Physiological determinants of fertile florets in wheat: variation between elite cultivars and effects of Ppd and Eps genes

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TESI DOCTORAL

Physiological determinants of fertile florets in wheat: variation between elite cultivars and effects of *Ppd* and *Eps* genes

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Memòria presentada per optar al grau de Doctor per la Universitat de Lleida
Programa de Doctorat en Ciència i Tecnologia Agrària i Alimentària

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2016

To Eduardo y Ana

Agustín, Mariana y Corina

Juan, Ester y Cacha

Acknowledgments

First of all, I would like to appreciate Dr. Roxana Savin and Dr. Gustavo A. Slafer for giving me an incomparable opportunity in my professional and personal life oversea. Although this meant to leave everyone and everything far away, I made one of the most important steps in my life until now. Thanks to you two, for helping me to carry out my PhD leading me during these years and for giving me your point of view about science life. I could live such different experiences inside and outside the lab, thank you for that also.

To University of Lleida who supported most of my work.

To ADAPTAWHEAT the project in which I worked in and which gave me the space to carry out my Thesis and allowed me to meet great researchers. Specially thanks to Simon Griffiths.

To the Ministry of Economy and Competitiveness of Spain.

To Dr. Fernanda Dreccer and the Commonwealth Scientific and Industrial Research Organisation (CSIRO) for one of the best experiences I had during my PhD, allowing me to work in a totally different environment showing me a completely different way to work on science and in an incredible and wonderful country. To Mary Anne for all her kindness, advices and her help and to the nice people I met there.

To my friend, half-sister and co-worker: Helga Ochagavía Orbegozo who shared every single good and bad experience I have lived during these years since I arrived. Cause without her I wouldn't have been able to arrive until here, thank you for each moment, word, laugh and tear, for made me feel as part of your family and group of friend. thanks to all of them. Cheers for the sharpeners, the three stooges and for the writers, we are above, we found the door! Lucky to meet you here. To my friend Andreu to whom I want to appreciate his patience, for listening and helping me at life and work, for every single hour we shared. To Ángel for his sincerely friendship, for every joke, for taking care of me, for the advices and help. To Elías for the funny moments shared inside and outside the uni, thank you two for arriving in the precisely moment. To all the nice people I met here and shared many experiences and, in different ways, made me easy all these years, specially, Raziel, Eva and Marisol.

To the people from the lab, Ariel, Jordi, Nicolás, Mónica, Carlos, Guifre, Pau, Addy, Elena, Ester, Edu, Dunia, Eloi, Laura and Harry for the times we shared and for all their help.

Specially and most important, to my family. Thank you for being so far away but so near to me, thank you for giving me the strength to follow, for being here with me in all possible ways avoiding me to give up even in the worst moments. I would like to thank my parents: Ana y Eduardo for growing me up prioritizing the responsibility and effort but most important to be a good person. To my brother Agustín, my sisters Mariana y Corina, my sister in law Georgina, each of you took a very special part of this experience, as you know your are pieces of myself. To my aunt Lau, my truly (fairy)

godmother and all the rest of my huge family who walked with me, particularly my grandparents who are waiting for me. To my Spanish family the Ochagavía-Orbegozo for considering me as part of them, for all the support and the special and very nice moment they shared with me.

To all my friends, almost family: Anto, Flor, Lula, Loli, Mary, Sam and So; my "comu" group: Anita, Ber, Benja, Caro, Dai, Emis, Euge, Feli, Flor, Lucas, Manu, Nico, Pau and Tomi and my TOCs: Anita, Cin, Flore and Marie. Thanks all of you for walking next to me so far away without releasing my hand, for pushing me out from the deeply. For making me feel so near.

Finally, to all the people that were part of my life in Lleida and in the University that in one way or other they contribute me to arrive until here.

Table of contents

List of tables

Chapter II:

Chapter III:

Chapter IV:

Chapter V:

Chapter VI:

List of figures

Chapter I:

Chapter II:

Chapter III:

Chapter VI:

Chapter VII:

Abstract/Resumen/Resum

Wheat yield is linearly related to grain number per m^2. As wheat is a cleistogamus plant, understanding the physiological determinants of fertile florets resulted of the different floret development dynamics due to genetic variability and ascertaining the role the particular genes: Photoperiod (*Ppd*) and earliness *per se* (*Eps*) might be useful to achieve improvements in grain number and yield.

Three field studies (each comprising at field experiments conducted across two growing seasons) were carried out mainly to characterize the dynamics of floret primordia initiation and degeneration in a set of modern cultivars of hexaploid wheat and in *Ppd* and *Eps* near isogenic lines (NILs) and to determine which are the most critical parameters of such dynamics in establishing genotypic differences in the number of fertile florets at anthesis. In addition, to evaluate the possible effects due to the interaction between the *Eps* genes and temperature experiments under controlled conditions were also conducted. Across all the experiments, a huge amount of dissections were periodically made determining not only the number but also de developmental stage of floret primordia, providing an exceptionally detailed database of dynamics of floret development.

High variability was found in the number of fertile florets due to different genetic background among elite cultivars and due to the introgression of the photoperiod insensitivity alleles. Effects on the number of fertile florets among *Eps* NILs were subtle as expected for these genes used for fine tune flowering time and presented a strong relationship with the temperature. However in both cases of near isogenic lines, results showed the dependency of the source (*Ppd* and *Eps*) and chromosome (*Eps*) of the particular alleles. Differences in the number of fertile florets were mainly explained by differences in the floret generation/degeneration dynamics and in most cases well associated with floret survival. In most cases, these differences well correlated with differences in the late reproductive phase due to differences among modern cultivars or due to the reduction associated with the introgression of the photoperiod insensitivity alleles.

Advantageous cultivars with higher number of fertile florets among the elite population tested could be used for the next breeding programs, in the case of manipulating photoperiod insensitivity or the earliness *per se*, breeders might be careful due to the effects that can caused on the setting of the spike fertility.

El rendimiento del cultivo de trigo se encuentra linealmente relacionado con el número de granos por m^2. Debido a que el trigo es una planta cleistógama, entender los determinantes fisiológicos del número de flores fértiles, que es resultado de las diferentes dinámicas del desarrollo floral dadas por variabilidad genética, y determinar el rol particular de los genes de fotoperiodo (*Ppd*) y precocidad intrínseca (*Eps*) podría ser útil para lograr mejoras en el número de granos y por lo tanto en el rendimiento.

Se llevaron a cabo tres ensayos a campo durante dos años consecutivos, con el objetivo de caracterizar las dinámicas de iniciación y degeneración de primordios florales en un set de cultivares modernos de trigo hexaploide y en líneas isogénicas para *Ppd* y *Eps*, y determinar cuáles son los parámetros críticos de esas dinámicas que establecen las diferencias genotípicas en el número de flores fértiles en antesis. Además, para evaluar los posibles efectos de la interacción entre los genes *Eps* y la temperatura, se llevaron a cabo ensayos bajo condiciones controladas. En todos los experimentos, se hicieron periódicamente una gran cantidad de disecciones determinando no sólo el número, sino también, el estado de desarrollo de los primordios florales. Esto generó una base de datos detallada de las dinámicas de desarrollo floral.

Se encontró gran variabilidad en el número de flores fértiles debido a distinto fondo genético entre cultivares elite y debido a la introgresión de alelos insensibles al fotoperiodo. Los efectos en el número de flores fértiles en las líneas isogénicas para *Eps* fueron sutiles, como era esperable para este tipo de genes usados para ajustar la fecha de floración, y resultaron estar fuertemente relacionados con la temperatura. Sin embargo, los resultados indicaron la dependencia de la fuente (*Ppd* y *Eps*) y del cromosoma (*Eps*) de cada alelo particular. Las diferencias en el número de flores fértiles fueron explicadas principalmente por diferencias en las dinámicas de generación/degeneración de flores y en la mayoría de los casos asociadas a la supervivencia de flores. Mayormente estas diferencias correlacionaron bien con las diferencias en la fase reproductiva tardía entre cultivares modernos o debido a la reducción asociada la introgresión de alelos insensibles al fotoperiodo.

Aquellos cultivares que presentaron la ventaja de tener un mayor número de flores fértiles en las poblaciones elite, podrían ser utilizados en los próximos programas de mejoramiento; en el caso de manipular la insensibilidad a fotoperiodo o la precocidad intrínseca, los mejoradores deberían ser cautelosos debido a los efectos que puedan causar sobre el establecimiento de la fertilidad de la espiga.

El rendiment del cultiu de blat es troba linealment relacionat amb el nombre de grans per m^2. Degut al fet que el blat és una planta clistògama, entendre els determinants fisiològics del nombre de flors fèrtils, el qual és resultat de les diferents dinàmiques del desenvolupament floral donades per variabilitat genètica, i determinar el rol particular dels gens de fotoperíode (*Ppd*) i precocitat intrínseca (*Eps*) podria ser útil per tal d'obtenir millores en el nombre de grans i, per tant, en el rendiment.

S'han dut a terme tres assaigs de camp durant dos anys consecutius, amb l'objectiu de caracteritzar les dinàmiques d'iniciació i degeneració de primordis florals en un set de conreus moderns de blat hexaploide i en línies isogèniques per *Ppd* i *Eps,* i determinar quins són els paràmetres crítics d'aquestes dinàmiques què estableixen les diferències genotípiques en el nombre de flors fèrtils en antesi. A més a més, per avaluar els possibles efectes de la interacció entre els gens *Eps* i la temperatura, s'han dut a terme assaigs sota condicions controlades. En tots els experiments, s'han realitzat periòdicament una gran quantitat de disseccions determinant no solament el nombre, sinó que també, l'estat de desenvolupament dels primordis florals. Això va generar una base de dades detallada de les dinàmiques de desenvolupament floral.

Es va trobar gran variabilitat en el nombre de flors fèrtils donat el diferent fon genètic entre conreus d'elit i degut a la introgressió d'al·lels insensibles al fotoperíode. Els efectes en el nombre de flors fèrtils en les línies isogèniques per *Eps* van ser subtils, com era d'esperar per aquest tipus de gens utilitzats per ajustar la data de floració, i van resultar estar freqüentment relacionats amb la temperatura. No obstant això, els resultats van indicar la dependència del donant (*Ppd* i *Eps*) i del cromosoma (*Eps*) de cada al·lel particular. Les diferències en el nombre de flors fèrtils van ser explicades principalment per diferències en les dinàmiques de generació/degeneració de flors i en la majoria dels casos associats a la supervivència de flors. Majoritàriament aquestes diferències van correlacionar bé amb les diferències en la fase reproductiva tardana entre conreus moderns o degut a la reducció associada a la introgressió d'al·lels insensibles al fotoperíode.

Aquells conreus que presentaven l'avantatge de tenir un major nombre de flors fèrtils en les poblacions elit, podrien ser utilitzats en els pròxims programes de millora; en el cas de manipular la insensibilitat a fotoperíode o la precocitat intrínseca, els milloradors haurien de ser cautelosos degut als efectes que puguin causar sobre l'establiment de la fertilitat de l'espiga.

Chapter I: General introduction

1. Why wheat? Crop relevance

Wheat is one of the most important cereals in human diet since the ancient times. Nowadays, wheat contributes *c.* 19% of the calories and *c.* 20% of the total protein consumed all over the world (Braun *et al*., 2010).

The importance of wheat is also revealed by it growing area, that represents a large proportion of the world-wide arable lands (218.7 million ha; Fig. 1.1). In addition, it was the third cereal with higher production quantity of *c.* 26.7% (663.4 million tonnes) after maize and rice between these last years (Fig. 1.1).

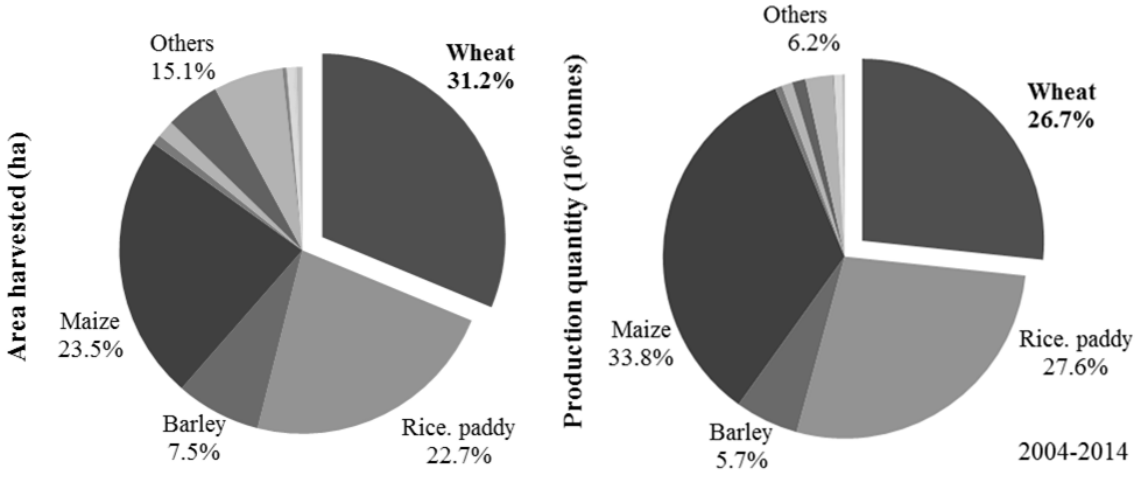

Figure 1.1. Average area harvested and production quantity of the most common cereals between 2004-2014 all over the world. Data obtained from http://faostat3.fao.org.

Due to the predictions of population increase by 2050 (*c.* 9.4 billion; Foulkes *et al*., 2011) together with an increase in average individual requirements, there is an urgent requirement to increase wheat production in order to satisfy the demand. Wheat yield gains are currently not large enough, being estimated in 0.54% per year between 1997 and 2007, less than half of that required in the near future (1.32% annual increase) (González *et al*., 2011) and being quite distance to the increases achieved during the Green Revolution, with growth average wheat yields of 3.6% per annum in developing countries (Dixon *et al*., 2009). The possible solutions may include: i) incrementing crop lands, although the availability is scarce and brings about issues on the long-term sustainability of agro-ecosystems (Reynolds *et al*., 2012); ii) developing management strategies as in the use of water and fertilizers but it is limited due to economic and environmental issues, iii) improving genetically yields through plant breeding focused on adaptation and yield potential. The latter seems to be the only successful way out to maintain the balance between wheat production and demand in the near future.

The background conditions in which exceptionally large genetic gains must be achieved makes the requirement even more challenging. It is predicted a mean global warming of *c*. 1.5 to 5.8°C by the end of the century (Rosenzweig *et al*., 2001 and references therein) and it is expected a reduction in wheat production of 6% per degree of temperature increment (Asseng *et al*., 2015).

Therefore, understanding the physiological mechanisms that control grain yield plays a relevant role in order to further increase grain yield. In fact, the study of the crop developmental phases and the dynamics of the different organs generation/degeneration and how they are affected by genotypic differences or environmental conditions could be critical in order to achieve yield increments.

2. Wheat developmental phases

Wheat physiology can be analyzed in terms of wheat development and wheat growth. Even though yield is a measure of growth, crop development is critical in determining yield. Wheat crop cycle is divided into different developmental phases which can be defined due to micro and macroscopic changes (Miralles and Slafer, 1999) (Fig. 1.2). During the vegetative phase leaves are being developed from sowing to double ridge stage when the early reproductive phase starts and the spikelet primordia are developed. Following, the late reproductive phase starts at the terminal spikelet stage lasting until anthesis or flowering time, during this phase, floret primordia develop (this includes the floret generation and the degeneration processes) and the number of fertile florets per spike is set. Around anthesis, the number of fertile tillers is also set determining the final number of spikes. After pollination, grain number is set and the grain filling period starts until physiological maturity.

The number of grains per unit land area is defined from sowing to anthesis with a critical period from the emergence of the penultimate leaf (20-30 days before anthesis) until *c*. 7-10 days post anthesis (Fischer 1985; Slafer and Savin, 1991), while the average weight is defined from some days before anthesis until maturity (Calderini *et al*., 2001; Ugarte *et al*., 2007). Normally, there is a negative relationship between grain number and grain weight but the nature of this negative relationship is not necessarily competition for limited assimilates during grain filling (Miralles and Slafer, 1995; Acreche and Slafer, 2006), as grains growth is most frequently sink-limited (Slafer and Savin, 1994; Kruk *et al*., 1997; Borrás *et al*., 2004; Shearman *et al*., 2005; Cartelle *et al*., 2006; Acreche and Slafer, 2009; Serrago *et al*., 2013, González *et al*., 2014; Sanchez-Bragado *et al*., 2014). Consequently, yield gains can be achieved by improving any of its major components, but significant gains would be almost exclusively dependent on increments in the number of grains per unit land area (Slafer *et al*., 2014).

Therefore, understanding the mechanisms involved in defining the grain number would allow to identify traits which might be critical to increase grain yield. Although some fertile florets may not set grains, the usual grain setting ranges achieved under field conditions are very large (>70 %; Savin and Slafer, 1991; Ferrante *et al*., 2013*a*), which is expected in a cleistogamous plant species. Therefore grain number would be largely determined by the number of fertile florets and the latter is simply the consequence of floret generation/degeneration dynamics. Thus, the importance of how the environmental conditions affects the developmental phases lies on the effect on the organs that are being generated and the define the potential grain yield (Slafer *et al*., 1996; Slafer, 2003). Manipulating the critical period becomes an important tool to increase the spike fertility (Fischer 2011; Foulkes *et al*., 2011; García *et al*., 2011).

Although soil fertility, water and nutrients availability and radiation were eventually reported to affect wheat development rates (Rawson 1993; Evans 1987; Rodriguez *et al*., 1994) their impact resulted weak and not always consistent (e.g. Hall *et al*., 2014). The main factors affecting the crop developmental phases are: temperature, photoperiod, vernalisation and their interaction (Slafer *et al*., 2015). When all are fully satisfied for a normal development, differences among cultivars can be the result of the apparently intrinsic effects of earliness *per se* genes (Slafer *et al*., 2015, and references therein).

Temperature affects the development rate, at low temperatures the rate is reduced and the phases length is increased, at high temperatures the rate is increased and the phases length reduced (Slafer and Rawson, 1994). The rate of development increases linearly between the base and optimum temperatures in all crops, that is why the thermal time model (Monteith, 1984) is used to follow the development, when the thermal ranges explored are between these two cardinal temperatures. Temperature affects all the developmental phases during the whole growth cycle (Miralles and Slafer, 1999) in all crops.

Photoperiod response is more complex. Wheat plants are classified as "long-day" due to a reduction in the developmental phases when the photoperiod is lengthened (Slafer *et al*., 2015). Most reports described the effects on time to anthesis due to different photoperiod sensitivity (Law, 1987; Worland, 1996; Worland *et al*., 1998); moreover, some studies reported the effect on time to anthesis/heading due to photoperiod manipulation under field conditions (Stelmakh, 1998; Whitechurch and Slafer 2001). Photoperiod sensitivity is the delay in duration of certain stage of development per hour difference between actual and optimum photoperiod that is why it is considered more a quantitative (delayed development) than a qualitative (no further development) response. It has been already recognized that photoperiod sensitivity changes throughout wheat development (Slafer and Rawson, 1996; Miralles and Richards, 2000; González *et al*., 2002). However little research has focused on the effect of photoperiod on the individual pre-anthesis phases (González *et al*., 2005*c*) and this represents a powerful tool for plant breeding to fine-tune the flowering time avoiding any stress which can impact on grain yield.

Other important factor affecting the developmental phases is vernalisation, the accumulation of coldness which accelerates the development of sensitive cultivars (winter vs spring) (Slafer *et al*., 2015). In winter wheats lack of vernalisation delays the flowering while when vernalisation is saturated they flower coincidently with spring wheats (Trevaskis *et al*., 2003). Optimal vernalisation temperature was defined between 3.8 and 6ºC with temperatures in the range from -1.3 and 15.7ºC having some vernalisation effect, being it more reduced as the thermal condition is further away from the optimum vernalisation values (Porter and Gawith, 1999, an references therein). Vernalisation response affects the duration of the vegetative phase (Flood and Halloran, 1986; Ritchie, 1991; Robertson, *et al*., 1996) and the early and late reproductive phases in interaction with photoperiod (González *et al.*, 2002, 2003*b*).

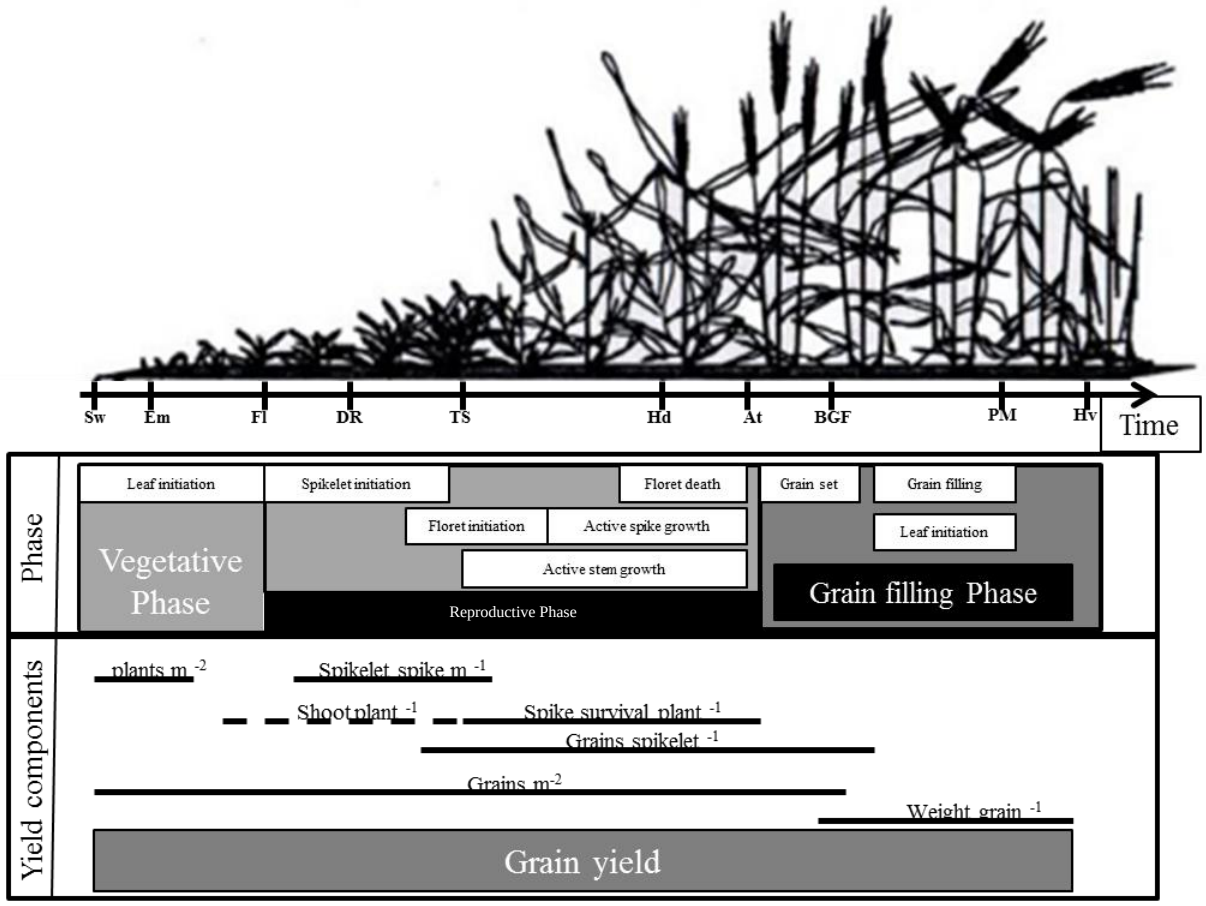

Figure 1.2. Diagram shows the stage of sowing (Sw), emergence (Em), floral initiation (FI), initiation of the first double ridge (DR), terminal spikelet initiation (TS), heading (Hd), anthesis (At), beginning of the grain-filling period (BGF), physiological maturity (PM), and harvest (Hv). Boxes indicate the periods of differentiation or growth of some organs within the vegetative, the reproductive and grain-filling phases and timing when different components of grain yield are produced. Extracted and adapted from Miralles and Slafer, 1999.

When all the crop requirements of vernalisation and photoperiod are satisfied, there are still residual differences in time to heading among cultivars. These differences are referred to as intrinsic earliness which reflects the basic development rate of a genotype, a term referring to differences in development that are intrinsic and therefore independent on the environment. Then, genetic factors determining differences in intrinsic earliness were termed earliness *per se* (Slafer *et al.*, 2015, and references therein). However, this independence of the environment (which is relevant as the earliness *per se* of genotypes would be maintained in any growing condition) is true for vernalizing temperatures and daylength, but it has been assumed respect to temperatures. As temperature has an universal effect on developmental rates, it has been implicitly assumed that there would not be significant genetic differences in sensitivity to temperature; an issue that is debatable (Appendino and Slafer, 2003; Karsai *et al.*, 2013), and will be considered in the present thesis as well.

3. Fertile florets

As mentioned above, grain number is mostly associated to the number of fertile florets produced (Kirby, 1988; Slafer and Andrade, 1993; Miralles *et al*., 1998, 2000) mainly due to wheat is a cleistogamous species. The number of fertile florets was reported to be affected by i) genotypic differences (Miralles *et al*., 1998), and ii) environmental conditions including both resources such as availability of nutrients (Sibony and Pinthus, 1988; Ferrante *et al*., 2010), and signals, such as red/far-red light ratios (Ugarte *et al*., 2010) and photoperiod (Miralles *et al*., 2000; González *et al*., 2003*b*; González *et al*., 2005*a*; González *et al*., 2005*b*; Ghiglione *et al*., 2008; Serrago *et al*., 2008).
Floret development starts in the central spikelets in the positions most proximal to the rachis and progresses from there to most distal positions (Sibony and Pinthus, 1988) and continue until around booting (Kirby, 1988; González *et al*., 2003*a*, González *et al*., 2005*a*, Ferrante *et al*., 2010, 2013*a*). Usually, from 6 to 12 floret primordia per spikelet are being developed (Sibony and Pinthus, 1988; Youssefian *et al*., 1992; Miralles *et al*., 1998), but most of them degenerate before anthesis indicating a possible competition for assimilates against the stems and spikes determining the rate (González *et al*., 2005*a*; Ghiglione *et al*., 2008) and the onset of floret mortality (González *et al*., 2011; Ferrante *et al*., 2013*b*).
The rate of development of the floret mortality period was reported to be accelerated by extending photoperiod, reducing the duration of the phase (González *et al*., 2003*b*, González *et al.,* 2005*a*) and was associated to the expression of genes linked to floral development cell proliferation and programmed cell death. Aborting primordia anatomy including the vacuoles formation and authophagosomes suggested a programmed cell death rather than passive death while another mechanism involved in floret abortion is the decrease of soluble sugars during the spike growth (Slafer *et al*., 2015).

3.1 Floret development dynamics

To analyze differences in floret primordia development among genotypes and detailed analysis of the generation and degeneration dynamics, the quantitative scale proposed by Waddington *et al*. (1983) is most frequently used. This scale considers the changes in the morphology of the barley and wheat pistil until anthesis (Fig. 1.3). Floret primordia stages are considered individually from when the scale reaches the stage of W3.5 and follows developing through time when the successively floret primordia start to develop. Floret initiation in central spikelets occurs around the apical stage of terminal spikelet. The initiation continues until reaching the maximum number of floret primordia initiated and then it follows the floret mortality, with the consequence of that a little proportion of initiated primordia survives and reaches the fertile floret stage at anthesis, defining the final number of fertile floret per spikelet. Floret survival tends to be low (Miralles *et al*., 1998; Ghiglione *et al*., 2008; González *et al*., 2011; Ferrante *et al*., 2013*b*) but critical to establish spike fertility (González-Navarro *et al*., 2015).

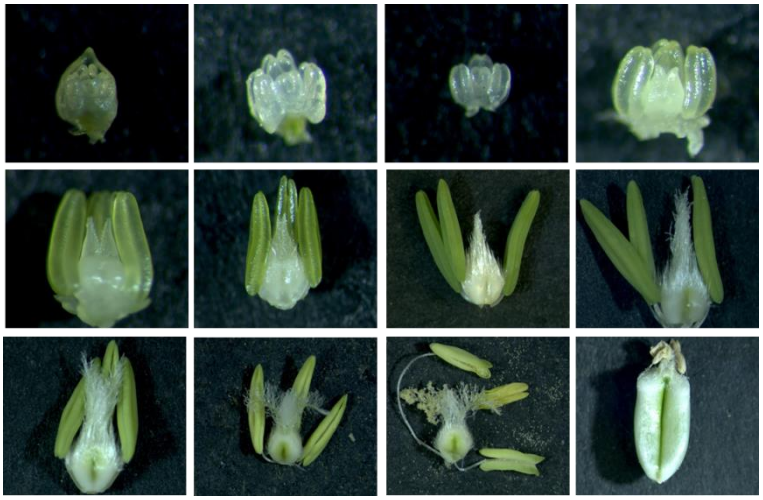

Figure 1.3. Illustration of different floret development stages. Changes in the pistil morphology is followed and characterized using the Waddington *et al*., 1983 scale allowing establishing differences among florets positions within a particular genotype and among different genotypes.

3.2 Wheat development as affected by genetic control

Allohexaploid wheat presents three genomes, A, B and D which confers high variability an more complex genetic regulation which is mostly based on the effect of different genes along the cycle: the photoperiod response (*Ppd*), vernalisation (*Vrn*) and the earliness *per ser* (*Eps*) (Slafer, 2012; Gomez *et al*., 2014).

Photoperiod response is regulated by a group of genes in the short arm of chromosome 2: *Ppd-D1* (in D genome), *Ppd-B1* (in B genome) *Ppd-A1* (in A genome). Photoperiod insensitivity is a dominant response mainly conferred by *Ppd-D1a* allele (Worland and Law, 1985; Worland, 1996; Beales *et al*., 2007) although *Ppd-B1* was also reported to be an important source (Scarth and Law, 1984; Tanio and Kato, 2007) while Bentley *et al*., 2011 reported a reduction in flowering time due to photoperiod insensitivity in the A genome in synthetic hexaploid wheats with the strength intermediate between that of the insensitivities in the D and B genomes. Besides, photoperiod insensitivity in 2D is associated with a 2089-bp deletion upstream of the coding region (Beales *et al*., 2007) while in B genome no sequence mutation was found; but Díaz *et al*., 2012 reported alleles conferring early flowering had an increased copy number an altered gene expression and in the A genome of tetraploid wheat there is a 1027 or 1117 bp deletions upstream of the coding region (Wilhelm *et al*., 2009).

Vernalisation response is regulated by *Vrn-A1 (Vrn1)*, *Vrn-B1* (*Vrn2*) and *Vrn-D1 (Vrn3)*, in the middle of the long arm of chromosomes 5A, 5B, 5D. Dominance in one or more loci removes the vernalisation requirement giving a spring phenotype while the winter ones present recessive alleles. It was reported that alelles like *Vrn-A1a* conferred vernalisation insensitivity (Appendino *et al*., 2003; Yan *et al*., 2004). Besides, additional loci like: *Vrn2*, *Vrn3* and *Vrn4* are involved but less information about how they act in wheat is reported. In barley $VrnH_3$ was characterized to play an important role between the vernalisation and photoperiod pathways and polymorphisms in its promoter and in the intron 1 were shown to contribute to variation in flowering time under field conditions, particularly with evident effects after jointing stage (Casas *et al*., 2011).

Earliness *per se* gene response is more quantitative with many genes detected across different chromosomes. These genes have, in most cases, effects of a magnitude much smaller than the *Ppd* and *Vrn* genes and are useful for fine-tuning adaptation. In addition they may interact with photoperiod and vernalisation. The earliness *per se* locus *Eps-A^m1*was recently mapped within 0.8 cM interval on chromosome 1A^mL of diploid *Triticum monococcum* L., and it was shown that its effect was modulated by temperature (Bullrich *et al*., 2002). *Eps-A^m1* and the *Eps-3A^m* loci were reported to determine the number of spikelets and grains per spike and regulate flowering time (Bullrich *et al*., 2002; Lewis *et al*., 2008; Gawroński *et al*., 2014). However, few works are available due to they were mapped in crosses segregating for *Ppd* and *Vrn* masking their effects (Zikhali *et al*., 2015). Recently, Zikhali *et al*., 2015 described a deletion of the chromosomal region ELF3 was linked to the earliness *per se* locus *Eps*-D1 which causes early flowering in bread wheat.
As it was previously mentioned, there are many reports of the effects of these genes on developmental rates towards heading/anthesis and on yield components, however, few works considered the same genetic background using near isogenic lines and, to the best of my knowledge, virtually none analyzed the effects of these genes on the floret generation/degeneration dynamics.

This Thesis presents the detailed analyses of the effects of i) different genetic background among modern cultivars, ii) *Ppd* and iii) *Eps* genes on floret development determining the spike fertility level in wheat.

4. Objectives

The main aim of this Thesis was to understand the complex floret generation/degeneration mechanisms that define the number of fertile florets at anthesis as affected by the genetic variability among modern cultivars, the *Ppd* and *Eps* genes.
The general hypothesis was that the number of fertile florets at anthesis resulted from the floret generation/degeneration dynamics would be reduced as the duration of the phase of floret development is reduced by genetic differences or the introgression of *Ppd*-insensitivity alleles or *Eps*-early alleles.

To contrast the hypothesis different specific objectives were outlined:
To analyze the dynamics of floret primordia initiation and degeneration in a set of modern cultivars of hexaploid wheat and to determine which were the most critical parameters of such dynamics in establishing genotypic differences in the number of fertile florets at anthesis and to find out a possible role of synchrony in the floret primordia development in determining genotypic differences in floret survival (**Chapter III**).
To evaluate the effect of the different allelic combination into the same background of the *Ppd* (**Chapter IV**) and *Eps* (**Chapter V**) genes on the final number of fertile florets at anthesis characterizing floret development patterns during the stem elongation phase, and quantifying the dynamics of floret primordia initiation and degeneration to shed

light on some physiological causes of the photoperiod insensitivity and the *Eps* early response on the reproductive fertility of the crop.
To evaluate the possible interaction between the *Eps* genes and temperature on the number of fertile florets and the floret development dynamics (**Chapter VI**).

5. Outline of the present Thesis

This Thesis is divided into seven chapters. These chapters include the general introduction (Chapter I), a description of the general procedures and the methodology that was used in most chapters (Chapter II). This chapter was thought to avoid being repetitive, although specific materials and methods used only in particular chapters are included within the corresponding chapter. Then, four experimental chapters, Chapters III, IV, V and VI, are included presenting the results of the different experiments carried out to text the hypothesis. Finally, Chapter VII contains a general discussion ending with the conclusions of the thesis. This last chapter highlights key results and conclusions of each chapter whilst exhibits the consistencies and inconsistencies across the different studies reported throughout the thesis.

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Chapter II: General procedures

The research in which this thesis is based on has been conducted on different experiments. The details of each experiment will be provided in the Materials and Methods section of each research chapter, but as several methods were common to many if not all the experiments to avoid unnecessary repetitions across the different chapters, I prepared this specific Chapter aimed to describe and to explain (and in occasions to illustrate) all the methods (determinations, measurements and analyses) that were common to more than one experiment. Naturally determinations, measurements and analyses done only in one experimental setup were described in the Materials and Methods section of the particular corresponding experimental Chapter. Avoid the repetitions will both reduce the length of the experimental chapters and improve the readability of the thesis (by consistently referring to a single methodological chapter from the different experimental chapters).

1. Background conditions

In all the experiments, the treatments consisted essentially of the genotypes grown in field conditions (with the exception of the experiment in controlled conditions reported in Chapter VII where treatments were the factorial combination of selected genotypes and growing temperatures). The background conditions in these experiments were as stress-free as possible: (i) weeds, insects and diseases were controlled or prevented using conventional commercial pesticides with doses recommended by their manufacturers; (ii) irrigation was applied when needed in order to avoid water stress: flood-irrigation (Experiments in Chapter III, IV and last growing season of experiments in Chapter V) or sprinkle-irrigation (First and second growing seasons of the experiments in Chapter V) during the whole growing cycle and plots (pots in the controlled environment experiment) were fertilised to avoid any nutrient deficiency.

2. Measurements and analyses

In table 2.1, the experiments are summarized and listed in order to show the amount of treatments within each chapter; however the details of the experimental conditions and plant material used are provided in the Materials and Methods section of the specific experimental Chapter.

2.1 Developmental stages

To determine stages of development, plots were individually inspected from once a week to every second day (depending on temperatures and closeness of the stages) and the timings of terminal spikelet and anthesis (DC 6.5 of the scale of Zadocks *et al.*, 1974) were recorded. The timing of anthesis stage in each experimental unit was taken when 50% of plants in each experimental unit reached that stage. Thermal time was calculated from sowing to terminal spikelet and DC 6.5 as well as for the duration of the phase between them: the late reproductive phase (LRP) when floret development takes place. For this purpose, the daily average temperature [(Tmax+Tmin)/2] was summed between the intervals considered. This computation of thermal time assumed a base

temperature of 0ºC and that Tmin was never below this threshold and Tmax was never above the optimum temperature for developmental progress.

Table 2.1. Location, growing season and plant material of the experiments carried out in the present Thesis (and the specific chapter in which results from them are presented).

Chapter	Location	Growing season	Plant material
III	Field experiment, Bell·lloc d'Urgell, Spain	2012-2013	9 modern cultivars
III	Field experiment, Bell·lloc d'Urgell, Spain	2013-2014	9 modern cultivars
IV	Field experiment, Bell·lloc d'Urgell, Spain	2012-2013	12 *Ppd* NILs + Paragon
IV	Field experiment, Bell·lloc d'Urgell, Spain	2013-2014	12 *Ppd* NILs + Paragon
V	Field experiment, Algerri, Spain	2012-2013	20 *Eps* NILs (4*)
V	Field experiment, Algerri, Spain	2013-2014	32 *Eps* NILs (6*)
V	Field experiment, Bell·lloc d'Urgell, Spain	2014-2015	12 *Eps* NILs (2*)
VI	Growth chamber experiment, Lleida, Spain	2015-2016	4 *Eps* NILs at 6, 9, 15, 21, 24ºC (2*)
VI	Growth chamber experiment, Norwich, United Kingdom	2015-2016	4 *Eps* NILs at 12 and 18ºC (2*)

*Number of contrasts considered for the analysis: *Eps*-late vs -early for each particular parental cross and chromosome.

2.2 Number of fertile florets at anthesis

Samples of aboveground biomass were taken at anthesis from each replicate of each genotype from a sample area of 0.5 m long of a central row, which had been labelled few weeks after emergence warranting that the plant density and uniformity was that ideally expected in the sample area and its borders. The number of fertile florets (style and stigmatic branches spreading and green or yellow anthers visible) was counted in main-shoot and tiller spikes, within each spikelet from one side and all along the entire spike as it is illustrated in Fig. 2.1.

2.3 Floret development and living primordia dynamics

From the onset of stem elongation onwards, three plants from each genotype were sampled frequently (normally 2-3 times a week, depending on temperature). The spikes (Fig. 2.2A) of the main shoots were dissected under microscope (Leica MZ 7.5, Leica Microscopy System Ltd, Heerbrugg, Switzerland) and then within particular central spikelets (Fig. 2.2B) floret primordia were counted and the stage of development of each primordium (Fig. 2.2C) was determined following the scale described by Waddington *et al*., (1983).

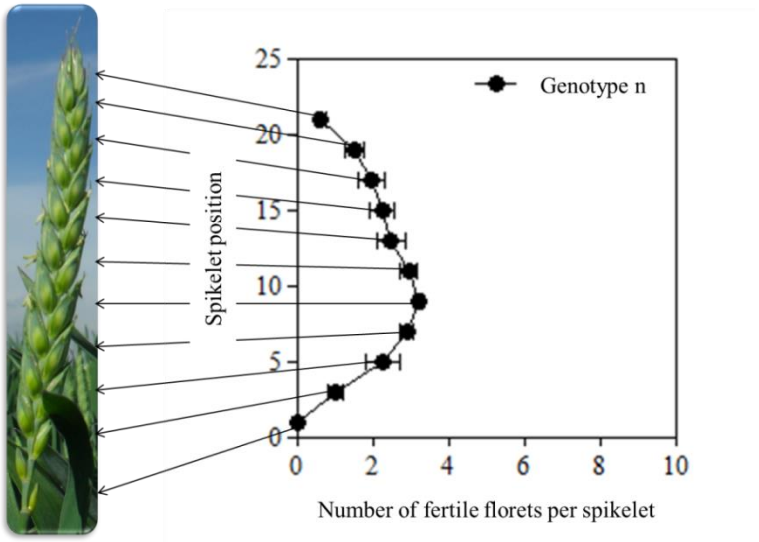

Figure 2.1. Illustration of mapping fertile florets. The number of fertile florets in each spikelet position from one side of the spike was counted from the basal to the apical positions including the terminal spikelet.

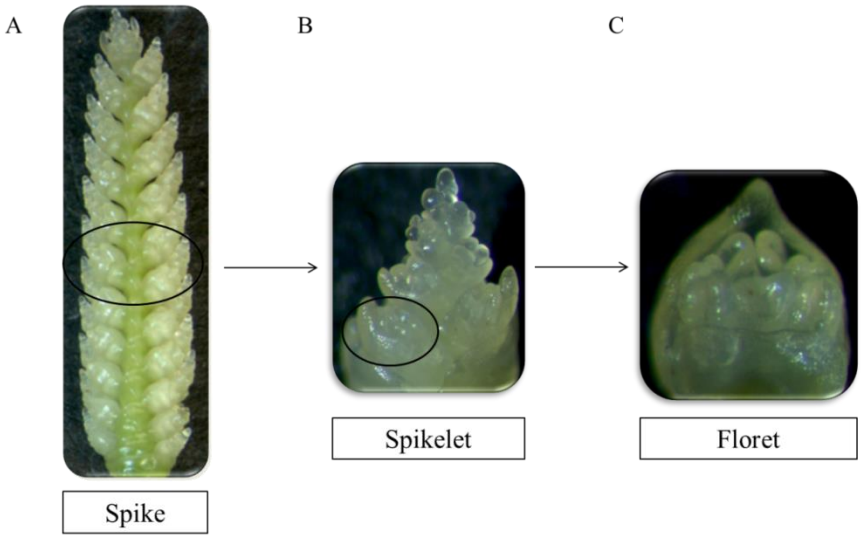

Figure 2.2. View of: the entire spike with all the spikelets (A), a particular spikelet with the floret primordia (B) and a floret primordium (C) under microscope.

Pistil development progress was observed in each floret primordia as shown in Fig. 2.3 from the early stages until W10, when florets were considered fertile or until the maximum Waddington stage reached by each floret primordium that did not reached W10. Florets were numbered from 1 to n, from the closest to the most distal positions respect to the rachis, respectively. The Waddington score used for describing the pistil development was plotted against the thermal time from anthesis describing the floret development dynamics for each floret primordium within the same genotype (Fig. 2.4) and for all the genotypes. This allowed determining the timing of the onset and the duration of development for each particular floret primordium. It also showed the florets which reached the fertile stage (W10) as well as the most advanced developmental stage reached by the primordia that never became fertile florets.

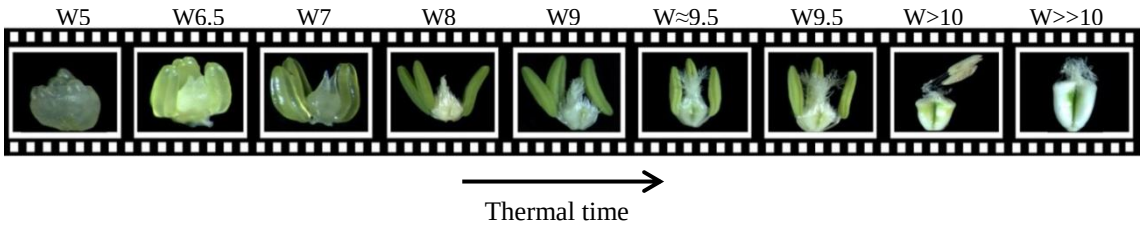

Figure 2.3. Floret development along thermal time from early stages until post anthesis stages when the fecundation has already occurred. Above each picture the stage of development is indicated following the scale of Waddington *et al*., (1983).

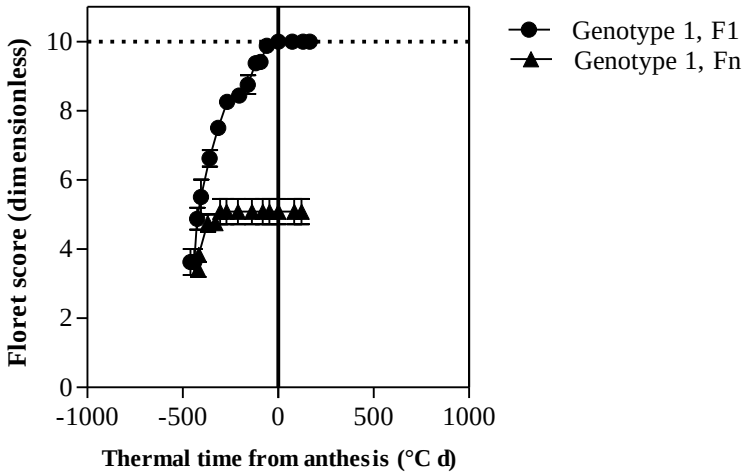

Figure 2.4. Floret score using Waddington scale against thermal time from anthesis for the floret primordium closest to the rachis (F1) of the hypothetical genotype 1, chosen as example, (closed circles) and for a distal floret primordium (Fn) of the same genotype (closed triangles).

In order to analyze and compare each floret development dynamics among treatments, each floret of each genotype in the same position (F1, F2,.., Fn) were plotted together (Fig. 2.5). Differences between genotypes could be observed either as shifts among the curves for the same floret (Fig. 2.5, left panel), meaning differences in the time of development initiation and on the duration of the floret development phase, or as different scores reached by the same florets (Fig. 2.5, right panel).

The number of living floret primordia within spikelets was plotted against thermal time from anthesis for each genotype. This shows the number of primordia that were developing normally at each sampling timing in the central spikelets from the beginning of floret generation (florets were considered to be a single primordium at W3.5, before that stage what the scale refers to the stage of development of the spike as a whole, not of the individual florets) until the maximum of floret primordia is reached (floret development generation phase: TT_G). In addition, floret initiation was defined when floret 1 reached the stage of Waddington (W) 3.5. After the maximum number of primordia initiated is reached, the floret mortality or degeneration starts (TT_D) until the number of fertile florets is finally established around anthesis. This number of fertile florets is therefore the end result of the generation and degeneration process and then

has as components the maximum number of floret primordia and the floret survival (Fig. 2.6, left panel) which was calculated as the ratio between the number of fertile florets at anthesis and the maximum number of floret primordia developed. In order to compare genotypes, living floret dynamics were plotted together showing differences in the duration of the floret generation/degeneration phase length, in the maximum number of floret primordia developed and the final number of fertile florets (Fig. 2.6, right panel).

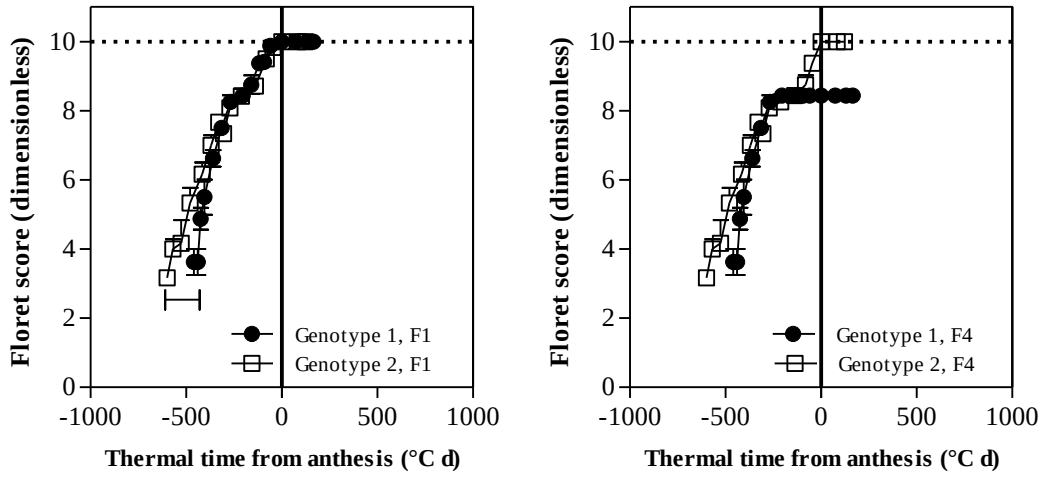

Figure 2.5. Floret score using Waddington scale against thermal time from anthesis for floret 1 (left panel) and floret 4 (right panel) of hypothetical genotypes 1 (closed circles) and 2 (open square), the line inset the right panel indicates the shift among the curves indicating differences in the timing of the onset of floret initiation (left panel).

2.4 Synchrony in floret primordia initiation

The likelihood of a relatively distal floret primordia to become a fertile floret might be related to the synchrony of initiation of different florets. To estimate the degree of synchrony among the floret primordia initiation for each particular treatment, I determined the timing (thermal time) of the onset of floret initiation, as when each primordium reached W3.5, in the cases that did not coincide with any microscope observation; it was estimated using each floret development dynamics fitting a linear regression between floret score and thermal time, while the progression of development was clearly linear (Fig. 2.7), and estimating with the parameters of the linear regression the timing of W3.5 for that particular primordium. Once the timing of Waddington stage 3.5 of each floret primordium was calculated, each floret position was plotted against thermal time to its stage W3.5 (Fig. 2.8, left panel) and fitted a linear regression whose slope was the rate of floret primordia initiation, and its reciprocal the "plastochron" for floret primordia (i.e. the average thermal time interval between the initiation of two consecutive floret primordia). Even though the Waddington scale is strongly qualitative in nature, this allowed comparing genotypes in terms of synchrony of floret initiation events comparing the slopes of the linear regressions (Fig. 2.8, right panel, a_1 *vs* a_2) and the plastochrons for floret primordia.

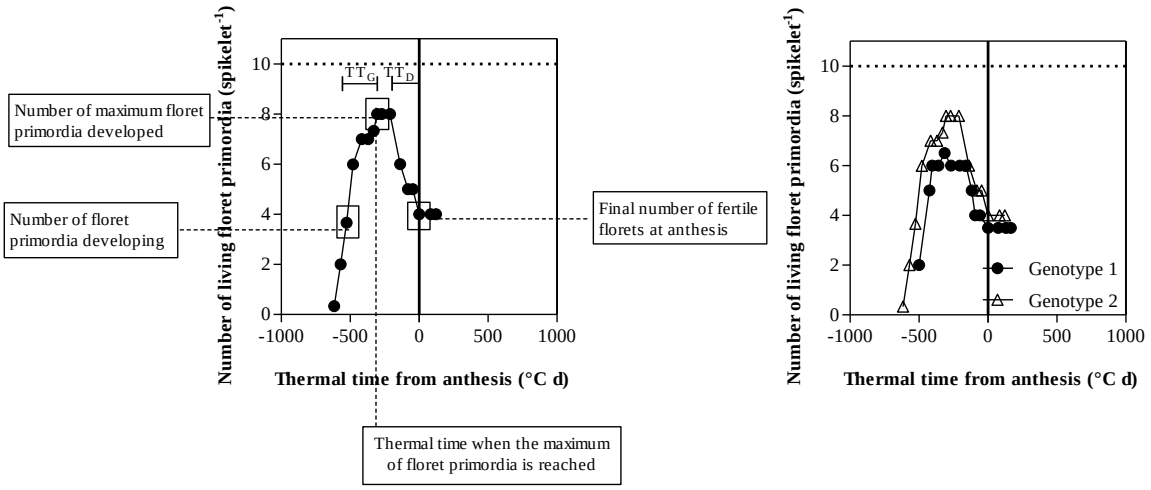

Figure 2.6. Number of living floret primordia against thermal time from anthesis for an hypothetical genotype. The number of living floret primordia for a particular thermal time, the number maximum of floret primordia developed and the final number of fertile florets are indicated, and the segments on the top indicate the length of the phases (in thermal time) of floret primordia generation (TT_G) and degeneration (TT_D) (left panel). Comparison of the number of living floret primordia per spikelet against thermal time between hypothetical genotypes 1 (closed circles) and 2 (open triangle) (right panel).

2.5 Statistical analyses

To determine the significance of genotypic differences I subjected the data to analysis of variance (ANOVA) or t-tests and to *a posteriori* contrasts, depending on the experiments: Fishers Least Significant Difference (LSD) or LSMeans Differences Student's t test to determine when differences between particular treatments were significant using JMP® Pro version 12.0 (SAS Institute Inc., Cary, NC, USA). To determine the degree of relationships between variables, linear regression analyses were performed.

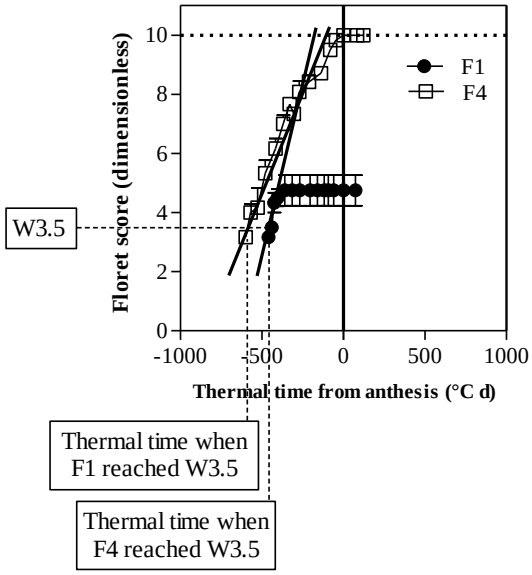

Figure 2.7. Illustration of the estimation of the timing of W3.5 for the floret primordium (F1, F4) through linear regression of each particular primordium developmental dynamics in the cases in which W3.5 did not coincide with a direct microscopic observation.

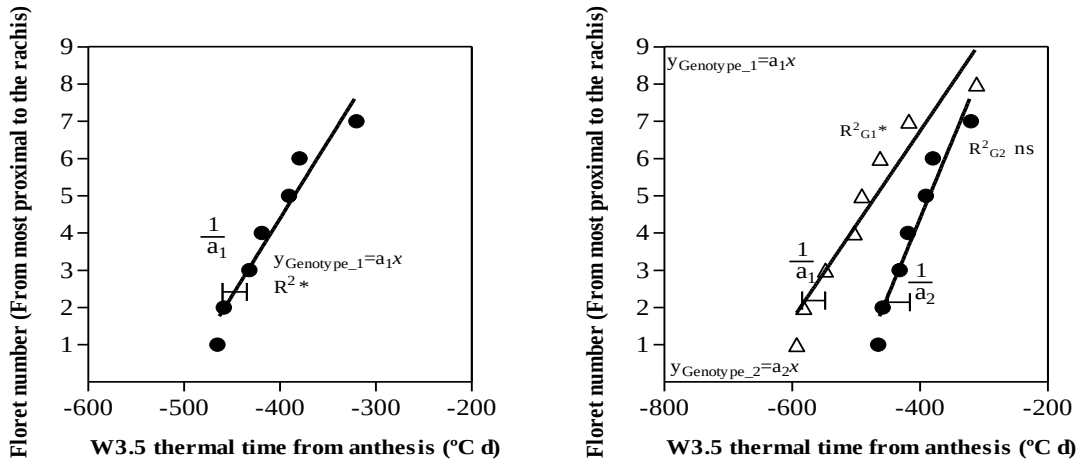

Figure 2.8. Illustration of the timing of W3.5 for each floret primordium in an hypothetical genotype. Slope indicated the rate of floret initiation (a_1), the R^2, the significance of the linear regression and the reciprocal of the slope to calculate the thermal time average between the appearance of two successive floret primordia are shown (left panel). Illustration of the timing of W3.5 for each floret primordium comparing two genotypes. Slopes of the linear regressions a_1 and a_2, R^2 and significances and the reciprocals of the slope for each genotype are also shown (right panel).

