 8.39 in ESP at 75 days. In other words, the number of reproductive nodes was greater at LSP than ESP at 60 days from seeding, but the average number ended up being smaller in LSP compared to ESP at physiological maturity. At the same time, the number of reproductive nodes varied among cultivar accordingly to the age of the plants. CDC Mozart and Argus had the greatest number of reproductive nodes at 45 days, but highest were Carneval and TMP15213 at 60 and 75 days from seeding (Table A3.3). Specifically, CDC Bronco and Nitouche had 0.9 nodes more at LSP compared to ESP, whereas CDC Striker had 0.8 nodes more at ESP compared to LSP (Table A3.2).

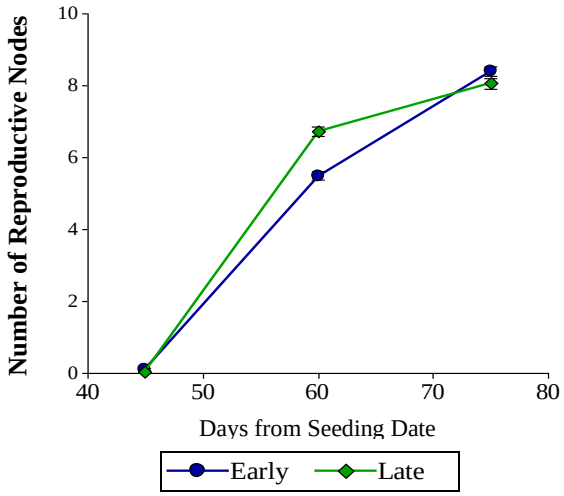

Fig. A3.3 Effect of seeding date on the number of reproductive nodes per plant evaluated at 45, 60, 75 days after seeding, evaluated in 18 field pea cultivars under field conditions. Means of 4 replications (n=144 plants) with their respective error bars are shown.

Table A3.3 Means (± SE) of number of reproductive nodes and total number of nodes per plant from 18 field pea cultivars at 45, 60, and 75 days after seeding (DAS).

Cultivar	**Total number of nodes per plant**			**Number of reproductive nodes per plant**		
	45 DAS	**60 DAS**	**75 DAS**	**45 DAS**	**60 DAS**	**75 DAS**
40-10	12.5±0.16 w-y†	19.8±0.73 r	23.1±0.68 c-k	0.00±0.00 m	4.4±0.38 l	7.7±0.59 de
Aggasiz	13.4±0.31 uv	21.5±0.69 k-q	23.7±0.66 a-i	0.04±0.04 m	6.6±0.42 g-i	8.8±0.34 ab
Argus	13.9±0.40 tu	21.7±0.87 k-q	23.5±0.79 b-j	0.30±0.15 m	6.2±0.54 h-k	8.0±0.45 cd
Carneval	14.4±0.31 t	23.0±0.69 d-k	24.6±0.50 a-e	0.00±0.00 m	7.4±0.41 d-f	9.1±0.29 a
CDC Bronco	12.0±0.28 y	20.5±0.66 o-r	23.1±0.52 c-k	0.00±0.00 m	5.6±0.35 k	8.2±0.39 b-d
CDC Centennial	12.2±0.23 xy	20.2±0.66 qr	22.1±0.48 i-o	0.04±0.04 m	6.1±0.31 h-k	7.9±0.36 c-e
CDC Golden	14.5±0.30 st	22.4±0.39 h-m	24.8±0.21 a-c	0.00±0.00 m	5.8±0.41 jk	8.2±0.45 b-d
CDC Meadow	13.6±0.35 u	22.1±0.74 i-n	23.9±0.60 a-h	0.00±0.00 m	6.3±0.45 h-k	8.0±0.52 cd
CDC Mozart	13.5±0.47 uv	20.8±0.40 n-r	23.1±0.16 c-k	0.40±0.17 m	6.2±0.28 h-k	8.6±0.42 a-c
CDC Patrick	13.5±0.27 uv	22.4±0.61 h-m	24.6±0.31 a-d	0.00±0.00 m	5.9±0.45 i-k	8.1±0.28 b-d
CDC Sage	12.8±0.45 v-x	21.0±0.63 l-r	22.7±0.46 g-k	0.00±0.00 m	5.9±0.48 i-k	7.6±0.31 de
CDC Striker	13.2±0.31 u-w	20.9±0.43 m-r	23.0±1.04 d-k	0.00±0.00 m	5.6±0.26 k	7.7±0.56 de
CDC Treasure	15.1±0.17 s	22.6±0.26 h-l	24.6±0.55 a-f	0.09±0.09 m	6.5±0.19 g-j	8.5±0.38 a-c
Cutlass	13.4±0.25 uv	21.0±0.59 l-r	22.8±0.57 g-k	0.13±0.13 m	6.2±0.34 h-k	7.9±0.35 c-e
MFR043	13.2±0.19 u-w	22.3±0.43 h-n	25.3±0.57 a	0.00±0.00 m	5.5±0.41 k	8.5±0.27 a-c
Naparnyk	12.8±0.30 v-x	21.9±1.50 j-p	22.9±1.44 f-k	0.16±0.09 m	6.8±0.33 f-h	7.7±0.37 de
Nitouche	13.5±0.27 uv	22.1±0.42 i-n	24.9±0.55 ab	0.00±0.00 m	5.7±0.53 k	8.5±0.37 a-c
TMP15213	13.3±0.31 u-w	22.5±0.52 h-l	24.4±0.36 a-g	0.00±0.00 m	7.2±0.28 e-g	9.1±0.24 a

†Values within a column and within variable followed by the same letter are not significantly different at P<0.05.