3. References

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Zadoks, J. C., Chang T. T., Konzak, C. F. 1974. "A Decimal Code for the Growth Stages of Cereals." *Weed Research* 14 (6): 415–21.

Chapter III: Variation between elite cultivars on the physiological determinants of fertile florets

1. Introduction

Large grain yield gains are necessary to satisfy the current and future food demands. Even though grain yield is a multifactorial trait, large increases seem impossible without increasing grain number (Slafer *et al*., 2014). As wheat is a cleistogamous species, grain number is largely associated with the number of fertile florets (Kirby, 1988; Slafer and Andrade, 1993; Miralles *et al*., 1998; 2000). It was already explained how the number of fertile florets is affected by environmental factors such as availability of resources and photoperiod conditions, as well as by genotypic factors such as the introgression of *Rht* alleles (Chapter I). However few works reported in detail the dynamics of processes determining the number of fertile florets at anthesis: onset and rate of floret primordia initiation, number of maximum floret primordia developed and the floret mortality process determined by duration and rate of floret primordia degeneration. A recently published study, Guo *et al*., (2016), reported that even though there was clear variability among cultivars of European wheat under field conditions in the maximum number of floret primordia initiated per spikelet, genotypic differences in number of fertile florets was much more strongly dependent upon differences in floret survival than in maximum number of primordia developed. This is consistent with previous reports (e.g. Ferrante *et al*., 2013, with very few durum wheat cultivars; González-Navarro *et al*., 2015, with lines of hexaploid wheat of a germplasm panel of CIMMYT).

Modern cultivars adapted to a particular region are the most valuable germplasm used by breeders aiming to improve yield (or any other complex agronomic trait), by crossing elite x elite expecting transgressive segregation for that complex trait but within a progeny with proven agronomic value (parents would be already well adapted, tolerant to main diseases and with appropriate "agronomic type"). As these genotypes are all high-yielding, the likelihood of achieving that transgressive segregation would increase if the parents crossed possess particular physiological traits determining yield, and eventually two different physiological traits may be complemented in some of the offspring. In this context, characterizing the main physiological traits responsible for yield is relevant for a proper, analytical process for selecting prospect parents for a cross. The dynamics of floret development (i) is one of the critical physiological processes determining differences in grain number and yield, (ii) has been analyzed only very few occasions (due to the complexity and difficulty in determining such dynamics), and to the best of my knowledge never before in a set of modern cultivars. Therefore, it was aimed to characterize the dynamics of floret primordia initiation and degeneration in a set of modern cultivars of hexaploid wheat and to determine which are the most critical parameters of such dynamics in establishing genotypic differences in the number of fertile florets at anthesis. The study also allowed making an analysis of a possible role of synchrony in the floret primordia development in determining genotypic differences in floret survival.

2. Materials and methods

2.1 General description

Two field experiments were carried out in fields (Table 3.1) very close to Bell·lloc d'Urgell, Lleida, NE Spain (Lat. 41°38N, Long. 0°47´E, 196 m above sea level). In both fields the soil type was a complex of Calcisol petric and Calcisol haplic (soil information collected from IUSS Working Group WRB, 2006), with soil Nitrogen availability warranted for crop growth.
Sowing rates were within the ranges used to optimize yield potential in the area (300 seeds m^{-2} during the first growing season and 350 seeds m^{-2} during the second one). Plots size was 4.8 m^2 (6 rows 0.2 m apart and 4 m long) in both growing seasons.

Table 3.1. Experiments field details: growing seasons, date of sowing and modern cultivars grown as treatments.

Experiment	Growing season	Sowing date	Cultivars (classified as)
1	2012-2013	22/11/2012	Nogal (winter)
			Ingenio (winter)
			Garcia (winter)
			Califa Sur (spring)
2	2013-2014	12/11/2013	Arthur Nick (spring)
			Sensas (spring)
			Atae (spring)
			Rodolfo 413MO (intermediate)
			Tribat I33* (intermediate)

* at the time of the experiments, this was an experimental advanced and promising breeding line (SAR32) that has now been registered as the commercial cultivar Tribat I33.

2.2 Treatments and design

Treatments were the 9 modern genotypes of wheat well adapted to the growing conditions of the region. They were at the time of the initiation of the study 8 commercial cultivars, released between 1999 and 2014 (GENVCE-Semillas Battle), and one advanced breeding line; but later the line ended registered as a new cultivar (Table 3.1). Please note that even though they were classified as winter, spring or intermediate (facultative) cultivars, in Mediterranean regions with mild winters, this classification is not relevant as agronomically all types are sown normally before the onset of winter (between mid-October and mid-December, most frequently in mid-November) and they reach anthesis with only few days of difference, in all cases within the optimal window of time for flowering.
In both experiments treatments were arranged in a randomized complete block design with three replicates.

2.3 Measurements and analyses

We determined the number of floret primordia periodically from terminal spikelet initiation to anthesis, when the number of fertile florets was counted in detail for each spikelet in both main-shoot and tiller spikes. The general procedures and all measurements and analyses were presented in detail in Chapter 2.
In order to illustrate with some details, and help to further understand, the physiological mechanisms behind the differences observed among modern commercial cultivars, we selected the two cultivars with extreme behaviors (considering the average over the two growing seasons in the number of the fertile florets at anthesis) to show genotypic differences in physiological determinants of fertile floret number, though the results are offered for each of the cultivars in each growing season in the Annex 3. Again to reduce the number of panels in each figure of the main text of this chapter, the floret development dynamics are shown for florets number 1, 3, 4 and 6 to summarize the effects, but the rest of floret dynamics of each floret primordia and for the rest of cultivars can be also seen in Annex 3. These floret positions were chosen because of their contrasting behavior in most cases: Floret 1 always is fertile in all genotypes; F3 and F4 are labile florets and therefore are the most sensitive to treatments, including genotypic differences, and are responsible for the differences in fertile floret number; and F6 never becomes fertile in any genotype. In addition, the timing of Waddington 3.5 stage, which allowed analyzing the floret initiation rate, for each floret primordia for the rest of each genotype is also shown in Annex 3.

2.4 Weather conditions

Weather data were recorded hourly at standard meteorological stations provided by the agro-meteorological network of Catalonia. Although both the first (264.3 mm) and the second (303.0 mm) growing seasons were rainier than the average of the previous six years (246.5 mm), flood irrigation was needed around flowering to avoid water stress. Temperatures were in general similar in both growing seasons (in turn similar to the average of the previous 6 years; Fig. 3.1). There was however a major difference in the average of the maximum temperature during April (when anthesis occurred for all cultivars in both growing seasons) which was clearly warmer in the second than in the first growing season (22.5 and 18.8ºC, respectively). In addition, both experimental seasons had warmer Aprils than the average of the maximum temperature in the six previous years (from 2007 to 2012) for that month (17.4ºC).

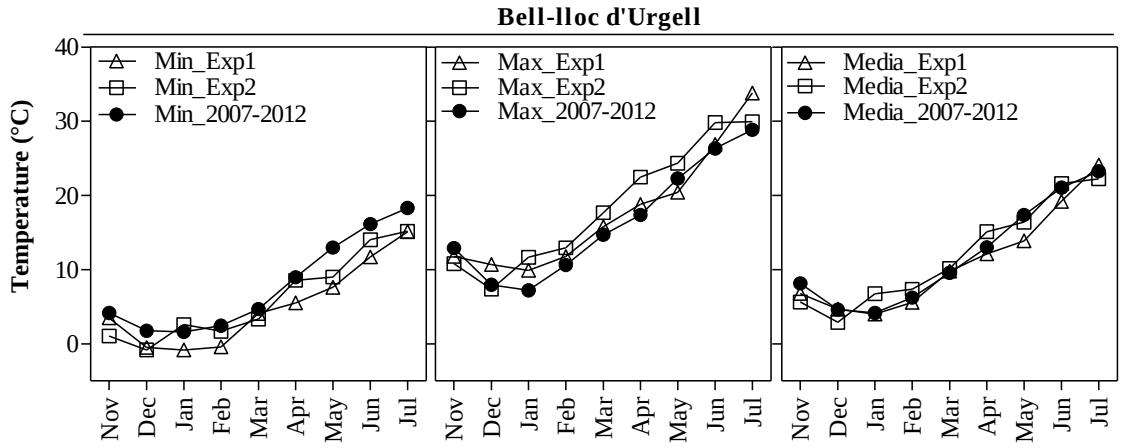

Figure 3.1. Weather conditions: Minimum (left panel), maximum (middle panel) and mean (right panel) temperatures averaged monthly in the first (Exp1) and in the second (Exp2) growing seasons and in the six years previous to the experiments (2007-2012).

3. Results

3.1 Number of fertile florets at anthesis

High variability was found in the number of fertile florets at anthesis among elite cultivars during both growing seasons (Fig. 3.2). Cultivar Nogal presented the highest number of fertile florets while Atae presented the lowest value with differences between them of *c.* 46% during the first growing season (Fig. 3.2, left panel). In the second growing season, the largest differences were found between Sensas and Arthur Nick cultivars of *c.* 30% (Fig. 3.2, right panel). In addition and consistently, important and significant differences of *c.* 21% between Nogal and Atae cultivars were found in the second growing season as well (Fig. 3.2, right panel). In fact, in the second growing season the number of fertile florets of Nogal was not significantly lower than that of Sensas, and that of Atae was not significantly higher than that of Arthur Nick (Fig. 3.2, right panel). The average of the number of fertile florets at anthesis for each genotype across growing seasons showed that the largest and most significant differences were those between cultivars Nogal and Atae. This reasonable consistency across seasons is further supported by the magnitude and the nature of the GxE interaction. Regarding the magnitude, the mean square of the GxE interaction for the number of fertile florets per m^2 (43.878 fertile florets m^{-2}), even when statistically significant, was negligible compared with the mean square of the genotypic effect (154.791 fertile florets m^{-2}), more than 3-fold greater. Regarding the nature, much of the significance of this interaction is given by a single cultivar, Arthur Nick (Fig. 3.2)[1].

Thus, we selected cultivars Nogal and Atae to illustrate in detail the more complex traits measured in the main body of this Chapter, but the data characterizing each of the rest of cultivars are presented in Annex 3, and relationships between traits are performed considering the attributes for the 9 cultivars. Naturally all conclusions are supported by the overall results, not just those of Nogal and Atae.

[1] if the analysis is carried out disregarding Arthur Nick the mean square of genotype becomes almost 6-fold than that of the GxE

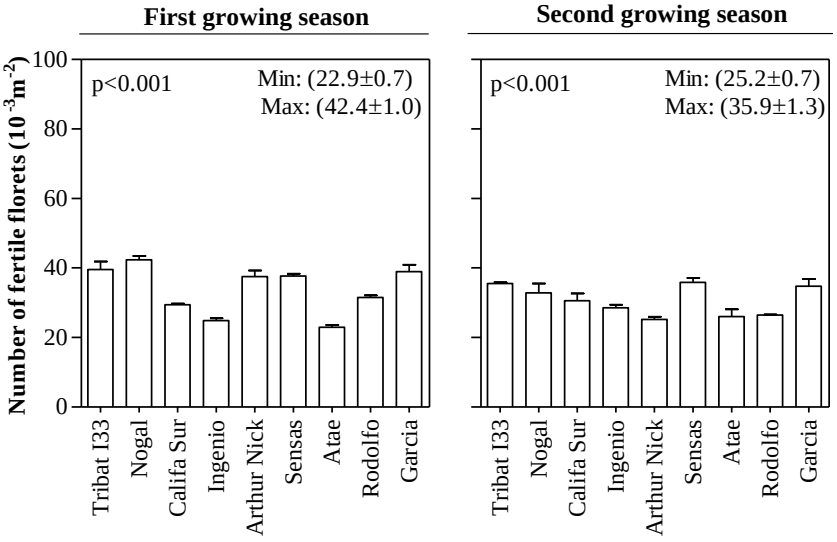

Figure 3.2. Number of number of fertile florets per square meter among the modern cultivars during the first (*left panel*) and second growing seasons (*right panel*). Mean ± SEM are represented, p-value of genotype source resulted from ANOVA. Besides, the minimum and maximum number of fertile florets among the genotypes are indicated inset each panel.

3.2 Relevance of fertile florets per spike and spikelet

The differences among genotypes in the number of fertile florets per m^2 were reasonably well explained by their differences in number of fertile florets per spike. In the first growing season the relationship was highly significant (Fig. 3.3, left panel) while in the second growing season it was significant only with 5.6% of probability (Fig. 3.3, right panel). In both growing seasons the differences in fertile florets per m^2 between Nogal and Atae reflected well these relationships (Fig. 3.3). Thus, the understanding of the genotypic differences in fertile florets per m^2 requires a more detailed analysis of the fate of florets in the spikes because differences among the modern cultivars in fertility of the spikes seemed more relevant than in fertility of tillers.

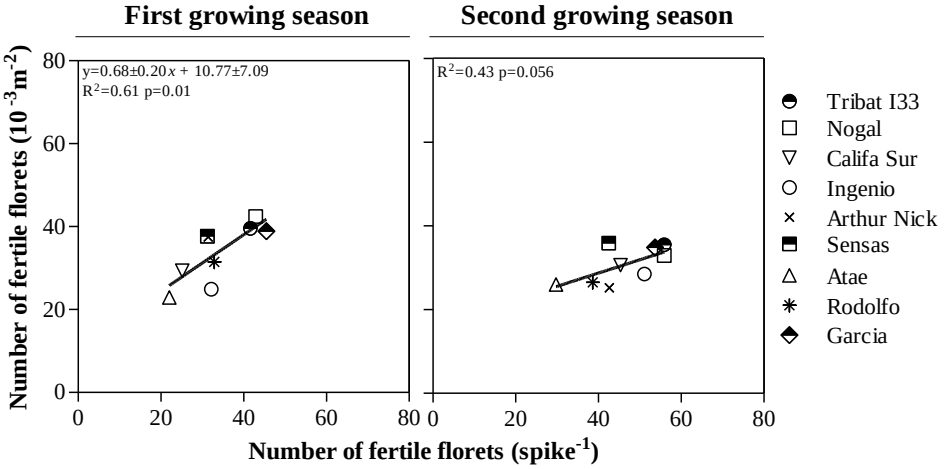

Figure 3.3. Relationship between the number of fertile florets at anthesis per square meter and the number of fertile florets per spike among the modern cultivars during the first (*left column*) and the second growing seasons (*right column*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

The number of fertile florets per spikelet was found to be consistently higher in Nogal than in Atae: the consistency of the difference was outstanding: it appeared along all the spikelets of the spikes of both main-shoots and tillers and in both growing season (Fig. 3.4) in line with the differences seen in the number of fertile florets per unit land between them (Fig. 3.2). Expectedly, the number of fertile florets "mapped" in main-shoot spikes was consistently higher than those in tiller spikes across most cultivars (Figs. 3.4 and A3.1-2). Thus, the number of fertile florets per spike in the main-shoot spikes reflected well the situation of all spikes in the canopy; naturally with values falling above the 1:1 ratio (spike fertility is higher in main-shoot than in tiller spikes (Fig. 3.5).

Moreover, as the differences in the mapping of fertile florets evidenced that they were fairly distributed along the different spikelets of the spike (see above, Fig. 3.3) the differences observed in the spike fertility among the modern cultivars were explained at least by *c.* 51% during the first growing seasons and even more during the second growing season (*c.* 65%) by differences in the fertility of the central spikelets (Fig. 3.6), where the floret development starts and the number of florets per spikelet is accordingly highest. Therefore, analyzing in detail the floret developmental processes determining at the end the level of fertility in the central spikelets of the main-shoot spikes seems a reasonable approach to understand the mechanisms behind the differences among modern cultivars in floret fertility.

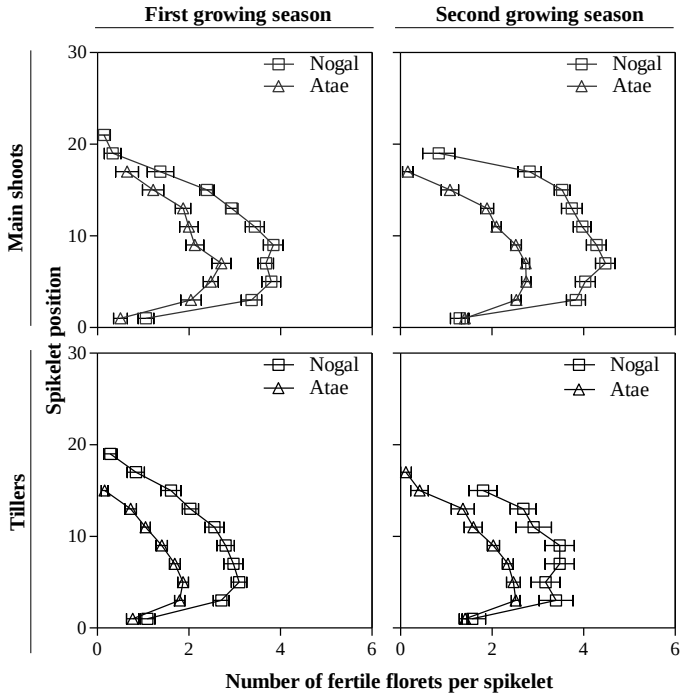

Figure 3.4. Mapping of fertile florets (fertility of each spikelet position on the main-shoot and tiller spikes) (top and bottom panel, respectively) for the selected cultivars Atae (open triangles) and Nogal (open squares) during the first (*left columns*) and the second growing seasons (*right column*). Each data-point is the average of all replicates and within each replicate the value was the average of 5 plants and the segment in each data-point stands for the standard error of the means.

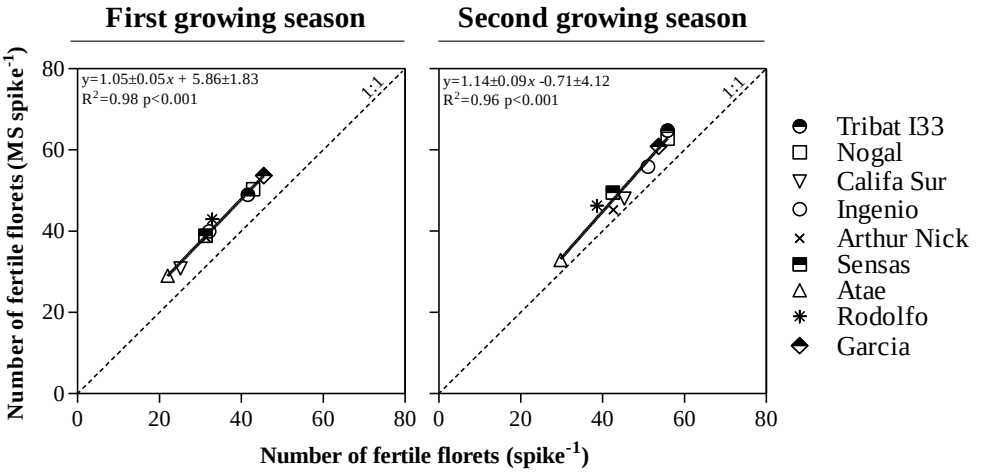

Figure 3.5. Relationship between the number of fertile florets per main-shoot spike and the number of fertile florets per spike among the modern cultivars during the first (*left column*) and the second growing seasons (*right column*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

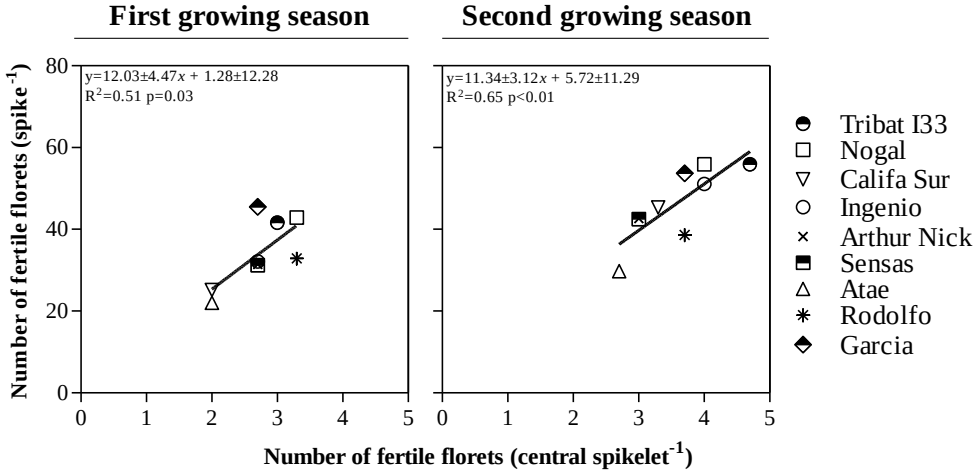

Figure 3.6. Relationship between the number of fertile florets per spike and the number of fertile florets per central spikelet among the modern cultivars during the first (*left column*) and the second growing seasons (*right column*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

3.3 Floret development and living floret primordia dynamics in the central spikelets

During both growing seasons, no major differences were found in the dynamics of development if the most proximal floret (Floret 1) in the two cultivars with extreme values of number of fertile florets at anthesis, beyond the fact that it seemed to have started a bit earlier (respect to the anthesis stage) in Nogal than in Atae. In both cultivars Floret 1 reached the fertile floret stage (stage 10) in all plants examined. On the other hand, Floret 3, which again started to develop earlier in Nogal than in Atae, finally reached the fertile floret stage in all plants examined only in Nogal (Fig. 3.7A) while in Atae this floret position did not reach the fertile floret stage in any of the plants examined (Fig. 3.7A, top panels) or did so only in some of them, that is why the most advanced stage of development averaging across all replicates was between 9 and 10 (Fig. 3.7A, bottom panels). When considering the fourth floret position, Nogal seemed to have started to develop earlier and it distinctly reached more advanced stages of

development at anthesis than (Fig. 3.7A). Floret 4 was fertile only in few (Fig. 3.7A, top panels) or in all (Fig. 3.7A, bottom panels) plants of Nogal whilst it did never reach the stage of fertile floret in Atae (i.e. in none of the plants examined in either of the two growing seasons). Floret 6 was always infertile (for the two cultivars and the two growing seasons), but even in this case the primordia of this floret position reached always more advanced stages in Nogal than in Atae, though the difference was more clear in the first than in the second growing season (Fig. 3.7A). All other floret primordia not shown in the main text of the chapter exhibited similar differences between Nogal and Atae: Floret 2 was also fertile in all plants of both cultivars in both seasons, floret 5 was intermediate between florets 4 and 6 and floret 7 was a bit less developed than floret 6 in both cultivars but again it developed a bit more in Nogal than in Atae (Fig. A3.3,4,5,6); and in the second season only Nogal started to develop the floret primordia 8 (Fig. A3.4). The dynamics of floret generation and degeneration (considering the living florets at each timing of development progress) can be analyzed through integrating the dynamics of development of each floret primordia (Figs. 3.7B; A3.21; and A3.22). Overall primordia considered and cross growing seasons, it can be summarized that Nogal presented a longer floret development phase, with a particular longer floret mortality phase in the first growing season (when differences in fertile florets was largest), reaching a slightly higher maximum number of living floret primordia and a higher number of final fertile florets than Atae (Fig. 3.7B). As in both growing seasons the magnitude of the difference in number of fertile florets at anthesis seemed much higher than that in maximum number of floret primordia (Fig. 3.7B) it seems clear that floret survival was more relevant than floret initiation in determining the genotypic differences in number of fertile florets between Nogal and Atae. Developmental patterns of each floret primordia in each of the two growing seasons for each of the other 7 cultivars can be found in Annex 3 (Fig. A3.7-A3.20). The dynamics of floret generation and degeneration derived for each of these other cultivars in both years also showed a large mortality rate: most floret primordia initiated did not progress in development enough to reach the stage of fertile florets (Fig. A3.21-A3.22). Consequently, even though there was variation in the maximum number of primordia initiated, differences among cultivars in number of fertile florets which was not (first growing season) or was slightly related to the their maximum number of florets[2] (Fig. 3.8, top panels). On the other hand, the number of fertile florets it was very strongly related to floret survival in both growing seasons (Fig. 3.8, bottom panels).

To elucidate possible causes of variation among elite material in failure of floret primordia to keep developing normally towards becoming fertile florets, we analyzed (i) synchrony of development of different primordia, being the conjecture that a major cause for genetic variation in labile primordia failing to become a fertile florets could be genotypic differences in synchrony of development of the different floret primordia; and (ii) overall duration of the process of floret development, being the alternative

[2] The term "slightly" refers not only to a relatively low coefficient of determination (compared with the one obtained with floret survival) but also that the range of variation in maximum number of floret primordia initiated was much smaller than the range of variation in number of fertile florets

conjecture that a genetic variation in phenological time for floret development would drive the genetic variation in critical determinants of the number of fertile florets.

3.4 Synchrony in floret primordia initiation

To estimate the degree of synchrony we analyzed both (i) the rate of initiation (stage 3.5) of different individual floret primordia at different positions within the spikelet (the higher the rate the more synchronized the development as the time elapsed between the initiation of successive floret primordia is the reciprocal of such rate), and (ii) stage of development of Floret 1 at the onset of initiation of Florets 3 or 4 (the most frequent labile floret primordia) (the earlier the stage more synchronized the development between the most advanced floret and those labile florets determining the number of fertile florets).

The former estimate represents an overall assessment of synchrony, generalized over all floret primordia (including the most proximal which are always fertile and the most distal ones that are never fertile). For its quantification we fitted linear regressions between the cumulative number of florets reaching the stage of development 3.5 in the Waddington *et al.*, (1983) scale and thermal time from anthesis. All regressions fitted (all in all 18 regressions) showed a strong linear pattern (R^2 from 0.88 to 0.99 and p-values from <0.001 to <0.01) (Fig. 3.9, A3.23-24). The latter is a more direct reflection of the specific synchronization between the most developed floret primordia and the labile florets. For its quantification we determined the thermal time before anthesis when each of these primordia (Floret 3 or Floret 4) started their individual development (at stage 3.5) and then determined the stage of development of Floret 1 at that time.

Interestingly, the floret primordia initiation rate and its reciprocal, the "plastochron" for floret primordia (the average thermal time interval between the initiation of two consecutive floret primordia), differed among cultivars (Fig. 3.9, A3.23-24). During both growing seasons floret primordia of Atae tended to initiate at a faster rate than those from Nogal (Fig. 3.9), although differences in the slope were statistically significant only during the second growing season.

Despite the existence of genotypic differences in synchrony of floret initiation, it failed in being a critical contributor to the genetic differences in either number of maximum floret primordia (Fig. 3.10, top panels), floret survival (Fig. 3.10, middle panels) or number of fertile florets (Fig. 3.10, bottom panels) in any of the two growing seasons. The genotypic differences in stage of development of Floret 1 at the onset of development of Florets 3 (ranging from or 3.7 to 5) or 4 (ranging from or 4 to 5.9) were not responsible for differences in either of these three parameters of the dynamics of living florets (Figs. A3.25 and A3.26). There was no relationship between these parameters and the Waddington stage of the most advanced floret (F1) at the thermal time when the maximum number of primordia is reached: the beginning of the floret death either (Fig. A3.27).

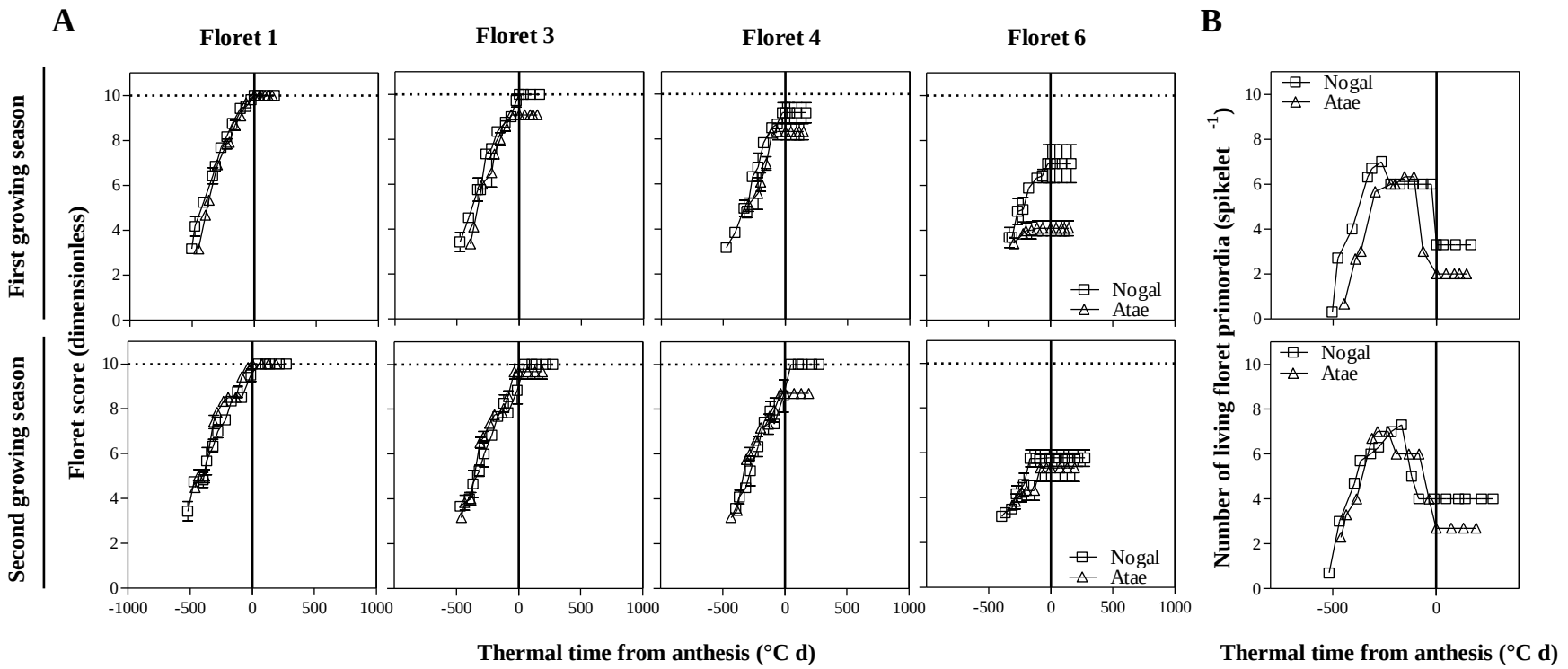

Figure 3.7. Dynamics of the floret development of F1, F3, F4 and F6 in central spikelets (A) and the number of living floret primordia (B) through thermal time from anthesis for the selected Atae (open triangles) and Nogal (open squares) cultivars during the first (*top panels*) and the second growing seasons (*bottom panels*). Data average from 3 plants, bars stands for the standard error of the means.

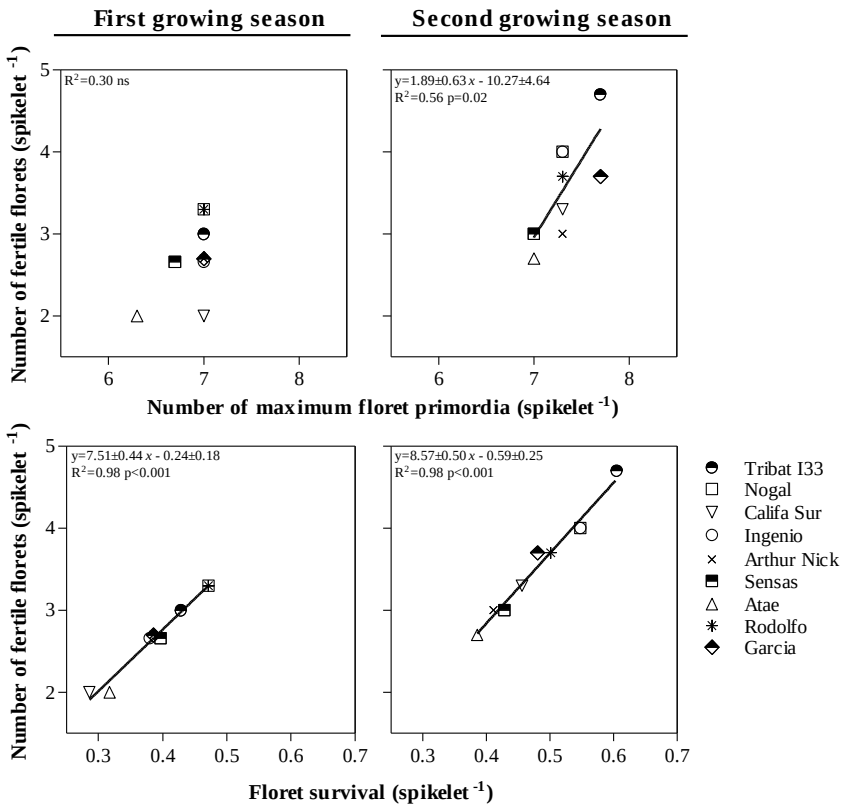

Figure 3.8. Relationship between the number of fertile florets in the central spikelets at anthesis and the number of maximum floret primordia developed in the central spikelets of the main-shoots spikes (*top panels*) and the relationship between the number of fertile florets in the central spikelets at anthesis and the floret survival in the central spikelets (*bottom panels*) among the modern cultivars during the first (left column) and the second growing seasons (right column). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

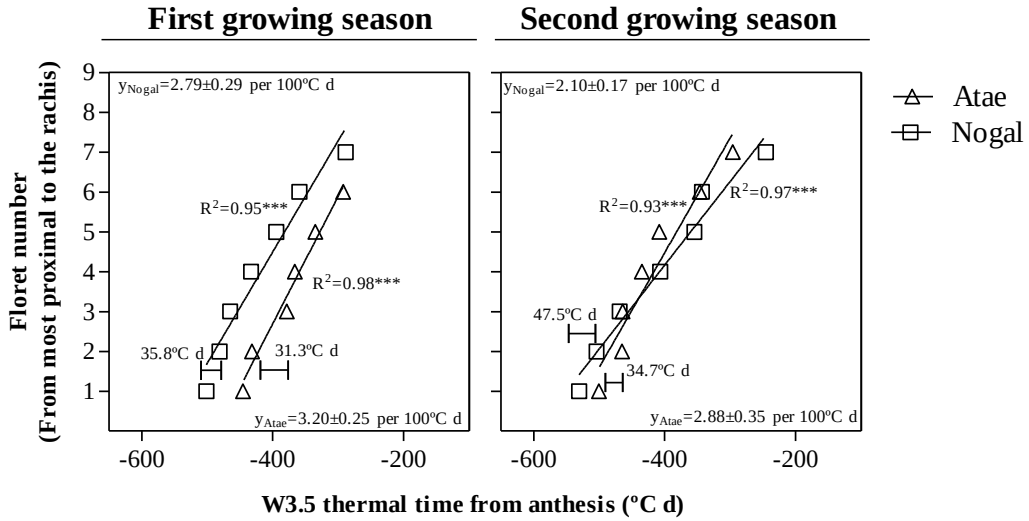

Figure 3.9. Timing of W3.5 for each floret primordium through thermal time from anthesis in Atae (open triangle) and Nogal (open square) cultivars during the first (left panel) and the second growing seasons (right panel). Inset each panel, the rate of floret initiation expressed in florets per 100°C d and the thermal time between the appearance of two following floret primordia are indicated. The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are also shown.

As the cultivars were all modern well adapted materials, variation in time to anthesis was not large (maximum difference was *c.* 150ºC d). But most of the difference was concentrated in genotypic differences in the length of the late reproductive phase, from terminal spikelet to anthesis (Fig. 3.11). This relationship seems relevant as what is a slight difference in duration for the whole period from sowing to anthesis seems a rather relevant difference in duration of the late reproductive phase, as it is during this phase when floret development takes place. The genotypic differences in duration of the period from the onset of individual floret initiation (when F1 reached the stage 3.5) to anthesis seemed to have influenced positively both floret survival and number of fertile florets. Although the relationships were significant in the first growing season (Fig. 3.12, left panels) and only a trend in the second growing season (Fig. 3.12, right panels), the trends became much more solid (p=0.06 in floret survival and p=0.07 in the number of fertile florets) if Garcia, the single cultivar having the longest duration of this phase -and one of the highest number of fertile florets-, were not taken into account.

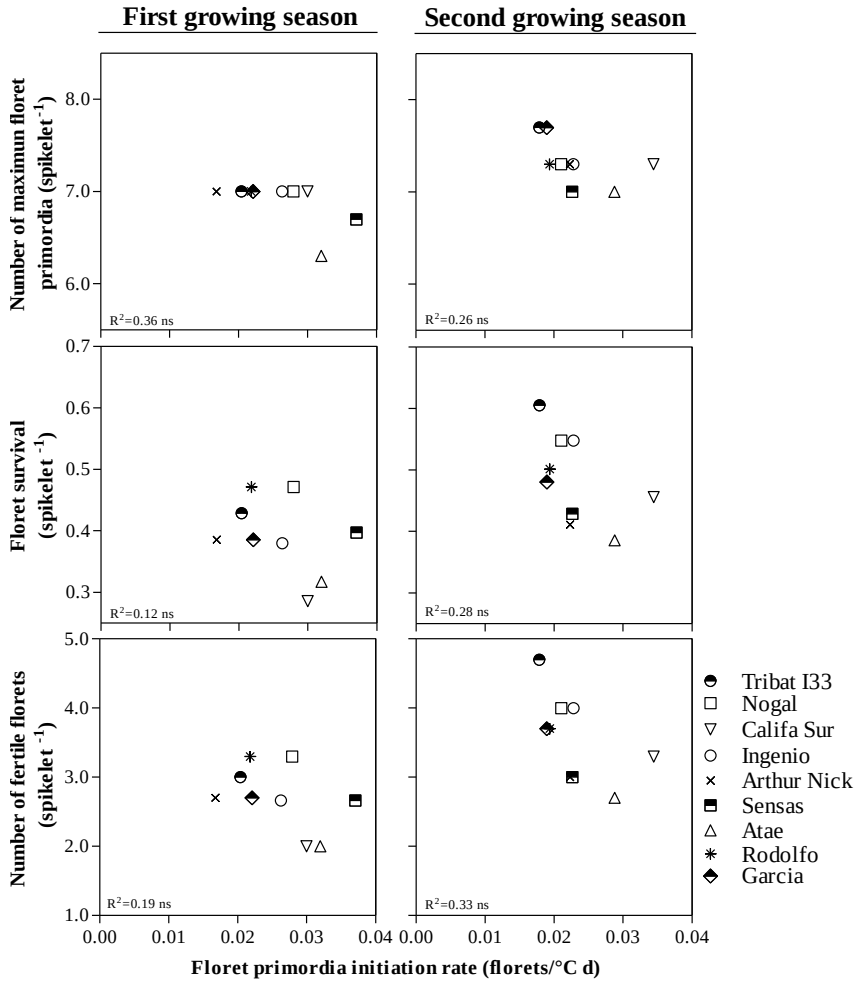

Figure 3.10. Relationship between the number of maximum floret primordia developed (*top panels*), the floret survival (*middle panels*) and the number of fertile floret (*bottom panels*) in the central spikelets against floret primordia initiation rate among the modern cultivars during the first (*left column*) and the second growing seasons (right column). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

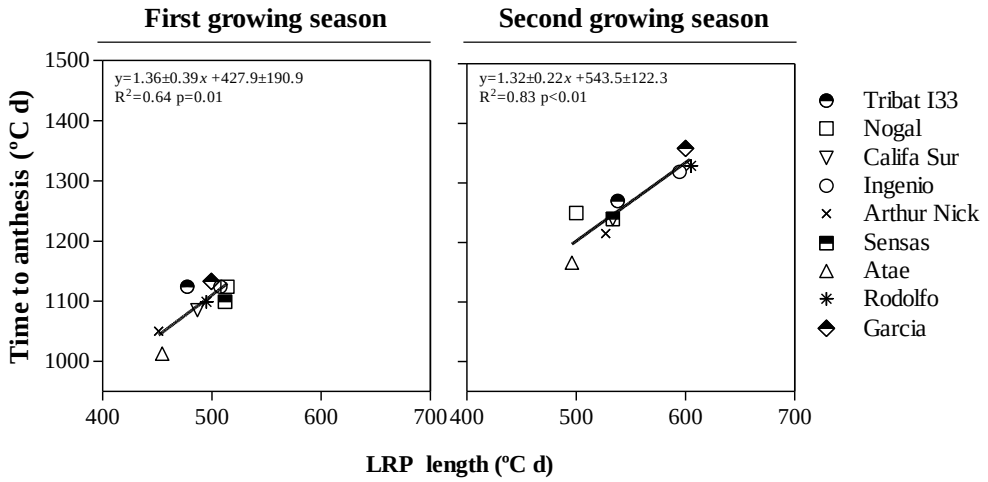

Figure 3.11. Relationship between time to anthesis and late reproductive phase (LRP) length among the modern cultivars in the first (*left panel*) and second growing seasons (*right panel*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

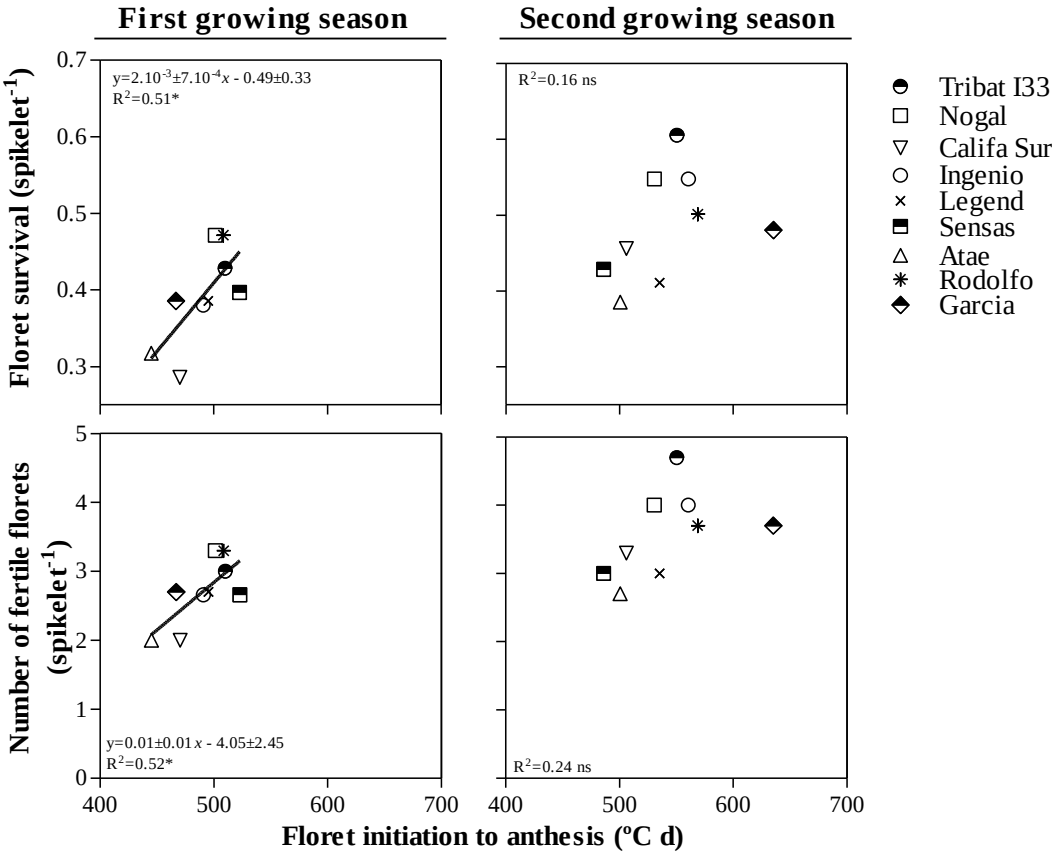

Figure 3.12. Relationship between the floret survival in the central spikelets (*top panels*) and the final number of fertile florets in the central spikelets (*bottom panels*) against the floret initiation to anthesis phase among the modern cultivars during the first (*left column*) and the second growing seasons (*right column*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

4. Discussion

Modern cultivars were tested to understand the mechanisms that control the number of fertile florets at anthesis, whose relevance is critical in determining the number of grains at maturity (which in turn dominates yield determination; Peltonen-Sainio *et al*., 2007; Slafer *et al*., 2014) in cleistogamous plants such as wheat. Even though differences in most traits (and particularly so in very detailed ones like floret development patterns and the dynamics of floret generation/degeneration) might be much smaller among well adapted modern cultivars than among genotypes selected to represent wider ranges of variation (e.g. old and modern cultivars, lines derived from wide crosses) or even among related but different species, I preferred to identify (likely relatively minor) differences in lines that represents actual valuable material for realistic breeding programs. In particular, I know that this set of modern cultivars did differ in fruiting efficiency (Elía *et al*., 2016), and therefore they constitute a relevant material to study dynamics of floret development. Indeed, significant differences were seen in the number of fertile florets at anthesis among the elite cultivars during both growing seasons, and although there was a significant GxE interaction, (i) the magnitude of the mean squares of GxE was much smaller (*c*. one third) than that of the genotypes, and (ii) it was mostly due to the inconsistent performance of only one cultivar. Thus, not only there were differences among elite material for breeders but also these differences were reasonably consistent between environments.

The cultivars studied, even when they were all modern, high-yielding, well adapted materials, differed in the number of fertile florets produced and the difference was more related to differences in floret survival than in the maximum number of floret primordia developed. This is consistent with previous a large body of evidences based on environmental effects on spike growth before anthesis, such as shading (Fischer and Stockman, 1980), nitrogen availability (Sibony and Pinthus, 1988; Ferrante *et al*., 2010), photoperiod condition (González *et al*., 2003), and combination of some environmental treatments (Langer and Hanif, 1973; Whingwiri and Stern, 1982; González *et al*., 2003, González *et al*., 2005). Much less has been analyzed in terms of the organogenesis bases for genotypic differences in spike fertility, and the few works available are also consistent with the results reported in this Chapter for a set of modern wheat cultivars all well adapted to the region. For instance, analyzing the relationships between number of fertile florets and either maximum number of floret primordia or floret primordia survival (i) between near-isogenic lines for *Rht* genes (Miralles *et al*., 1998), (ii) among genotypes of a panel selected in CYMMMIT (González-Navarro *et al*., 2015) and (iii) among a wide range of European wheats (Guo *et al*., 2016) it was consistently found that, again, floret survival was more relevant than floret initiation in determining differences in spike fertility. Therefore, it seems that results from both environmental (being them either resources -like radiation or N- or signals –like photoperiod-) and genetic (being these a collection of materials from different regions or well adapted cultivars to the same region) effects are consistently evidencing that floret survival is pivotal to determine the number of fertile florets.

We tested whether differences in synchrony in the initiation of proximal and distal floret primordia could part of the explanation, focused on a pure developmental origin of genetic variation. To the best of my knowledge this is the first time this sort of association is tested in wheat (the idea of synchrony in determining ear fertility in maize –with a radically different reproductive system- had been proposed before Cárcova *et al*., 2000). We failed in identifying synchrony of development among florets being initiated through time as a relevant origin of genotypic differences in floret survival (and consequently in the number of fertile florets), indicating a possible trade-off between the rate of initiation and the length of the phase when the initiation and survival of primordia takes place. Indeed, during both growing seasons, differences in time to anthesis were well explained by differences in the duration of the late reproductive phase (in addition, during the first growing season it seemed that floret survival and the number of fertile florets were related to the phase from floret intiation until anthesis). These results are also in line with previous reports evidencing that a longer floret developmental phase may increase floret survival (González *et al*., 2003; Serrago *et al*., 2008). There must be an evolutionary signature in making floret survival so critical for the determination of fertile florets: it seems wheat plants grossly overproduce floret primordia and then allows a certain number to survive (being this number tightly governed by environmental and genetic factors); and this might be seen as unexpectedly wasteful and therefore unacceptable in evolutionary terms, unless clear advantages of such strategy can be put forward (see General Discussion in the last Chapter of this thesis).

Others experiments were carried out using near isogenic lines (NILs) to refine the analyses on the bases of fertile floret determination due to genetic differences in wheat, whose results are presented in the following chapters.

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6. Annex 3: Supplementary data

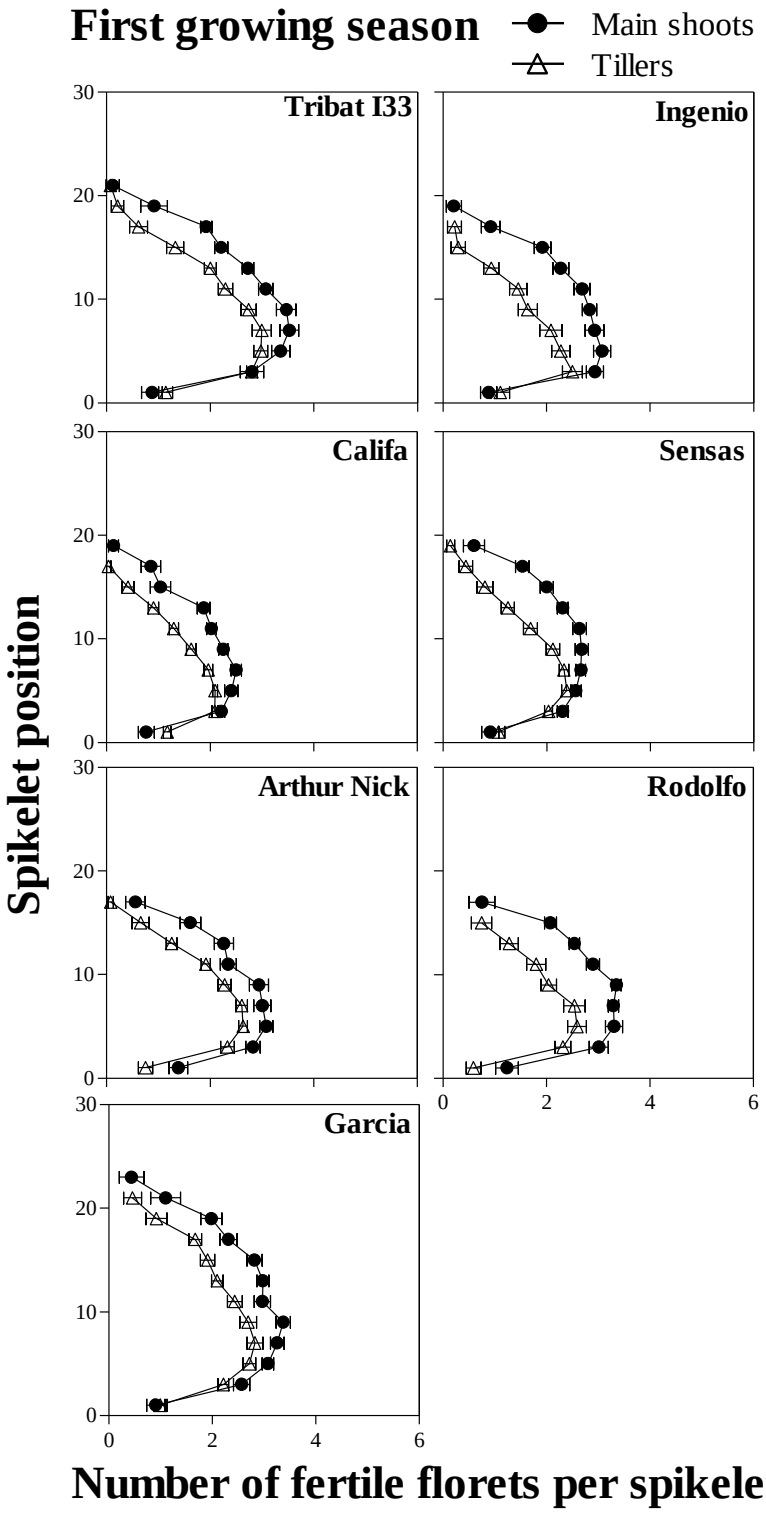


Figure A3.1. Fertile florets in each spikelet position on the main-shoots (closed circles) and tillers spikes (open triangles) for the different modern cultivars during the first growing season. Average of 5 plants and the standard error of the means are represented.

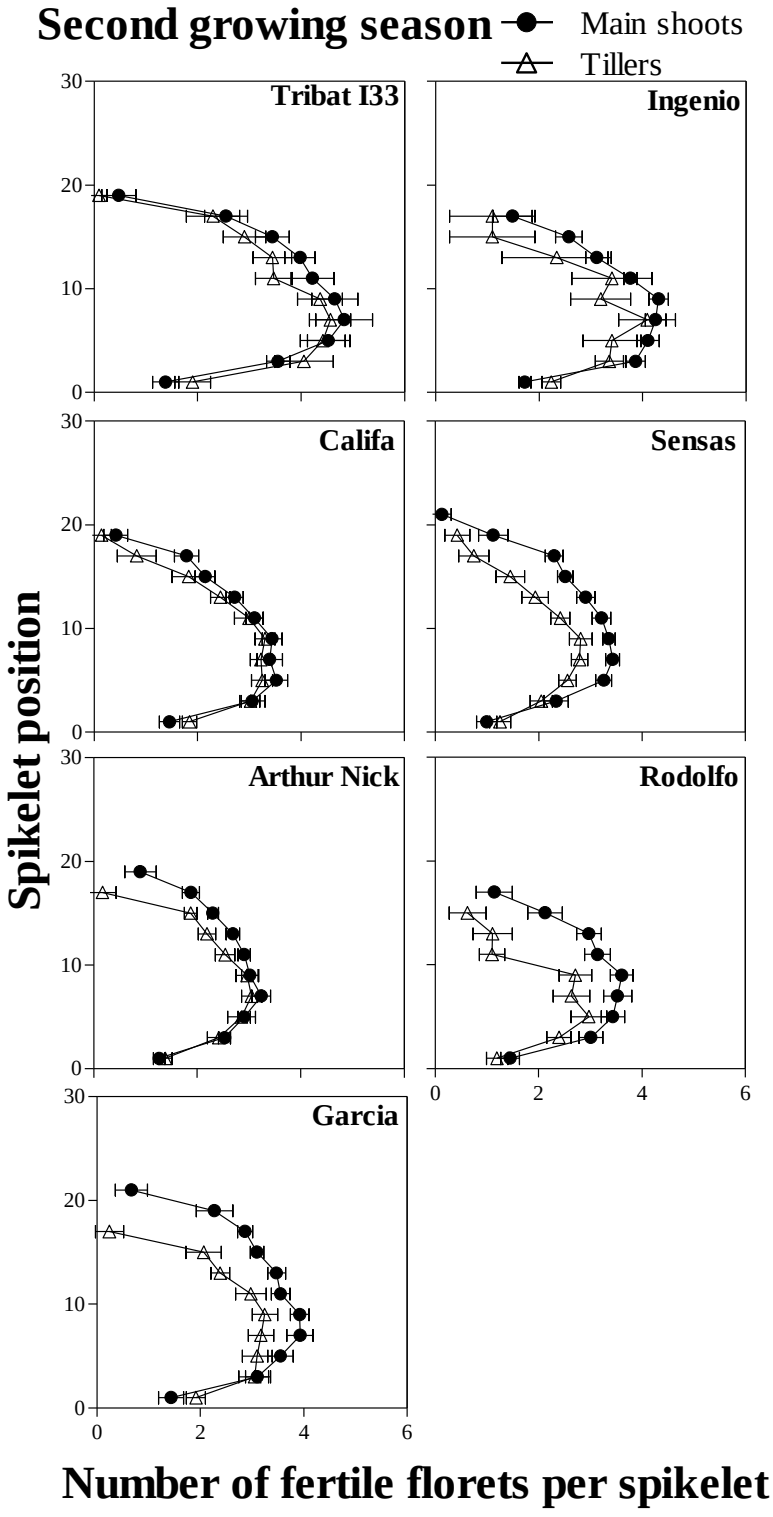


Figure A3.2. Fertile florets in each spikelet position on the main-shoots (closed circles) and tillers spikes (open triangles) for the different modern cultivars during the second growing season. Average of 5 plants and the standard error of the means are represented.

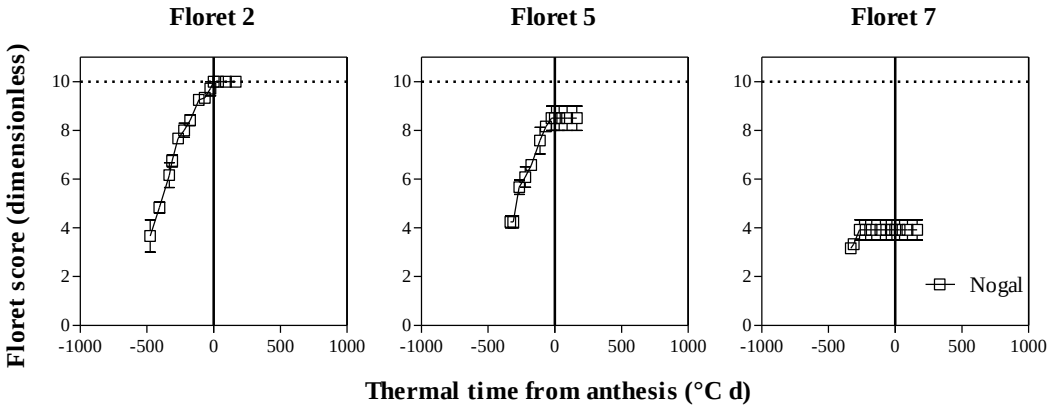


Figure A3.3. Dynamics of the floret development of F2, F5 and F7 in central spikelets of the main-shoot spikes in Nogal cultivar during the first growing season. Data average from 3 plants, bars stands for the standard error of the means.

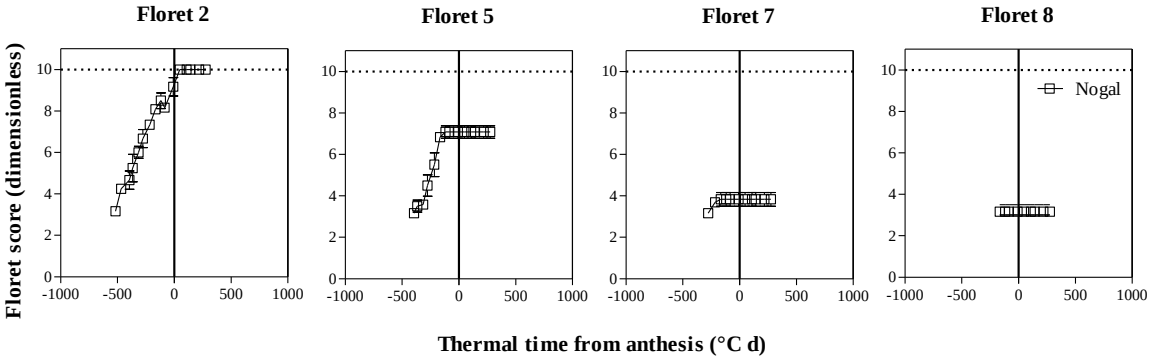


Figure A3.4. Dynamics of the floret development of F2, F5, F7 and F8 in central spikelets of the main-shoot spikes in Nogal cultivar during the second growing season. Data average from 3 plants, bars stands for the standard error of the means.

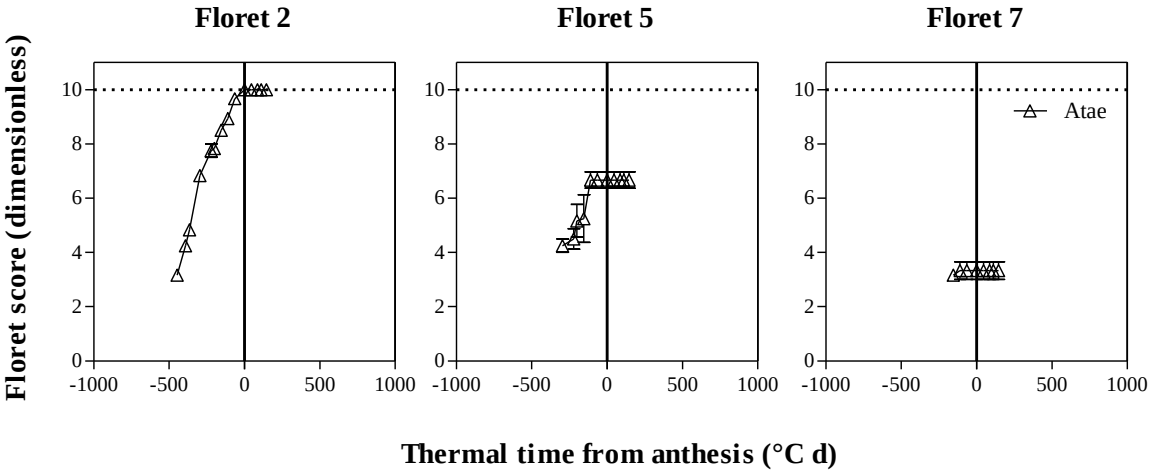


Figure A3.5. Dynamics of the floret development of F2, F5 and F7 in central spikelets of the main-shoot spikes in Atae cultivar during the first growing season. Data average from 3 plants, bars stands for the standard error of the means.

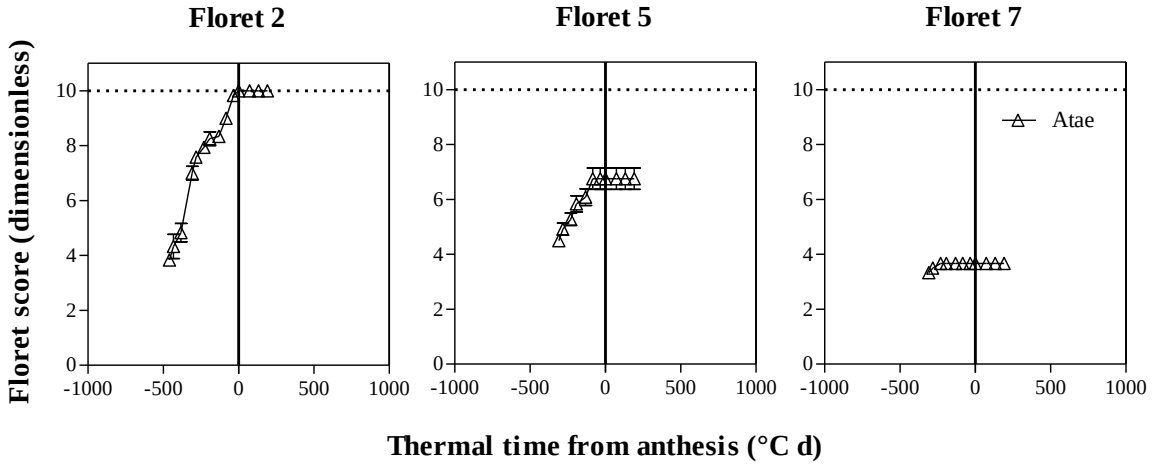


Figure A3.6. Dynamics of the floret development of F2, F5 and F7 in central spikelets of the main-shoot spikes in Atae cultivar during the second growing season. Data average from 3 plants, bars stands for the standard error of the means.

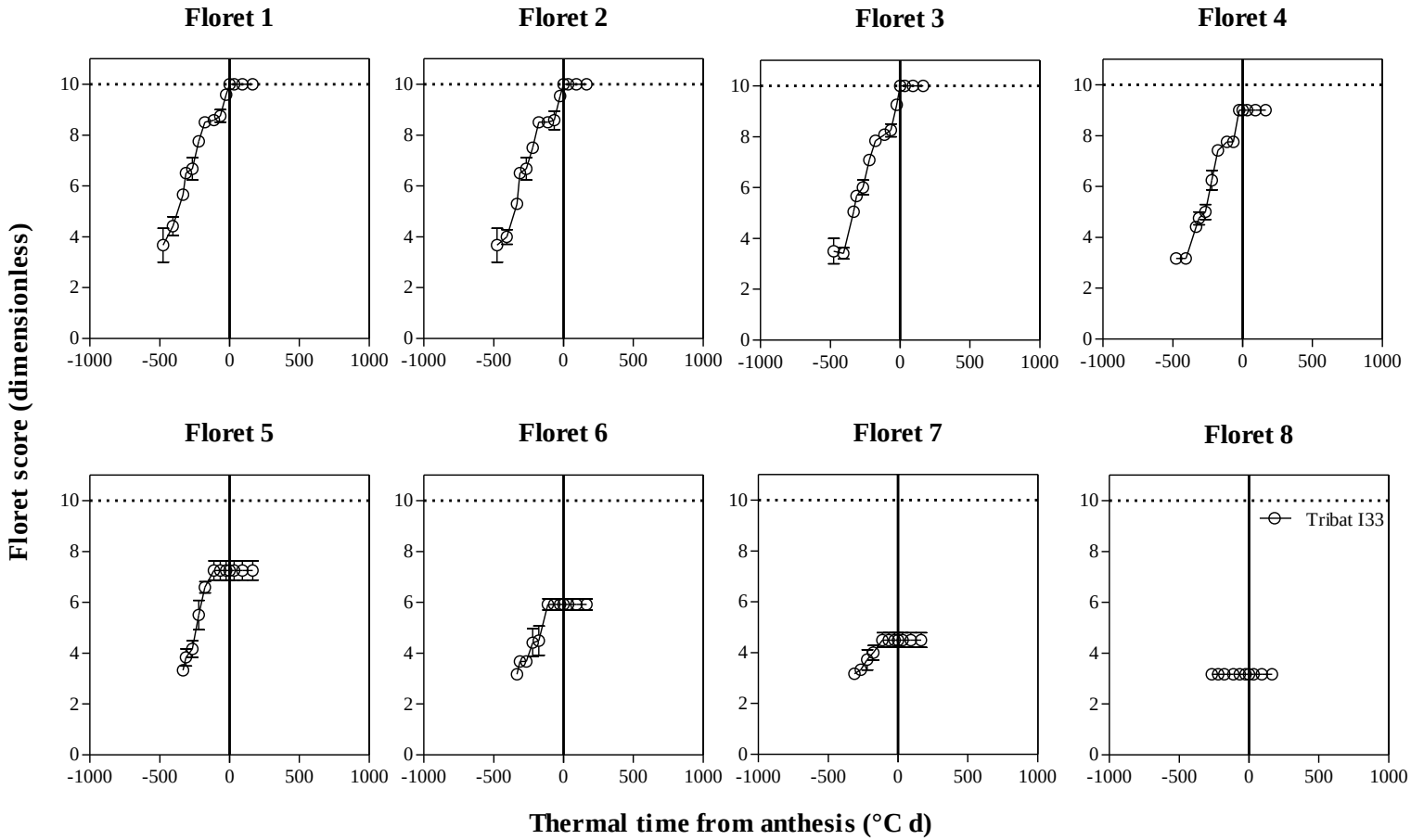


Figure A3.7. Dynamics of the floret development of F1, F2, F3, F4, F5, F6, F7 and F8 in central spikelets of the main-shoot spikes in Tribat I33 cultivar during the first growing season. Data average from 3 plants, bars stands for the standard error of the means.

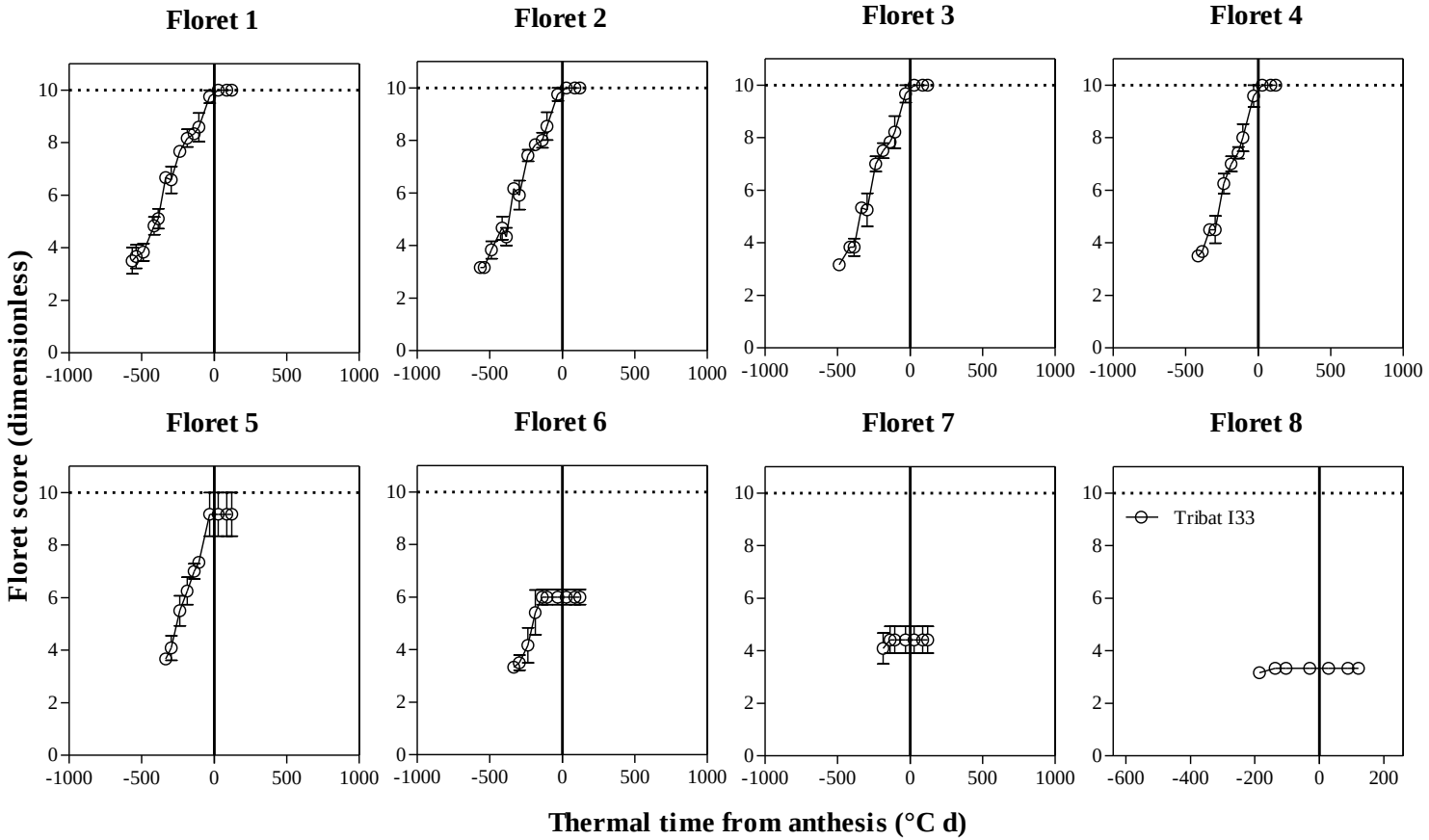


Figure A3.8. Dynamics of the floret development of F1, F2, F3, F4, F5, F6, F7 and F8 in central spikelets of the main-shoot spikes in Tribat I33 cultivar during the second growing season. Data average from 3 plants, bars stands for the standard error of the means.

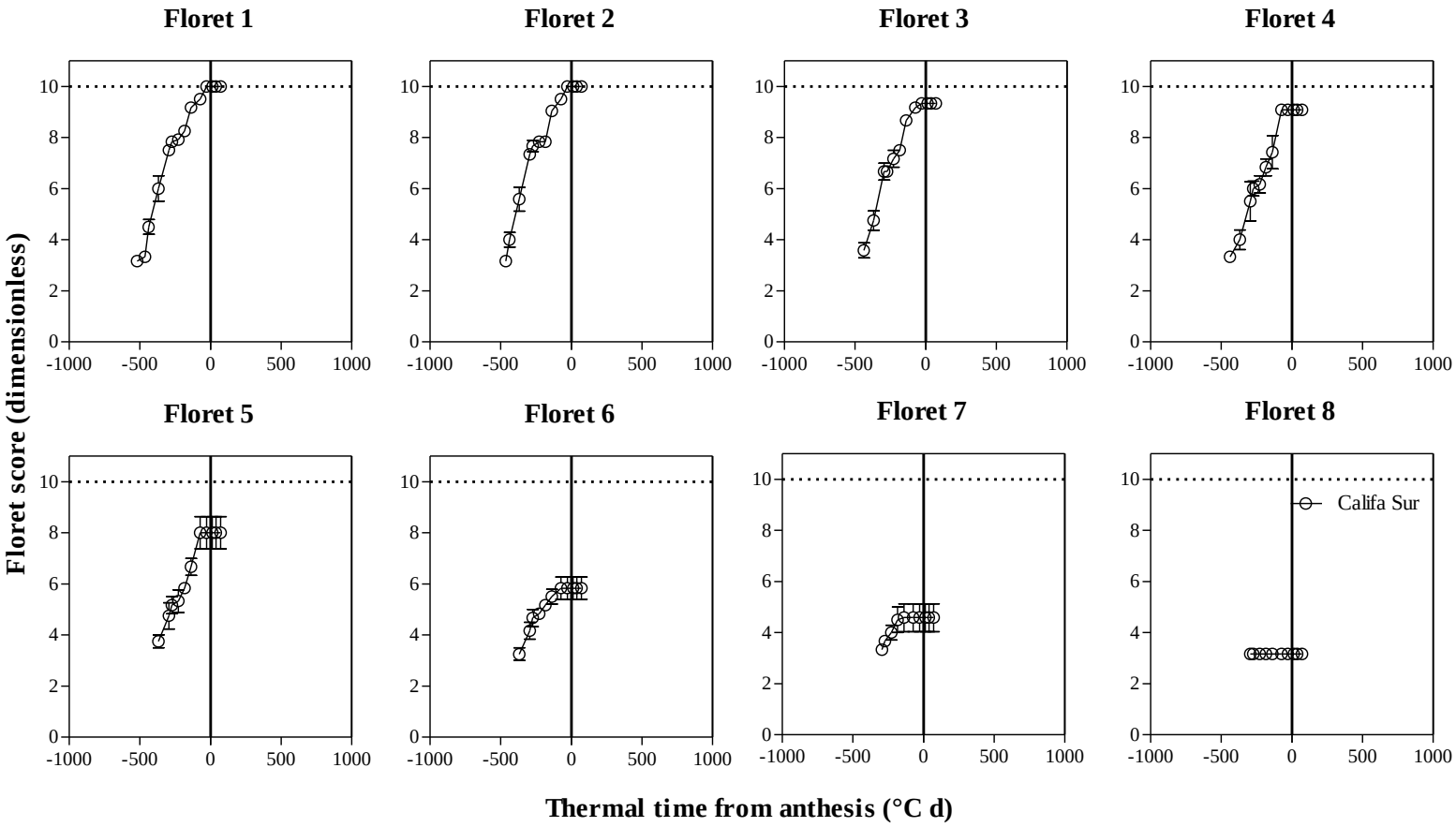


Figure A3.9. Dynamics of the floret development of F1, F2, F3, F4, F5, F6, F7 and F8 in central spikelets of the main-shoot spikes in Califa Sur cultivar during the first growing season. Data average from 3 plants, bars stands for the standard error of the means.

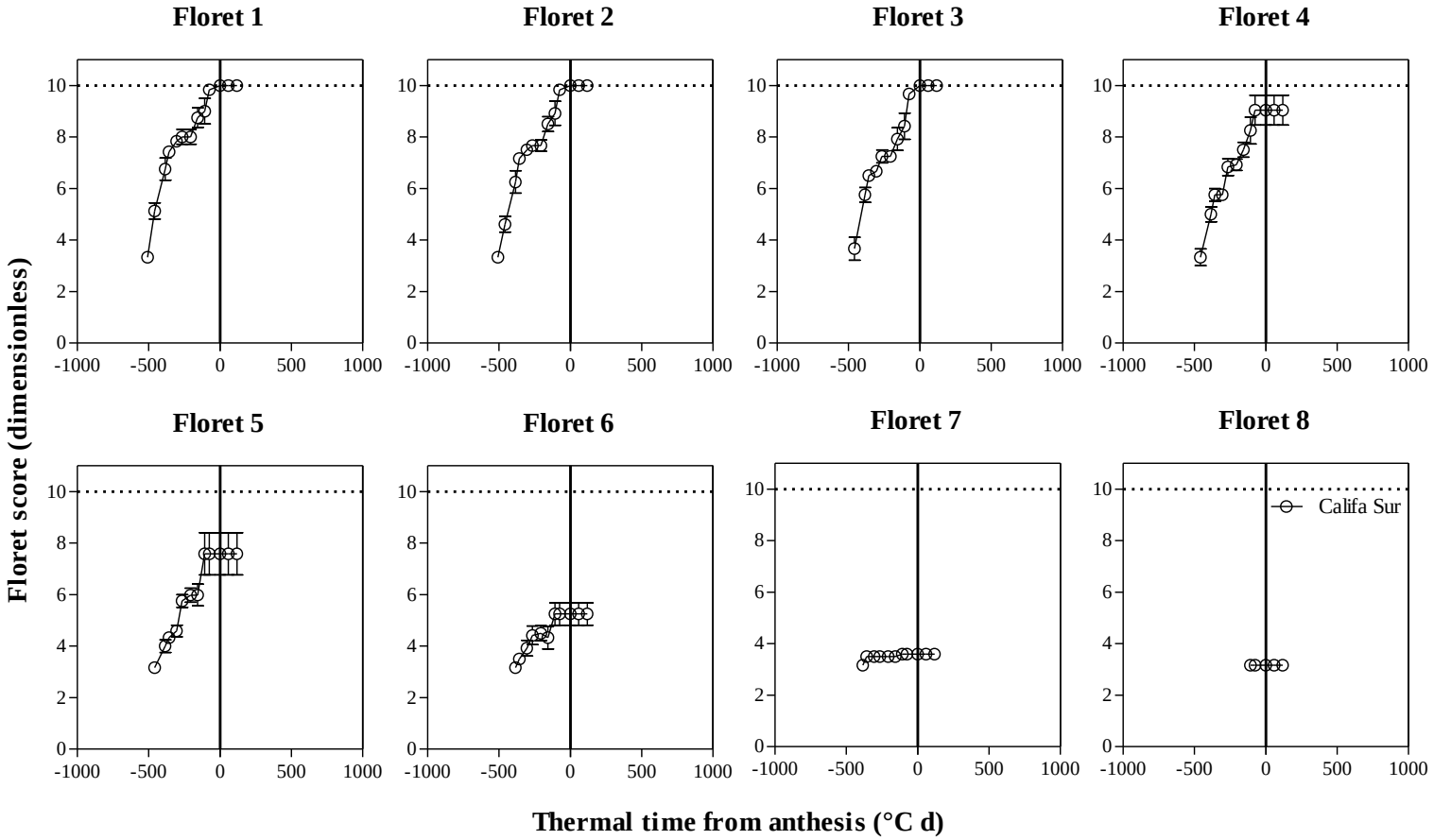


Figure A3.10. Dynamics of the floret development of F1, F2, F3, F4, F5, F6, F7 and F8 in central spikelets of the main-shoot spikes in Califa Sur cultivar during the second growing season. Data average from 3 plants, bars stands for the standard error of the means.

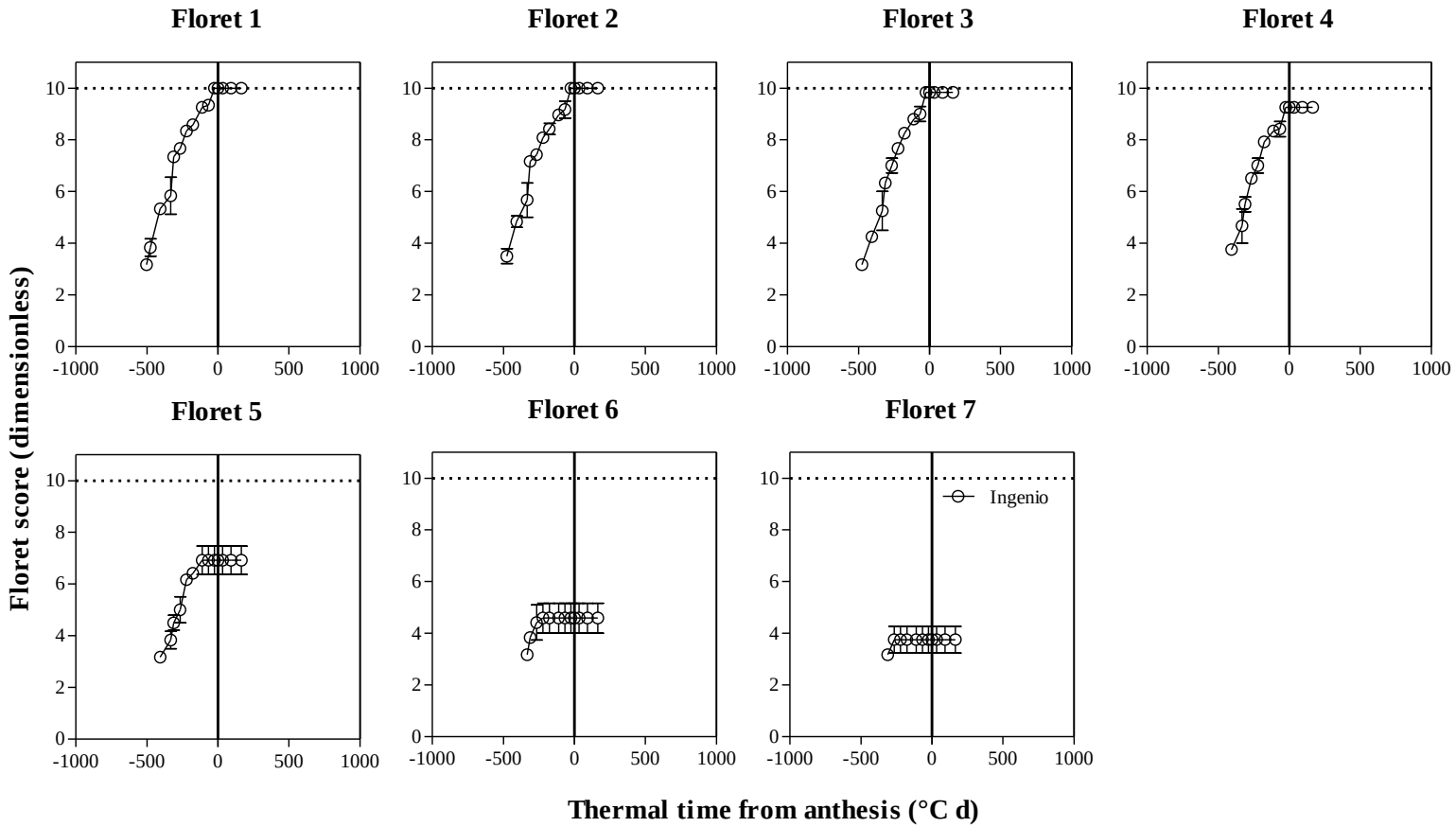


Figure A3.11. Dynamics of the floret development of F1, F2, F3, F4, F5, F6 and F7 in central spikelets of the main-shoot spikes in Ingenio cultivar during the first growing season. Data average from 3 plants, bars stands for the standard error of the means.

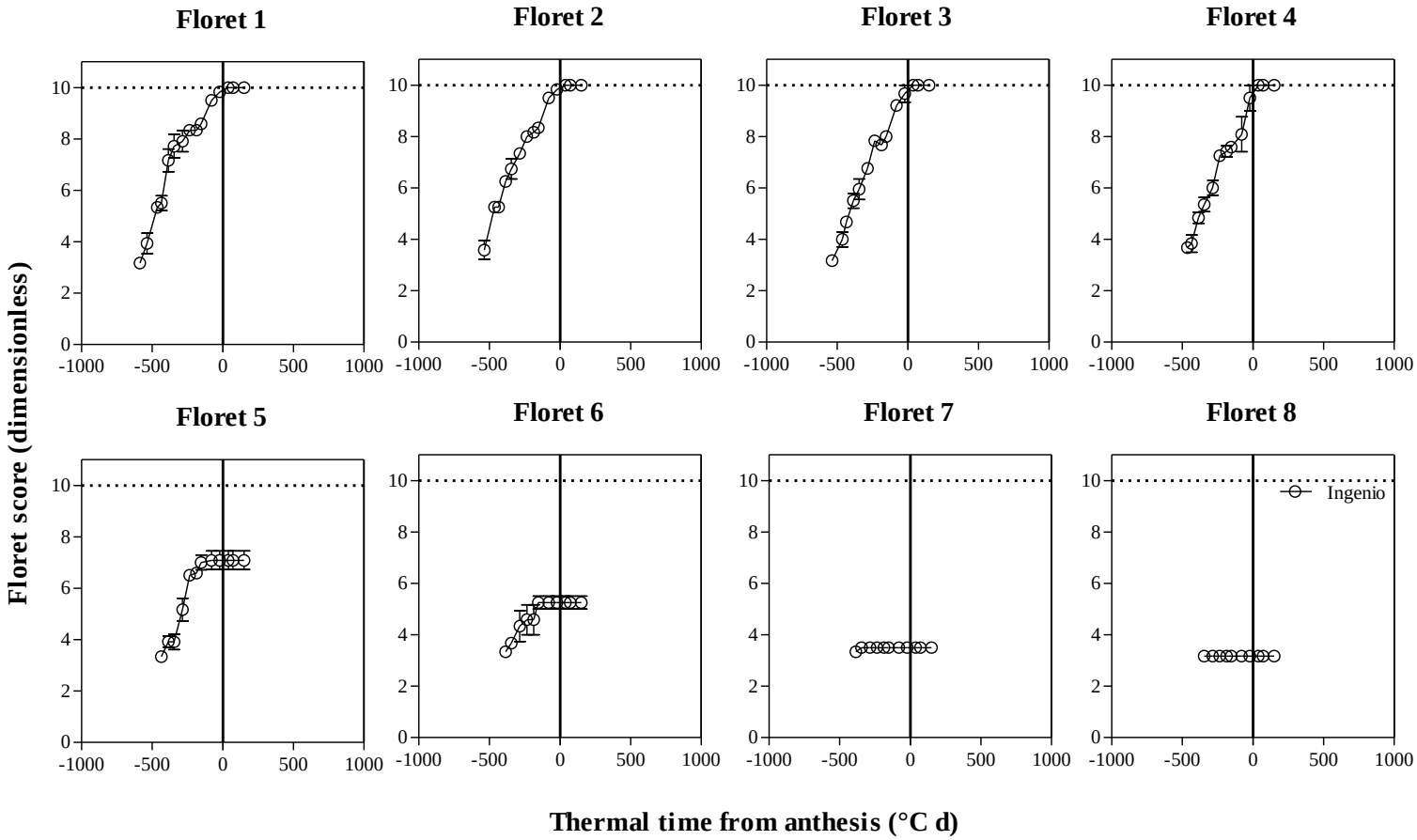


Figure A3.12. Dynamics of the floret development of F1, F2, F3, F4, F5, F6, F7 and F8 in central spikelets of the main-shoot spikes in Ingenio cultivar during the second growing season. Data average from 3 plants, bars stands for the standard error of the means.

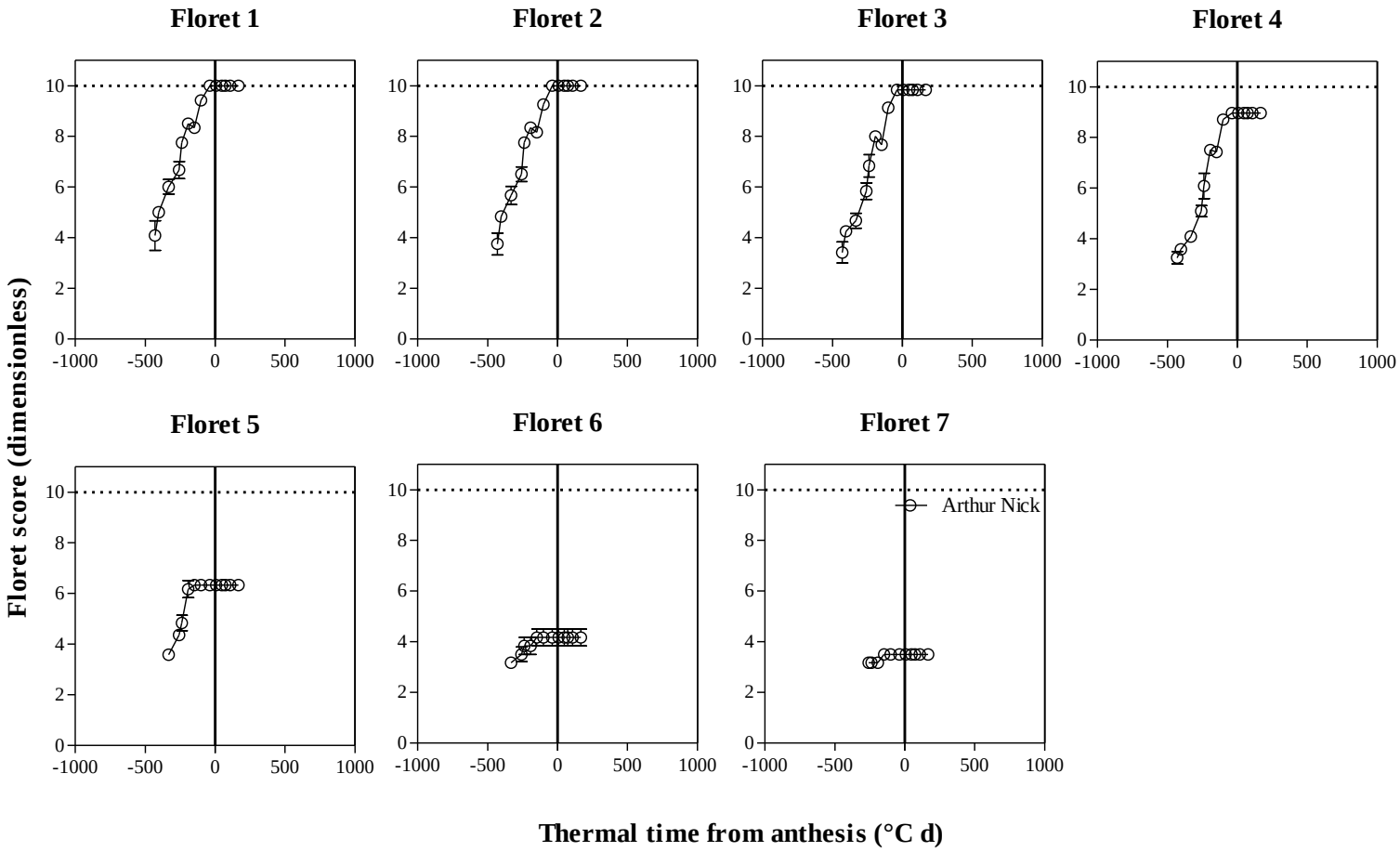


Figure A3.13. Dynamics of the floret development of F1, F2, F3, F4, F5, F6 and F7 in central spikelets of the main-shoot spikes in Arthur Nick cultivar during the first growing season. Data average from 3 plants, bars stands for the standard error of the means.

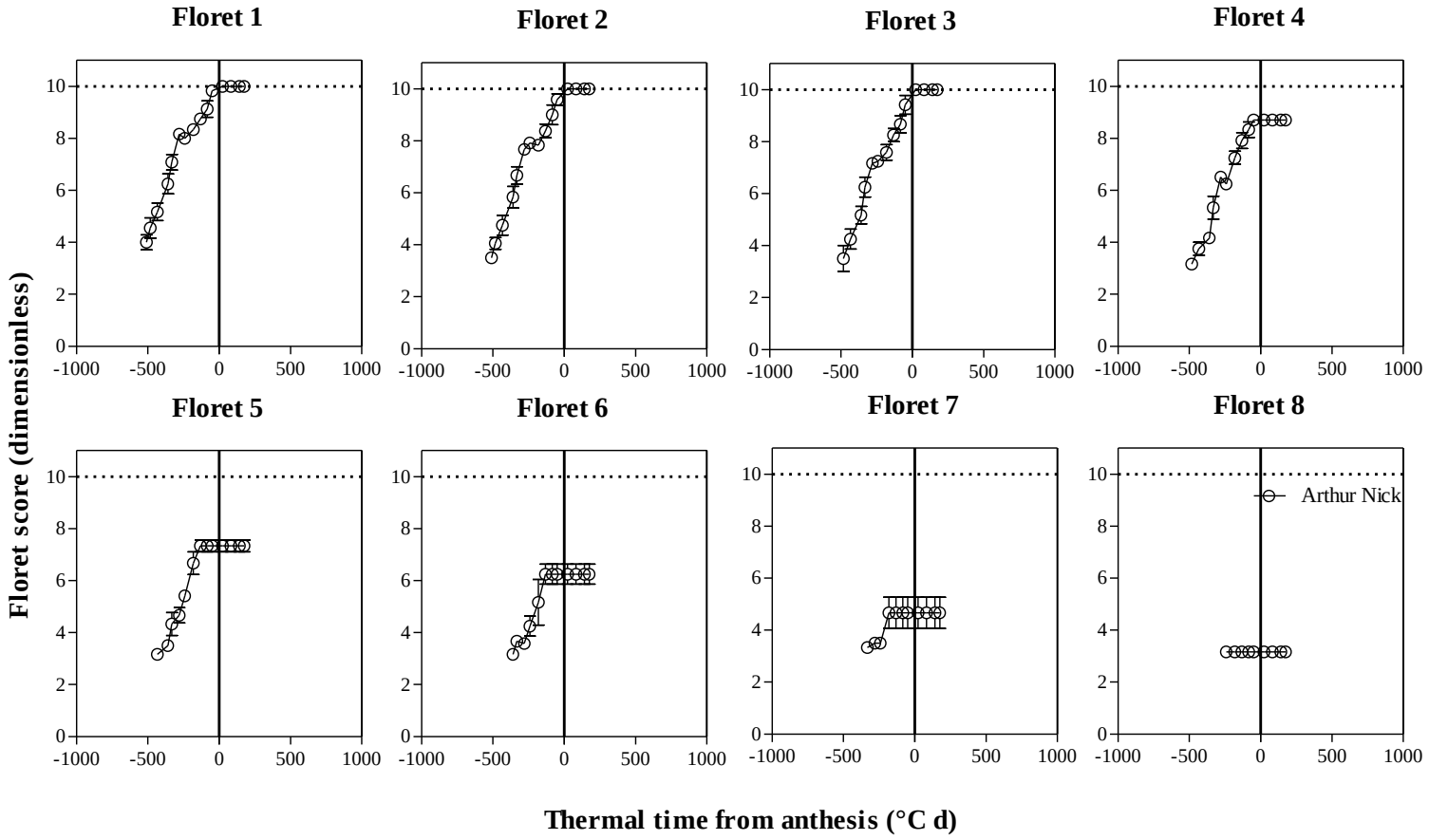


Figure A3.14. Dynamics of the floret development of F1, F2, F3, F4, F5, F6, F7 and F8 in central spikelets of the main-shoot spikes in Arthur Nick cultivar during the second growing season. Data average from 3 plants, bars stands for the standard error of the means.

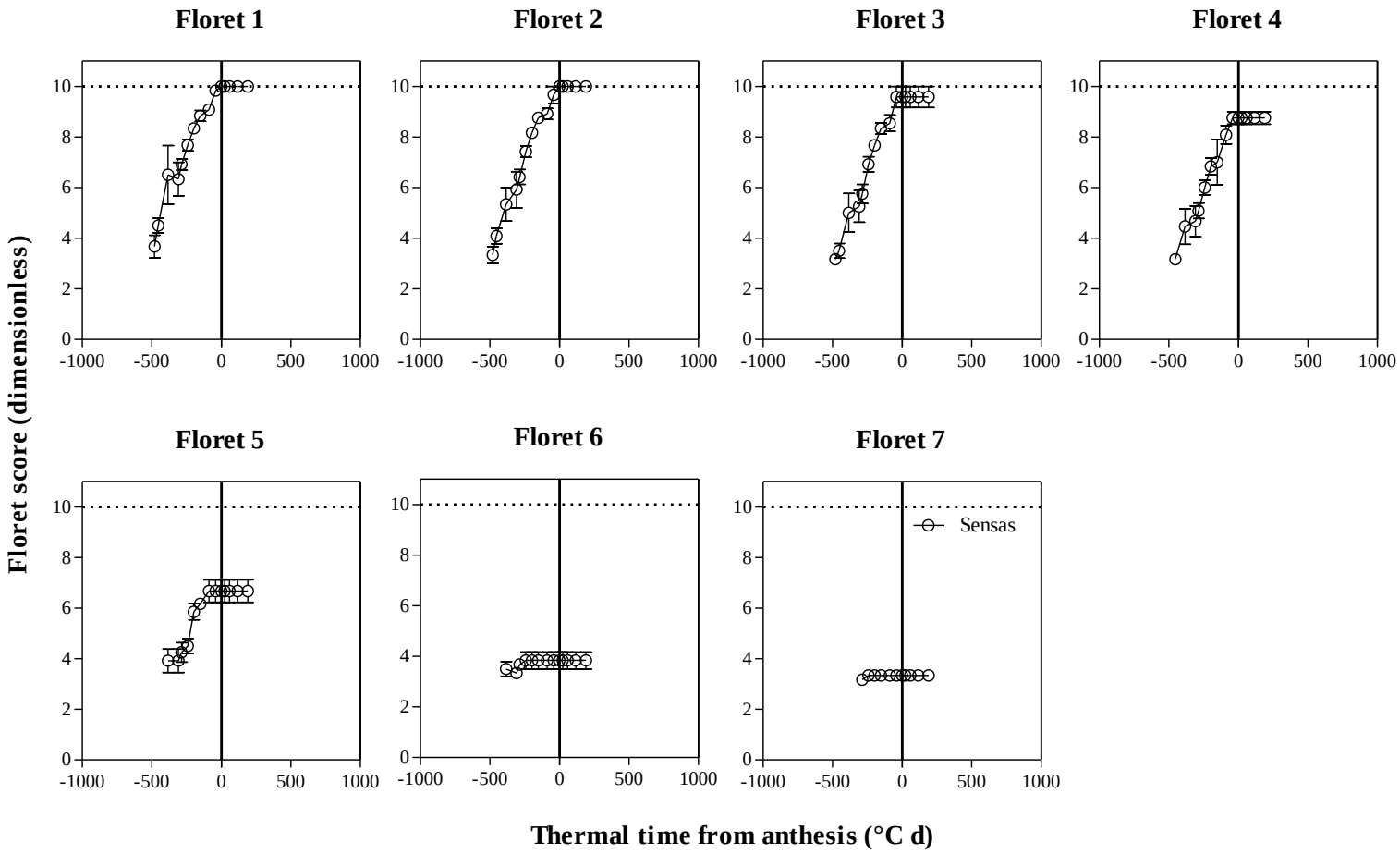


Figure A3.15. Dynamics of the floret development of F1, F2, F3, F4, F5, F6 and F7 in central spikelets of the main-shoot spikes in Sensas cultivar during the first growing season. Data average from 3 plants, bars stands for the standard error of the means.

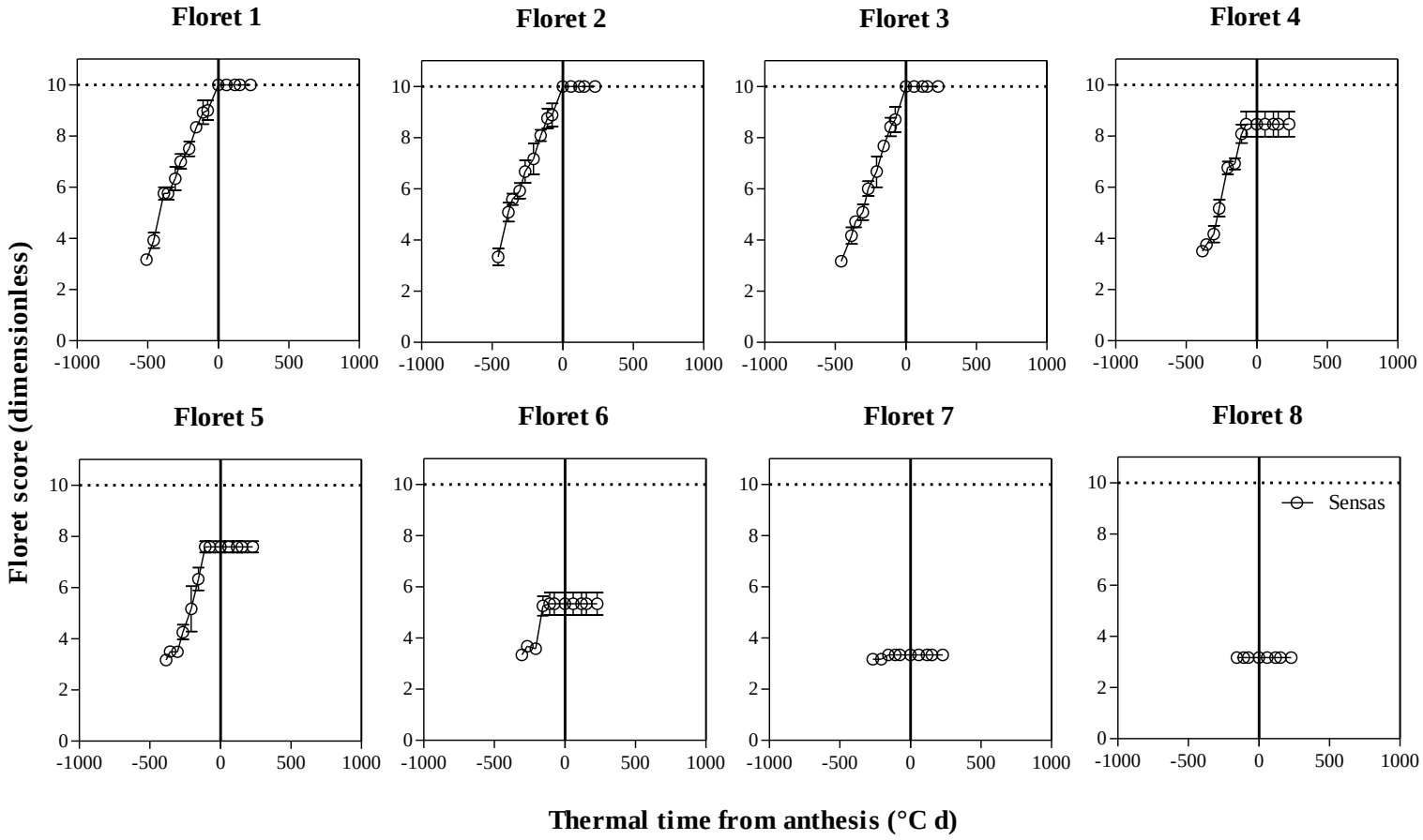


Figure A3.16. Dynamics of the floret development of F1, F2, F3, F4, F5, F6, F7 and F8 in central spikelets of the main-shoot spikes in Sensas cultivar during the second growing season. Data average from 3 plants, bars stands for the standard error of the means.

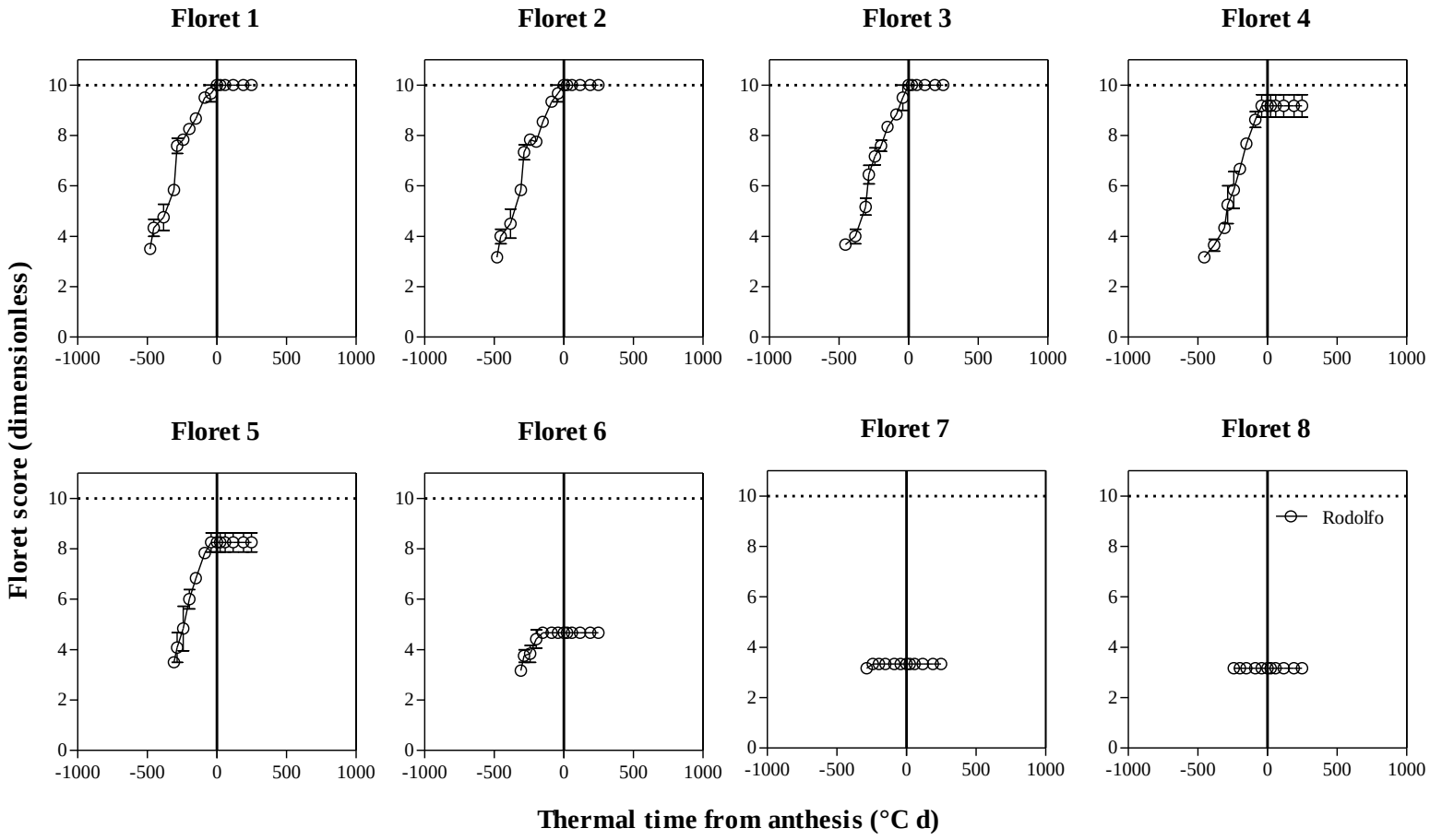


Figure A3.17. Dynamics of the floret development of F1, F2 F3, F4, F5, F6, F7 and F8 in central spikelets of the main-shoot spikes in Rodolfo cultivar during the first growing season. Data average from 3 plants, bars stands for the standard error of the means.

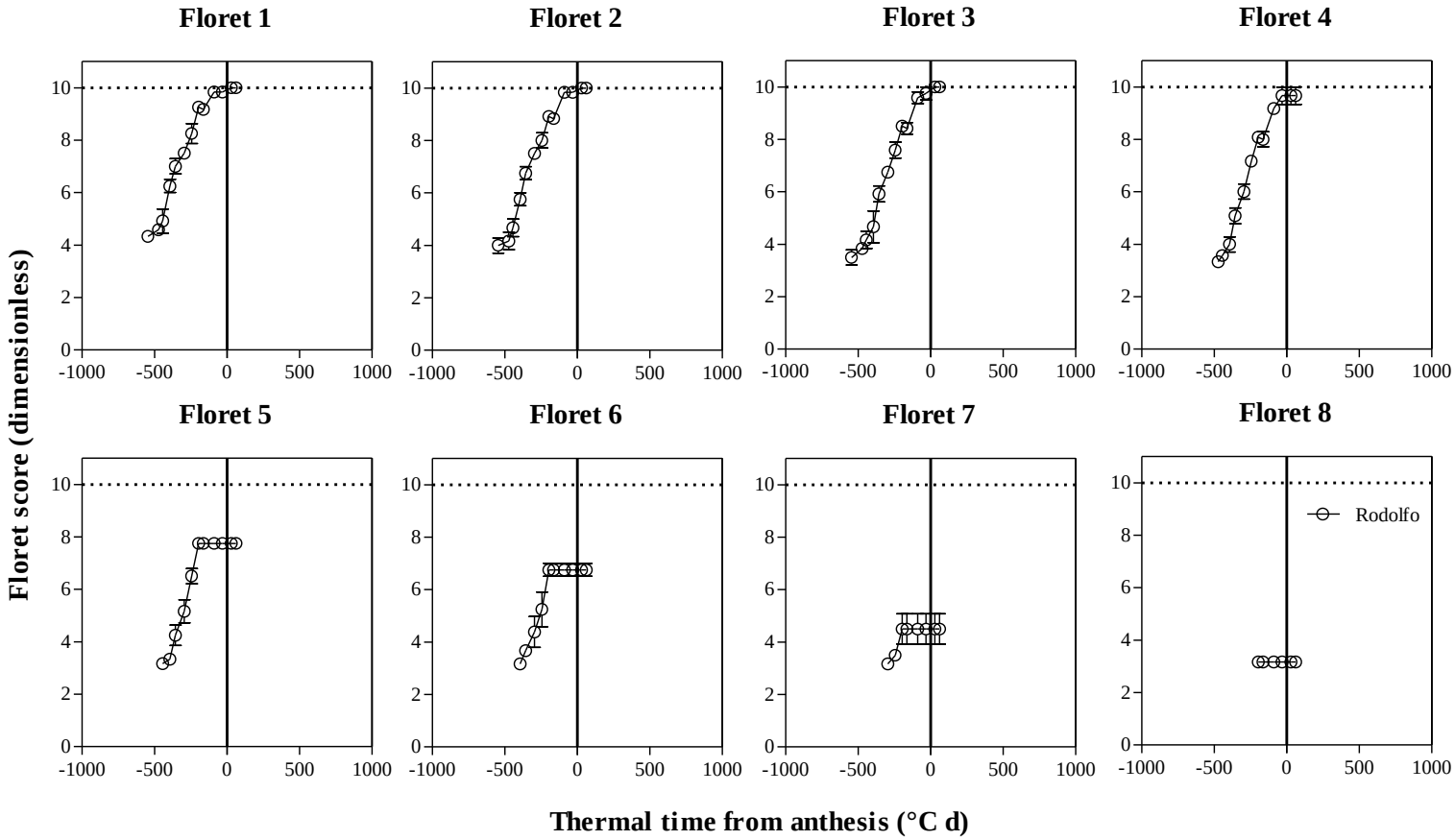


Figure A3.18. Dynamics of the floret development of F1, F2, F3, F4, F5, F6, F7 and F8 in central spikelets of the main-shoot spikes in Rodolfo cultivar during the second growing season. Data average from 3 plants, bars stands for the standard error of the means.

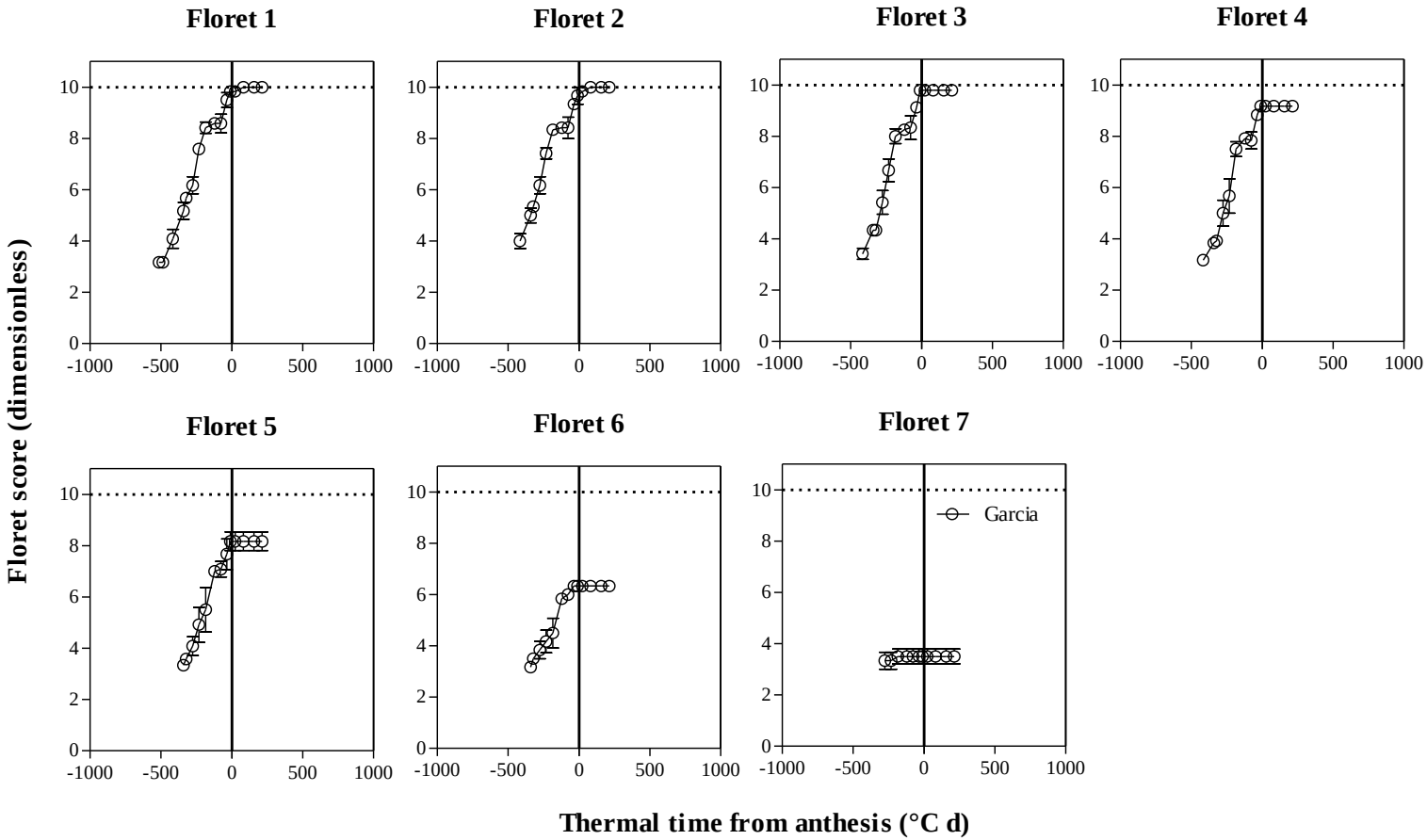


Figure A3.19. Dynamics of the floret development of F1, F2, F3, F4, F5, F6 and F7 in central spikelets of the main-shoot spikes in Garcia cultivar during the first growing season. Data average from 3 plants, bars stands for the standard error of the means.

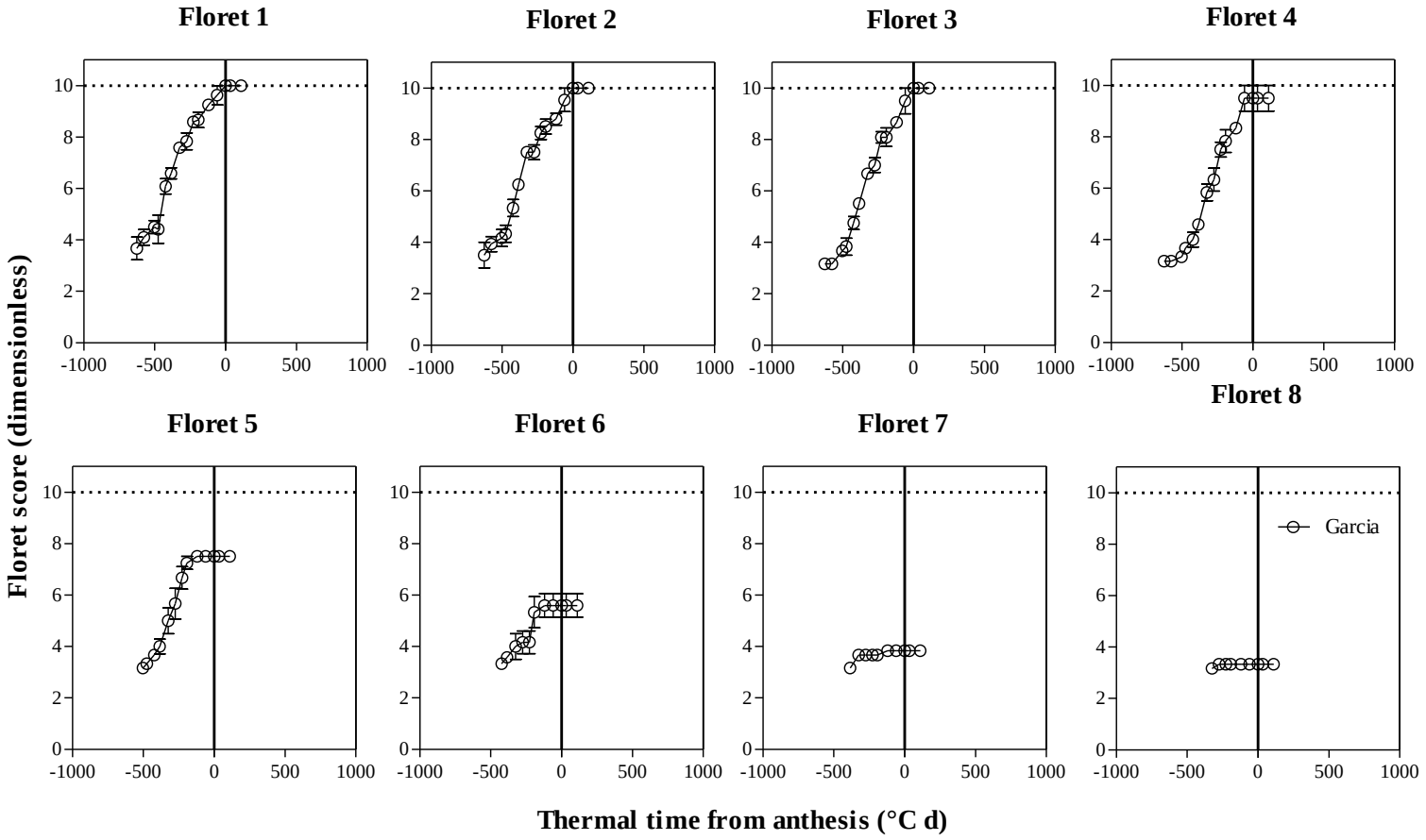


Figure A3.20. Dynamics of the floret development of F1, F2, F3, F4, F5, F6, F7 and F8 in central spikelets of the main-shoot spikes in Garcia cultivar during the second growing season. Data average from 3 plants, bars stands for the standard error of the means.

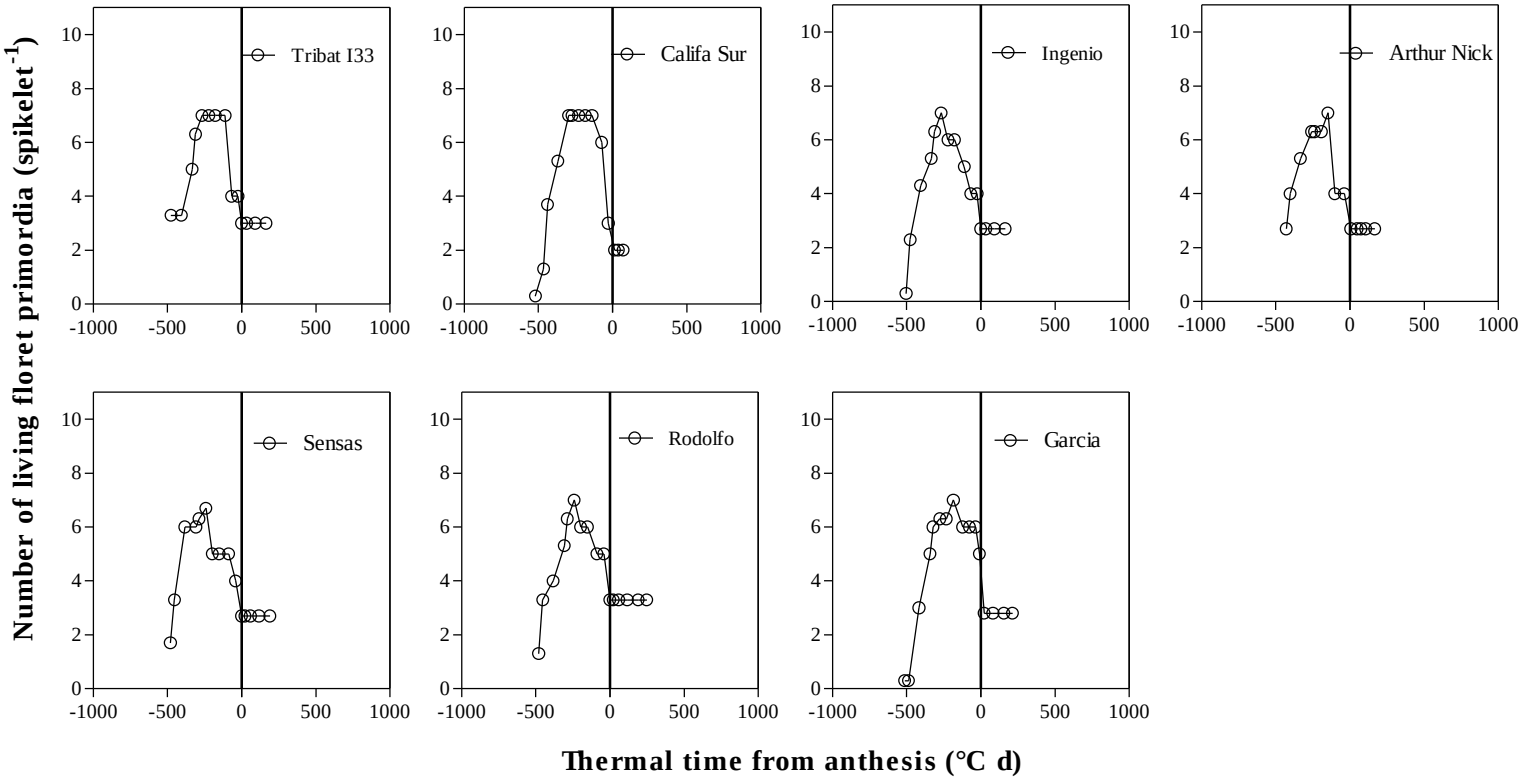


Figure A3.21. Number of living floret primordia in the central spikelets through thermal time from anthesis for Tribat I33, Califa Sur, Ingenio, Arthur Nick, Sensas, Rodolfo and Garcia cultivars during the first growing seasons.

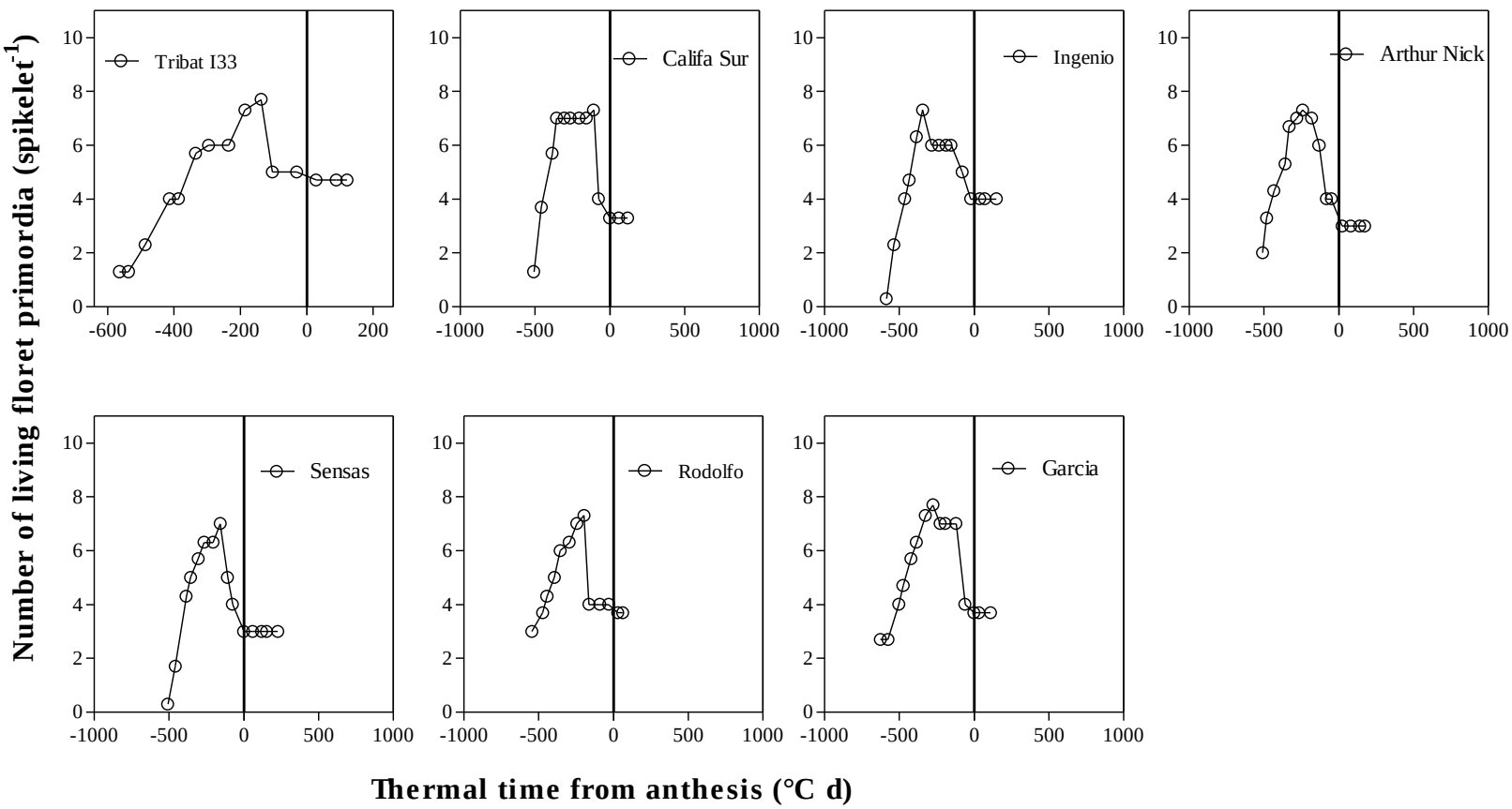


Figure A3.22. Number of living floret primordia in the central spikelets through thermal time from anthesis for Tribat I33, Califa Sur, Ingenio, Arthur Nick, Sensas, Rodolfo and Garcia cultivars during the second growing season.

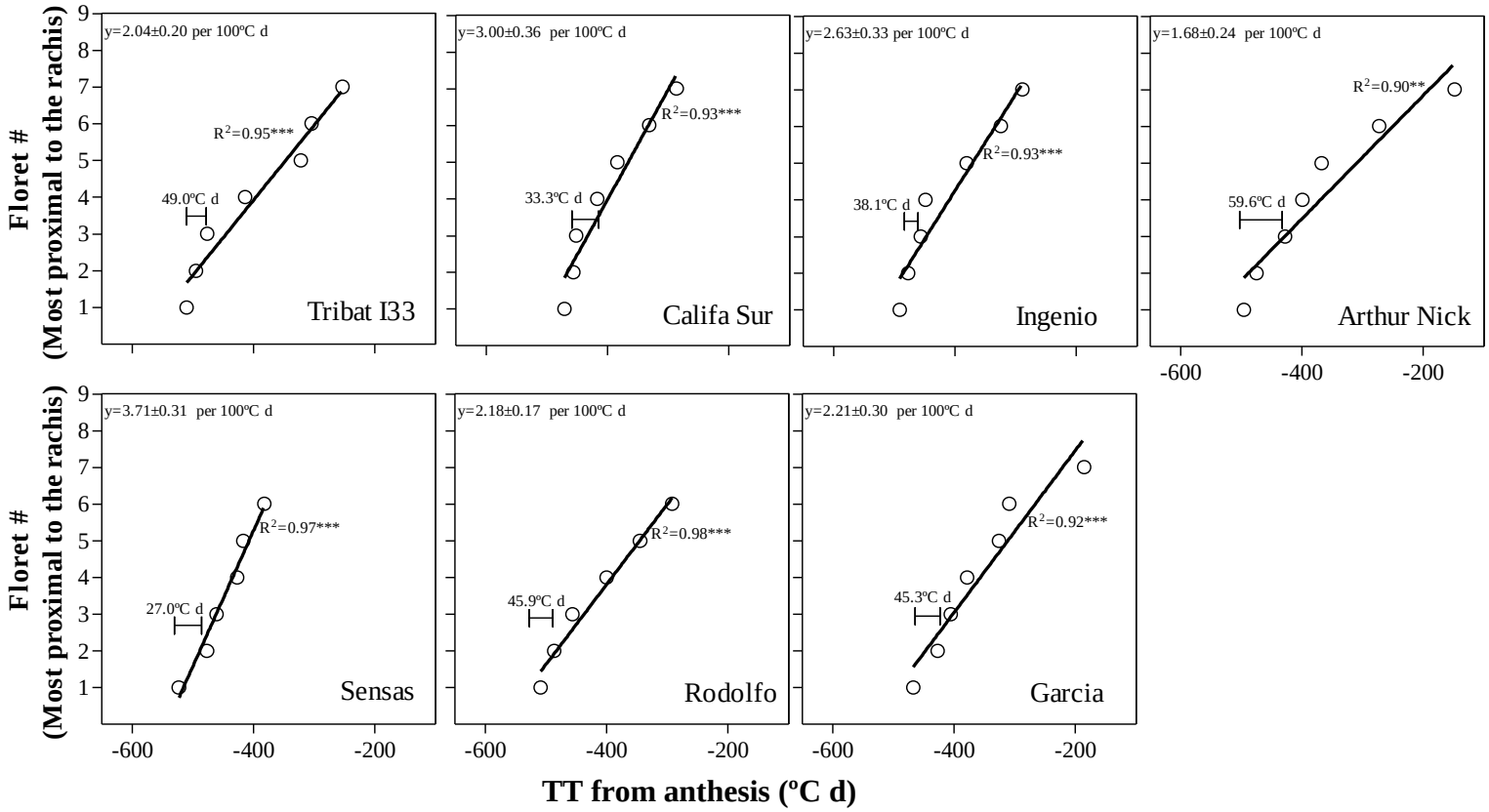


Figure A3.23. Timing of W3.5 for each floret primordium through thermal time from anthesis in Tribat I33, Califa Sur, Ingenio, Arthur Nick, Sensas, Rodolfo and Garcia cultivars during the first growing season. Inset each panel, the rate of floret initiation expressed in florets per 100°C d and the thermal time between the appearance of two following floret primordia are indicated. The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are also shown.

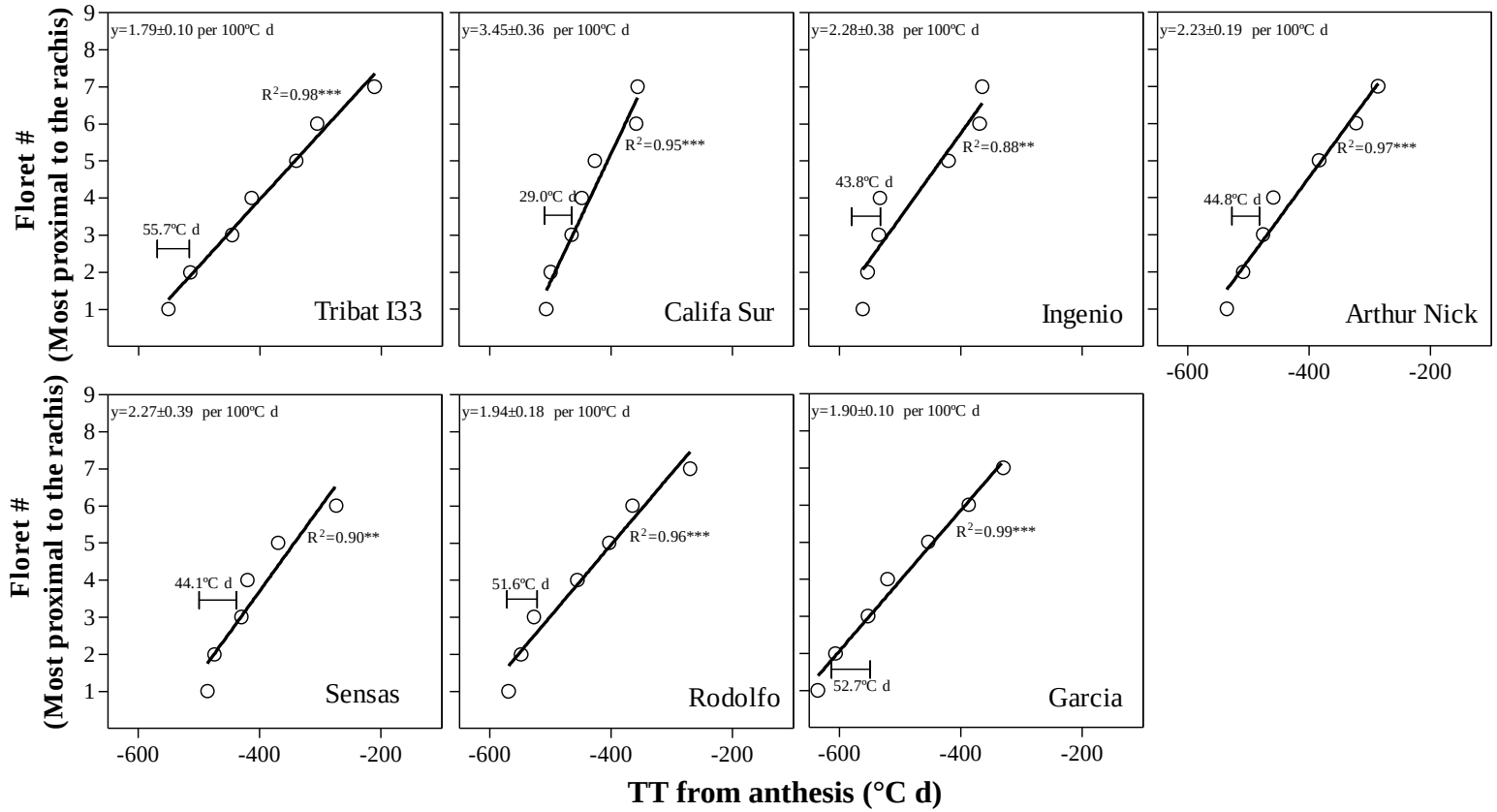


Figure A3.24. Timing of W3.5 for each floret primordium through thermal time from anthesis in Tribat I33, Califa Sur, Ingenio, Arthur Nick, Sensas, Rodolfo and Garcia cultivars during the second growing season. Inset each panel, the rate of floret initiation expressed in florets per 100°C d and the thermal time between the appearance of two following floret primordia are indicated. The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are also shown.

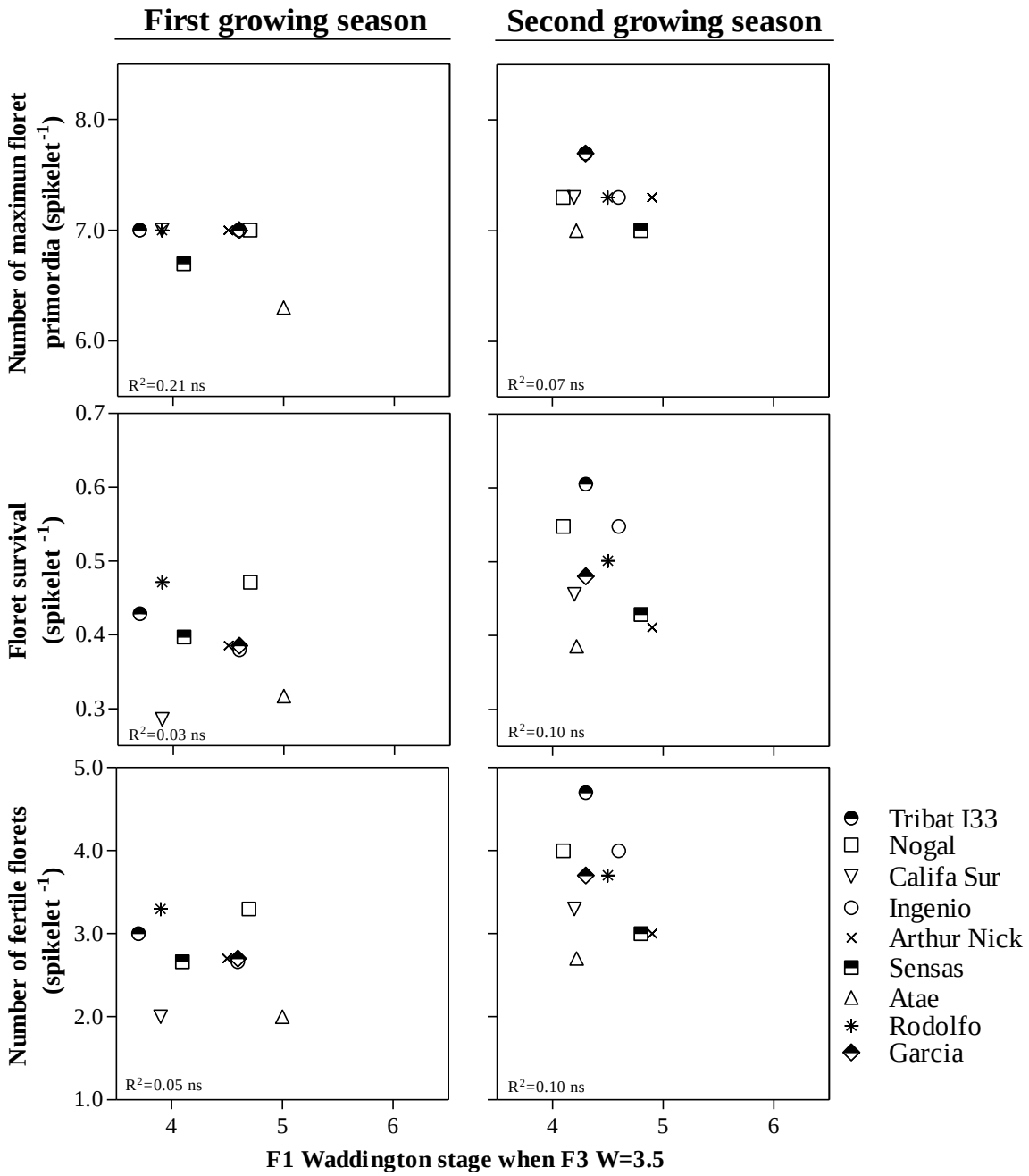


Figure A3.25. Relationship between the number of maximum floret primordia developed (top panels), the floret survival (middle panels) and the number of fertile floret (bottom panels) in the central spikelets against F1 Waddington stage when F3 reached W=3.5 among modern cultivars during the first (left column) and the second growing seasons (right column). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

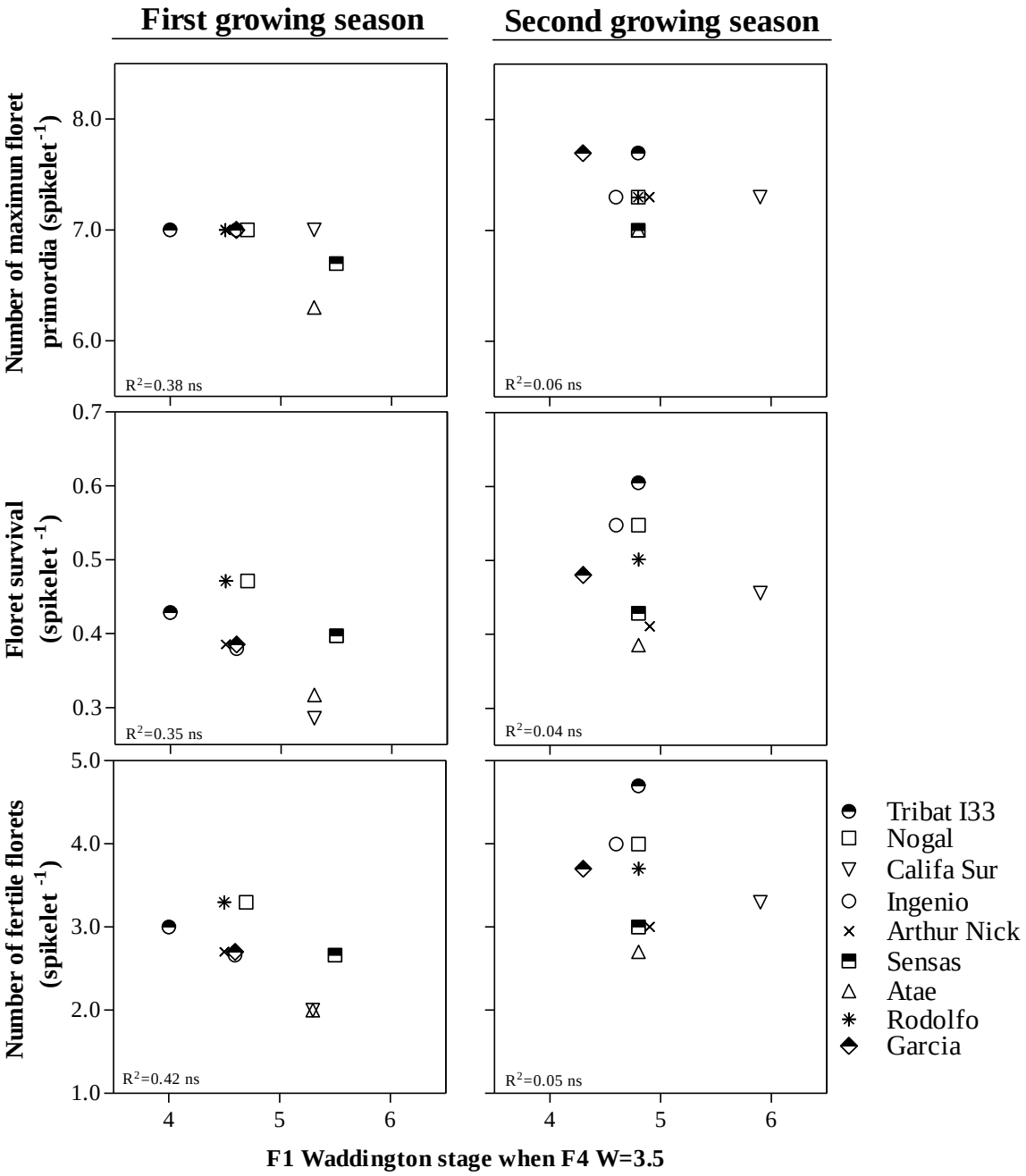


Figure A3.26. Relationship between the number of maximum floret primordia developed (top panels), the floret survival (middle panels) and the number of fertile floret (bottom panels) in the central spikelets against F1 Waddington stage when F4 reached W=3.5 among elite cultivars during the first (left column) and the second growing seasons (right column). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

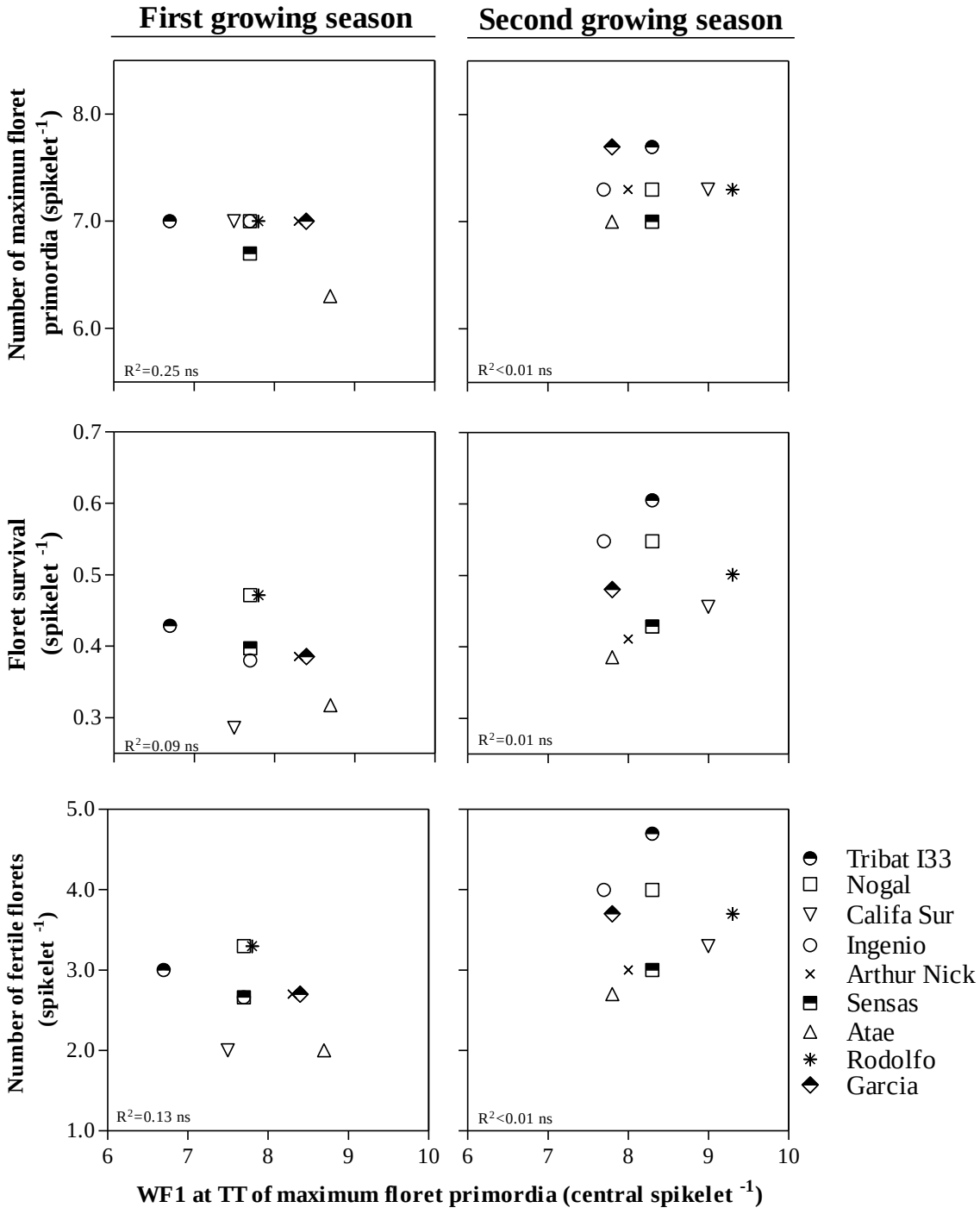


Figure A3.27. Relationship between the number of maximum primordia developed (top panels), the floret survival (middle panels) and the number of fertile florets (bottom panels) in the central spikelets against Waddington (W) stage of F1 at maximum floret primordia thermal time (onset of floret death) among the modern cultivars during the first (left panels) and the second growing seasons (right panels). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

Chapter IV: Physiological determinants of fertile florets as affected by Ppd genes

1. Introduction

The effects of major photoperiod sensitivity genes on the rate of development have been extensively reported for the total time from sowing or seedling emergence to anthesis (see Chapter I). However, due to the intrinsic difficulties associated with measurements required, the effects of these major *Ppd* alleles on the specific length of the floret development phase immediately preceding flowering have received much less attention, and those on the rates and dynamics of the floret development have been only exceptionally analyzed. This is rather relevant as wheat is a cleistogamous species and consequently most fertile florets, resulted from the survival of floret primordia initiated, become grains (Kirby, 1988); and yield is largely determined by the number of grains at maturity (Slafer *et al*., 2006; Reynolds *et al.*, 2009; Sadras and Slafer, 2012; Serrago *et al.*, 2013).

As stated above, there were very few attempts of quantifying the effects of *Ppd* alleles on the physiological bases of floret fertility. These attempts have considered a very limited number of isogenic lines (e.g. González *et al*., 2005*b*). As the genetic background may affect developmental responses (Jones *et al*., 2016), it is critical to count with more comprehensive studies of the effects of these alleles on the developmental processes determining floret fertility.

The main aim of the work reported in the present chapter was to determine the effects of *Ppd* alleles on the final number of fertile florets at anthesis characterizing floret development patterns during the stem elongation phase, and quantifying the dynamics of floret primordia initiation and degeneration to shed light on some physiological causes of these alleles on the reproductive fertility of the crop.

2. Materials and methods

2.1 General description

Two field experiments were carried out during 2012-2013 (first growing season) and 2013-2014 (second growing season) in two different fields close to Bell·lloc d'Urgell, Lleida, NE Spain (Lat. 41°38´11´´N, Long. 0°47´20´´E and Lat. 41º39´2´´N, Long. 0º46´23´´E, respectively). In both fields the soil type was a complex of Calcisol petric and Calcisol haplic, (IUSS Working Group WRB, 2006). Soil status at sowing in both growing seasons ensured that there was not a deficit of Nitrogen during the crop growth. Experiments were sown on 24 November 2012 and on 12 November 2013 at a rate of 300 seeds m^{-2} in both growing seasons. Plots size was 4.8 m^2 (6 rows 0.2 m apart and 4 m long) in both experiments.

2.2 Treatments and experimental design

Treatments consisted of wheat NILs differing in photoperiod allelic combinations (Table 4.1). These combinations were produced by having lines of the wild type Paragon (with sensitive alleles in all three genomes) and introgressing the insensitivity alleles in each of the three possible genomes A, B and D, with different doses: i.e. single (with insensitive allele in one of the genomes), double (with insensitive alleles in two of

the genomes) or triple dose (with insensitive alleles in the three genomes) of insensitivity. In addition, for particular loci different sources of the insensitivity alleles were used: sources of insensitivity were genotypes GS-100, Chinese Spring, Sonora 64 and Recital (donors). Photoperiod insensitivity alleles were introgressed in a common recipient parent, Paragon, by crossing followed by backcrossing to Paragon at least to BC_6. These NILs were produced and provided by the John Innes Centre (Norwich-UK).
The different lines were arranged in a completely randomized design in 2012-13 with an irregular number of replicates, from 1 to 5, depending on the amount of seeds available for each particular line. Only in two out of the 13 genotypes grown (Paragon with a single insensitive allele from Recital in chromosome 2B -a_P+B_R+d_P- and the triple insensitive NIL with insensitive alleles from GS-100 in chromosome 2A and from Sonora 64 in chromosomes 2B and 2D -A_{GS}+B_S+D_S-), the available seed was so scarce that I could only afford to sow one replicate. The ANOVAs were performed having the imbalance into account. As I multiplied the seed and produced our own stock in the first growing season, in the second experiment all lines were grown in a completely randomized block design with three replicates.

2.3 Measurements and analyses

Details of general procedures applied, measurements taken and analyses performed in the experiments of this chapter are detailed in Chapter II.
Results of this chapter are based on determination of the number of fertile florets at anthesis, the mapping of fertile florets in main-shoot and tillers spikes, and the dynamics of development of floret primordia in the central spikelets of the main-shoots spikes, analyzed for each floret position in each of the experimental units (each replicate of the 13 genotypes) in both experimental growing seasons. All in all the dynamics of floret development were analyzed for between seven and eight individual florets, depending on the genotype, in each experimental unit (see Chapter II for detail explanation on how these measurements were done).
To simplify the presentation of the results, in the main body of this chapter, the details are shown for 4 genotypes (which represent the ranges exhibited by the 13 lines) and for 4 floret positions (which represents roughly the range of lability of the floral organs). Regarding genotypes, I showed in the main body of the chapter the details for the wild type (with three sensitive alleles), and for one NIL with a single, one with a double and one with a triple substitution with insensitive alleles. For floret positions the details are shown in the main body of the chapter for floret primordia with either very stable final outcome (florets 1 and 6) or very labile (florets 3 and 4). Thus, the most proximal floret represent cases in which complete development is virtually always achieved, the sixth floret primordium from the rachis is never developed enough to reach the fertile floret stage at anthesis and florets 3 and 4 are the most labile positions in which the effects of treatments affecting floret development to reach a fertile floret can be seen most noticeably. However, all floret primordia were analyzed in detail in all the NILs in each growing season and the dynamics of the other florets and for each of all other NILs are also shown but in an annex to the chapter (Annex 4).

Table 4.1. Plant material used in the experiments consisting of NILs carrying one, two or three photoperiod insensitivity alleles in chromosome 2 of the different genomes A, B, D (the alleles conferring insensitivity are labelled with an "a" and those conferring sensitivity with a "b") and the parental Paragon. The code used for referring to the NILs indicating the genomes with insensitivity alleles (in capital) and with the source of such alleles (subscript) is also provided; in the case of the sensitive alleles (with the genome letter always in lowercase), the code was only P as the unique source of sensitivities is the wild type Paragon.

Genotype (donor insensitivity allele/alleles)	Number of insensitivity alleles	Genome	Allelic combination	Code
Paragon	0		*Ppd A1b Ppd B1b Ppd D1b*	$a_P+b_P+d_P$
P(GS-100 2A)	1	A	*Ppd A1a Ppd B1b Ppd D1b*	$A_{GS}+b_P+d_P$
P(Chinese Spring 2B)	1	B	*Ppd A1b Ppd B1a Ppd D1b*	$a_P+B_{CS}+d_P$
P (Sonora 64 2B)	1	B	*Ppd A1b Ppd B1a Ppd D1b*	$a_P+B_S+d_P$
P (Recital 2B)	1	B	*Ppd A1b Ppd B1a Ppd D1b*	$a_P+B_R+d_P$
P (Sonora 64 2D)	1	D	*Ppd A1b Ppd B1b Ppd D1a*	$a_P+b_P+D_S$
P(GS-100 2A+Chinese Spring 2B)	2	A+B	*Ppd A1a Ppd B1a Ppd D1b*	$A_{GS}+B_{CS}+d_P$
P(GS-100 2A+ Sonora 64 2B)	2	A+B	*Ppd A1a Ppd B1a Ppd D1b*	$A_{GS}+B_S+d_P$
P(GS-100 2A+ Sonora 64 2D)	2	A+D	*Ppd A1a Ppd B1b Ppd D1a*	$A_{GS}+b_P+D_S$
P(Chinese Spring 2B+ Sonora 64 2D)	2	B+D	*Ppd A1b Ppd B1a Ppd D1a*	$a_P+B_{CS}+D_S$
P(Sonora 64 2B+Sonora 64 2D)	2	B+D	*Ppd A1b Ppd B1a Ppd D1a*	$a_P+B_S+ D_S$
P(GS-100 2A+Chinese Spring 2B+Sonora 64 2D)	3	A+B+D	*Ppd A1a Ppd B1a Ppd D1a*	$A_{GS}+B_{CS}+D_S$
P(GS-100 2A+Sonora 64 2B+Sonora 64 2D)	3	A+B+D	*Ppd A1a Ppd B1a Ppd D1a*	$A_{GS}+B_S+D_S$

Furthermore, the dynamics of living floret primordia was estimated for the apical positions of all the NILs and Paragon in both growing seasons comparing the data of the dynamics of living floret primordia in the different spikelets positions of four cultivars from the bibliography (Ferrante *et al.*, 2013) and using my data of the central spikelets and final number of fertile florets in the apical positions measured in the NILs and Paragon.

2.4 Weather conditions

Main differences between growing seasons were that April and May were hotter (3.7 and 4.0ºC) in the second than in the first growing season: maximum temperatures averaged for April were 18.8ºC in the first growing season and 22.5ºC in the second one, and for May they were 20.4ºC in the first growing season and 24.4ºC in the second one (Fig. 4.1). All genotypes reached anthesis stage between 25 April and 21 May during the first growing season whereas during the second one they flowered between 21 April and 13 May. Comparing to the average of the six previous years (from 2007 to 2012, data provided by Xarxa d'Estacions Meteorològiques Automàtiques de Catalunya), April was hotter in both growing seasons (maximum temperatures: 1.4 and 5.1ºC above) while May maximum temperature in the first growing season was 1.8ºC lower than the historical maximum and during the second growing season it was 2.1ºC warmer (Fig. 4.1).

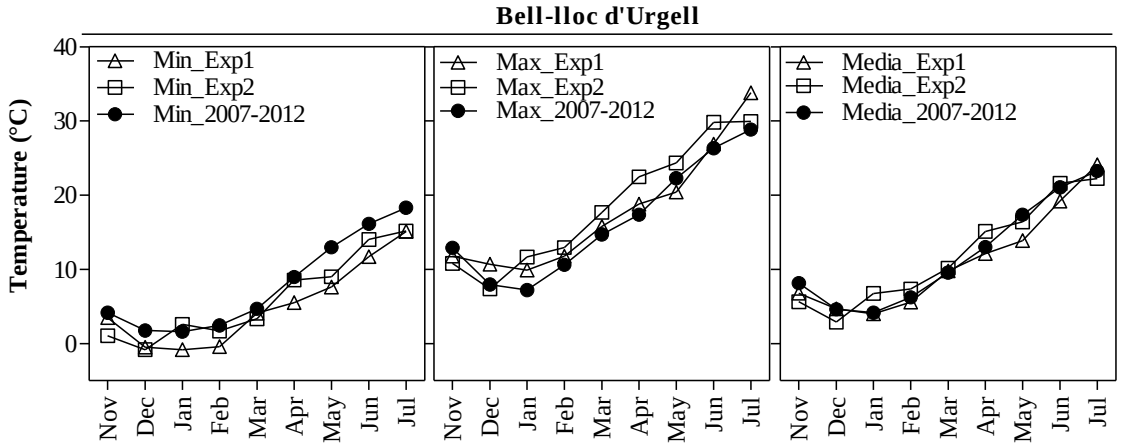


Figure 4.1. Weather conditions: Minimum (left panel), maximum (middle panel) and mean (right panel) temperatures averaged monthly in the first (Exp1) and second growing (Exp2) seasons and in the six years previous to the experiments (2007-2012).

3. Results

3.1 Number of fertile florets at anthesis

All NILs developed less fertile florets at anthesis than Paragon, although the differences were only consistently significant in the first growing season (Fig. 4.2, top-left panel) while only a consistent trend, though mostly non-significant, was evident in the second growing season (Fig. 4.2, top-right panel). In the first season the reductions ranged from less than 20 to more than 40% among the NILs with a single dose of insensitivity, and between almost 30 and 60% when 2 or 3 insensitivity alleles were introgressed (Fig. 4.2, bottom-left panel). In the second season all NILs exhibited less fertile florets at anthesis than Paragon as well, but only in one line, that with an insensitive allele in genome B introgressed from Chinese Spring, the reduction was significant and noticeable (Fig. 4.2, bottom-right panel).

Comparing the NILs with only one insensitivity allele, the strongest reduction in final number of fertile florets was observed with the insensitivity introgressed in the D genome in the first growing season (Fig. 4.2 bottom-left panel) but this allele was not consistently the stronger one in the second season (Fig. 4.2 bottom-right panel) and the effect of one of the NILs with insensitivity in the B genome introgressed from Sonora 64 was virtually equally strong to the insensitivity in the D genome (Fig. 4.2 left panels). Analyzing the data of the first growing season in which the introgression of *Ppd* alleles had a consistent significant effect, there was a clear overall inverse relationship between the number of insensitivity alleles and number of fertile florets (Fig. 4.2 top-left panel, inset); but that was true in average, while some individual NILs with a single insensitivity allele had stronger reductions in number of fertile florets than some particular NILs with more than a single introgression (Fig. 4.2 left-panels).

In order to find out possible causes behind the differences observed in the number of fertile florets at anthesis (i) the number (and the distribution) of fertile florets per spike (‘mapping’ the number of these florets at each spikelet position of the main-shoot and tillers spikes) and (ii) the developmental processes of floret primordia in the central spikelets of the main-shoots spikes were analyzed in detail. This was done for all lines,

but to illustrate the results in the main body of this chapter, representative NILs of the single (a_P+B_{CS}+d_P), double (A_{GS}+B_{CS}+d_P) and triple (A_{GS}+B_{CS}+D_S) insensitivity alleles were chosen: a_P+B_{CS}+d_P presented significant differences in the number of fertile florets compared to Paragon in both growing seasons, A_{GS}+B_{CS}+d_P presented the highest differences from Paragon during the first growing season and A_{GS}+B_{CS}+D_S was the triple one with significant differences compared to the parent during the first growing season. The data from the rest of the NILs are also presented with the same detail but in the Annex 4 of the present Thesis, and general relationships and conclusions are based on results of the whole set of lines analyzed not just those used to illustrate the results in the main body of the chapter.

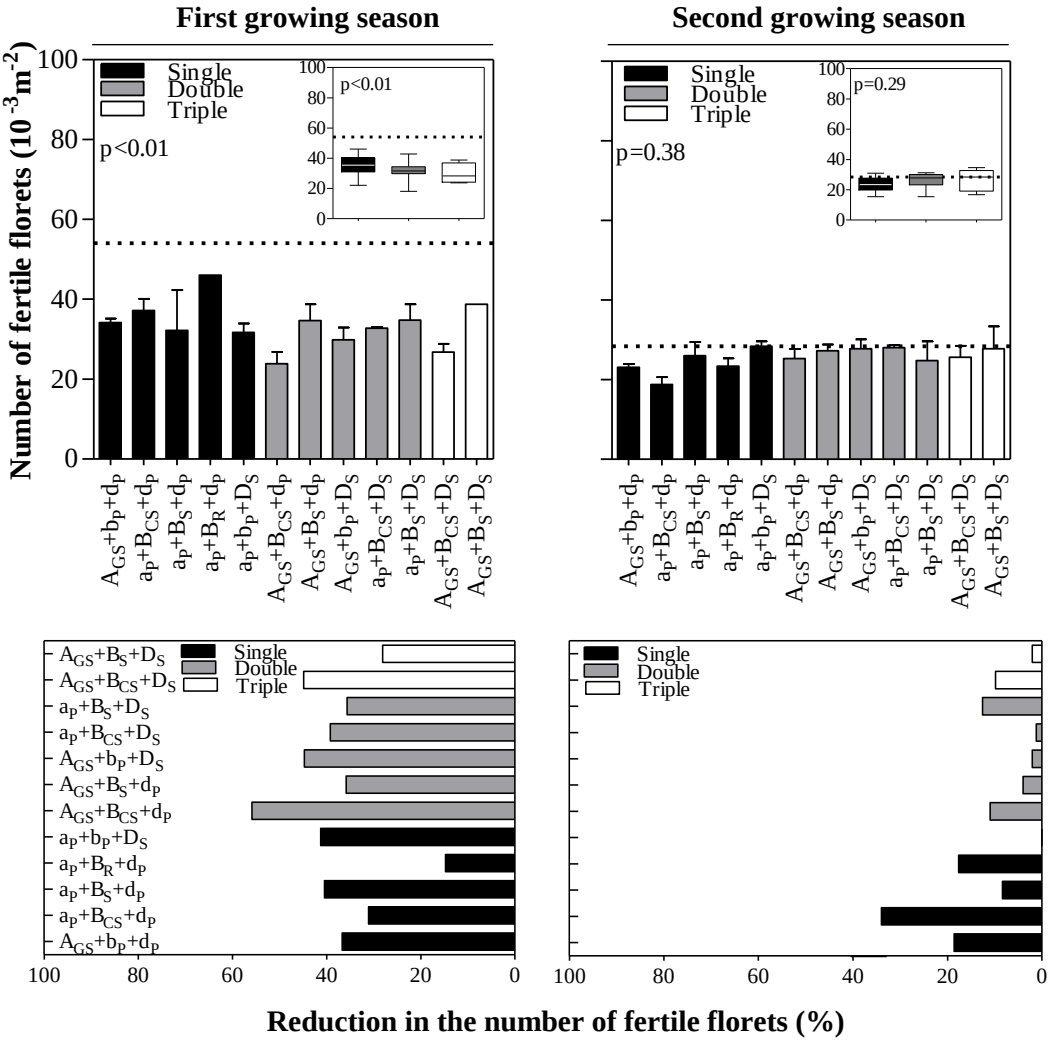


Figure 4.2. Top panels: Number of fertile florets for each of the *Ppd* NILs (bars) and the wild type Paragon (dotted line) during the first (*left panel*) and the second growing seasons (*right panel*). Error bars stand for the standard error of the means. Inset each panel are the box-plots grouping the NILs with the single, double or triple doses of *Ppd* alleles introgressed. *Bottom panels:* Reduction in the number of fertile florets between each of the *Ppd* NILs and the wild type Paragon during the first (*left panel*) and second growing seasons (*right panel*).

3.2 Mapping fertile florets

In general, differences in the number of fertile florets along the spikes between the NILs and Paragon were extremely clear only in the first growing season (Fig. 4.3, left columns of panels). This is in line with the highest reduction found in the total number of fertile florets at anthesis in this growing season (Fig. 4.2, left panels).

Compared with Paragon carrying one single insensitivity allele reduced the number of fertile florets more noticeably in the apical spikelets of the main-shoots and the tillers spikes in the first growing season (Fig. 4.3, top-left panel), but not in the second growing season, when the reduction was observed across most spikelets mainly in the main-shoots spikes (Fig. 4.3, top-right panels). The NILs carrying a double or triple doses of insensitivity alleles reduced the number of fertile florets more markedly making the difference with Paragon clear in almost all spikelets of both main-shoot and tiller spikes during the first growing season (Fig. 4.3, middle- and bottom-left panels), while differences in fertile florets per spike tended to be negligible in the second growing season (Fig. 4.3, middle- and bottom-right panels). Moreover, NILs spikes tended to be shorter than Paragon during the first growing season. Similar responses were found for main-shoot and tiller spikes for the rest of the NILs (see Fig. A4.1-4 in Annex 4). As the differences between NILs and Paragon in fertile florets per spike resembled reasonably well those in the number of fertile florets per unit land area (Fig. 4.4) the main effects of *Ppd* alleles were on the fertility of the spikes rather than on the fertility of tillers. Particularly, differences already explained in the number of fertile florets along the spikes in the NIL carrying one single insensitivity alleles may explain at least part of the effect on the reduction of the number of fertile florets at anthesis of *c.* 31 and 34% in comparison to Paragon in the first and second growing season, respectively (Fig. 4.2, bottom-left and right panels), and in the case of the NILs carrying double and triple insensitivity alleles may explain at least part of the highest differences of *c*. 56% and *c.* 45% respectively during the first growing season (Fig. 4.2, bottom-left panel).

In this context, explaining the causes for the differences in spike fertility between NILs with different levels of insensitivity becomes relevant. Due to the large number of determinations required for analyzing the developmental rate of single floret primordia (see Chapter II), the analysis of the likely origin of differences in floret fertility had to be restricted to florets of the central spikelets of the main-shoot spikes (as it was explained also in the chapter focused on differences in spike fertility among modern cultivars). Even though not perfect, fortunately the fertility of the main-shoot spikes reflected well that of the pool of all spikes in the canopy across all NILs (Fig. 4.5). Naturally the total number of fertile florets was consistently larger in the main-shoot spikes than in the average of all spikes (in all lines, in both growing seasons), but most (>86%; $p<0.001$) of the differences between lines in spike fertility for the average spike in the canopy were explained by differences in fertility of the main-shoot spikes. Likewise, differences in fertility pooling all the spikelets in the spike were highly related to the differences in fertility of the central spikelet (Fig. 4.6), in which I can

trace back the developmental process that would have given origin to differences in floret fertility for each NIL.

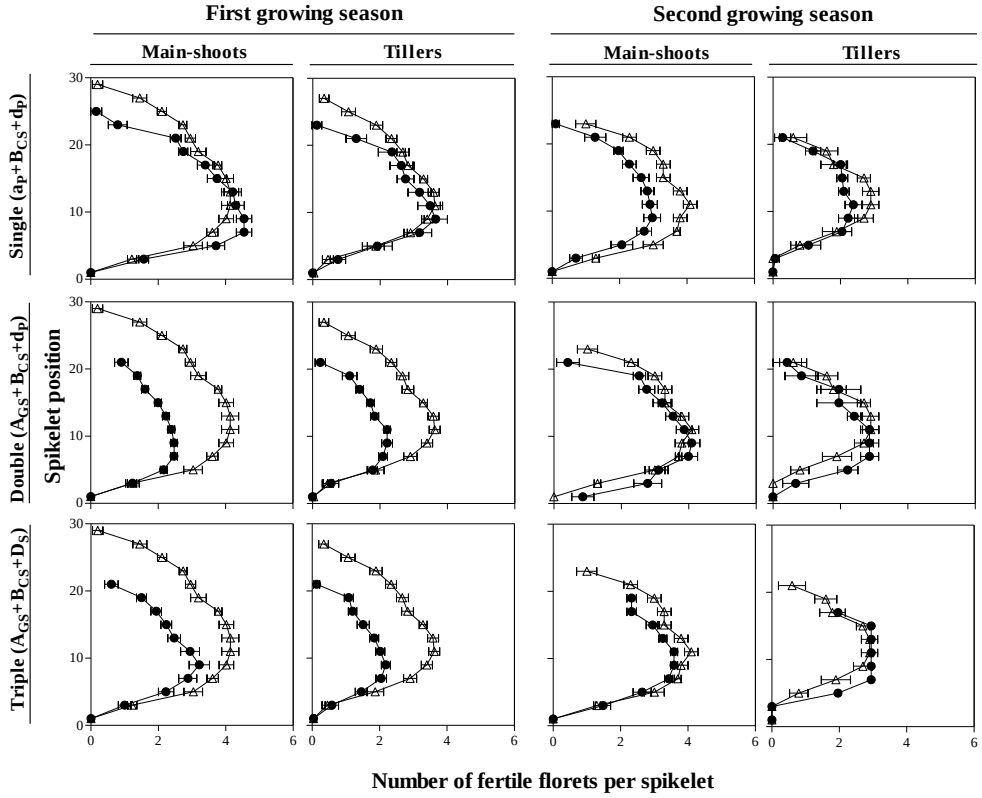


Figure 4.3. Mapping of fertile florets (fertility of each spikelet position on the main-shoot or tiller spikes) for the selected NILs (closed circles) with one (a_P+B_{CS}+d_P, *top panels*), two (A_{GS}+B_{CS}+d_P, *middle panels*) or three (A_{GS}+B_{CS}+D_S, *bottom panels*) insensitivity alleles in comparison to Paragon (a_P+b_P+d_P, open triangles) during the first (*two left columns of panels*) and the second growing seasons (*two right columns of panels*). Each data-point is the average of all replicates and within each replicate the value was the average of 4 plants and the segment in each data-point stands for the standard error of the means (not visible when smaller than the size of the symbol or absence of replicates in A_{GS}+B_{CS}+D_S tillers).

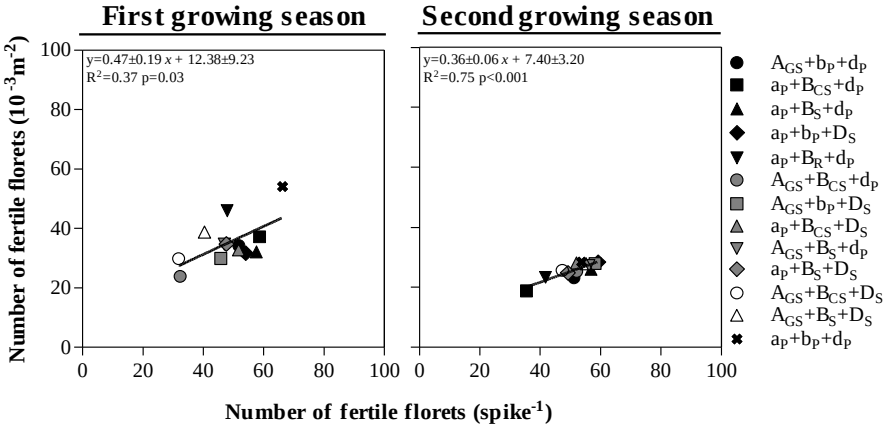


Figure 4.4. Relationship between the number of fertile florets at anthesis per square meter and the number of fertile florets per spike among the *Ppd* NILs and Paragon during the first (*left column*) and the second growing seasons (*right column*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

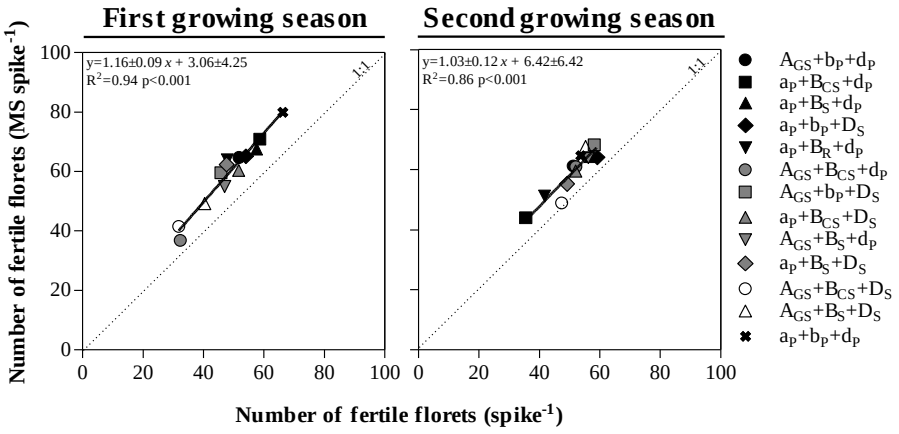


Figure 4.5. Relationship between the number of fertile florets per main-shoot spike and the number of fertile florets per spike among the *Ppd* NILs and Paragon during the first (*left column*) and the second growing seasons (*right column*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

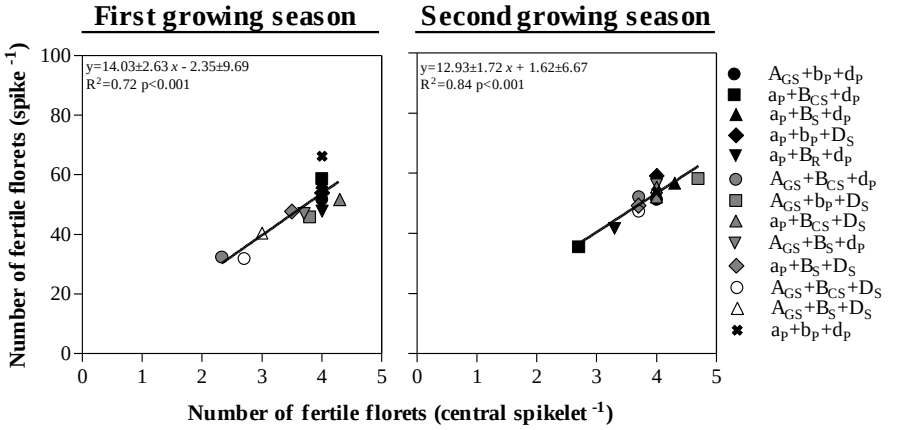


Figure 4.6. Relationship between the number of fertile florets per spike and the number of fertile florets per central spikelet among the *Ppd* NILs and Paragon during the first (*left column*) and the second growing seasons (*right column*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

3.3 Floret development and living floret primordia dynamics in the central spikelets

No relevant differences were found in the dynamics of developmental progress of florets 1, 3 and 4 between the NIL carrying a single insensitivity allele and Paragon during the first growing season (Fig. 4.7A, top panels). Although, floret 6 did not reach the fertile floret stage in any of these two genotypes, this floret primordium was clearly more developed in Paragon than in the NIL with a single insensitive allele (Fig. 4.7A, top panels). As the main difference in developmental rates of floret primordia in the first growing season was evidenced in floret positions which were not fertile in any case, the dynamics of floret initiation and degeneration showed a similar final number of fertile florets in the NIL and in Paragon, even though the period of floret degeneration (when survival or death of particular florets is determined) was slightly longer in the wild type (Fig. 4.7B, top panel). In the second growing season, no differences were found in the dynamics of floret 1 and 3 either, but the fourth floret developed normally until reaching the fertile floret stage in Paragon while it did not do so in the NIL with one insensitive

allele, and no differences were found in development of floret 6 (Fig. 4.7A, bottom panels). Consequently, carrying a single insensitivity allele lowered the number of fertile florets compared with Paragon, which again exhibited a longer period of floret degeneration which might be responsible for the reduced rate of floret mortality increasing the final number of fertile florets at the end of the floret development period (Fig 4.7B, bottom panel).

Comparing the floret development dynamics between a NIL carrying two insensitive alleles and Paragon, during the first growing season, no differences were found in floret 1 but floret 3 reached the fertile stage in all Paragon plants whereas it did so only in some of the plants of the NIL (Fig. 4.8A, top panels). In addition, floret 4 reached the fertile stage only in Paragon but never in the NIL with two insensitive alleles. Again, floret 6 which was not developed enough to reach the fertile floret stage in any genotype, presented in the first growing season a less advanced developmental stages in the NIL with two insensitive alleles than in Paragon (Fig. 4.8A, top panels). During the second growing season, no difference was found for F1 and 3 but floret 4 was fertile always in Paragon while it was so only in some plants of the NIL with two insensitive alleles (Fig. 4.8A, bottom panels). In this second season floret 6 was actually more developed in the NIL with two insensitive alleles than in the wild type (Fig. 4.8A, bottom panels). Regarding to the number of living floret primordia dynamics, in both growing seasons the NIL with two insensitive alleles had a shorter duration of floret death and a lower number of fertile florets at anthesis, in both cases the differences were clearer in the first than in the second growing season (Fig. 4.8B).

In the case of the NIL carrying three insensitivity alleles in comparison to Paragon results of developmental progress towards becoming fertile structures of individual florets were rather similar to the other two NILs, although more similar to the NIL with two than to that with one insensitive allele (Fig. 4.9A). Regarding the resulting dynamics of generation and degeneration of floret primordia the NIL with the three insensitive alleles presented a reduction in the number of fertile florets at the end of the process, which was more clear in the first than in the second growing season but the reduction in the duration of the floret death period was clear only in the second season (Fig. 4.9B).

Similar differences with the wild type for the overall patterns of the floret developmental progress for individual floret positions and of dynamics of living floret primordia were observed in most of the other NILs with also similar differences in magnitude of responses between growing seasons (see Annex 4, Fig. A4.5-34); although there were some exceptions in which the NILs with insensitivity finally produced more fertile florets that Paragon in the central spikelets (e.g. $a_P+B_{CS}+D_S$ in the first growing season; $a_P+B_S+d_P$ and $A_{GS}+b_P+D_S$ in the second growing season; see A4.30, 32 and 33). But these exceptions have to be taken carefully as they are more apparent than real: the insensitivity alleles also induced to produce less spikelets per spike and consequently the apparently higher fertility of these exceptional cases mostly reflects that difference in spike structure and in all cases introgression of insensitivity alleles reduced the overall fertility of the spikes. This can be supported by comparing the estimated dynamics of number of living floret primordia in the apical positions in which

photoperiod insensitivity caused a reduction in the number of fertile florets in all the NILs in comparison to Paragon in the first growing season (Fig. 4.10) and in most of them during the second one (4.11).

In general the effects of the photoperiod insensitivity alleles on the final number of fertile florets were mainly due to their effects on floret death determining the level of floret survival. Although there was variation in the maximum number of floret primordia initiated, overall NILs there was no relationship between the number of fertile florets at anthesis and the maximum number of floret primordia developed in any of the two growing seasons (Fig. 4.12, top panels). On the other hand, the number of fertile florets was highly and positively related to floret survival in both growing seasons (Fig 4.12, bottom panels).

At least in part the difference in likelihood of a floret primordium to become a fertile floret may be related to the differential level of competition that could be established between developing floret primordia governing the rate of floret death. Two major issues may be behind the establishment of different levels of competition, resulting in different levels of floret mortality: (i) the degree of synchronization of floret initiation and (ii) the timing available for floret development. To find out if photoperiod insensitivity alleles were affecting the synchrony I analyzed the linear regressions between the floret position, from 1 to 7-8 and thermal time (from anthesis) when these florets reached stage 3.5 in the Waddington *et al.*, 1983 scale (W3.5). The slopes of these relationships were compared (for more details see Chapter II).

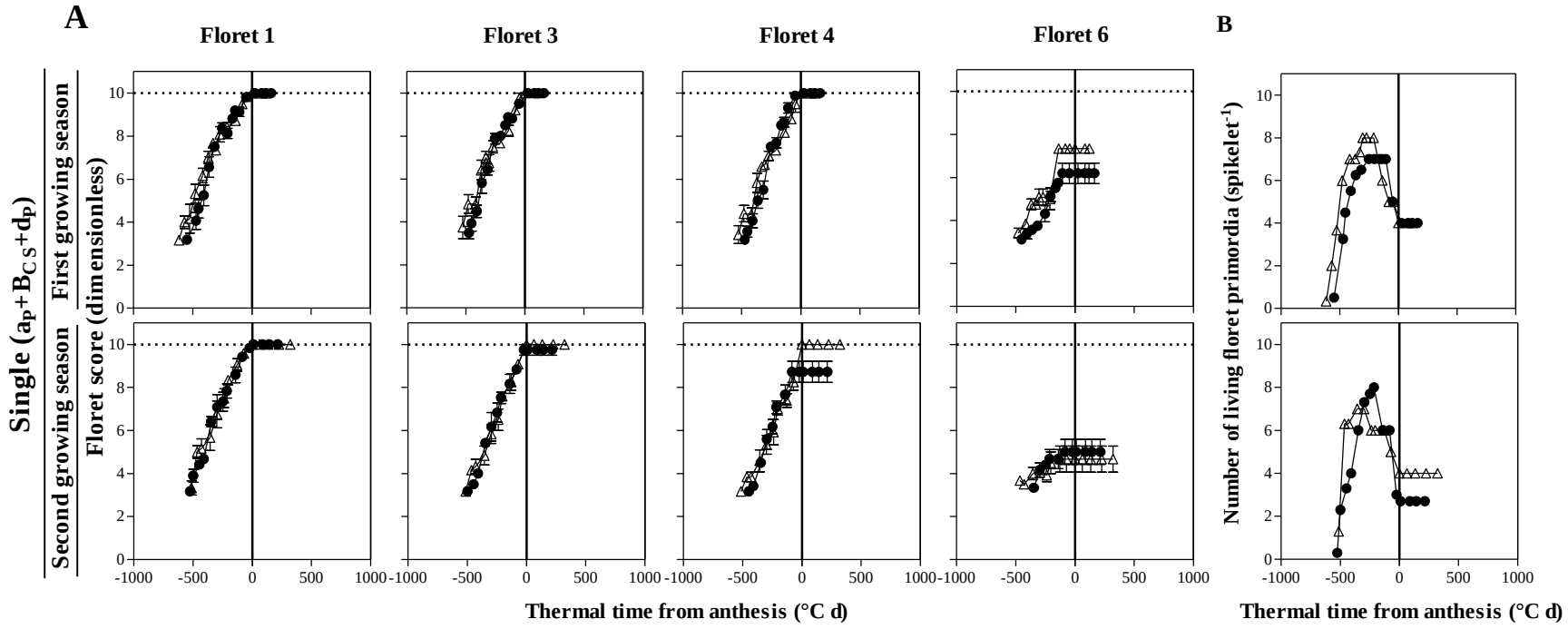


Figure 4.7. Dynamics of the floret development of F1, F3, F4 and F6 in central spikelets of the main-shoot (A) and the number of living floret primordia (B) through thermal time from anthesis in $a_P+B_{CS}+d_P$ carrying one single change (closed circles), in comparison to Paragon ($a_P+b_P+d_P$, open triangles) during the first (*top panels*) and the second growing seasons (*bottom panels*). Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

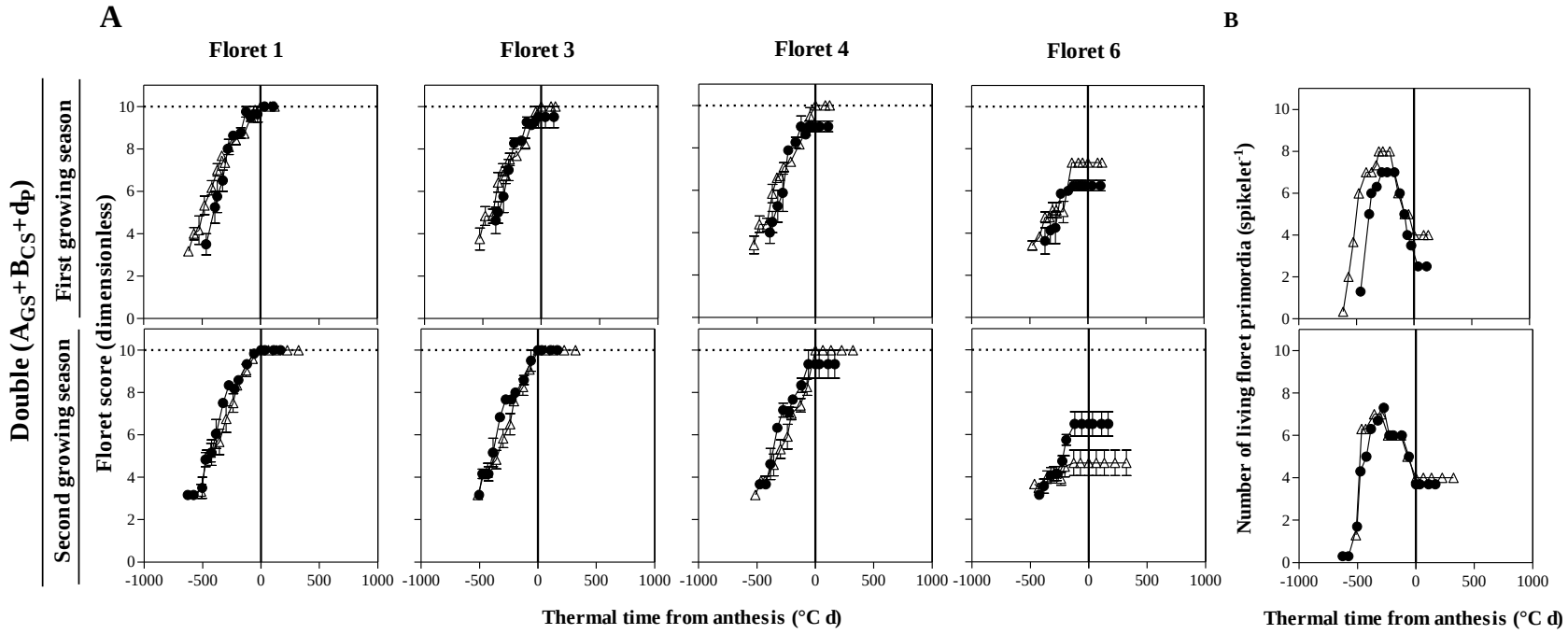
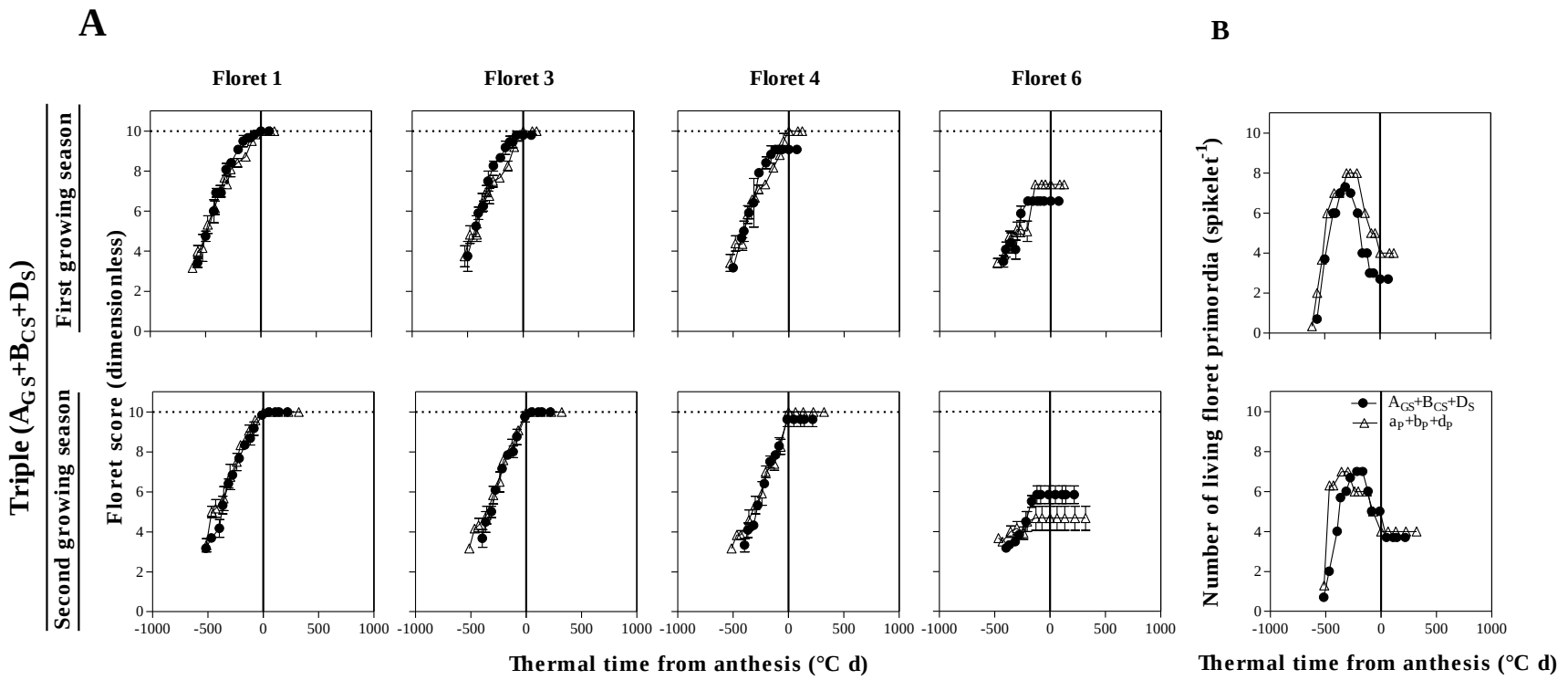


Figure 4.8. Dynamics of the floret development of F1, F3, F4 and F6 in central spikelets of the main-shoot (A) and the number of living floret primordia (B) through thermal time from anthesis in A_{GS}+B_{CS}+d_P carrying double change (closed circles), in comparison to Paragon (a_P+b_P+d_P, open triangles) during the first (*top panels*) and the second growing seasons (*bottom panels*). Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.



F*igure 4.9.* Dynamics of the floret development of F1, F3, F4 and F6 in central spikelets of the main-shoot (A) and the number of living floret primordia (B) through thermal time from anthesis in A_{GS}+B_{CS}+D_S carrying triple change (closed circles), in comparison to Paragon (a_P+b_P+d_P, open triangles) during the first (*top panels*) and the second growing seasons (*bottom panels*). Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

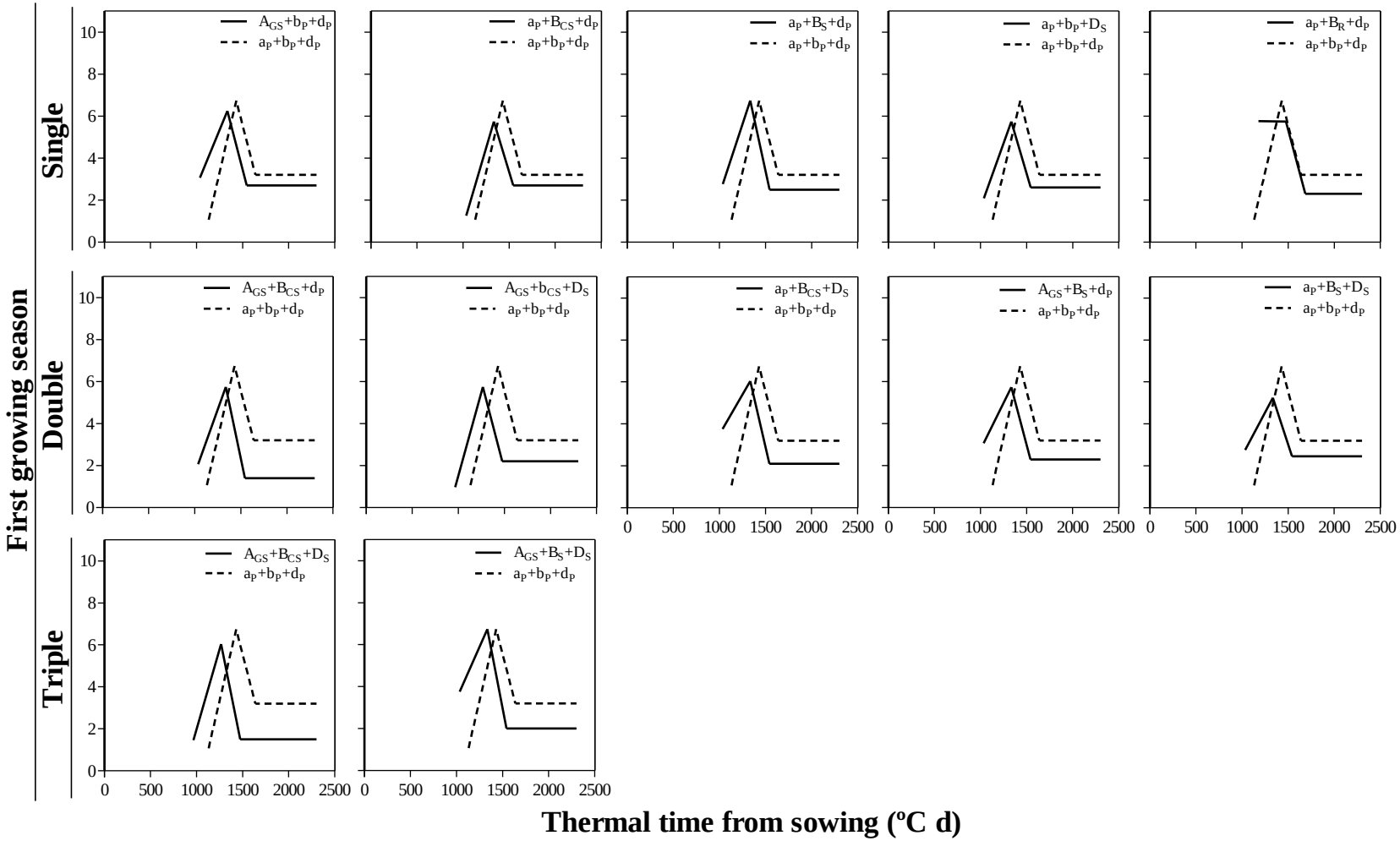


Figure 4.10. Number of living floret primordia in the apical spikelets through thermal time from sowing in NILs carrying single, double or triple changes (continuous line) in comparison to Paragon (dotted line) during the first growing season. Data was estimated using the data of the central spikelets measured and on the basis of the differences in the dynamics of living floret primordia among the different spikelets positions from Ferrante *et al*., 2013.

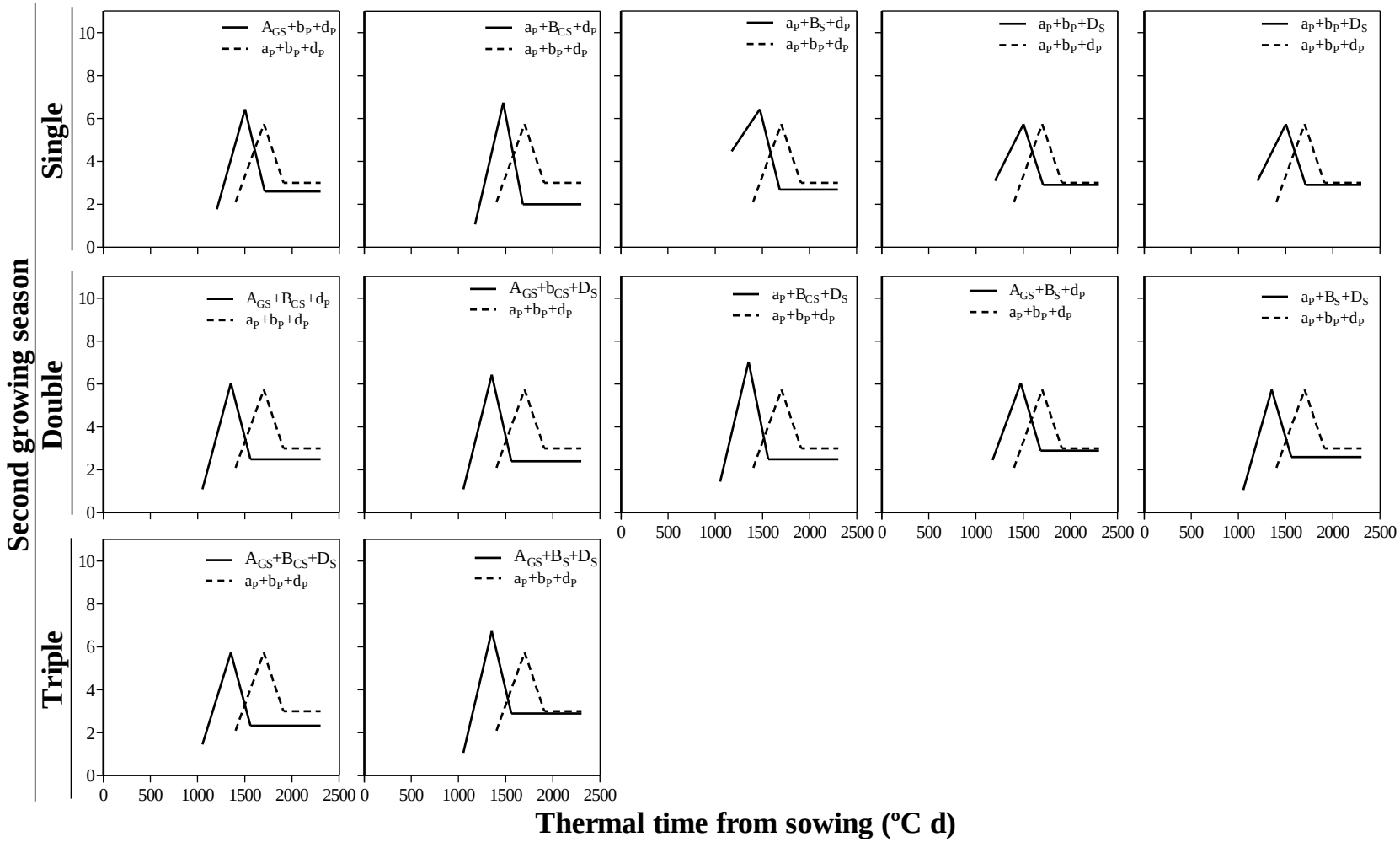


Figure 4.11. Number of living floret primordia in the apical spikelets through thermal time from sowing in NILs carrying single, double or triple changes (continuous line) in comparison to Paragon (dotted line) during the second growing season. Data was estimated using the data of the central spikelets measured and on the basis of the differences in the dynamics of living floret primordia among the different spikelets positions from Ferrante *et al*., 2013.

3.4 Synchrony in floret primordia initiation

The relationships between the floret position and the thermal time before anthesis when each floret initiated were clearly curvilinear in almost all cases, mainly because the most proximal floret primordia were initiated quickly after the previous primordium while the initiation of the most distal primordium tended to be noticeably delayed (remarkably so in the wild type in the second growing season) (Fig. 4.13). Disregarding this curvilinearity, as the trend has a strong linear component, in all cases the linear regression showed a highly significant coefficient of determination (R^2 ranging from 0.71, $p<0.05$, to 0.98, $p<0.001$; Figs. 4.13 and A4.35-36). During the first growing season, there was a trend to increase the rate of floret primordia initiation with the introgression of photoperiod insensitivity alleles,
the difference with the rate of the wild type was not significant for the NIL carrying a single dose of insensitivity alleles (Fig.4.13, top-left panel) but it was larger and significant for the NILs carrying double and triple doses of insensitivity alleles (Fig. 4.13, top-middle and -right panels). During the second growing season, differences were not significant (Fig.4.13, bottom panels). These results reflected well the overall results observed for each of the NILs (Fig. A4.35-36).
However, the differences in rates of floret initiation and consequently in synchrony between the initiation of early- and late-initiated primordia, which were clear in the first year and only minor in the second seemed largely inconsequential for the maximum number of floret primordia initiated (Fig. 4.14, top panels), the floret survival rate (Fig. 4.14, middle panels) and for the number of fertile florets at anthesis (Fig. 4.14, bottom panels).
Finally, there was not a clear relationship between (i) the maximum number of floret primordia initiated (Fig. 4.15, top panels), (ii) the floret survival rate (Fig. 4.15, middle panels), or (iii) the resulting number of fertile florets (Fig. 4.15, bottom panels) and the stage of development of the floret primordium nearest the rachis (the most advanced floret) at the timing when the maximum floret primordia is reached (the onset of floret death).
As it was seen among modern cultivars, the lack of consequences of synchrony on floret generation and survival would be likely reflecting a biological compensation between the rate of floret primordia initiation and the duration of the process.

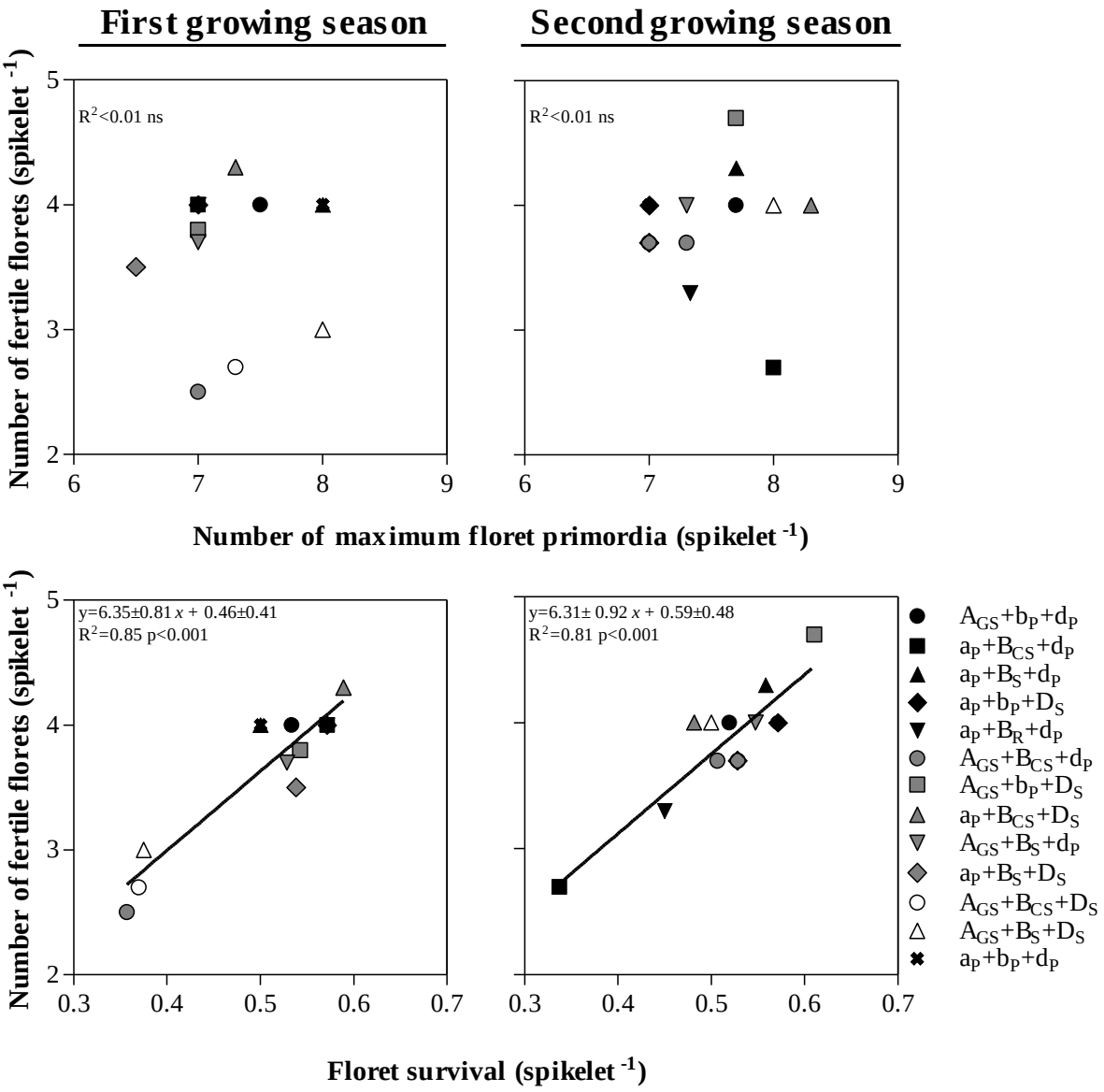


Figure 4.12. Relationship between the number of fertile florets at anthesis and the number of maximum floret primordia developed in the central spikelets of the main-shoots spikes (*top panels*) and relationship between the number of fertile florets at anthesis and the floret survival in the central spikelets from the main-shoots spikes (*bottom panels*) among *Ppd* NILs and Paragon during the first (*left panels*) and the second growing seasons (*right panels*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

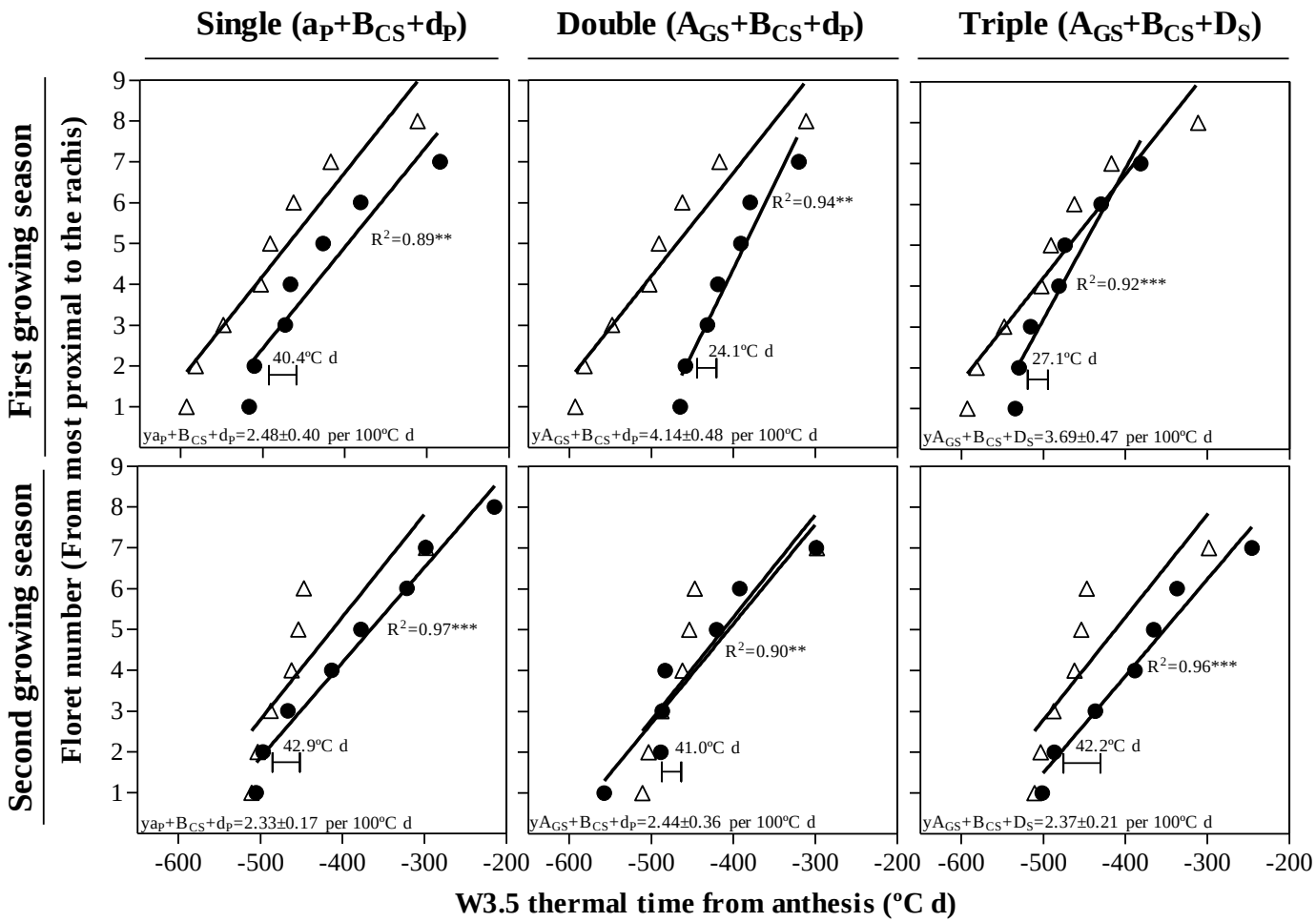


Figure 4.13. Timing of W3.5 for each floret primordium through thermal time from anthesis in selected NILs (closed circles) carrying one single (a_P+B_{CS}+d_P, left column), double (A_{GS}+B_{CS}+d_P, middle column) and triple change (A_{GS}+B_{CS}+D_S, right column) in comparison to Paragon (a_P+b_P+d_P, open triangles) which rate of floret initiation was: 2.53±0.30 (R^2=0.92***) during the first growing season (*top panels*) while it was 2.52±0.72 (R^2=0.71*) during the second growing seasons (*bottom panels*). In all the cases the floret initiation rates are expressed in florets per 100°C d. The coefficient of determination (R^2) and the level of significance for each NIL linear regression are shown. Inset each panel, thermal time between the appearance of two following floret primordia are indicated, for Paragon was 39.5 and 39.7°C d during the first and the second growing seasons, respectively.

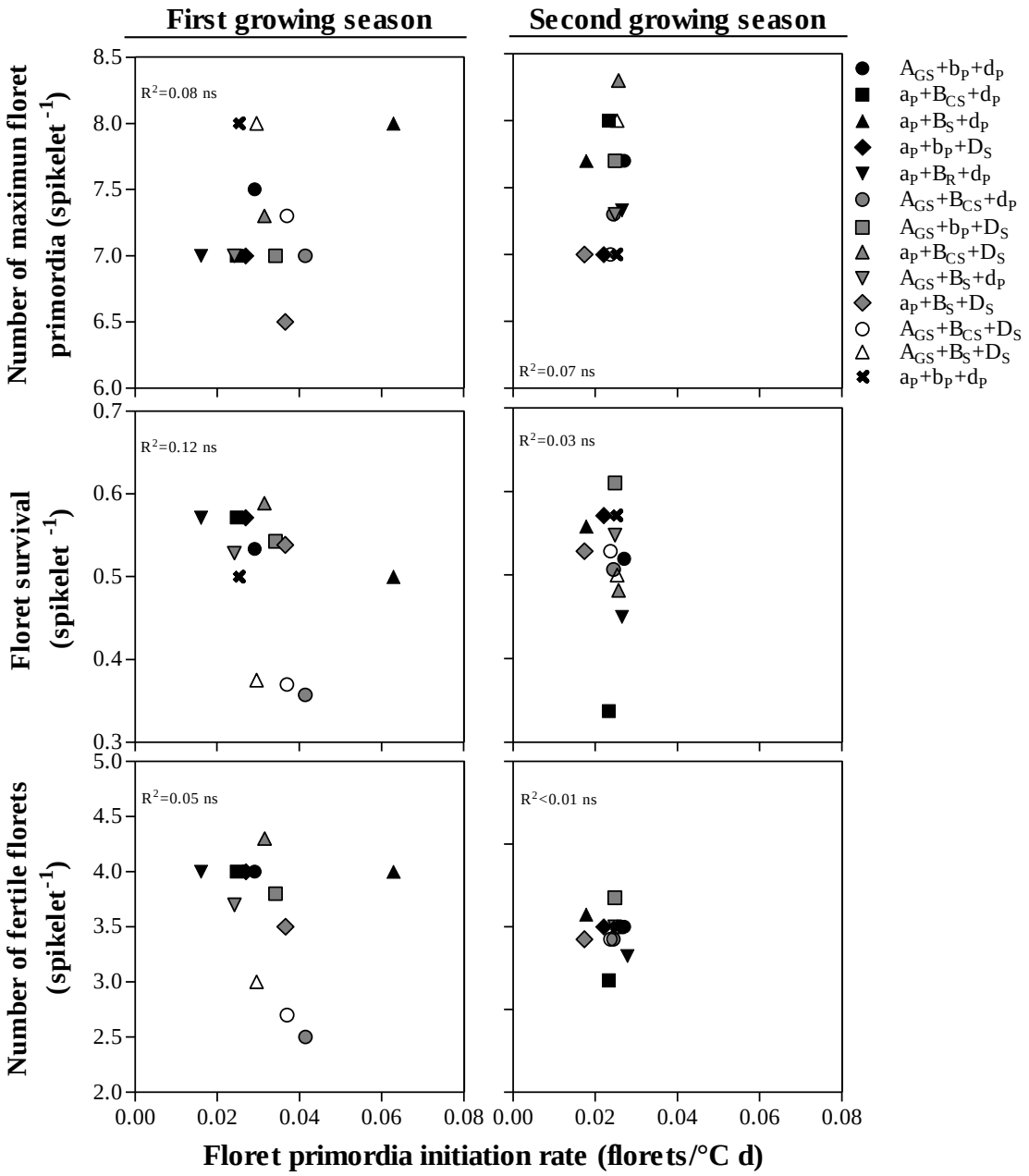


Figure 4.14. Relationship between the number of maximum floret primordia developed (*top panels*), the floret survival (*middle panels*) and the number of fertile floret (*bottom panels*) in the central spikelets against floret primordia initiation rate among the *Ppd* NILs and Paragon during the first (*left panels*) and the second growing seasons (*right panels*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

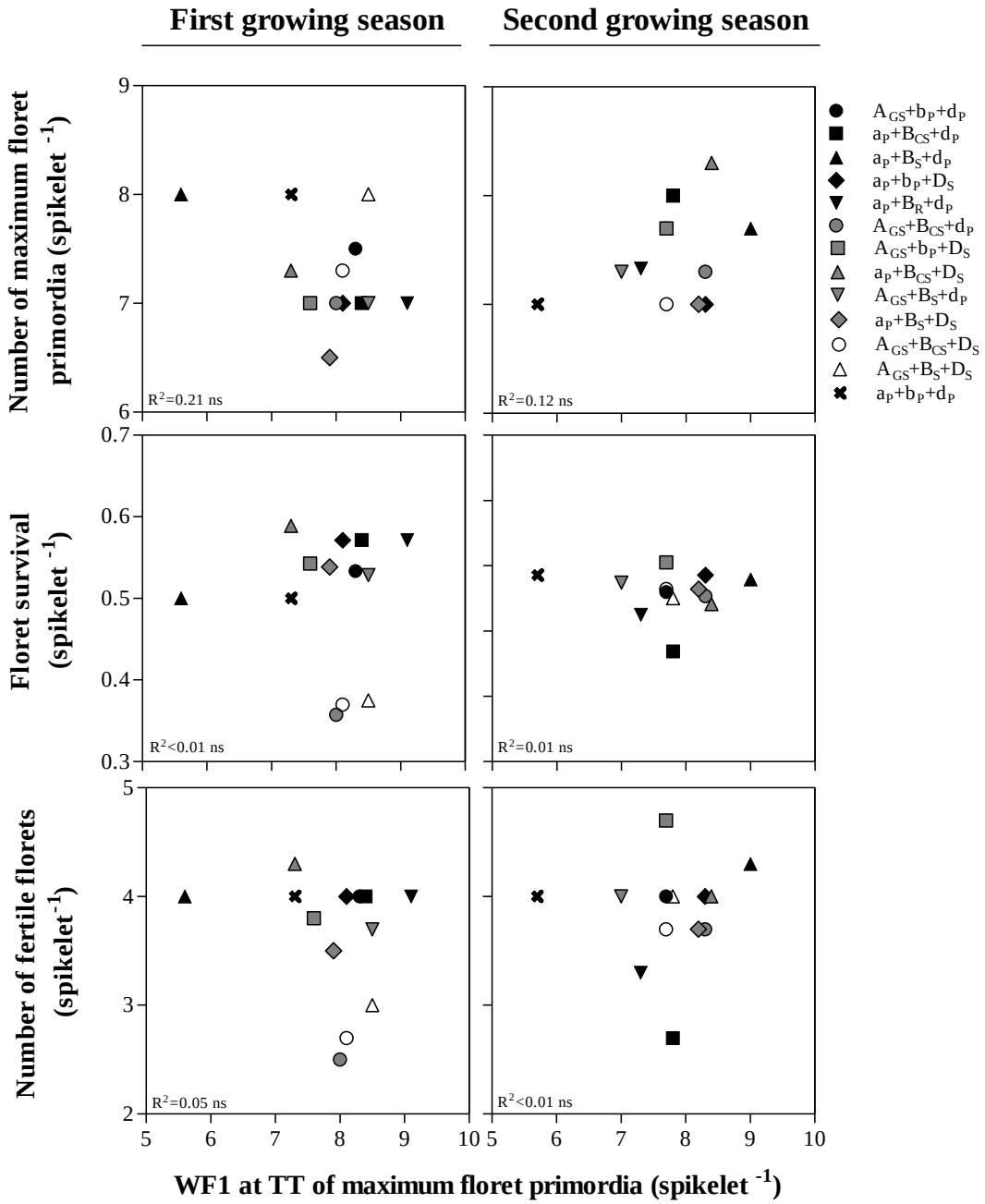


Figure 4.15. Relationship between the number of maximum floret primordia developed (*top panels*), the floret survival (*middle panels*) and the number of fertile florets (*bottom panels*) in the central spikelets against Waddington (W) stage of F1 at maximum floret primordia thermal time (onset of floret death) among the *Ppd* NILs and Paragon during the first (*left panels*) and the second growing seasons (*right panels*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

3.5 Duration of floret development

Carrying the insensitivity allele/s in most of the cases caused a reduction in time to anthesis due to a reduction in the duration of the late reproductive phase from terminal spikelet initiation to anthesis. This result was clear and highly significant in the first growing season but only a trend in the second growing season (Fig. 4.16).

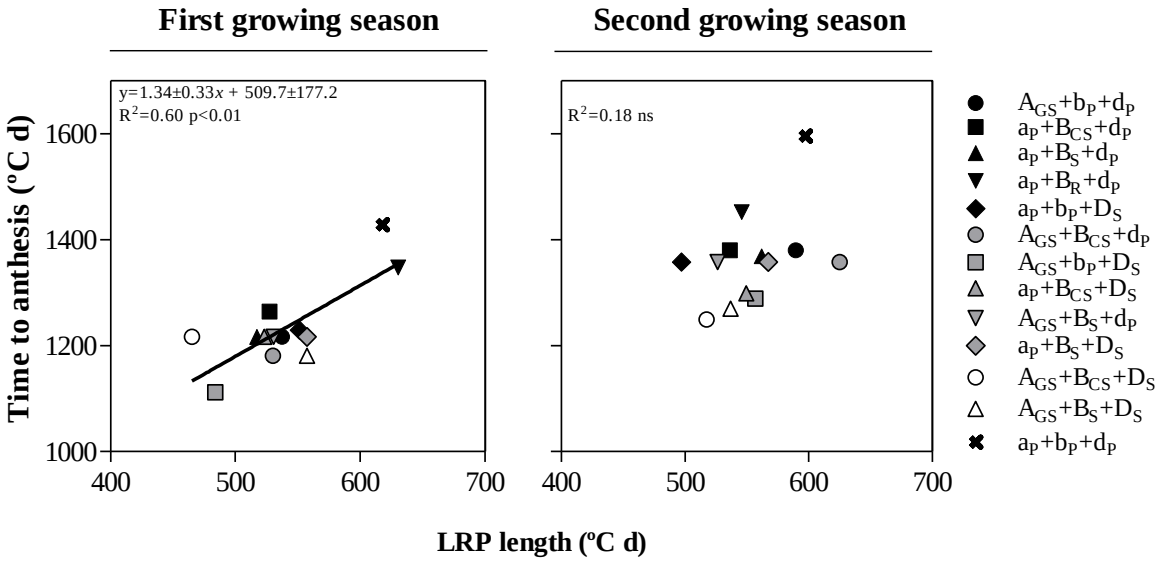


Figure 4.16. Relationship between time to anthesis and the late reproductive phase length (LRP) among the NILs and Paragon during the first (*left*) and the second growing seasons (*right*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

Alike what happened with the synchrony in floret development, there was no clear relationships between the rate of floret survival or its consequence, the number of fertile florets, and any of the durations of floret development. Neither the whole period of stem elongation, nor the specific period of floret initiation, or the period of floret primordia generation, or the period of floret degeneration were relevant for explaining by themselves the rate of floret mortality or the final number of fertile florets (Figs. 4.17-4.18).

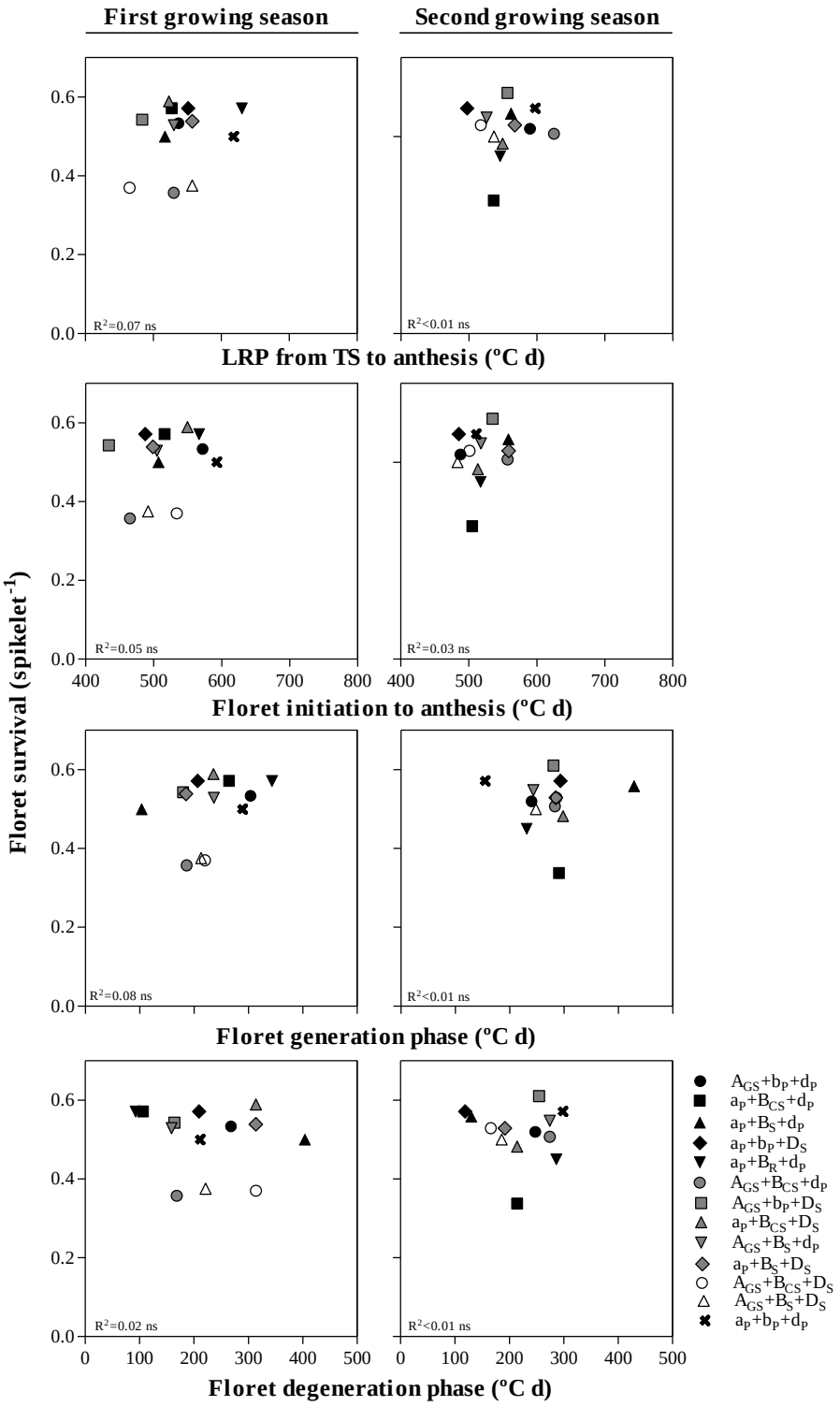


Figure 4.17. Relationship between the floret survival in the central spikelets and i) the late reproductive phase, ii) floret initiation to anthesis phase, iii) floret generation phase and i) floret degeneration phase among the *Ppd* NILs and Paragon during the first (*left column*) and the second growing seasons (*right column*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

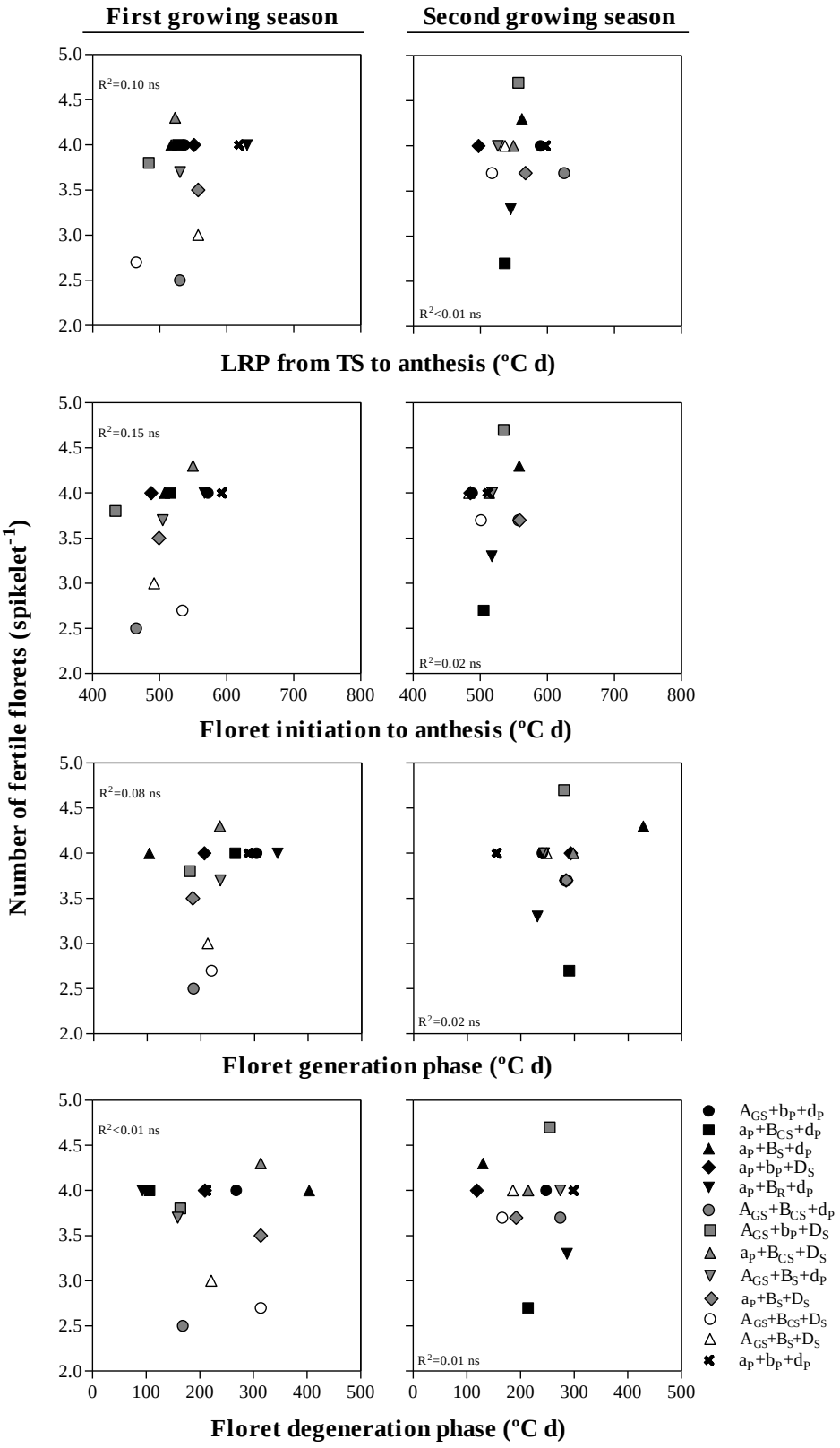


Figure 4.18. Relationship between the final number of fertile florets in the central spikelets and i) the late reproductive phase, ii) floret initiation to anthesis phase, iii) floret generation phase and i) floret degeneration phase among the *Ppd* NILs and Paragon during the first (*left column*) and the second growing seasons (*right column*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

4. Discussion

Ppd NILs and their parental Paragon represented a good population to study complex mechanisms that control floret development and consequently the bases of the determination of the number of fertile florets. This is because they are NILs backcrossed to the recurrent parent for at least 6 generations so genetic differences beyond the alleles involved in the study are minimized and the constitution of the NILs included an arrangement that allowed to analyze in detail not only the effect of the insensitivity in each of the three possible genomes and in their combinations (different doses), but also to elucidate the importance of the source of the alleles.

Carrying photoperiod insensitivity alleles caused a reduction in the number of fertile florets. This result is in agreement with the general effects of *Ppd* alleles on the number of organs being developed during the phase whose duration is affected by these genes. This has been established for leaf and spikelet primordia (i.e. that when photoperiod changes the duration of a phase it does change more or less proportionally the number of primordia initiated; Slafer and Rawson, 1994), but to the best of my knowledge the same effect on fertile florets was hypothesized (Slafer *et al.*, 2001) but not demonstrated and, as far as I am aware, this is the first time they are documented for floret primordia. It might not have been similar because, unlike the determination of leaves and spikelets in which all the primordia initiated will contribute to the final number achieved, the number of florets follows a process of generation followed by degeneration, determining the outcome of these two processes the number of fertile florets at anthesis. In fact, the processes are not parallel: in leaf and spikelet initiation phases it seems that *Ppd* alleles alter the rate of phasic development (changing the duration of the phase; González *et al.*, 2002; Whitechurch and Slafer, 2001; 2002; Foulkes *et al.*, 2004;) but do not noticeably affect the rate of organogenesis and consequently the number of organs initiated is rather linearly related to the duration of the phase when they are initiated (Slafer and Rawson, 1994). In this study it was shown that synchrony was improved with the introgression of insensitive alleles, which implies that unlike what happens with leaf and spikelet primordia, the rate of floret primordia initiation was positively affected by insensitivity to photoperiod, as much as these alleles positively affects the rate of phasic development of the late reproductive phase. Consequently the maximum number of floret primordia was not largely affected by *Ppd* alleles. Thus, the effect on the number of fertile florets seemed to have occurred indirectly, through the reduction in time making fewer resources available for the most labile floret primordia to continue developing normally towards producing fertile florets during the floret mortality phase. Thus, despite that insensitive alleles frequently improved the synchrony in floret primordia initiation there was compensation with the effects of these alleles in shortening the duration of the processes and finally synchrony was not related to floret survival or final number of fertile florets.

Even though the effect of *Ppd* insensitivity alleles on the number of spike-bearing tillers and on the number of leaves and spikelets developed in the previous phases can be contributing to the differences in the number of fertile florets per m^2 observed among the NILs and Paragon, the likelihood of the alleles to reduce the number of spikes per

unit land area is lower than reducing the number of florets per spike due to the number of floret per spike implicates a more refined regulation (Slafer *et al.*, 2014) more consistent with the strength of the effects of the alleles on the reproductive phases. In addition, the results showed that differences in the number of fertile floret per m^2 were well explained by differences in the number of fertile florets seen in the spikes.

In line with previous reports (González *et al.*, 2005*a*; González-Navarro *et al.*, 2015; Guo *et al.*, 2016) differences seen in the number of fertile florets in the spikelets were better related to the floret survival than to the maximum number of primordia developed and seemed not to be related to particular developmental stages of the more advanced florets.

The strength of the effects on the traits measured was rather independent of the particular genome of Paragon in which the sensitive allele was substituted by an insensitive allele. This is in line with differences reported in the order of strength of the genomes for time to heading or anthesis: while Scarth and Law, 1984; Law, 1987; Worland, 1996; Stelmakh 1998; Worland *et al.*, 1998; González et al., 2005*b*; Díaz *et al*., 2012; reported the *Ppd-D1* gene to be the strongest one; Tanio and Kato, 2007 remarked the importance of *Ppd-B1* comparable to *Ppd-D1*; and Bentley *et al.*, 2011 reported the strength in the A genome in synthetic hexaploid wheats with intermediate effects between D and B. This emphasizes the importance of the allelic form (source) being used for substitution in a particular genome. In addition, increasing the dose of insensitivity alleles tended to produce larger differences on traits although, it was not fully consistent. This lack of consistency may well be again due to the effect of the source of the alleles. These results and interpretations are in line with a previous discussion in González *et al*., 2005*b* who compared the effects of *Ppd*-D1 and *Ppd*-B1, mainly on phenology, that they found with those reported in other studies. That discussion highlighted lack of consistency implying that other elements would be more relevant than the genome in which the allele was substituted in determining the strength of the effect; remarking the importance of the source of the alleles over that of the genome in which the insensitive allele substituted the sensitive one, something already hypothesized long time ago (Scarth and Law, 1984).

As explained in the Results section, the effects of introgressing the *Ppd* alleles were of strongly different magnitude in the two growing seasons. Whilst major effects were evident in the first growing season, in the second growing season the results of the action of these alleles became only trends, mostly non-significant. Attempting to understand why the responses were different in the two seasons I analyzed different climatic characteristics (soils were similar and in both seasons experiments were well irrigated and fertilized). The hypothesis was that perhaps an interaction with temperature could be possible. This was based on the fact that temperature and photoperiod seem to act interactively, rather than additively (Slafer and Rawson, 1996). In fact, there was an increment in maximum temperatures at the field around the flowering time in the second growing season respect to the first one. Floral development has been identified as sensitive to climatic stress including pollen formation (Saini and Aspinall, 1982; Lalonde *et al.*, 1997) and floral abnormalities induced by heat stress (i.e. stamen hypoplasia and pistil hyperplasia) were also reported in rice (Takeoka *et al.*,

1991). A modelling exercise parameterized by flowering observations indicated that the temporal and spatial variability of anther activity within and between spikes may influence the relative resilience of wheat to sudden extreme climatic events (Lukac *et al.*, 2012). Furthermore, even the lines with different degrees of insensitivity and also Paragon presented lower number of fertile florets during the second growing season, the effect seemed to be stronger in Paragon rather than in the NILs (that is why significance of the differences were mostly lost in the second growing season) suggesting an interaction between temperatures and photoperiod sensitivity.

In general the main effects that explained the proposal aims are summarized and represented in Fig. 4.19 taking into account the effects of most NILs (*Ppd a*) in comparison to Paragon (*Ppd b*) in both growing seasons: Under moderate temperatures, carrying insensitivity (*Ppd a*) reduced time to flowering at least due to reducing the late reproductive phase, particularly the floret generation phase. This caused in most NILs the reduction in the maximum and final number of florets in central spikelets which together with possible decreases in floret primordia in other spikelets positions plus in the spikelets and tillers number caused a lower number of fertile florets per surface unit. However, when flowering happened under high temperature conditions even photoperiod insensitivity (*Ppd a*) still reduced time to anthesis and the late reproductive phase, the floret generation period was longer than in *Ppd b* and the floret degeneration shorter which caused the lack of the effects seen under moderate temperatures on the maximum and almost in the final number of florets in the central spikelets. Although still may exist reduction in the florets number in the other floret positions, spikelets and tillers number which contributed to reduce, even not significant, the number of fertile florets per unit surface. In addition, comparing *Ppd b*, which seemed to be more affected by temperature (temperature x photoperiod sensitivity), under moderate and high temperatures around anthesis, it could be seen that high temperatures reduced time to anthesis and the floret generation phase and increased the floret degeneration period lowering the number of fertile florets.

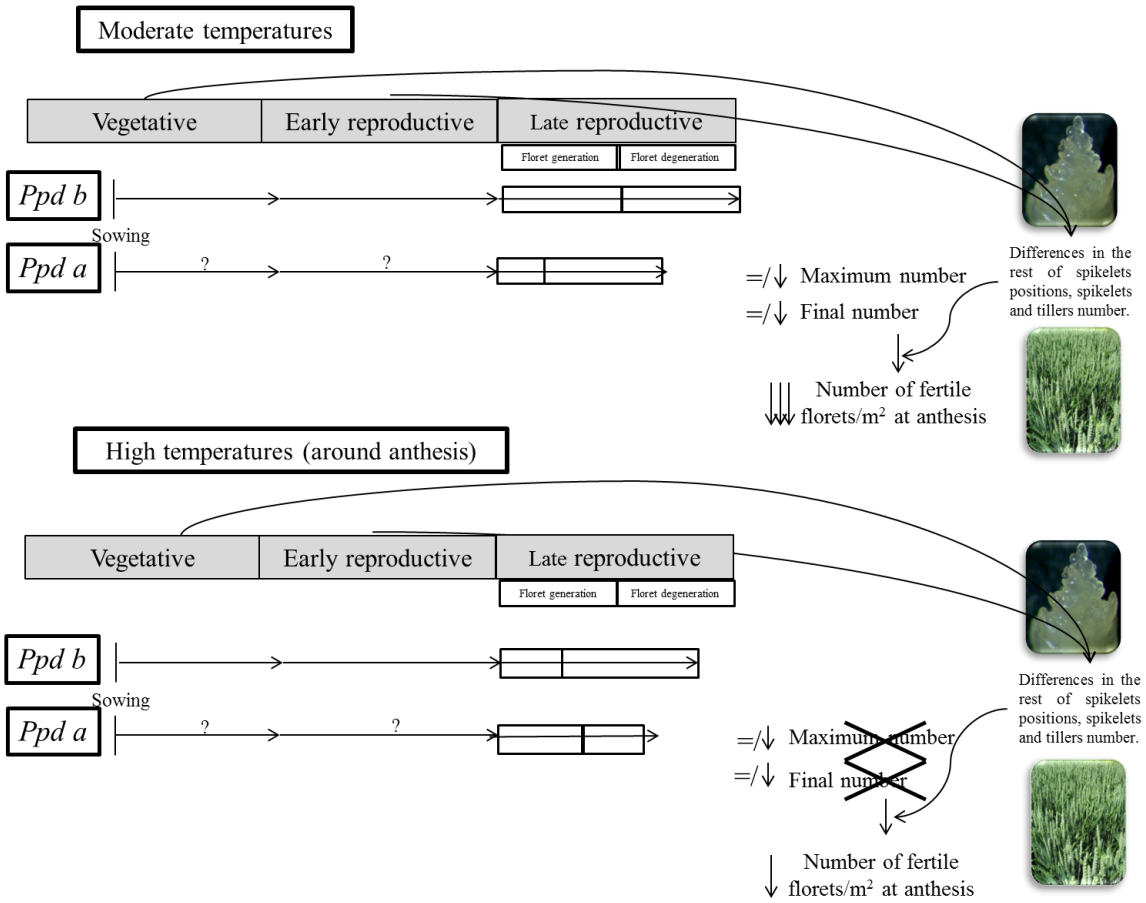


Figure 4.19. Schematic representation of the effects of carrying photoperiod insensitivity alleles (*-a-*), in comparison to Paragon (sensitive *–b-*), on developmental processes and the consequent overall effect on the number of fertile florets in two growing seasons contrasting in temperatures during the floret development period. *Top*: expected effects at moderate temperatures on the length of developmental phases until anthesis, particularly in that of floret primordia generation and degeneration determining the maximum and final (fertile) numbers of florets in central spikelets and their impact on the number of fertile florets per square meter. *Bottom*, at higher temperatures, the the differences due to the effect of photoperiod insensitivity on the duration of the floret development phases were minimized (becoming non-significant in most cases) with the concomitant loss of effect on the number of fertile florets.

5. References

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6. Annex 4: Supplementary data

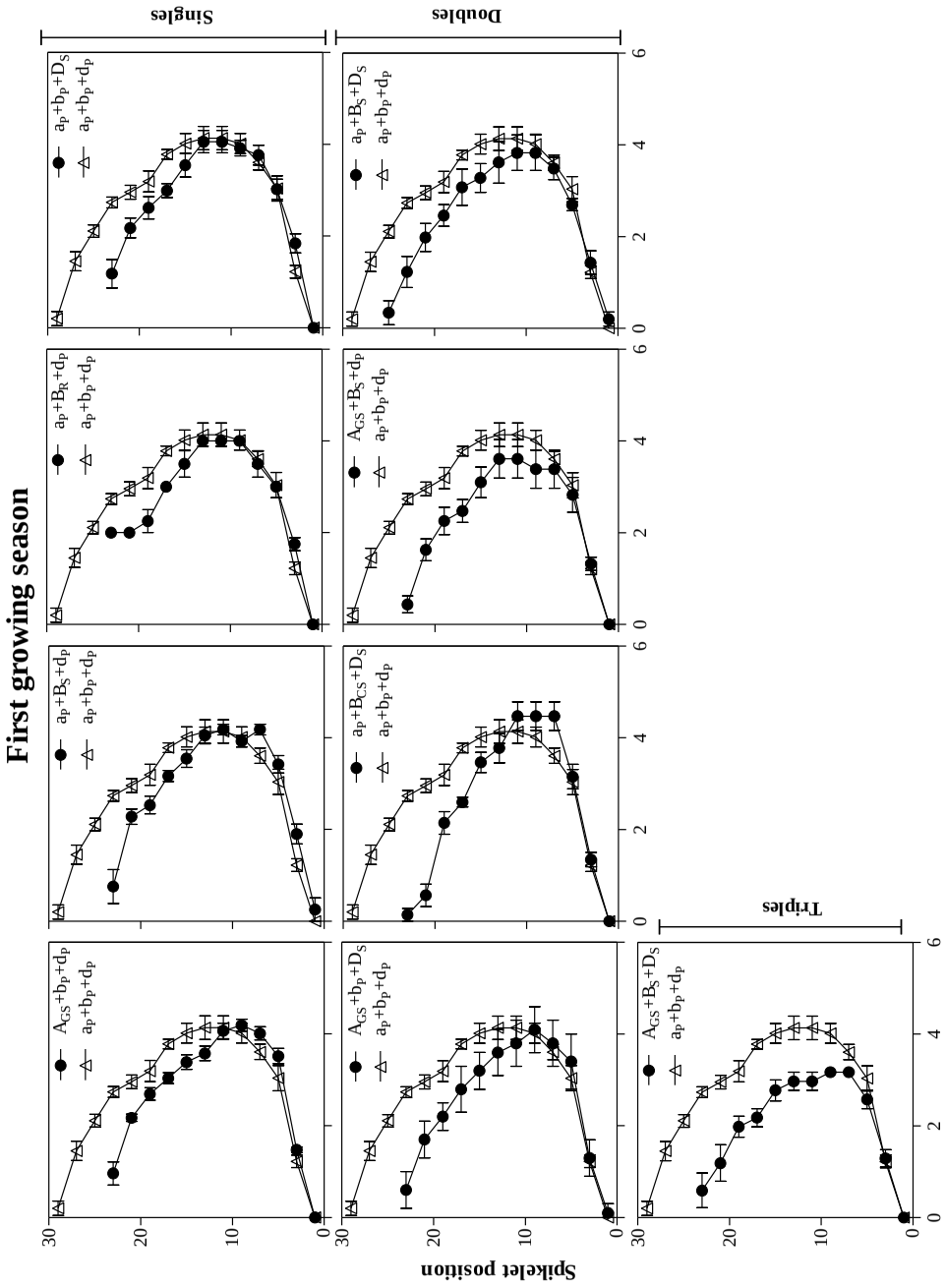

Figure A4.1. Mapping of fertile florets (fertility of each spikelet position on the main-shoot spikes) during the first growing season for the NILs (closed circles) with one, two or three insensitivity alleles in comparison to Paragon (open triangles). Each data-point is the average of all replicates and within each replicate the value was the average of 4 plants and the segment in each data-point stands for the standard error of the means (not visible when smaller than the size of the symbol or absence of replicates).

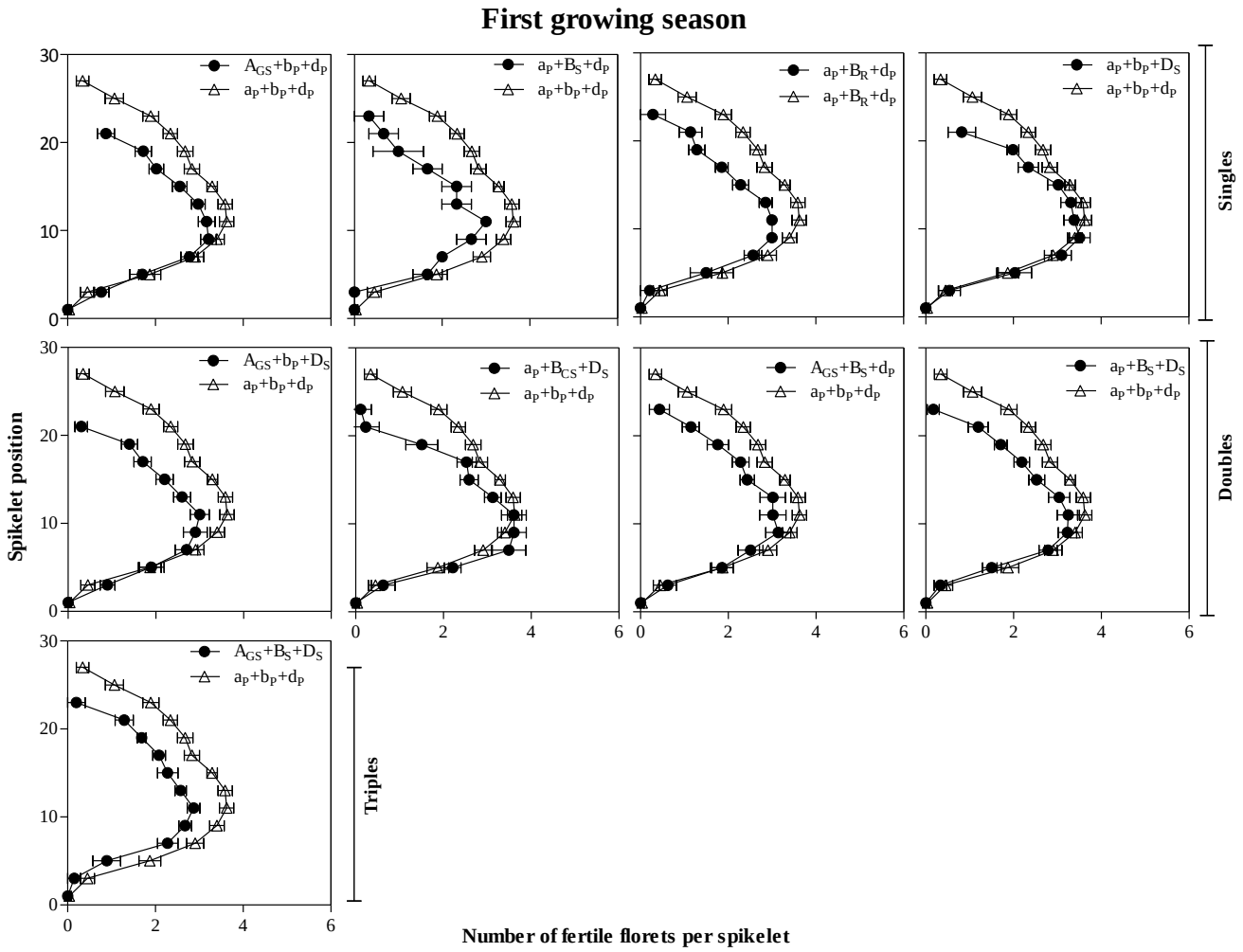

Figure A4.2. Mapping of fertile florets (fertility of each spikelet position on the tiller spikes) during the first growing season for the NILs (closed circles) with one, two or three insensitivity alleles in comparison to Paragon (open triangles). Each data-point is the average of all replicates and within each replicate the value was the average of 4 plants and the segment in each data-point stands for the standard error of the means (not visible when smaller than the size of the symbol or absence of replicates).

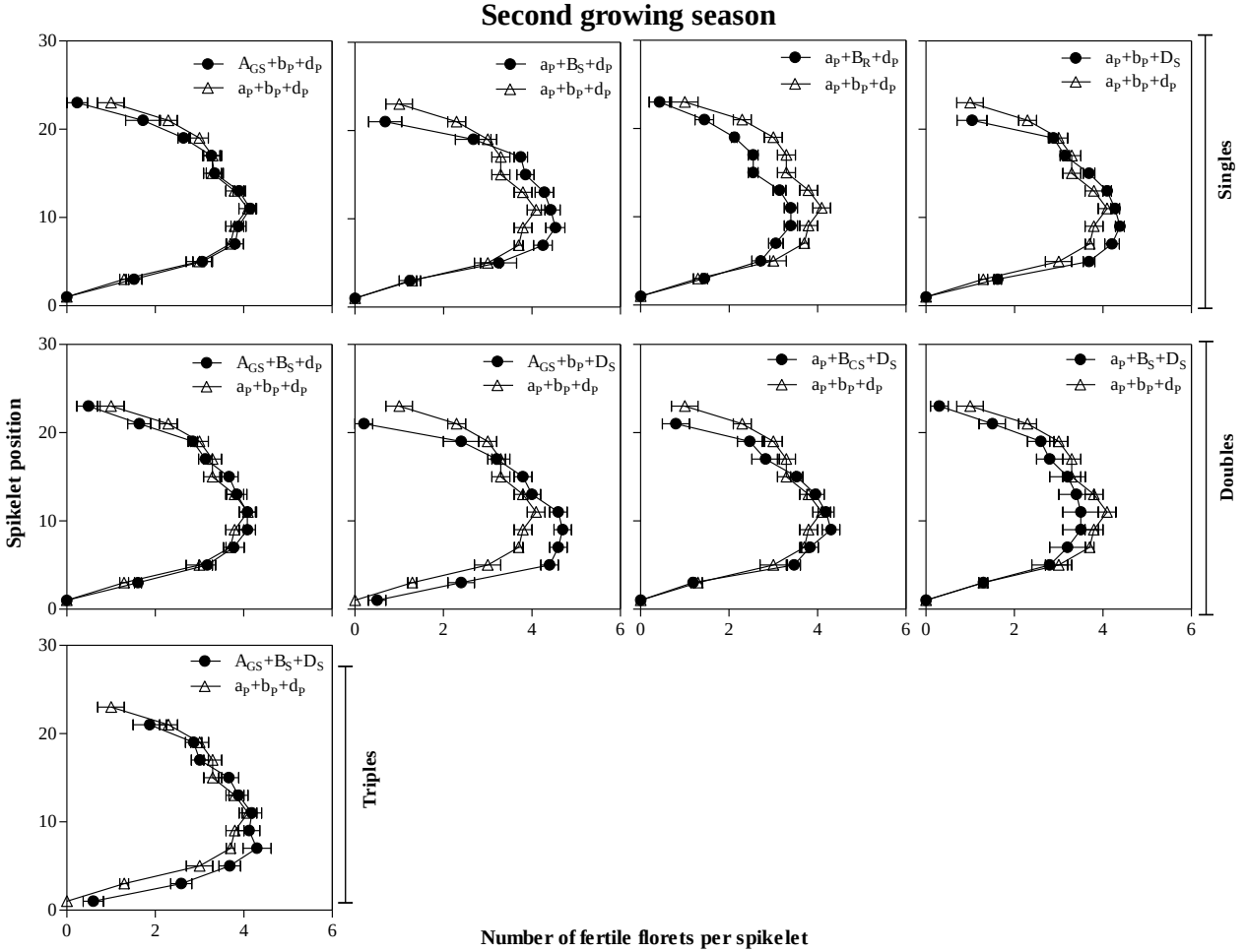

Figure A4.3. Mapping of fertile florets (fertility of each spikelet position on the main-shoot spikes) during the second growing season for the NILs (closed circles) with one, two or three insensitivity alleles in comparison to Paragon (open triangles). Each data-point is the average of all replicates and within each replicate the value was the average of 4 plants and the segment in each data-point stands for the standard error of the means (not visible when smaller than the size of the symbol or absence of replicates).

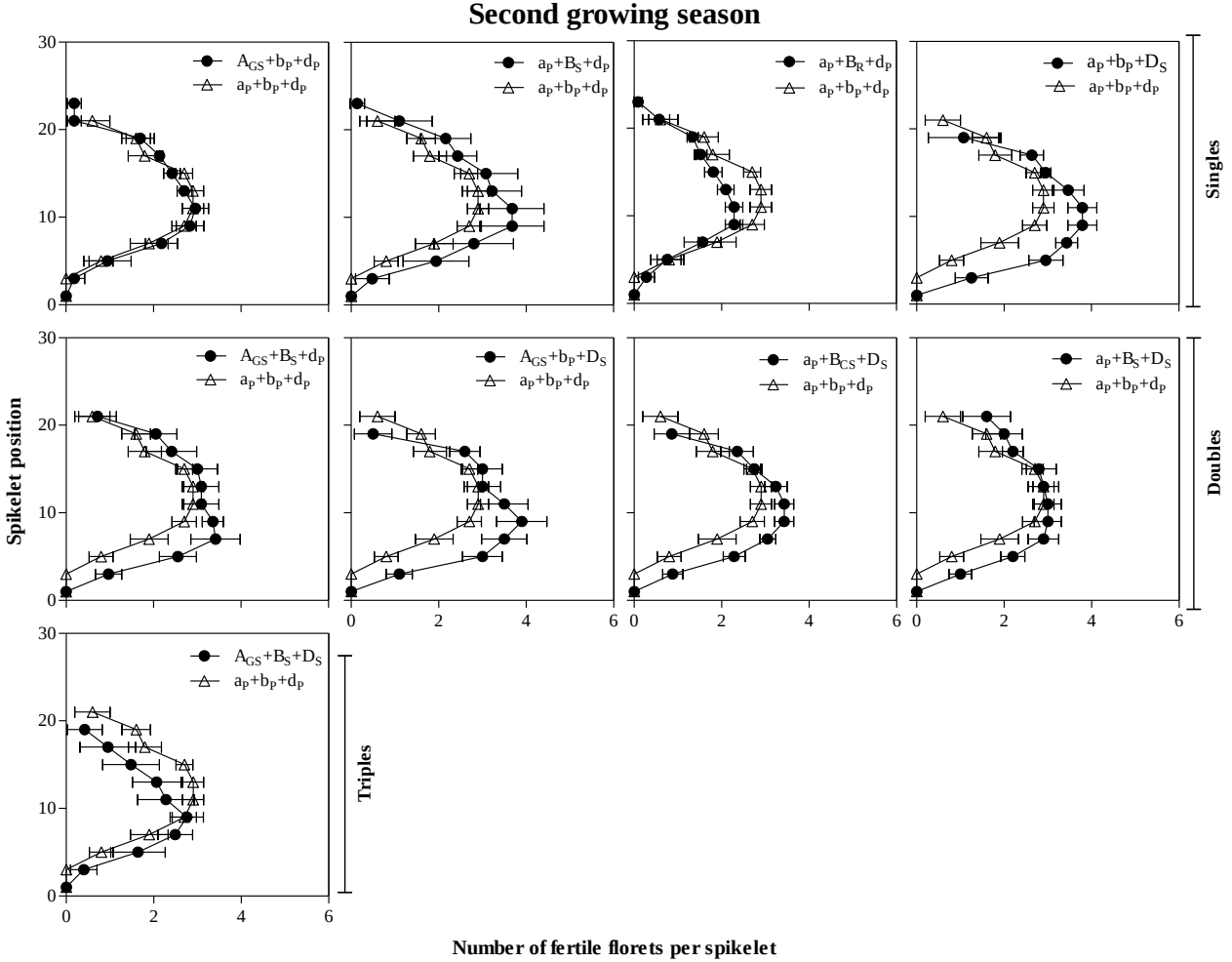

Figure A4.4. Mapping of fertile florets (fertility of each spikelet position on the tiller spikes) during the second growing season for the NILs (closed circles) with one, two or three insensitivity alleles in comparison to Paragon (open triangles). Each data-point is the average of all replicates and within each replicate the value was the average of 4 plants and the segment in each data-point stands for the standard error of the means (not visible when smaller than the size of the symbol or absence of replicates).

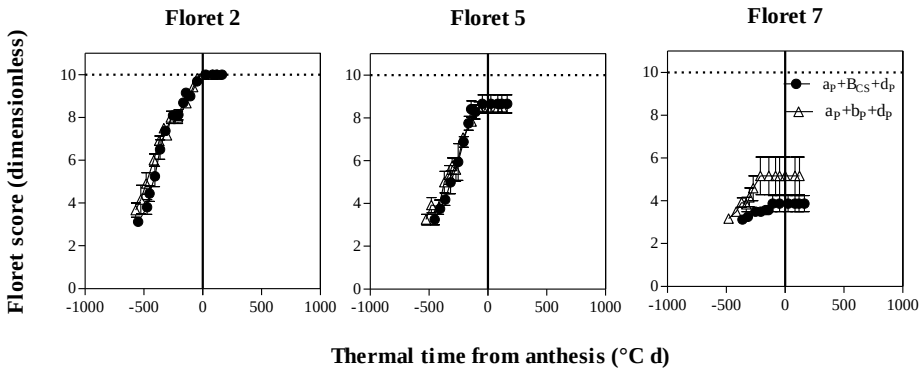

Figure A4.5. Dynamics of the floret development of F2, F5 and F8 in central spikelets of the main-shoots through thermal time from anthesis in $a_P+B_{CS}+d_P$ (closed circles), a NIL carrying one single change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

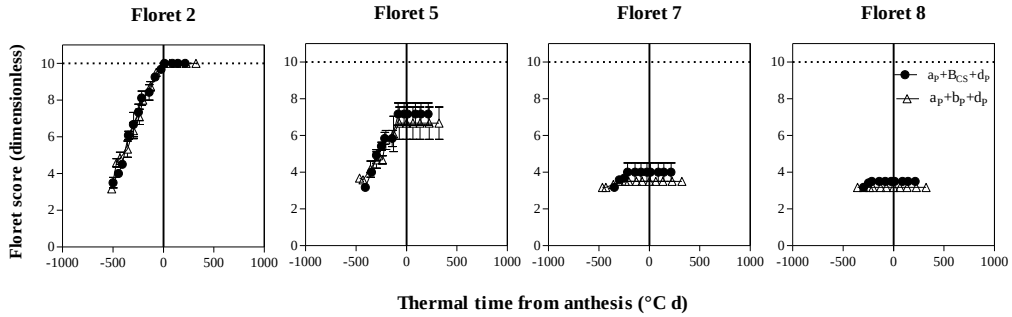

Figure A4.6. Dynamics of the floret development of F2, F5, F7 and F8 in central spikelets of the main-shoots through thermal time from anthesis in $a_P+B_{CS}+d_P$ (closed circles), a NIL carrying one single change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

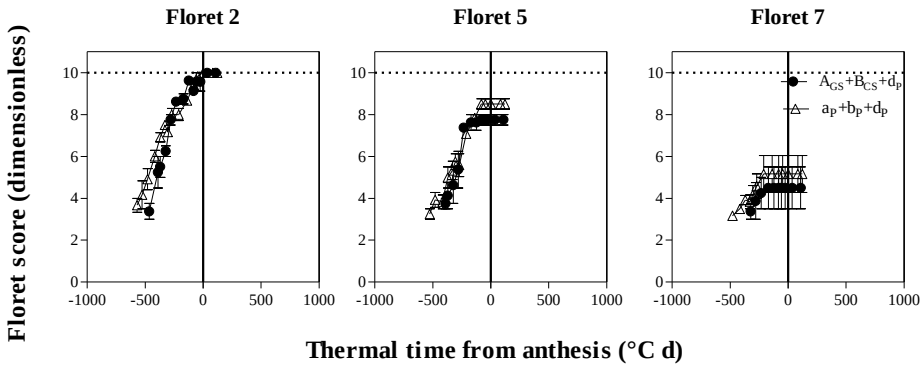

Figure A4.7. Dynamics of the floret development of F2, F5 and F7 in central spikelets of the main-shoots through thermal time from anthesis in $A_{GS}+B_{CS}+d_P$ (closed circles), a NIL carrying double change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

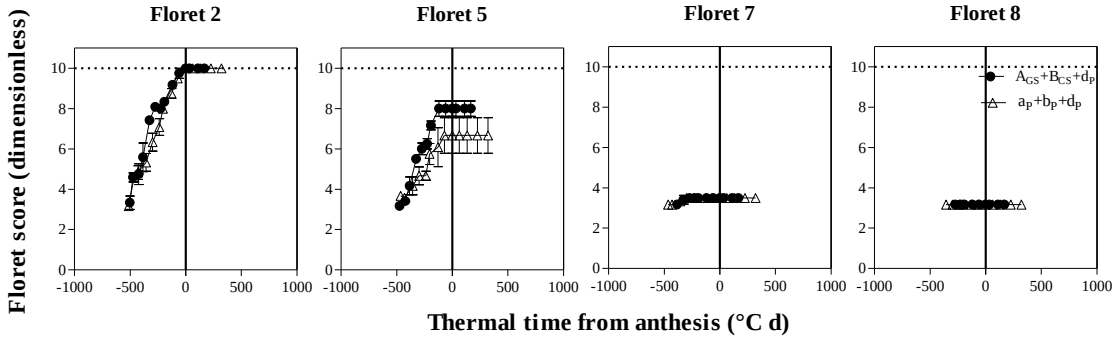

Figure A4.8. Dynamics of the floret development of F2, F5, F7 and F8 in central spikelets of the main-shoots through thermal time from anthesis in $A_{GS}+B_{CS}+d_P$ (closed circles), a NIL carrying double change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

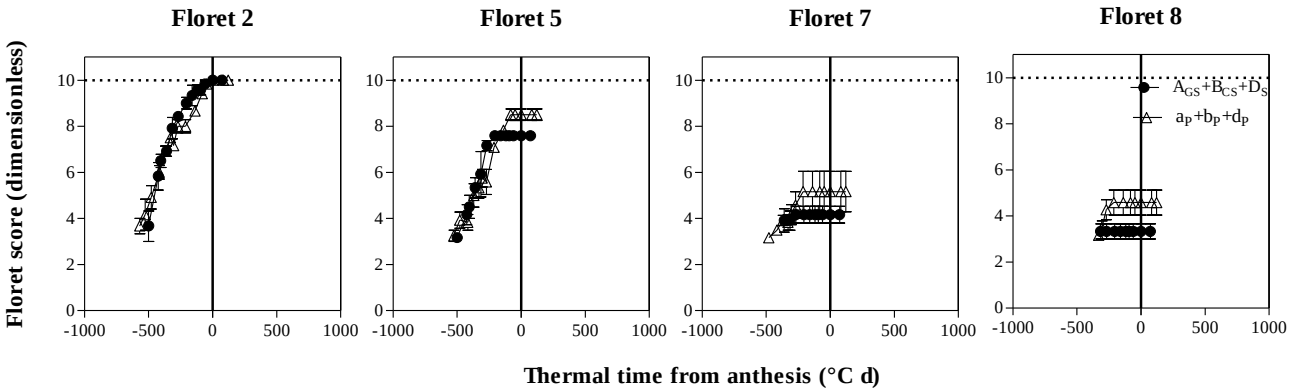

Figure A4.9. Dynamics of the floret development of F2, F5, F7 and F8 in central spikelets of the main-shoots through thermal time from anthesis in A_{GS}+B_{CS}+D_S (closed circles), a NIL carrying triple change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

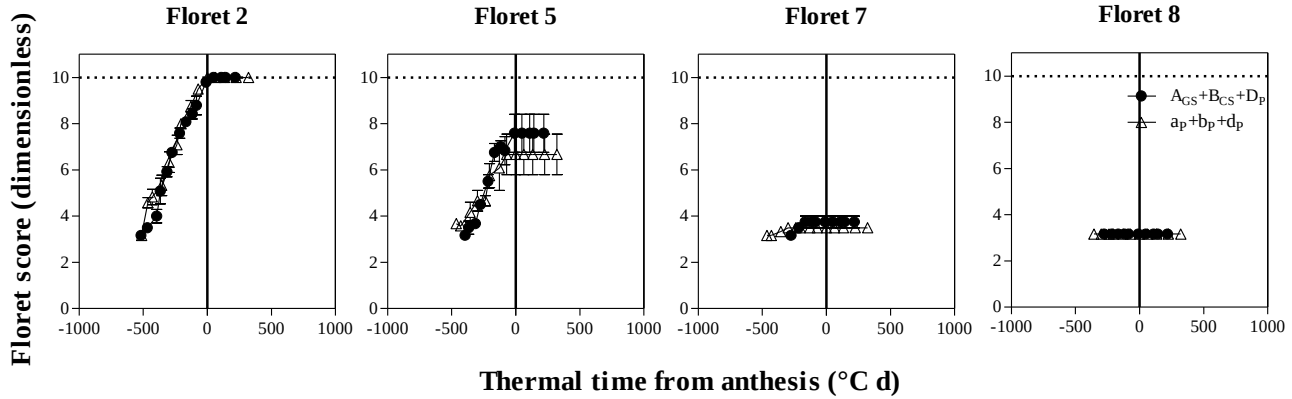

Figure A4.10. Dynamics of the floret development of F2, F5, F7 and F8 in central spikelets of the main-shoots through thermal time from anthesis in A_{GS}+B_{CS}+D_S (closed circles), a NIL carrying triple change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

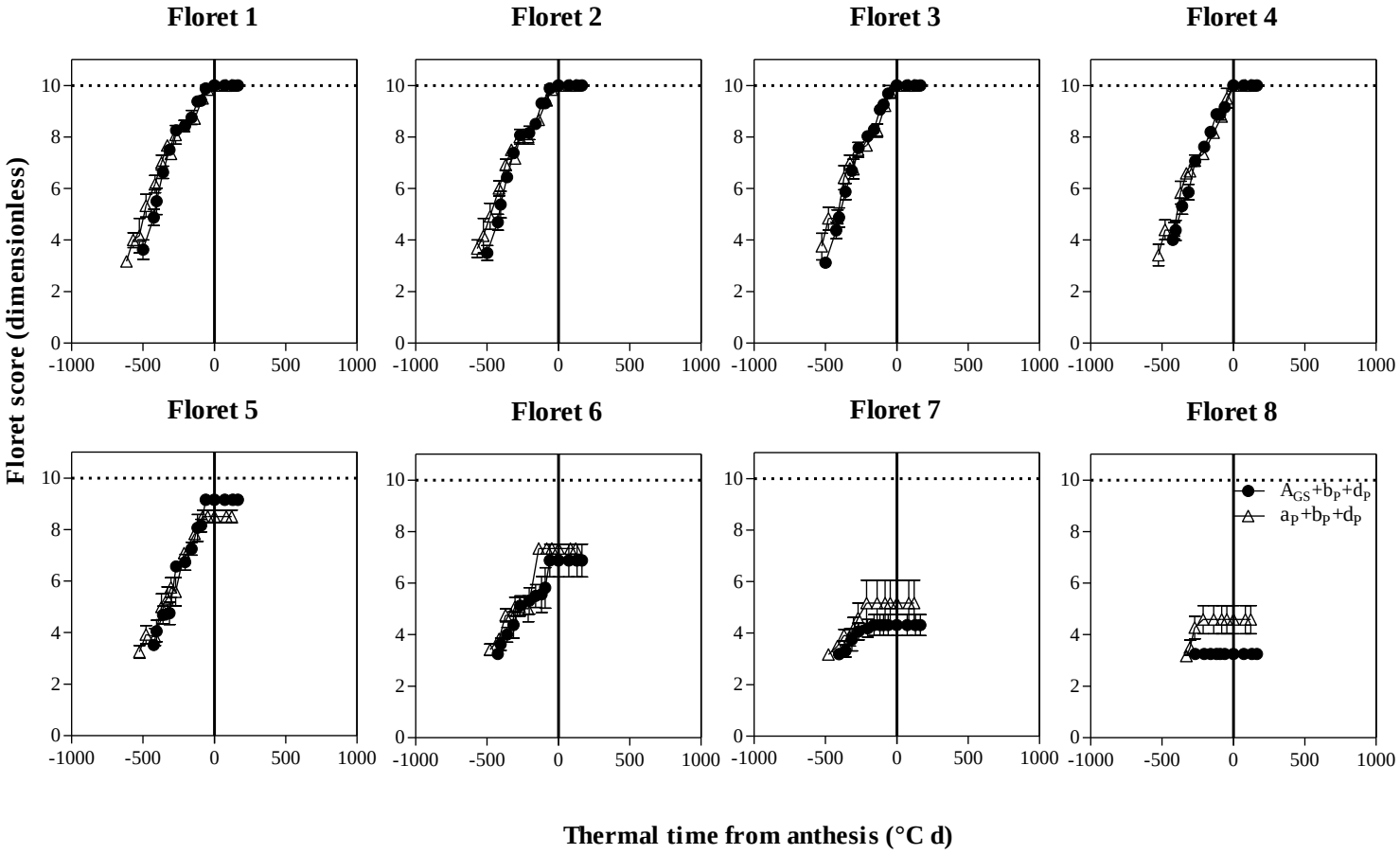

Figure A4.11. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $A_{GS}+b_P+d_P$ (closed circles), a NIL carrying one single change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

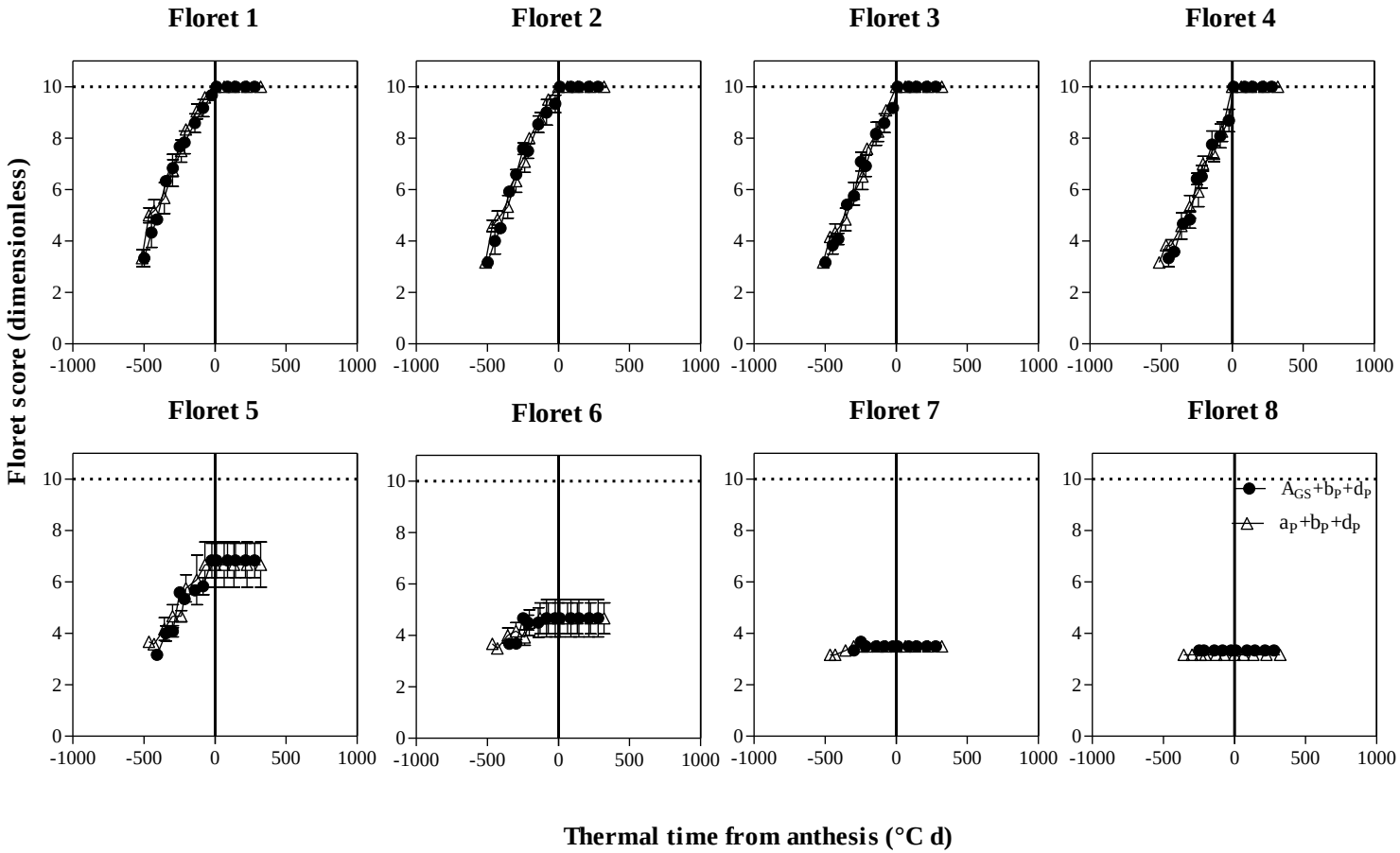

Figure A4.12. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $A_{GS}+b_P+d_P$ (closed circles), a NIL carrying one single change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

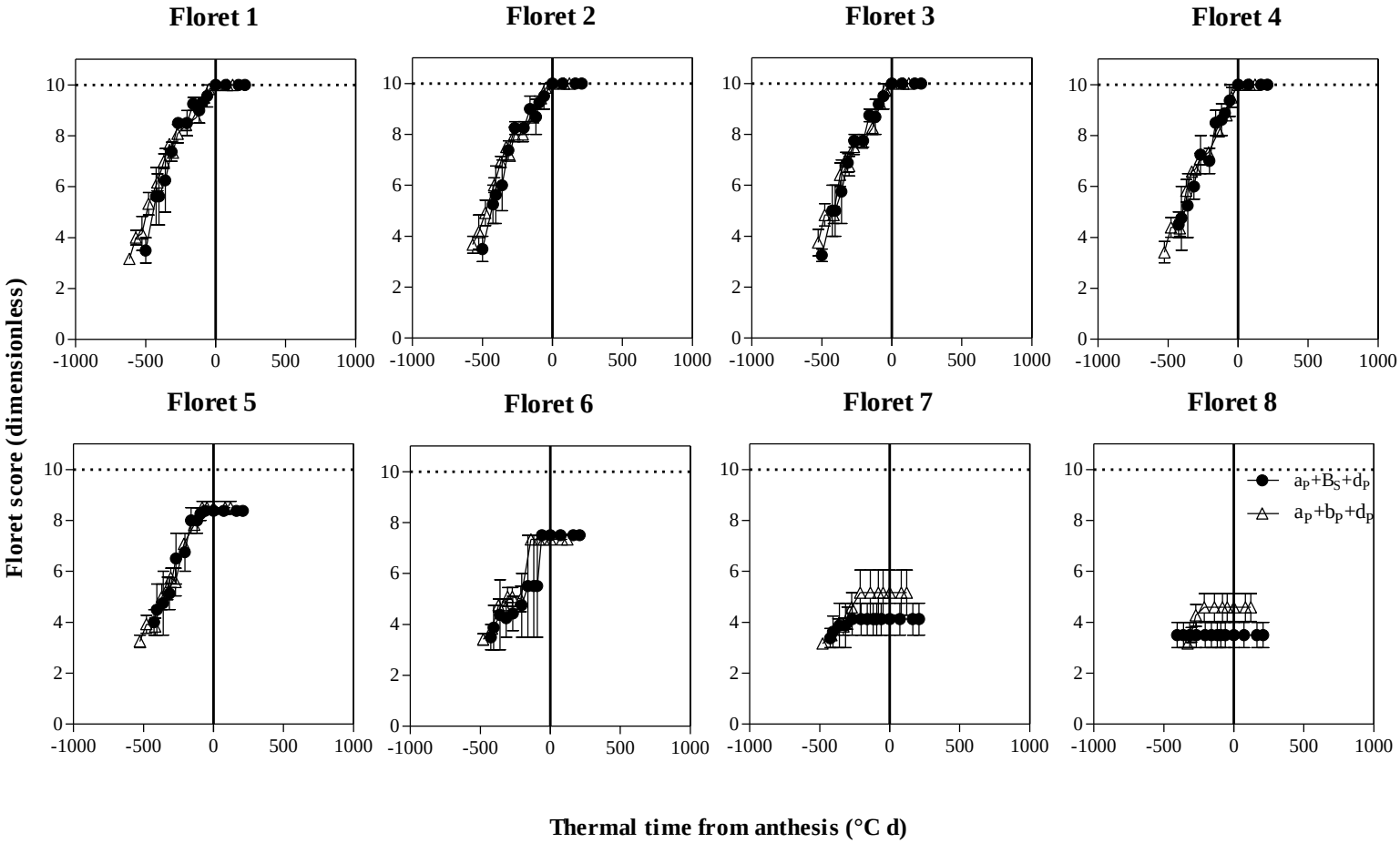

Figure A4.13. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $a_P+B_S+d_P$ (closed circles), a NIL carrying one single change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

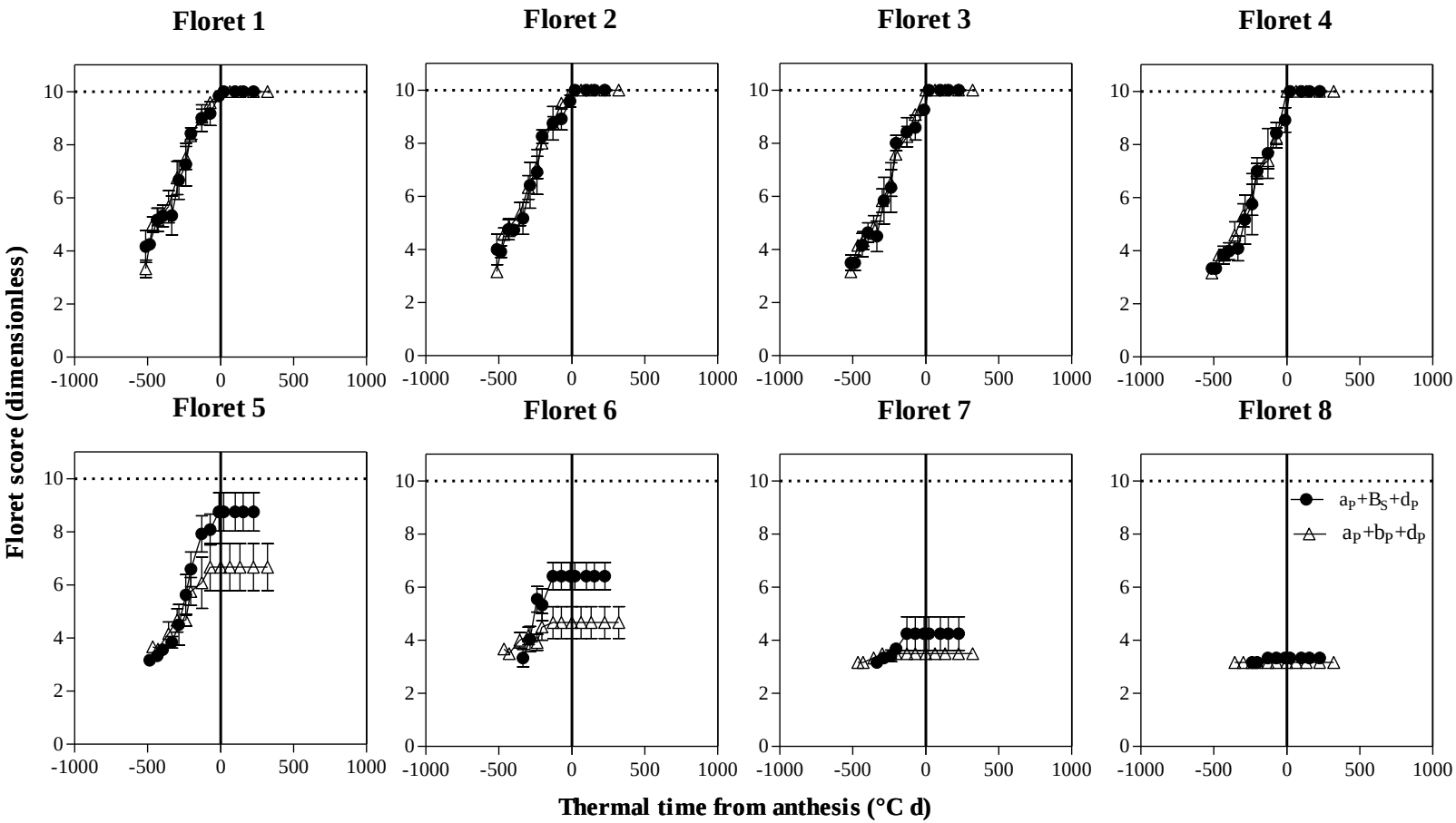

Figure A4.14. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $a_P+B_{S+}d_P$ (closed circles), a NIL carrying one single change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

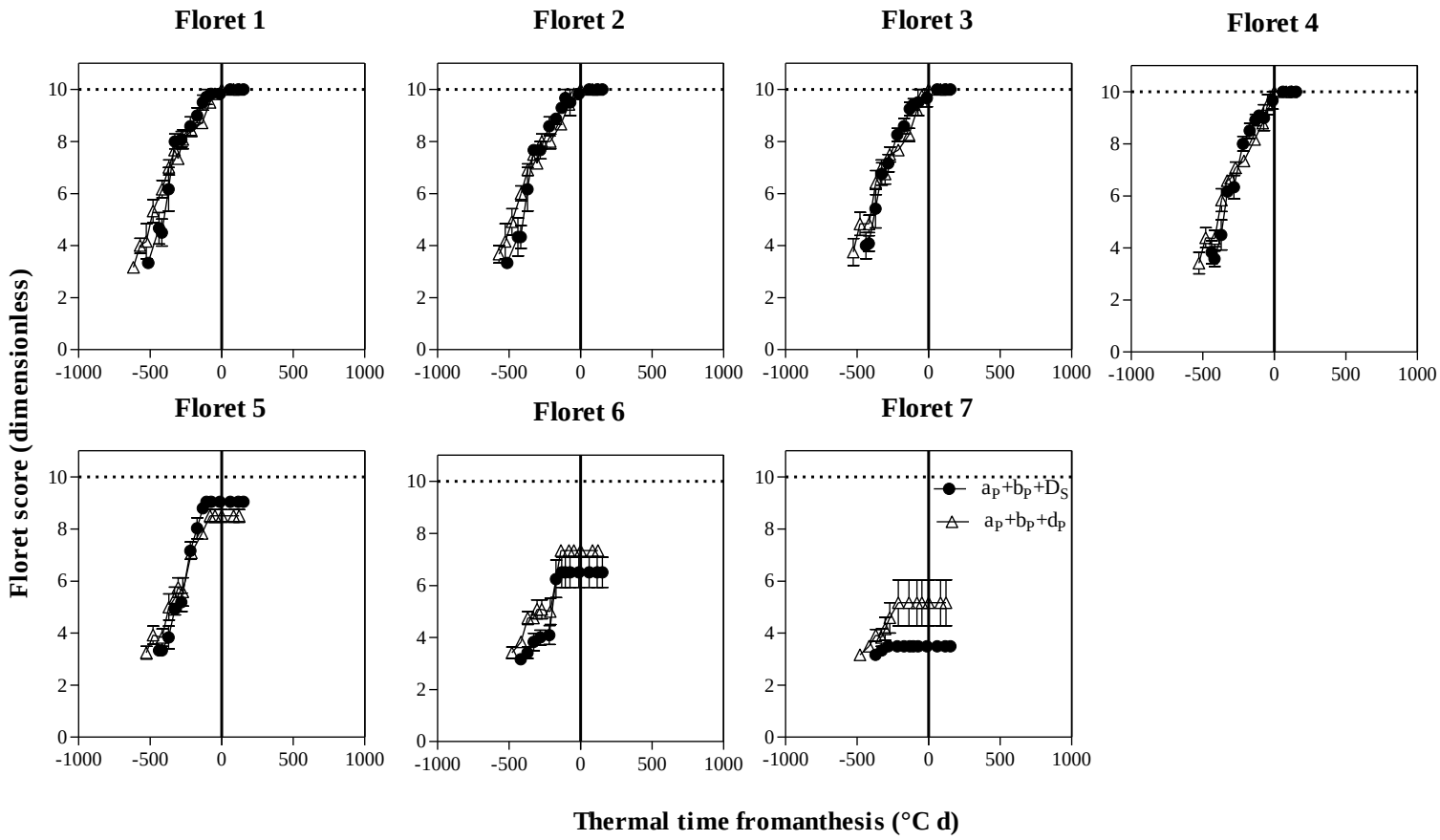

Figure A4.15. Dynamics of the floret development from F1 to F7 in central spikelets of the main-shoots through thermal time from anthesis in $a_P+b_P+D_S$ (closed circles), a NIL carrying one single change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

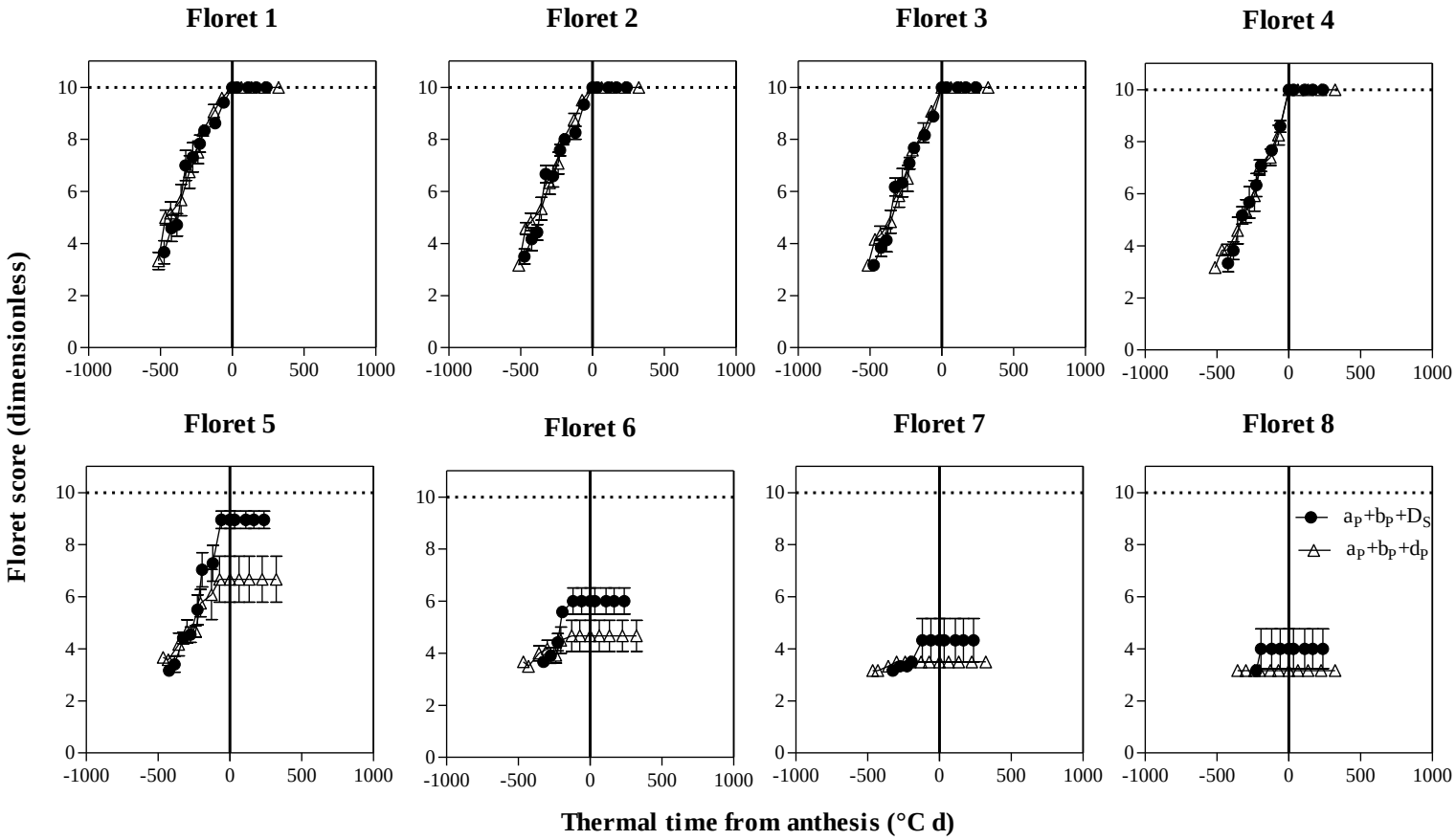

Figure A4.16. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $a_P+b_P+D_S$ (closed circles), a NIL carrying one single change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

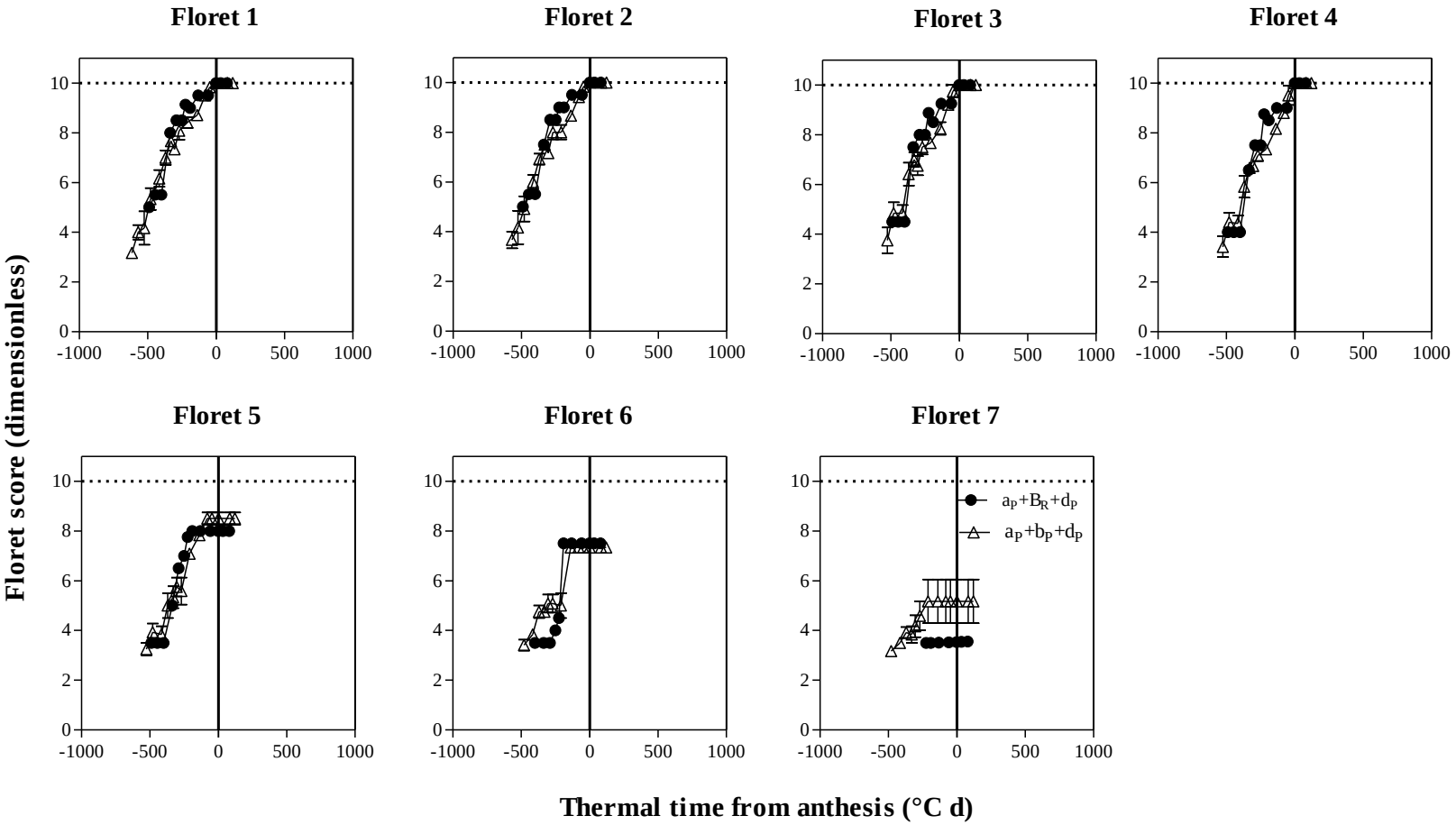

Figure A4.17. Dynamics of the floret development from F1 to F7 in central spikelets of the main-shoots through thermal time from anthesis in $a_P+B_R+d_P$ (closed circles), a NIL carrying one single change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

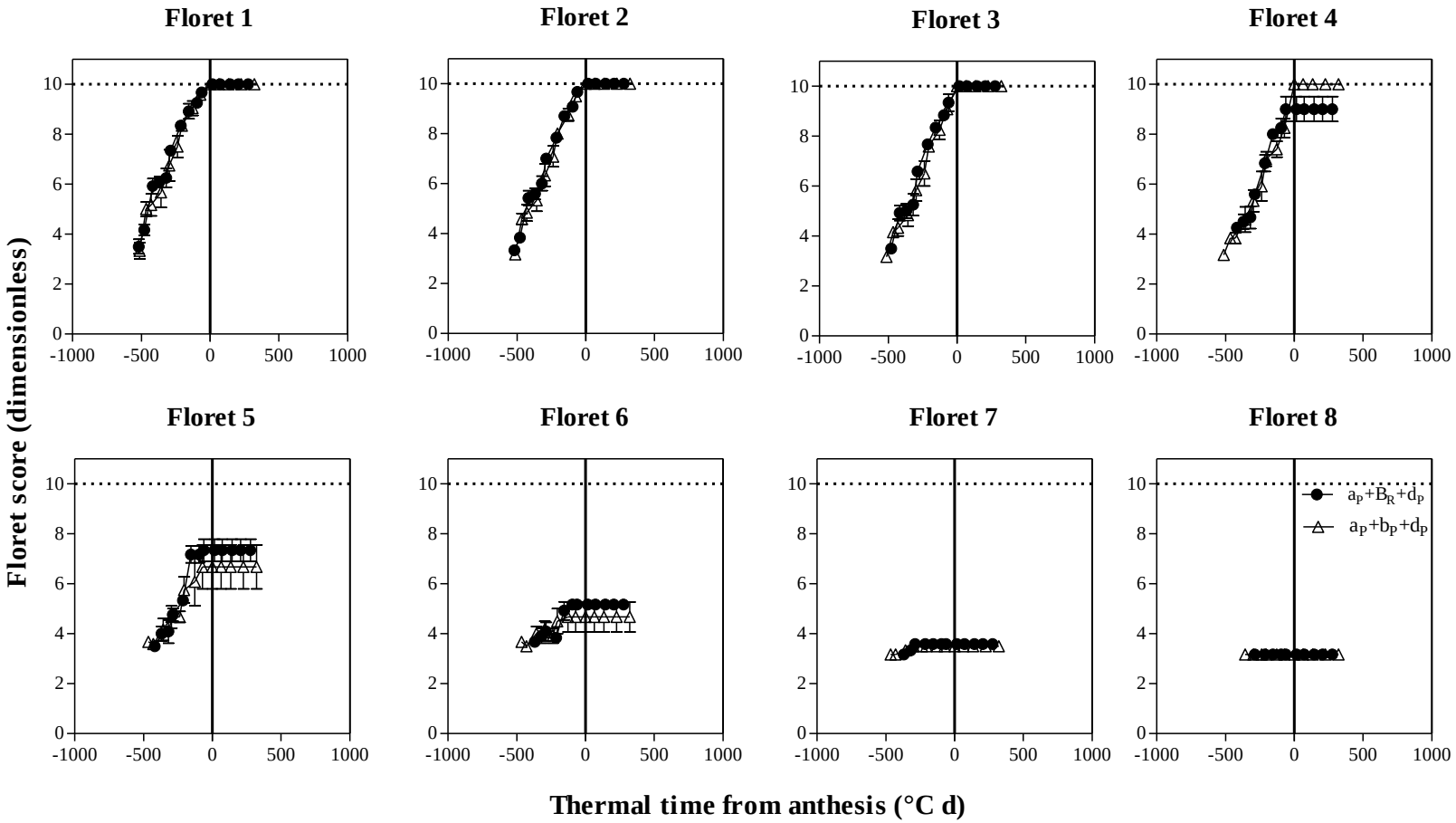

Figure A4.18. Dynamics of the floret development from F1 to F7 in central spikelets of the main-shoots through thermal time from anthesis in ap+$B_{R+}d_P$ (closed circles), a NIL carrying one single change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

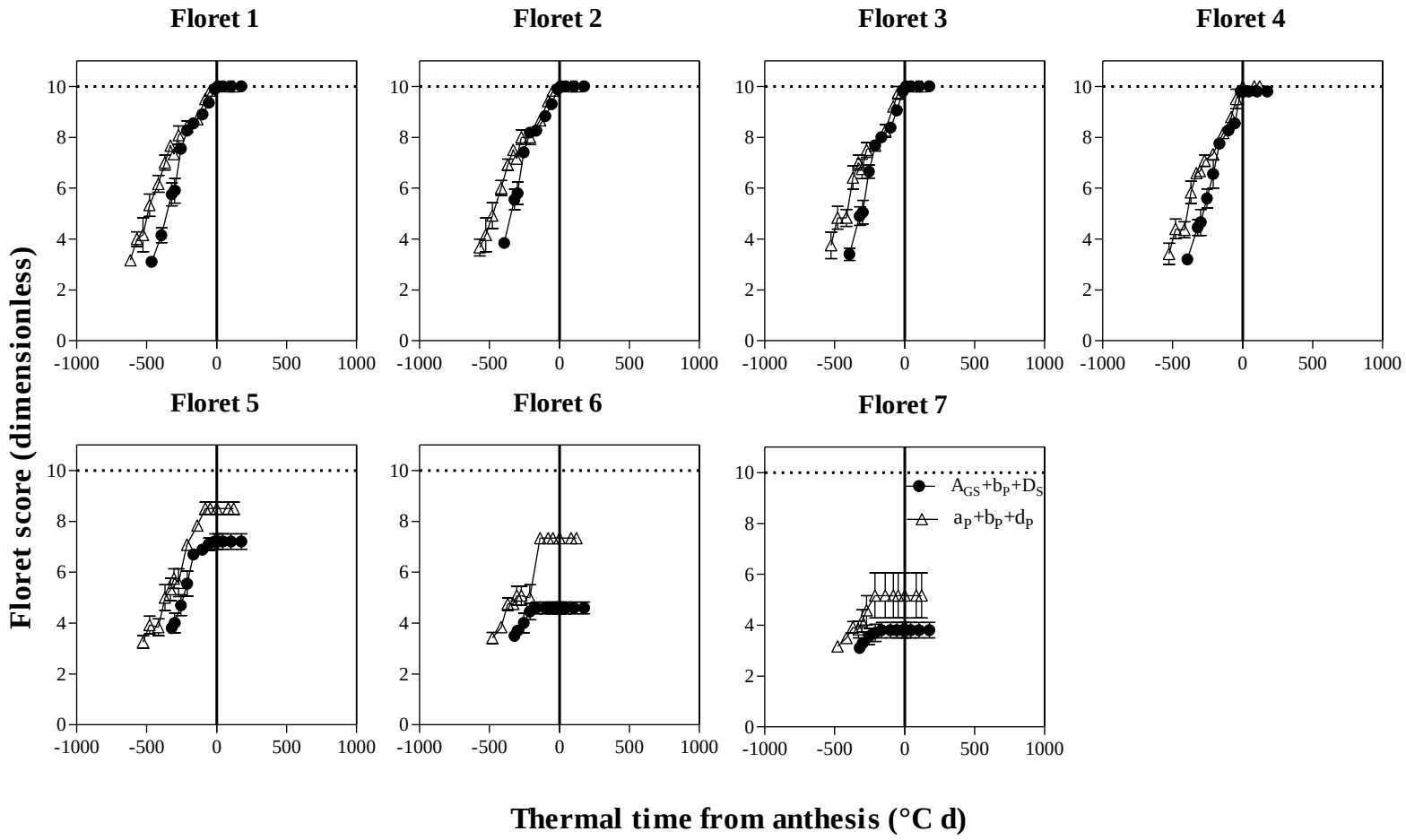

Figure A4.19. Dynamics of the floret development from F1 to F7 in central spikelets of the main-shoots through thermal time from anthesis in $A_{GS}+b_P+D_S$ (closed circles), a NIL carrying double change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

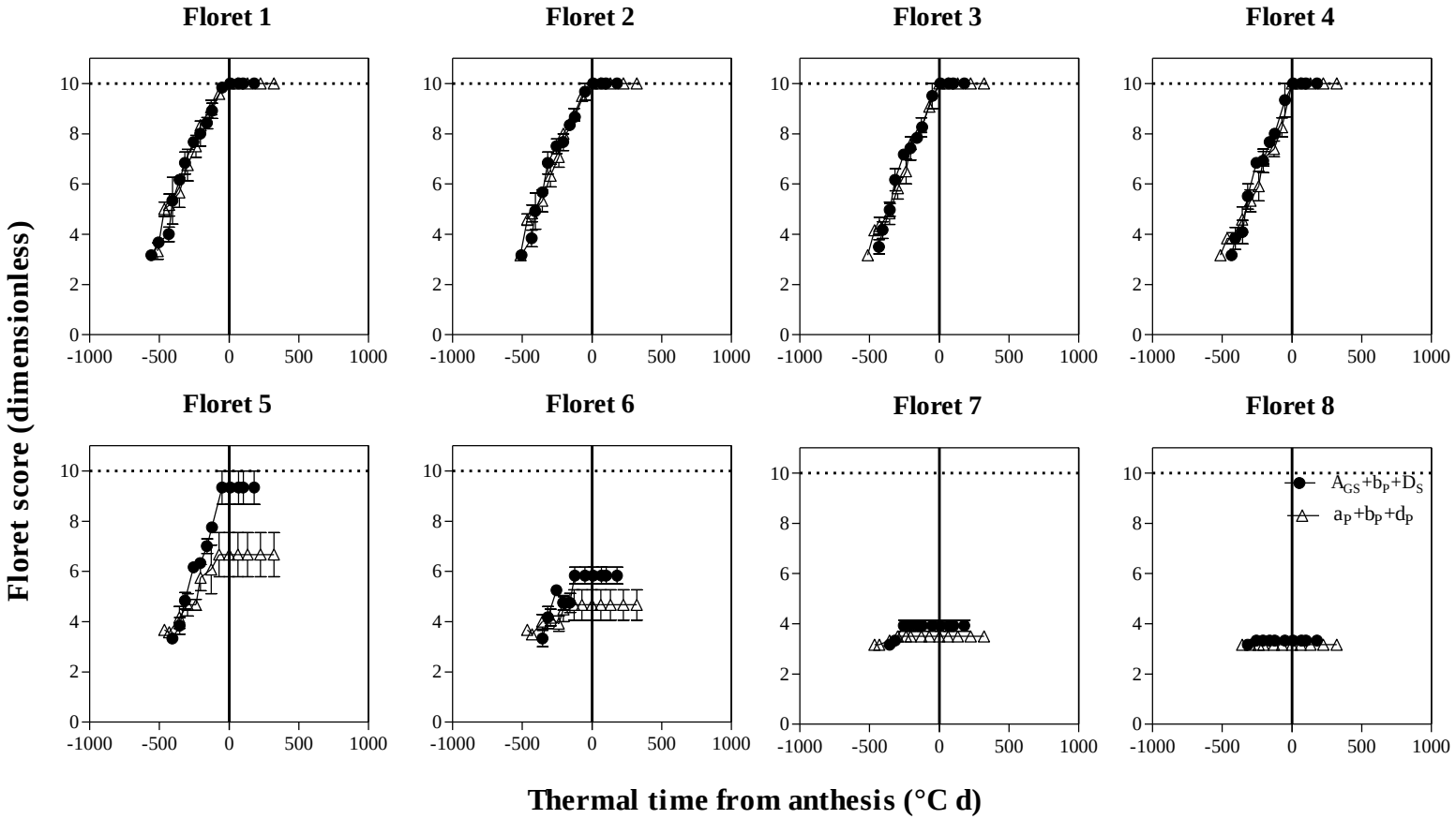

Figure A4.20. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $A_{GS}+b_P+D_S$ (closed circles), a NIL carrying double change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

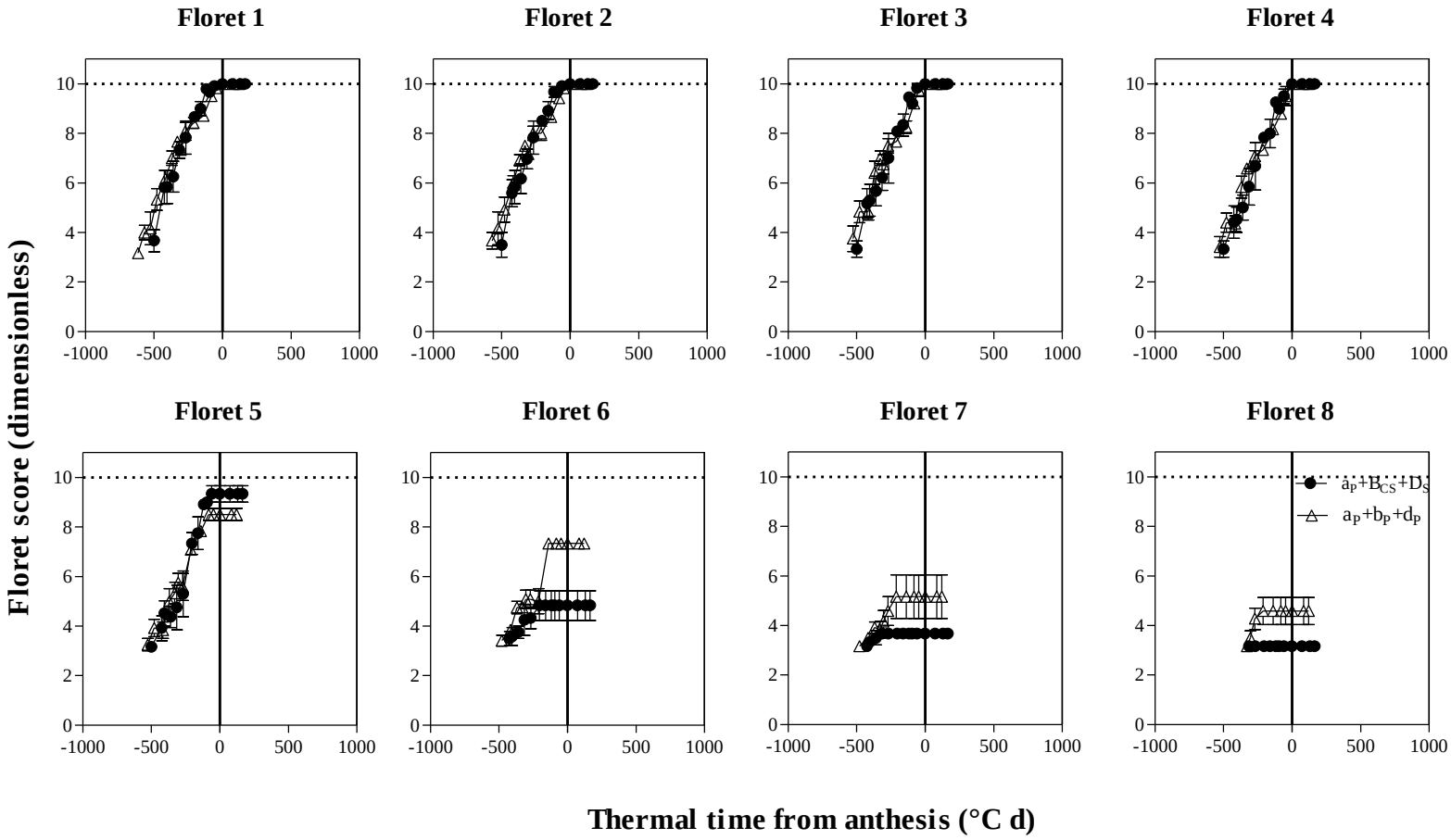

Figure A4.21. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $a_P+B_{CS}+D_S$ (closed circles), a NIL carrying double change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

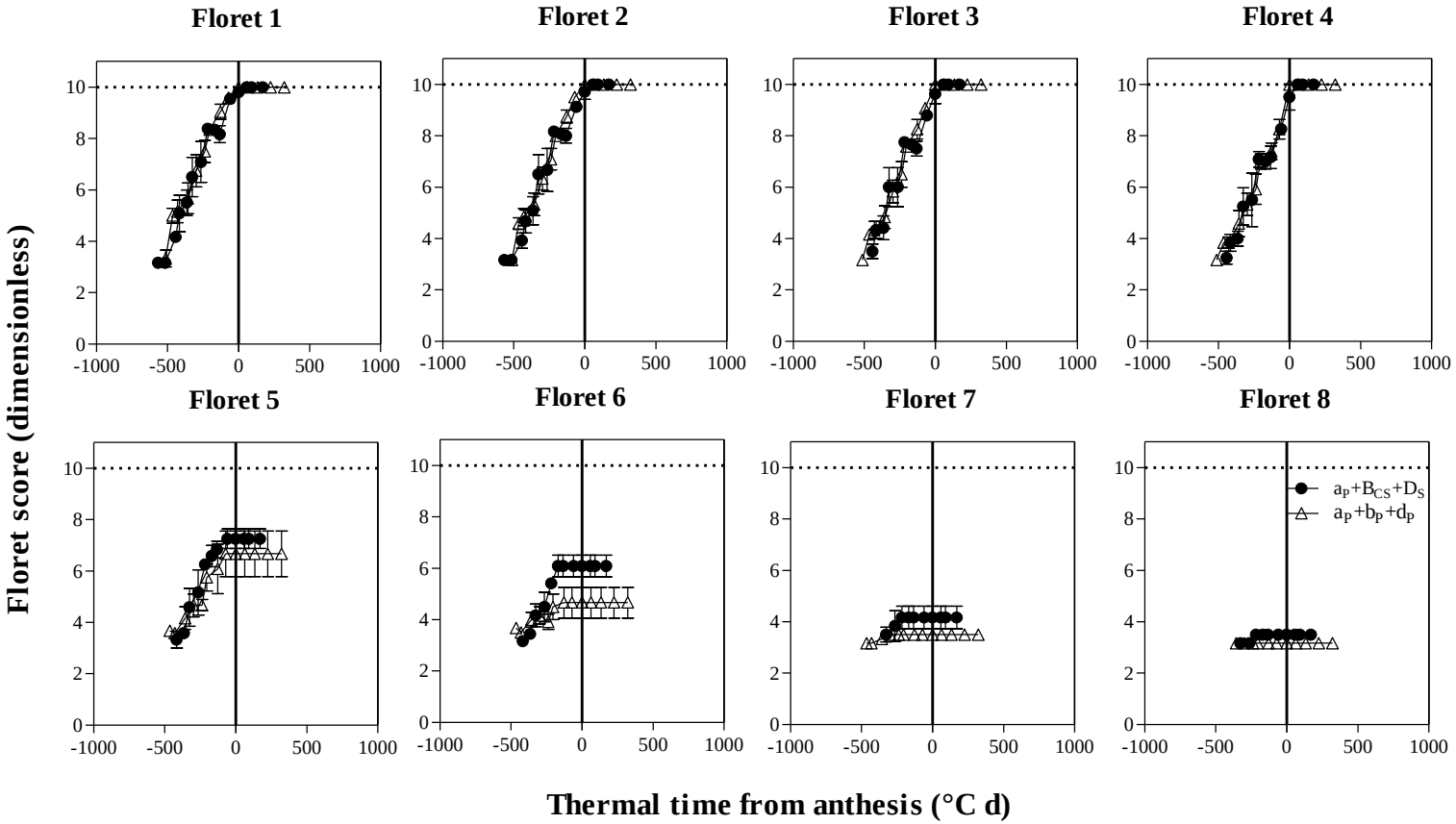

Figure A4.22. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $a_P+B_{CS}+D_S$ (closed circles), a NIL carrying double change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

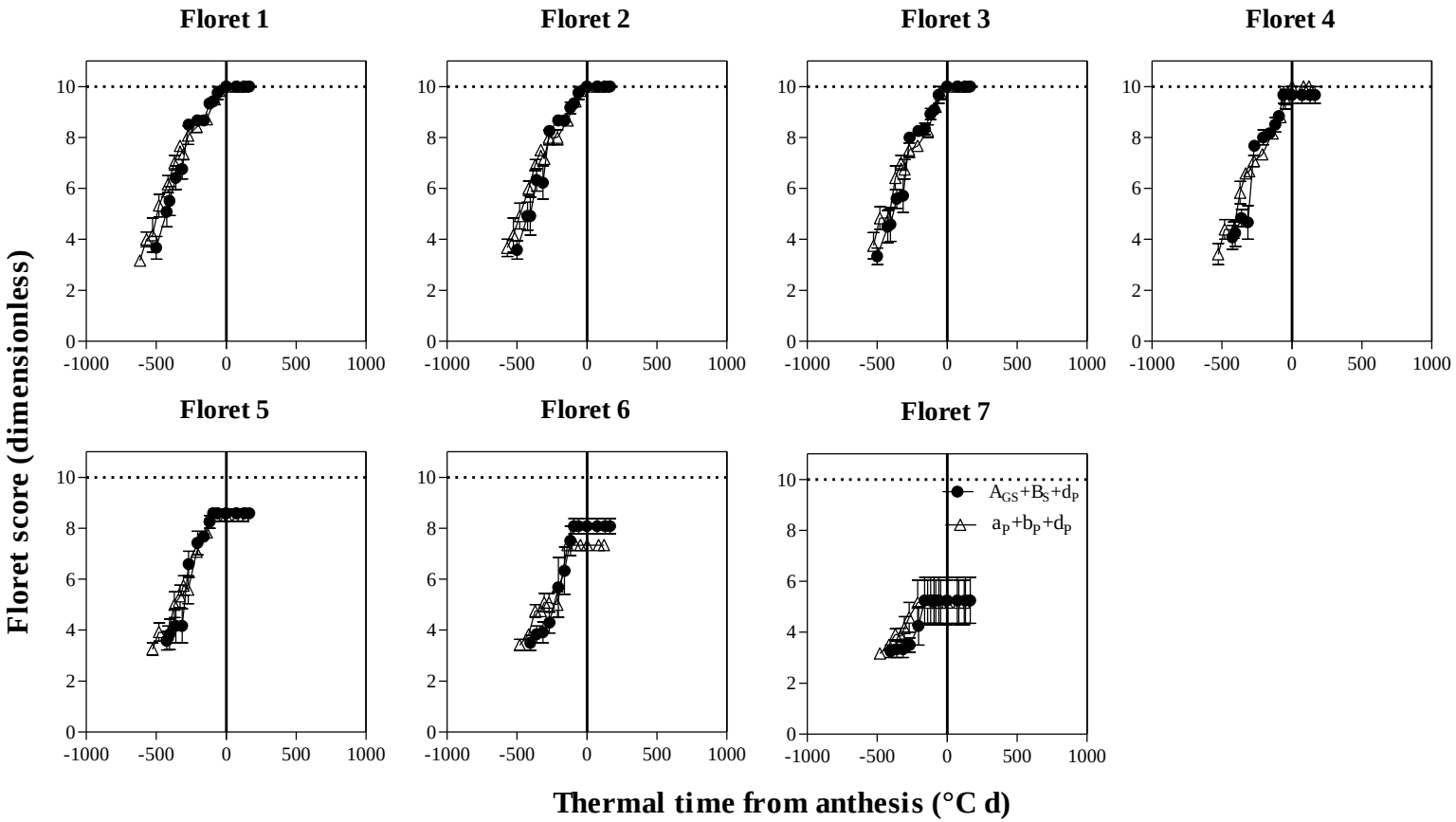

Figure A4.23. Dynamics of the floret development from F1 to F7 in central spikelets of the main-shoots through thermal time from anthesis in $A_{GS}+B_{S+}d_P$ (closed circles), a NIL carrying double change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

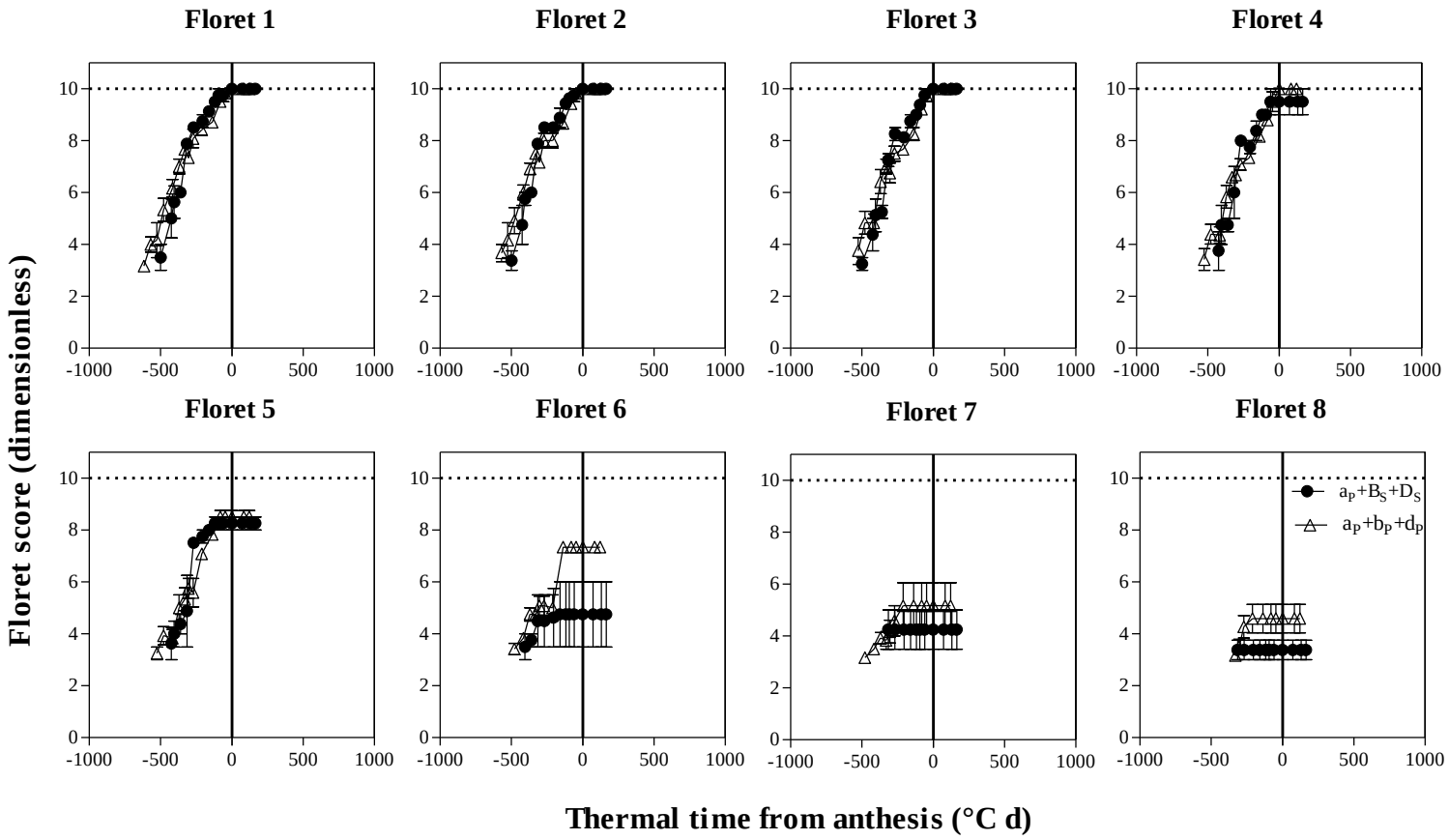

Figure A4.24. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in A_{GS}+$B_{S+}d_P$ (closed circles), a NIL carrying double change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

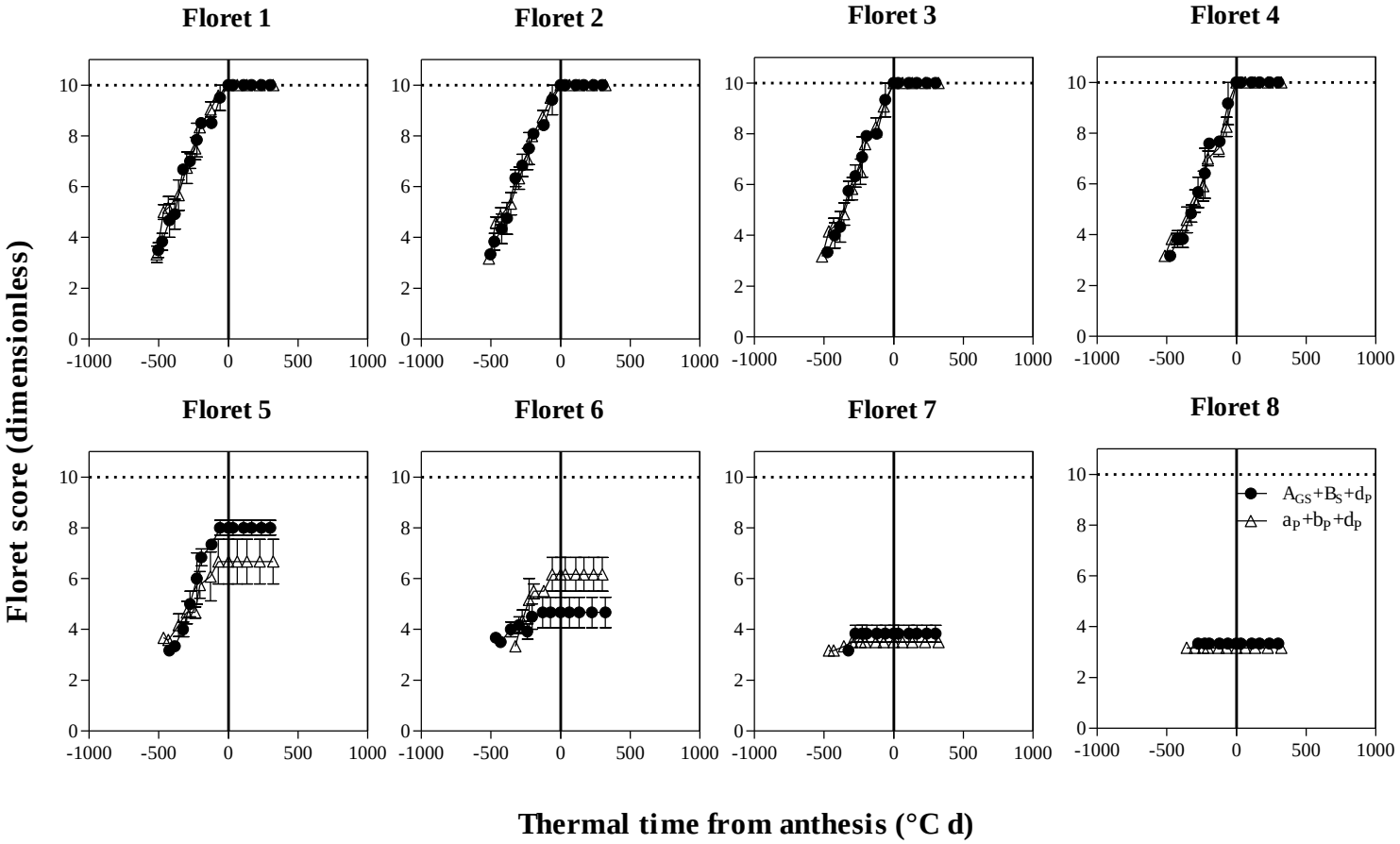

Figure A4.25. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $a_P+B_S+D_S$ (closed circles), a NIL carrying double change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

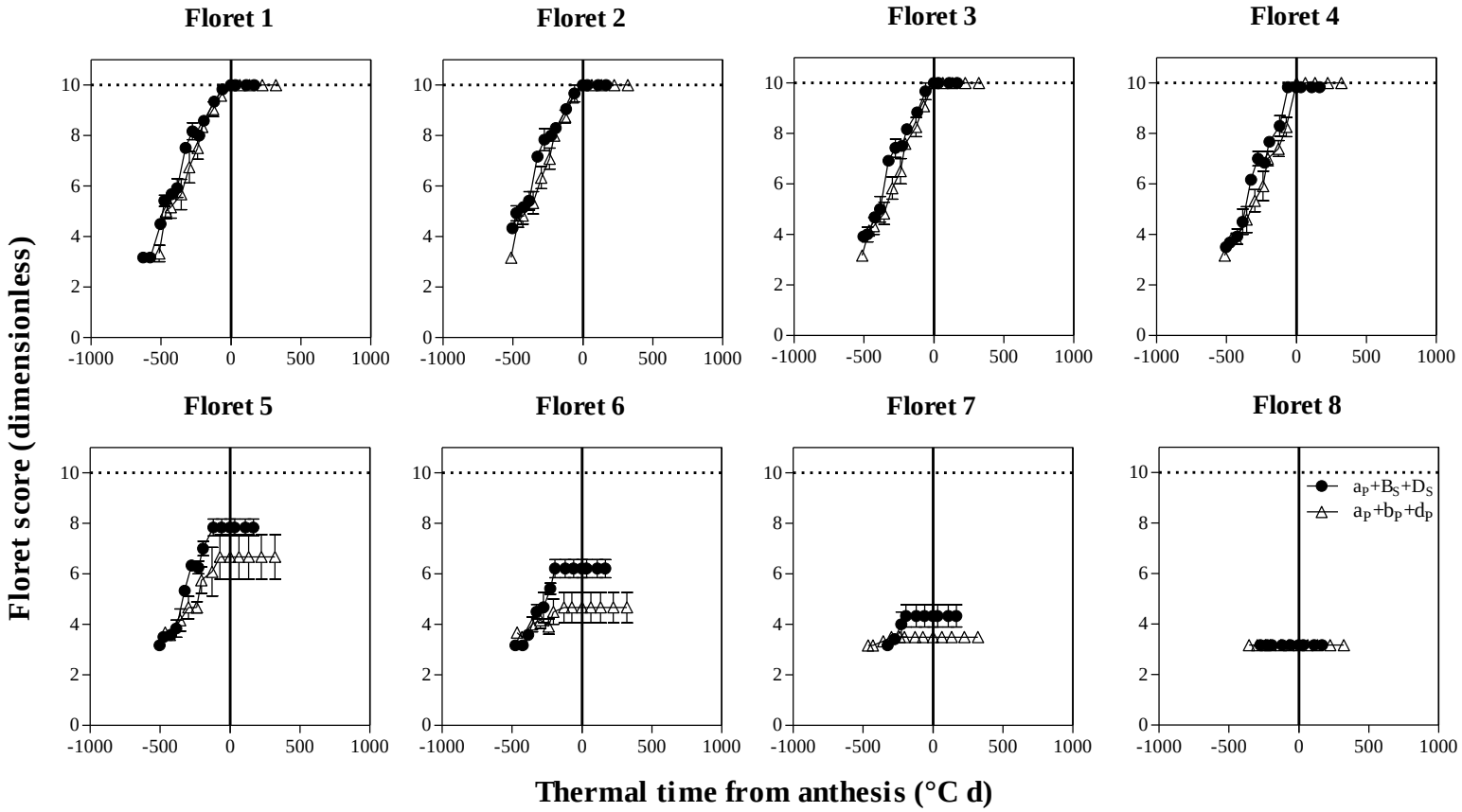

Figure A4.26. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $a_P+B_S+D_S$ (closed circles), a NIL carrying double change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

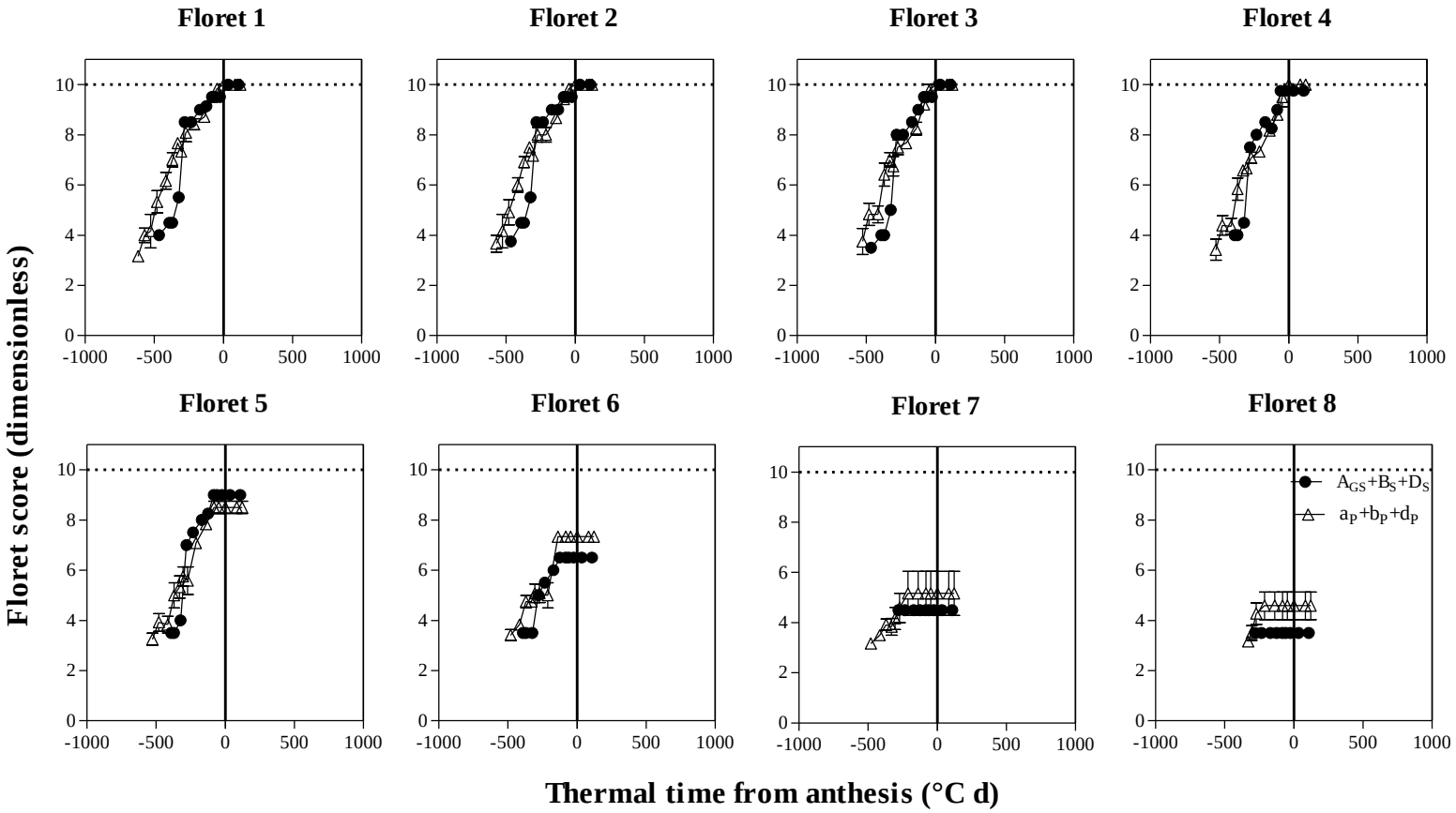

Figure A4.27. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $A_{GS}+B_S+D_S$ (closed circles), a NIL carrying triple change, in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

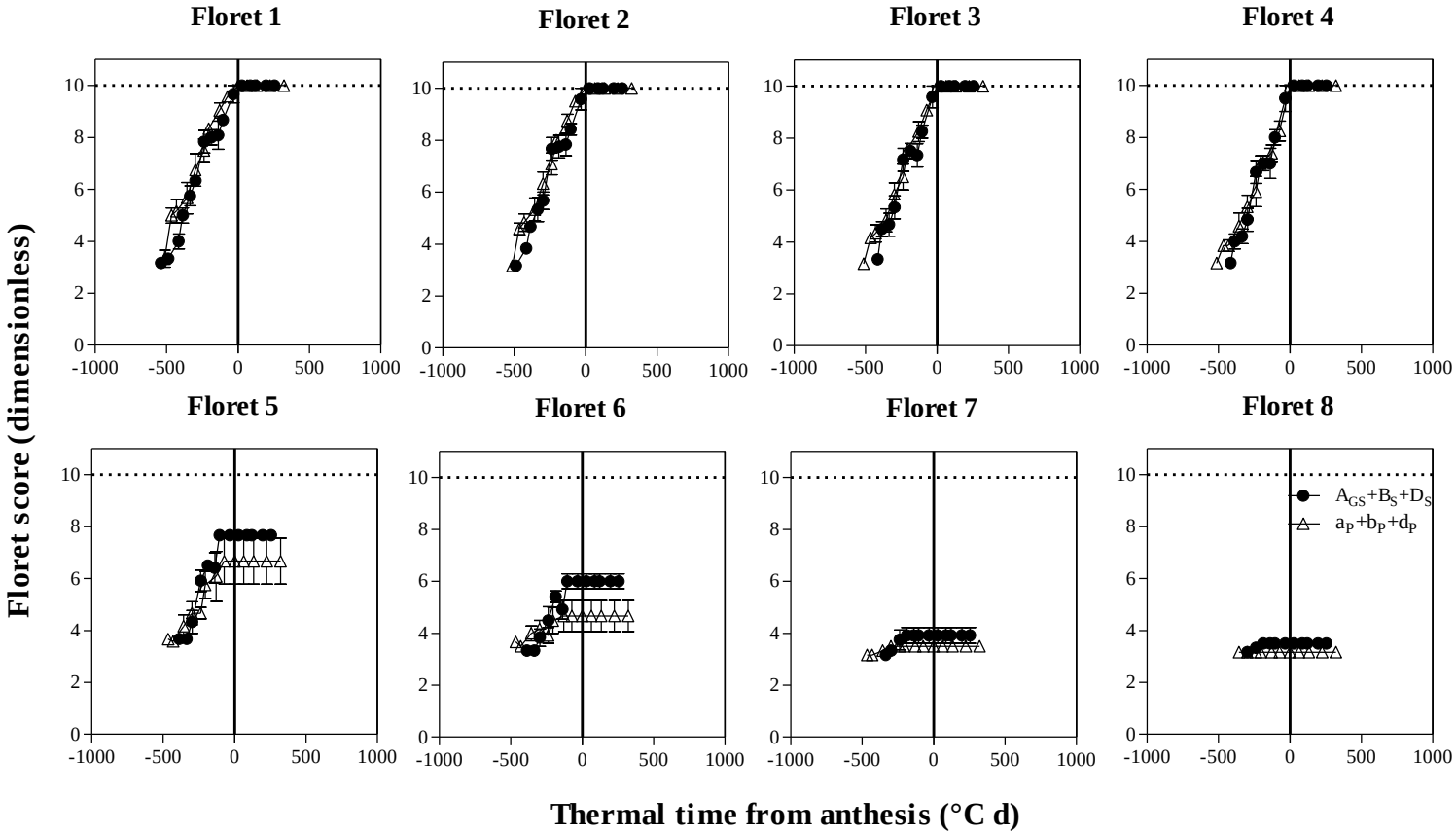

Figure A4.28. Dynamics of the floret development from F1 to F8 in central spikelets of the main-shoots through thermal time from anthesis in $A_{GS}+B_S+D_S$ (closed circles), a NIL carrying triple change, in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods), bars stands for the standard error of the means.

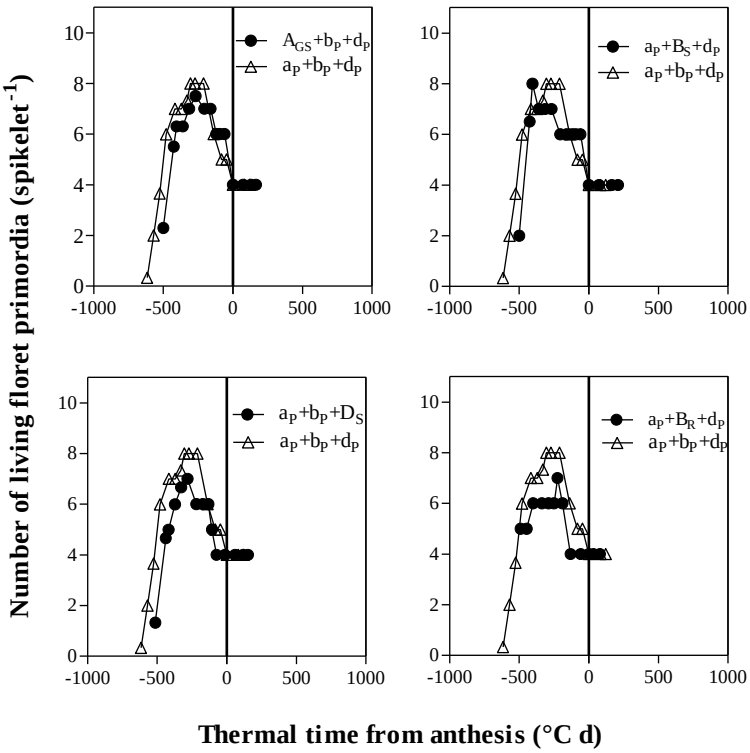

*Figure A4.29. N*umber of living floret primordia in the central spikelets through thermal time from anthesis in NILs carrying one change (closed circles), in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods).

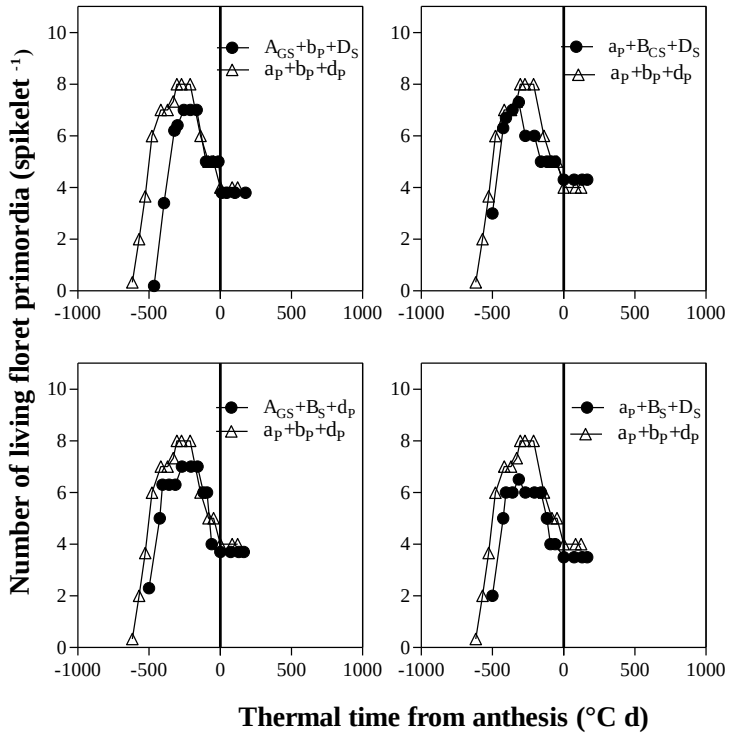

*Figure A4.30. N*umber of living floret primordia in the central spikelets through thermal time from anthesis in NILs carrying two changes (closed circles), in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods).

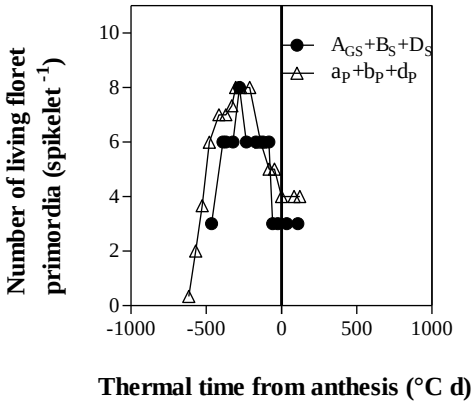

*Figure A4.31. N*umber of living floret primordia in the central spikelets through thermal time from anthesis in a NIL carrying three changes (closed circles), in comparison to Paragon (open triangles) during the first growing season. Data-points are average from replicates (see Materials and methods).

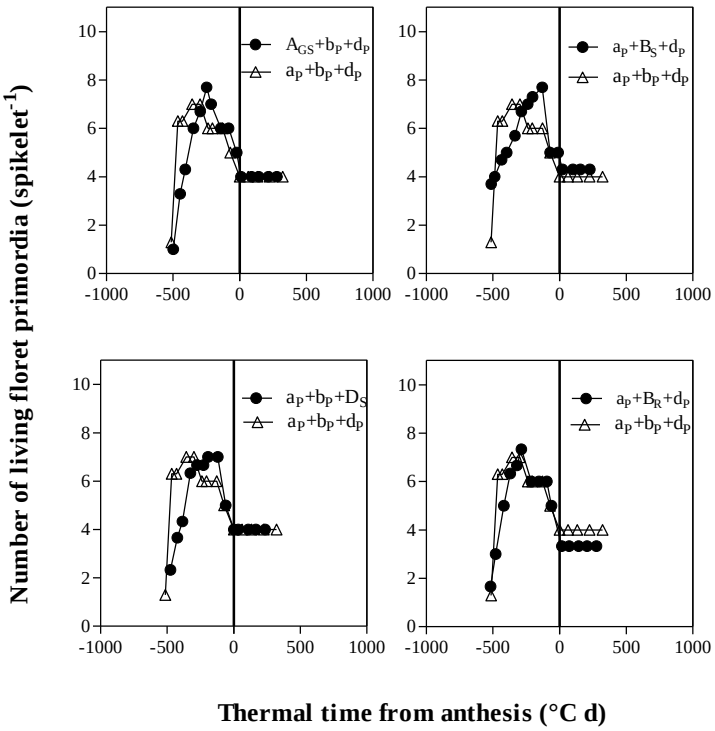

*Figure A4.32. N*umber of living floret primordia in the central spikelets through thermal time from anthesis in NILs carrying one change (closed circles), in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods).

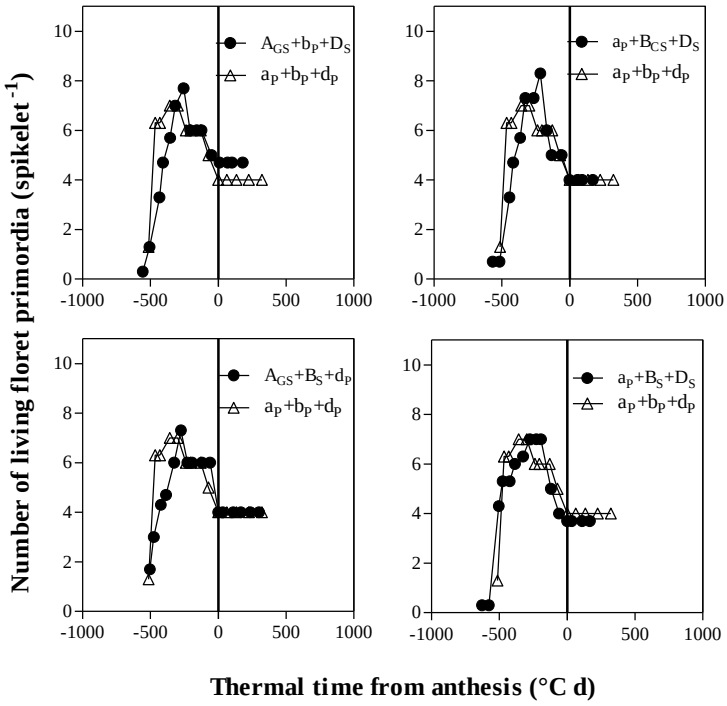

*Figure A4.33. N*umber of living floret primordia in the central spikelets through thermal time from anthesis in NILs carrying two changes (closed circles), in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods).

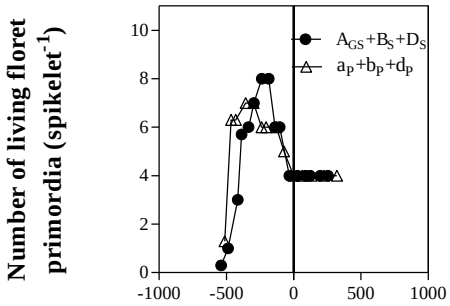

*Figure A4.34. N*umber of living floret primordia in the central spikelets through thermal time from anthesis in a NIL carrying three changes (closed circles), in comparison to Paragon (open triangles) during the second growing season. Data-points are average from replicates (see Materials and methods).

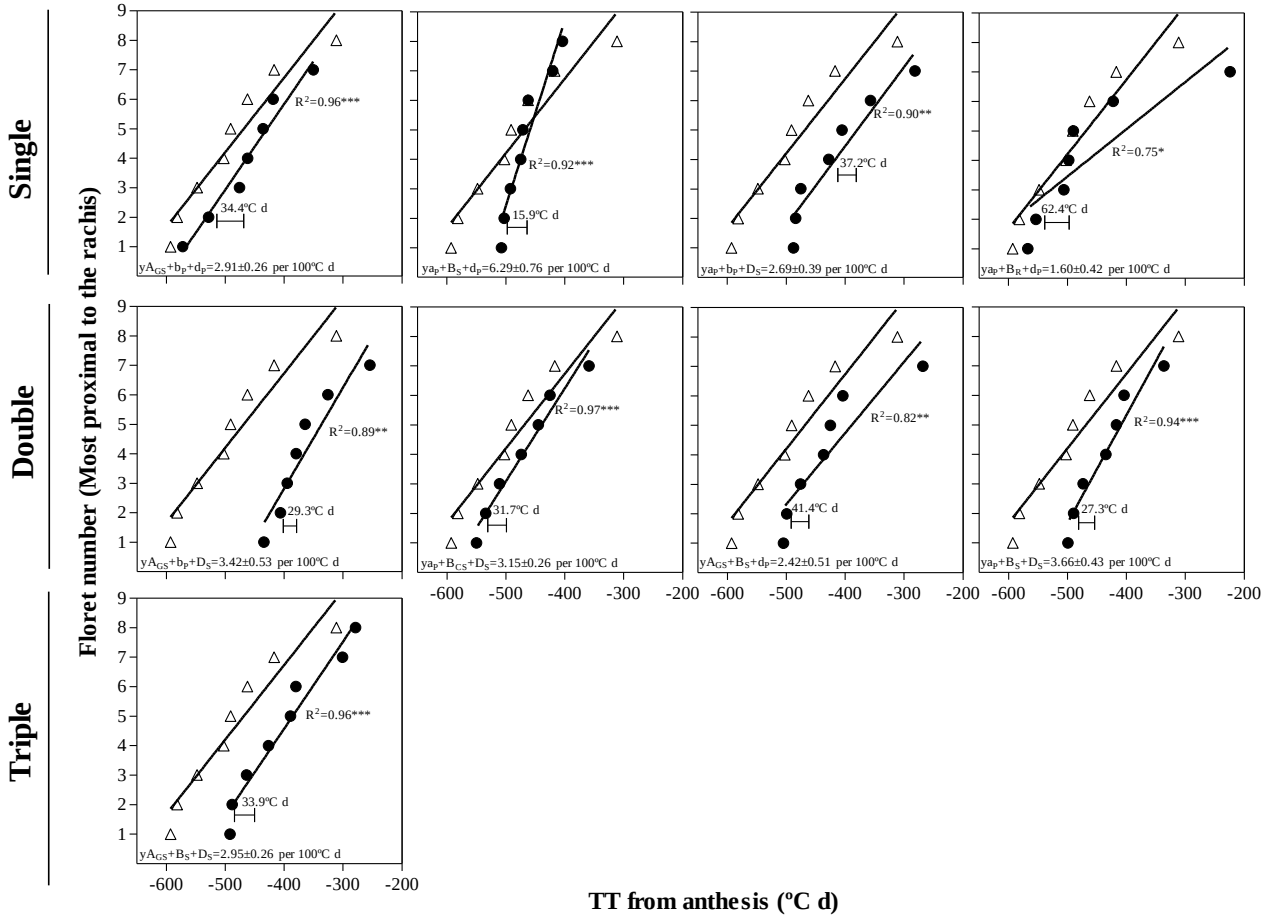

Figure A4.35. Timing of W3.5 for each floret primordium through thermal time from anthesis in NILs (closed circles) carrying one single (*top panels*), double (*middle panels*) and triple change (*bottom panels*) in comparison to Paragon ($a_P+b_P+d_P$, open triangles) which rate of floret initiation was: 2.53±0.30 (R^2=0.92***), during the first growing season. In all the cases the floret initiation rates are expressed in florets per 100°C d. The coefficient of determination (R^2) and the level of significance for each NIL linear regression are shown. Inset each panel, thermal time between the appearance of two following floret primordia are indicated, for Paragon was 39.5ºC d.

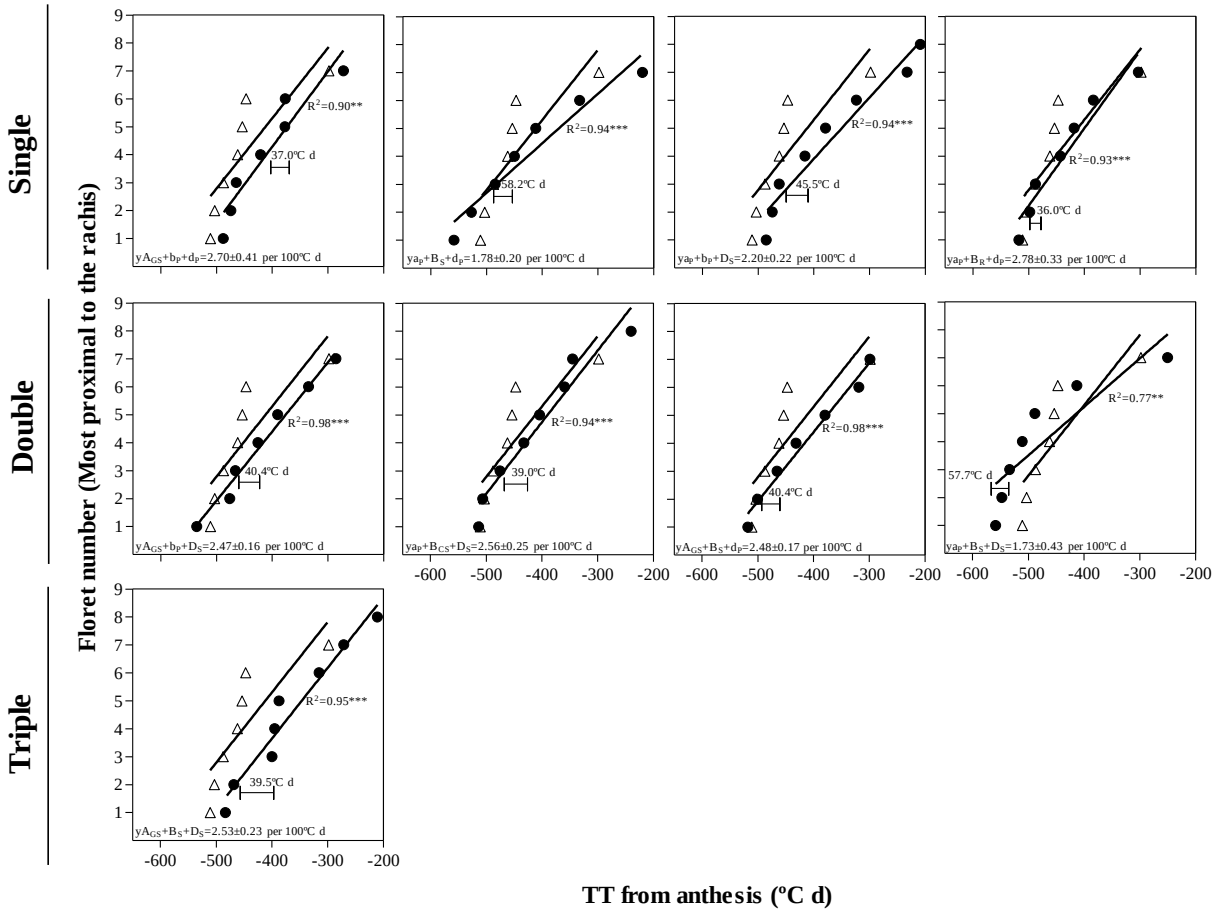

Figure A4.36. Timing of W3.5 for each floret primordium through thermal time from anthesis in NILs (closed circles) carrying one single (*top panels*), double (*middle panels*) and triple change (*bottom panels*) in comparison to Paragon ($a_P+b_P+d_P$, open triangles) which rate of floret initiation was: 2.52±0.72 (R^2=0.71*), during the second growing season. In all the cases the floret initiation rates are expressed in florets per 100°C d. The coefficient of determination (R^2) and the level of significance for each NIL linear regression are shown. Inset each panel, thermal time between the appearance of two following floret primordia are indicated, for Paragon was 39.7°C d.

Chapter V: Physiological determinants of fertile florets as affected by Eps genes

1. Introduction

Earliness '*per se*' (*Eps*) genes are within the most relevant genes that govern the wide wheat adaptability (Herndl *et al*., 2008) and it has been shown to affect the duration of the phases to flowering once the photoperiod and vernalisation are fully satisfied (Slafer *et al*., 2015, and references quoted therein). Although they are within the most relevant genes affecting development, the magnitude of their effects are frequently minor, being useful mainly for fine-tuning of flowering time in a regional adaptation (Griffiths *et al*., 2009).

Probably due to the complex experimental setup required (all photoperiod and vernalisation requirements must be fully satisfied to identify these genes), due to the expected relatively minor effects (which would be more difficult to detect for more detailed variables), or to assumptions commonly made in the literature (see Slafer, 1996), most reports on *Eps* effects focused on time to anthesis or heading, not normally on the effects these alleles might have on the development of particular organs. However, it was reported that the gene *Eps-A^{m}1* (an exceptional case of an *Eps* gene with a rather large effect on development in diploid wheat) not only affected the duration to heading, but also the number of spikelets and grains per spike (Lewis *et al.*, 2008). However, to the best of my knowledge there have been no studies reporting the effects of *Eps* genes in hexaploid wheat on the wheat floret development dynamics and on the setting of a particular level of the spike fertility.

The aim of this work was to identify (and if identified to quantify) changes in the number of fertile florets at anthesis, as well as in the dynamics of floret development among different wheat Near Isogenic Lines (NILs) for *Eps* genes under field conditions.

2. Materials and methods

2.1 General description

Fields experiments were carried out during three consecutive growing seasons: 2012-13 (first growing season), 2013-14 (second growing season) and 2014-15 (third growing season) as it is described in Table 5.1. In the first and second growing seasons the soil was classified as Fluvisol calcari (FAO, 1988) and Calcisol Petric (FAO, 1987) while in the third growing season, it was a complex of Calcisol petric and Calcisol haplic, (IUSS Working Group WRB, 2006). Soil status at sowing in both growing seasons ensured that there was not a deficit of Nitrogen during the crop growth. Plots size was 6m^2 (6 rows 0.20 m apart and 5 m long) in the first and second growing seasons while in the third one it was 4.8m^2 (6 rows 0.20 m apart and 4 m long). Sowing rates were 300 seeds m^{-2} during all growing seasons.

Table 5.1. Field experiments details: Growing season, location, coordinates and sowing date.

Growing season	Location	Coordinates	Sowing date
First	Algerri, Lleida, NE Spain.	lat. 41º 46' 49.19'' N, long. 0º 38' 18.49'' E	02/11/2012
Second	Algerri, Lleida, NE Spain.	lat. 41º48´12.85´´N, long. 0º37´8.85´´E	07/11/2013
Third	Bell-lloc d'Urgell, Lleida, NE Spain.	lat. 41º39´7.07´´ N, long. 0º46´19.70´´E	14/11/2014

2.2 Treatments and experimental design

Treatments consisted of wheat NILs differing in earliness *per se* alleles resulted from the crosses of Avalon x Cadenza (AxC) or Spark x Rialto (SxR), each pair of NILs carrying either the early or the late allele in Chromosome 1D (in both AxC and SxR) or in 3A (in AxC; Table 5.2). For the *Eps* in chromosome 1D of AxC there were 6 pairs of NILs (6 lines with the *Eps*-early and 6 with the *Eps*-late) and therefore all in all 12 lines. This was the same in the case of the *Eps* in chromosome 3A of AxC. Finally, for the *Eps* in chromosome 1D of SxR there were 4 pairs of NILs (4 lines with the *Eps*-early and 4 with the *Eps*-late) and therefore all in all 8 lines. In the cases of *Eps* alleles in chromosome 1D, all pairs were made on a single recurrent parent (Cadenza and Rialto, respectively), whilst in the case of *Eps* alleles in chromosome 3A, half of the pairs were made on Cadenza and the other half with Avalon as recurrent parents. Each individual NIL pairs were developed by making the double haploid to cross into the recurrent parent, a progeny of this cross was crossed twice into the recurrent parent (two back-crosses) and after the lines were selfed (3 cycles) (BC_2F_3), produced in Dr Simon Griffiths's lab at the John Innes Centre (Norwich-UK). Within each of the three families of NIL pairs, all differences could be only ascribed to the effect of that of the specific *Eps* gene, and therefore I analyzed the effect of each specific *Eps* as the average in the variables analyzed between the 2 lines in each of the NIL pairs within a family. Each of the three "families" of NILs (AxC 1D; SxR 1D; AxC 3A) were grown over two consecutive growing seasons and each family was sown in a different experiment in adjacent parts of the field (they were side-by-side). As treatments (lines) were arranged in three completely randomized blocks we had in each case either 18 or 12 replications (Table 5.2).

2.3 Measurements and analyses

We determined the number and Waddington stage of floret primordia periodically from terminal spikelet initiation to anthesis, when the number of fertile florets was counted in detail for each spikelet in both main-shoot and tiller spikes. The general procedures and all measurements and analyses were presented in detail in Chapter II. In all cases the analyses were done line per line individually but results are based on the average differences between late and early NIL pairs within families.

Table 5.2. Plant material used in the experiments consisting of *Eps* NILs resulted from the Avalon x Cadenza cross (AxC) differing in early or late alleles in chromosome 1D ($late_{AxC\_1D}$ vs $early_{AxC\_1D}$) or in chromosome 3A ($late_{AxC\_3A}$ vs $early_{AxC\_3A}$) and the *Eps* NILs resulted from the Spark x Rialto cross (SxR) differing in in early or late alleles in chromosome 1D ($Late_{SxR\_1D}$ vs $Early_{SxR\_1D}$). The growing seasons and the number of replicates are also given.

Parentals	Background	Source of *Eps* allele	Chromosome	Allele	Code	Growing seasons	Overall replicates
AvalonxCadenza	Cadenza	Avalon Cadenza	1D	*Eps-A^{m}1-l** *Eps-A^{m}1-e*	$late_{AxC\_1D}$ $early_{AxC\_1D}$	First and second	18
AvalonxCadenza	Cadenza & Avalon	Cadenza Avalon	3A	*Eps-A^{m}1-l* *Eps-A^{m}1-e*	$late_{AxC\_3A}$ $early_{AxC\_3A}$	Second and third	18
SparkxRialto	Rialto	Rialto Spark	1D	*Eps-A^{m}1-l* *Eps-A^{m}1-e*	$late_{SxR\_1D}$ $early_{SxR\_1D}$	First and second	12

**l* = allele conferring lateness; *e* = allele conferring earliness

The results showed in this chapter include the analysis of the effects of carrying the *Eps*-early allele in comparison to the *Eps*-late on the number of fertile florets along the spikes position due to changes in the floret development dynamics in the central spikelets. However, to summarize, only the dynamics for 4 contrasting floret positions are shown in the main body of the chapter, in which the effects of the treatment (genetic allelic variation) can be found. All the rest of floret positions can be found in Annex 5.

2.4 Weather conditions

Main difference on weather conditions among growing seasons is the averaged maximum temperature on May in which all the NILs from the different parental cross and chromosomes reached anthesis stage in all the growing seasons. Averaged maximum temperatures were: 20.0°C, 23.4°C and 27.6°C during the first, second and third growing seasons respectively (Fig. 5.1).

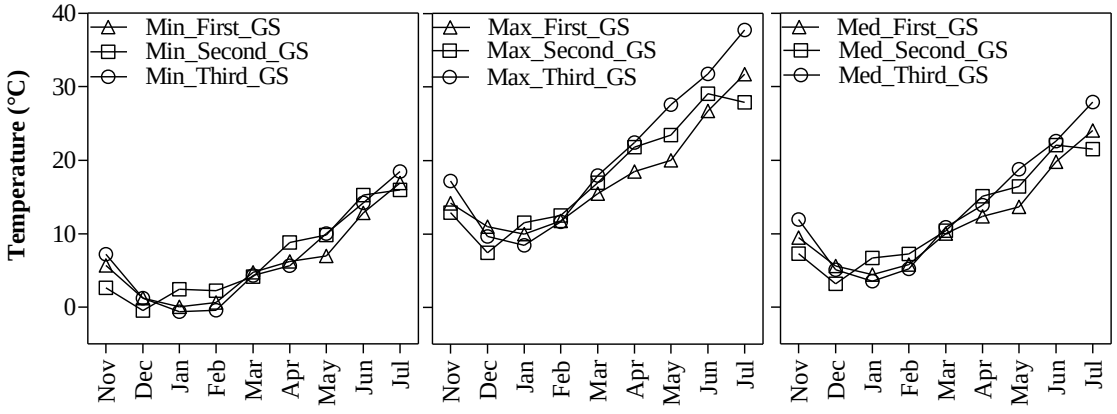

Figure 5.1. Minimum (left panel), maximum (middle panel) and mean (right panel) temperatures average monthly along the crop cycle at Algerri during the first and second growing seasons and at Bell-lloc d'Urgell during the third one.

3. Results

3.1 Spike fertility

The *Eps*-late alleles considered in this study increased the number of fertile florets respect the NILs with the *Eps*-early along the spikes of both main-shoots and tillers in at least one of the two growing seasons in which they were grown.
In the case of the *Eps* gene in chromosome 1D of AxC the number of fertile florets along the main-shoot and tiller spikes *Eps* were not significantly different in the first growing season (when curiously the *Eps* early variant tended to have a higher number than the late variant; Fig. 5.2, top panels). During the second growing season differences were higher and the *Eps*-late variant presented a higher number of fertile florets in the most spikelets than the early variant in both main-shoot and tiller spikes, which resulted in a significantly higher number of fertile florets per spike (Fig. 5.2, bottom panels).
The *Eps*-late allele in chromosome 3A of AxC showed similar results than that allele in chromosome 1D of the same cross but with more clear impact: in the first growing season in which it was grown the difference was not significant either, but the trend was for the late allele to induce higher fertility in the spike than the early allele (Fig. 5.3, top panels), while in the last growing season the difference became much larger and even more significant than in the case of the allele in 1D, being the spike fertility improved evident in almost all the spikelets in both the main-shoot and tiller spikes (Fig. 5.3, bottom panels).
When NILs from the SxR cross with contrasting alleles of the *Eps* gene in chromosome 1D were compared, again the lines carrying the *Eps*-early exhibited spikes with reduced fertility, though the number of fertile floret per spike was significantly higher in lines with the late allele than in those with the early allele in the main-shoots in the first growing season and in the tillers in the second growing season (Fig. 5.4).
As whenever a difference between NILs in spike fertility was detected, it was representative of most spikelets but clearer in the more fertile spikelets, those in the central part of the spikes, a detailed analysis of floret development in these spikelets could shed light on the origin of the differences in spike fertility.

3.2 Floret development and living floret primordia dynamics in the central spikelets

More detailed analysis was carried out to try to find out the likely origin of the overall slight increase in number of fertile florets due to the introgression of the late form of these *Eps* genes. Dynamics of development of each individual floret primordium as well as that of all living floret primordia were studied for each of the NILs. Florets 1, 3, 4 and 6 were chosen to illustrate the main results because they represent extreme cases of floret development: while floret 1 always reaches the fertile stage, floret 6 does never develop that much and florets 3 and 4 are those most commonly labile, determining their developmental dynamics whether or not the treatments affect floret fertility.
Although not in the main body of this chapter, the dynamics of other floret positions are shown in the Annex 5 (A5.1, 2, 3).

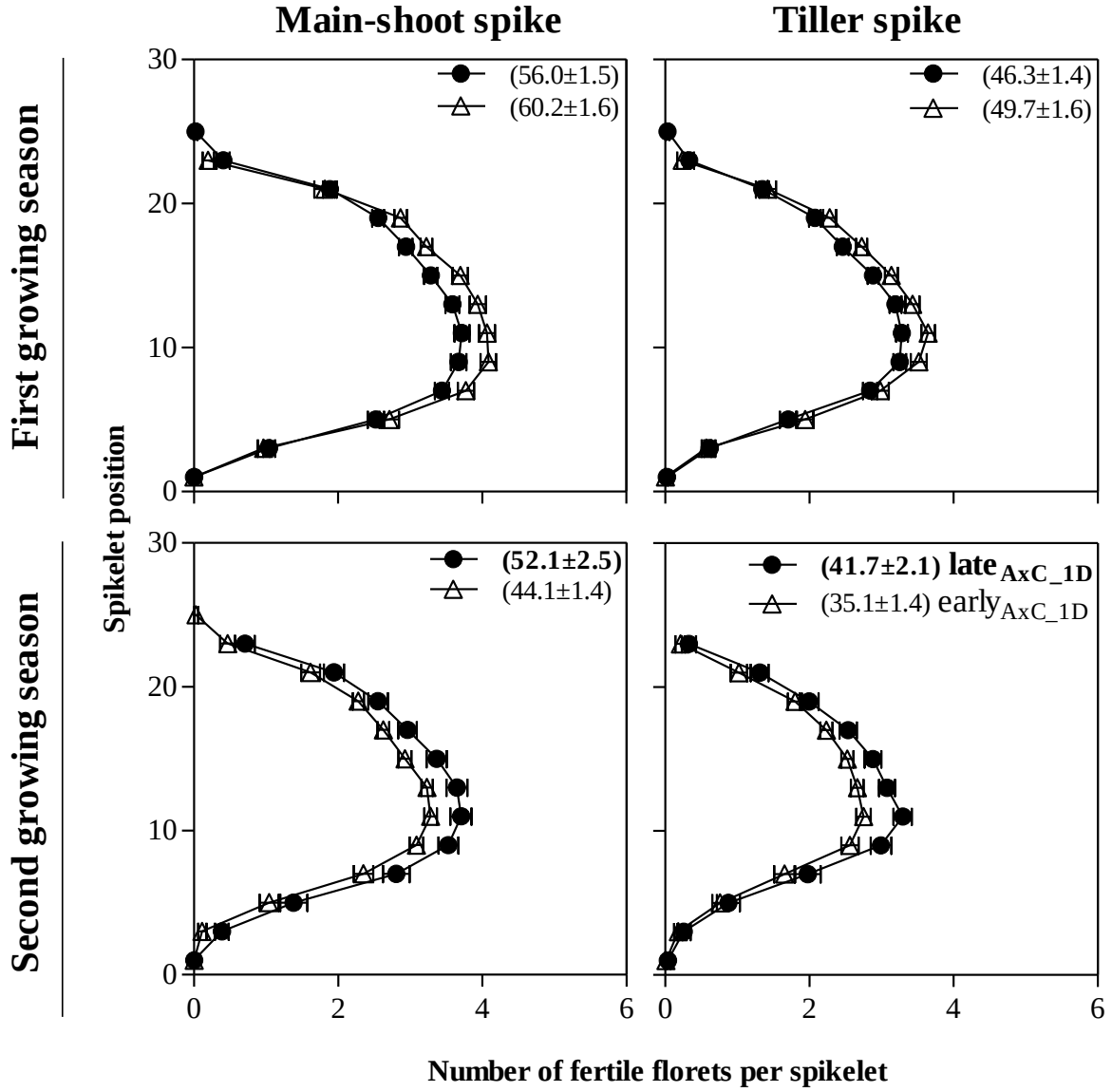

Figure 5.2. Mapping of fertile florets (fertility of each spikelet position on the main-shoot or tiller spike) (left and right columns) for the *Eps* NILs AxC 1D carrying the *Eps*-early allele (open triangle) or the *Eps*-late allele (closed circles) during the first (*top panels*) and the second growing seasons (*bottom panels*). Each data-point is the average of all replicates and within each replicate the value was the average of 4 plants and the segment in each data-point stands for the standard error of the means. The average of the number of fertile florets per spike and the standard error of the means are indicated in brackets, in bold when differences between early and late were significant.

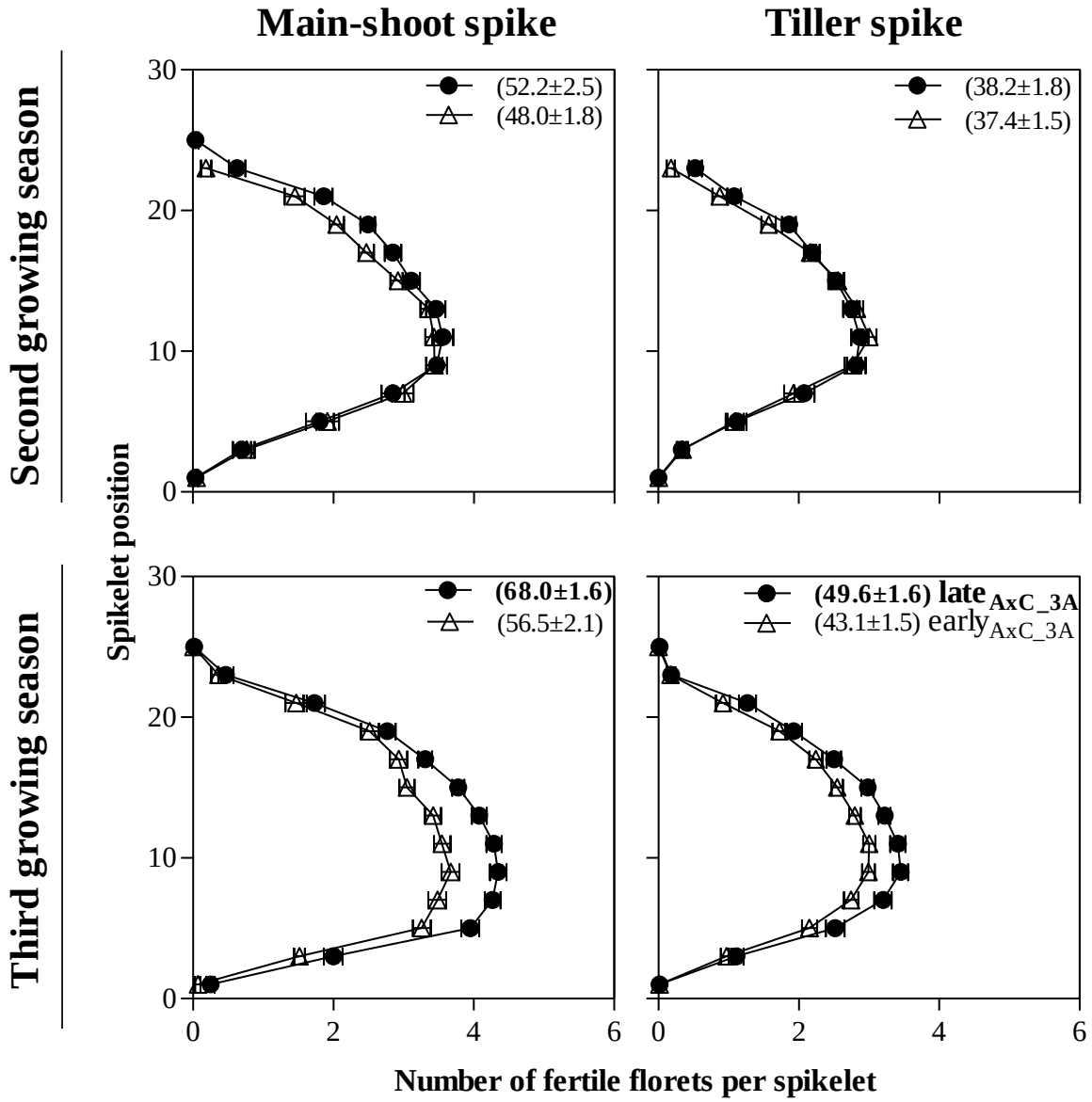

Figure 5.3. Mapping of fertile florets (fertility of each spikelet position on the main-shoot or tiller spike) (left and right columns) for the *Eps* NILs AxC 3A carrying the *Eps*-early allele (open triangle) or the *Eps*-late allele (closed circles) during the first (*top panels*) and the second growing seasons (*bottom panels*). Each data-point is the average of all replicates and within each replicate the value was the average of 4 plants and the segment in each data-point stands for the standard error of the means. The average of the number of fertile florets per spike and the standard error of the means are indicated in brackets, in bold when differences between early and late were significant.

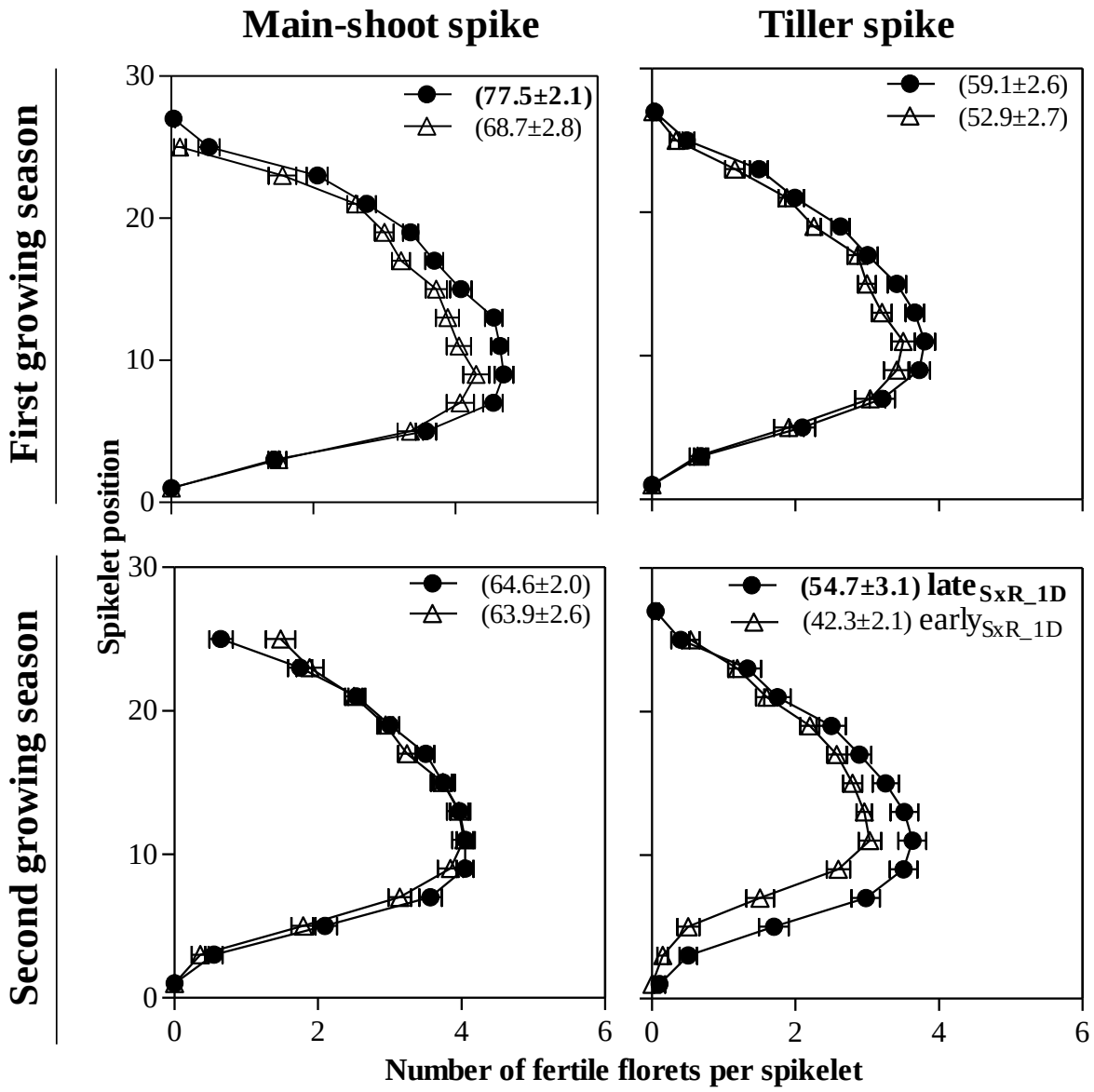

Figure 5.4. Mapping of fertile florets (fertility of each spikelet position on the main-shoot or tiller spike) (left and right columns) for the *Eps* NILs SxR 1D carrying the *Eps*-early allele (open triangle) or the *Eps*-late allele (closed circles) during the first (*top panels*) and the second growing seasons (*bottom panels*). Each data-point is the average of all replicates and within each replicate the value was the average of 4 plants and the segment in each data-point stands for the standard error of the means. The average of the number of fertile florets per spike and the standard error of the means are indicated in brackets, in bold when differences between early and late were significant.

There were no clear differences in the dynamics of development of the three most proximal floret primordia between NILs carrying the late and early *Eps* alleles in chromosome 1D resulted from the AxC cross in any of the two growing season in which all reached the stage of fertile floret (score 10) (Fig. 5.5A Florets 1 and 3; Fig. A5.1 Floret 2). On the other hand floret 4 reached that stage only in some of the plants of NILs carrying the *Eps*-early allele but not in others, whilst did so in most plants of NILs carrying the late allele in the second growing season (Fig. 5.5A) explaining the slight difference in spike fertility in that season (a difference not seen in the first growing season). Florets in more distal positions did never develop to stages even close to fertile florets in any NILs and growing seasons (Fig. 5.5A Floret 6; Fig. A5.1 Florets 5,7,8) and did not exhibit any difference in development between NILs, with the exception of Floret 5 in the second season that like in the case of floret 4, the NILs with the *Eps*-late allele tended to reach advanced stages than those with the early allele (Fig. A5.1 Florets 5, bottom panel). Consequently, there were no differences between the *Eps*-late or *Eps*-early NILs in the dynamics of generation and degeneration of floret primordia nor in the final number of fertile florets in the first growing season (Fig. 5.5B, top panel), whilst in the second season plants of both NILs initiated a similar number of floret primordia but the plants carrying the *Eps*-late allele showed a slightly slower rate of death towards the end of the floret mortality period, resulting in higher number of fertile florets at anthesis (Fig. 5.5B, bottom).

Similarly to what was described for the NILs varying in *Eps* from in chromosome 1D of AxC, when the effects of possessing the *Eps*-late vs -early allele in chromosome 3A, there were no clear differences in the dynamics of either developmental progress of particular floret primordia (Fig. 5.6A top panels; Fig. A5.2 top panels), or in the floret primordia generation/degeneration (Fig. 5.6B top panels), in the first growing season in which it was grown. Florets 1, 2 and 3 always reached the fertile stage simultaneously, and floret 4 did so only in part of the plants analyzed but not in the others, in the contrasting NILs; while more distal florets (5-8) (Fig. 5.6A top panels; Fig. A5.2 top panels) never reached this stage. In the second growing season in which it was grown, when spike fertility was more clearly affected (see above), developmental rates of florets 1-3 were again virtually unaffected by the *Eps* alleles (Fig. 5.6A bottom panels; Fig. A5.2 bottom panels), whilst floret 4 always was fertile in the plants of NILs with *Eps*-late allele while only in some plants with the *Eps*-early allele (Fig. 5.6A bottom panel); and more distal florets did not reach the stage of fertile florets in any case, though Floret 5 tended to reach advanced stages in the plants of NILs with the *Eps*-late allele (Fig. A5.2 bottom panel). Consequently, the *Eps*-late allele reduced the mortality of floret primordia (by maintaining further the development of labile florets), increasing the number of fertile florets at anthesis even when *Eps* NILs carrying either the early or late variant initiated the same number of floret primordia (Fig. 5.6B, bottom panel).

Finally, dynamics of floret 1 and 3 of the NILs carrying either the *Eps*-early or the late variant resulted from the SxR in 1D did not show any clear differences in either of the two growing season alike floret 4 in the second season, while floret 4 was fertile in all plants of the NILs carrying the *Eps*-late variant but only in several (though not all) plants carrying the *Eps*-early in the first growing season (Fig. 5.7A; Fig. A5.3). More

distal florets (5-8/9) (Fig. 5.7A; Fig. A5.3) did never reach the stage of fertile florets at anthesis in NILs with *Eps*-early alleles, in the lines with the *Eps*-late at least few plants had the fifth floret primordium developing normally to become a fertile floret at anthesis in the first growing season (Fig. A5.3, top panel). Therefore, the slight differences in the final number of fertile floret in the first growing season was again related to a reduced floret mortality due to an improved development of labile florets when the *Eps* allele was the late form (Fig. 5.7B, top panel) even though both NILs variants initiated the same maximum floret primordia in both growing seasons (Fig. 5.10B).

3.3 Duration of late reproductive phase

With the exception of the differences between NILs for *Eps* alleles in chromosome 1D, being significantly shorter in NILs carrying the *Eps*-early than in those with the *Eps*-late from AxC, during the first growing season (Fig. 5.8, left panel), differences between NILs in duration of the period from terminal spikelet to anthesis were small and not significant with the *Eps*-late only tending to increase duration of this phase (Fig. 5.8, right panel; Fig. 5.10). Although differences were not significant between NILs for *Eps* alleles in chromosome 3A in either of the two growing seasons, the trend was the opposite: the later reproductive phase tended to be longer in NILs with the *Eps*-early than in those with the *Eps*-late alleles (Fig. 5.9, right panel).

The differences in late reproductive phase, although slight between NILs in most cases, exerted a clear positive effect on spike fertility if the case of NILs for chromosome 3A in the second growing season are excluded (Fig. 5.11, left panel), and the relationship was even stronger if the actual duration of floret initiation (F1 at stage 3.5) to anthesis is considered (Fig. 5.11, right panel). The relationship was not only due to differences in backgrounds (genetic and environmental backgrounds) but also genuinely due to the action of *Eps*-late alleles in chromosome 1D: excluding the cases of chromosome 3A (squares in Fig. 5.11), in general it seems that within genetic backgrounds (AxC or SxR) and within growing seasons, the NILs with *Eps*-late alleles tended to exhibit a longer duration of the phases when floret development takes place and tended to increase the spike fertility as well (Fig. 5.11).

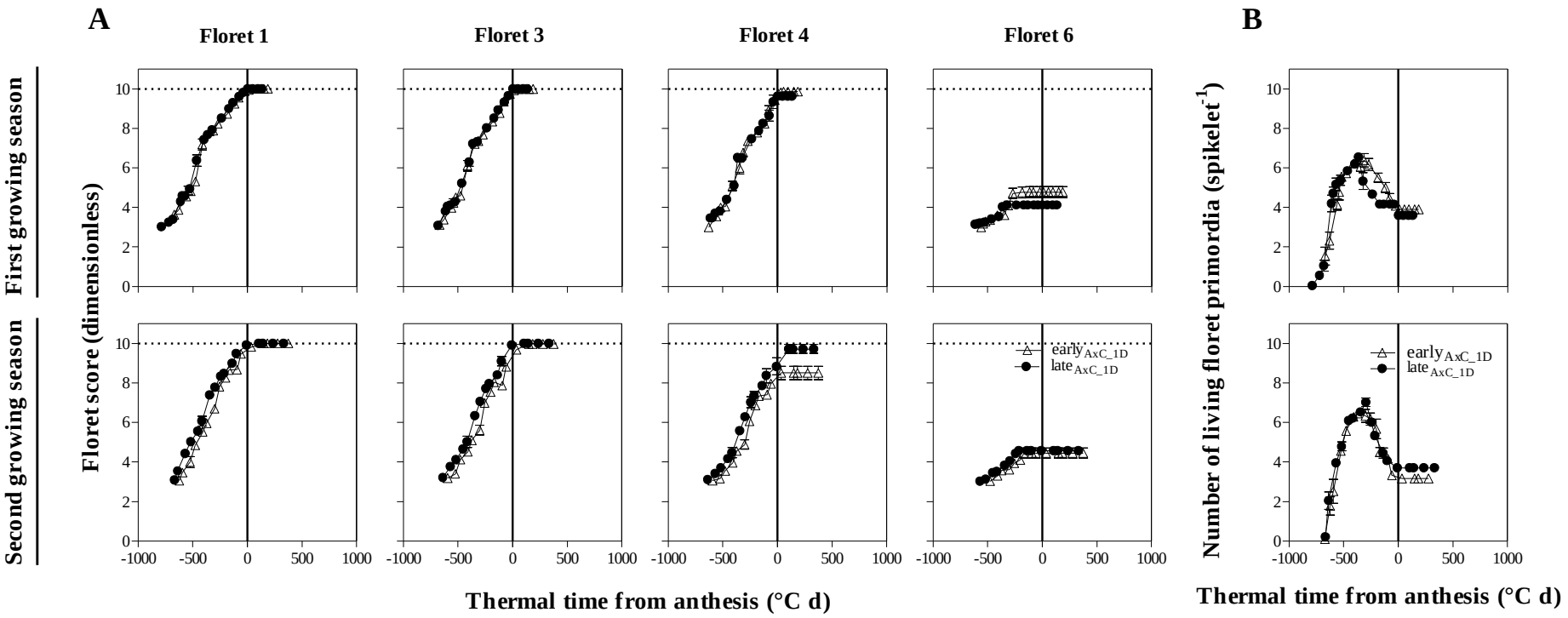

Figure 5.5. Dynamics of the floret development of F1, F3, F4 and F6 in central spikelets of the main-shoot (A) and the number of living floret primordia (B) through thermal time from anthesis in the *Eps* NILs carrying either the late (closed circles) or the early allele (open triangles) from the AxC in 1D during the first (*top panels*) and the second growing seasons (*bottom panels*). Each data-point is the average of all replicates and within each replicate the value was the average of 3 plants, bars stands for the standard error of the means.

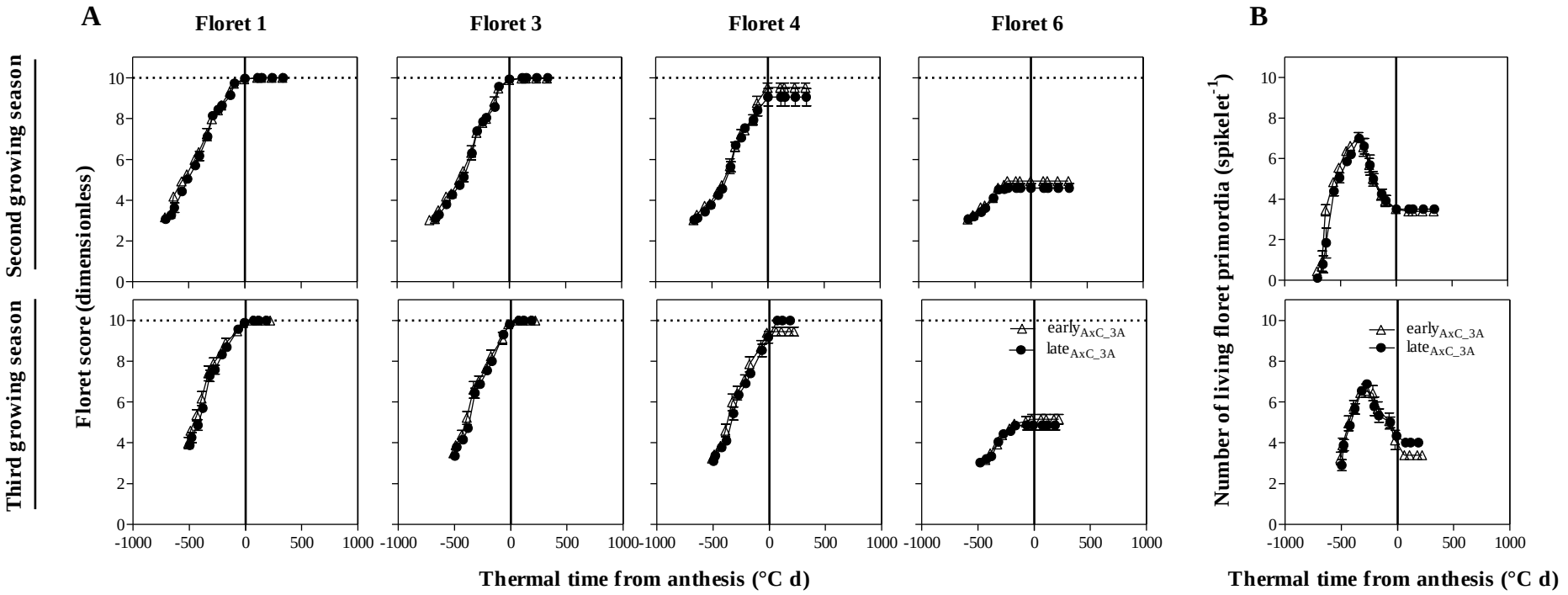

Figure 5.6. Dynamics of the floret development of F1, F3, F4 and F6 in central spikelets of the main-shoot (A) and the number of living floret primordia (B) through thermal time from anthesis in the *Eps* NILs carrying either the late (closed circles) or the early allele (open triangles) from the AxC in 3A during the first (*top panels*) and the second growing seasons (*bottom panels*). Each data-point is the average of all replicates and within each replicate the value was the average of 3 plants, bars stands for the standard error of the means.

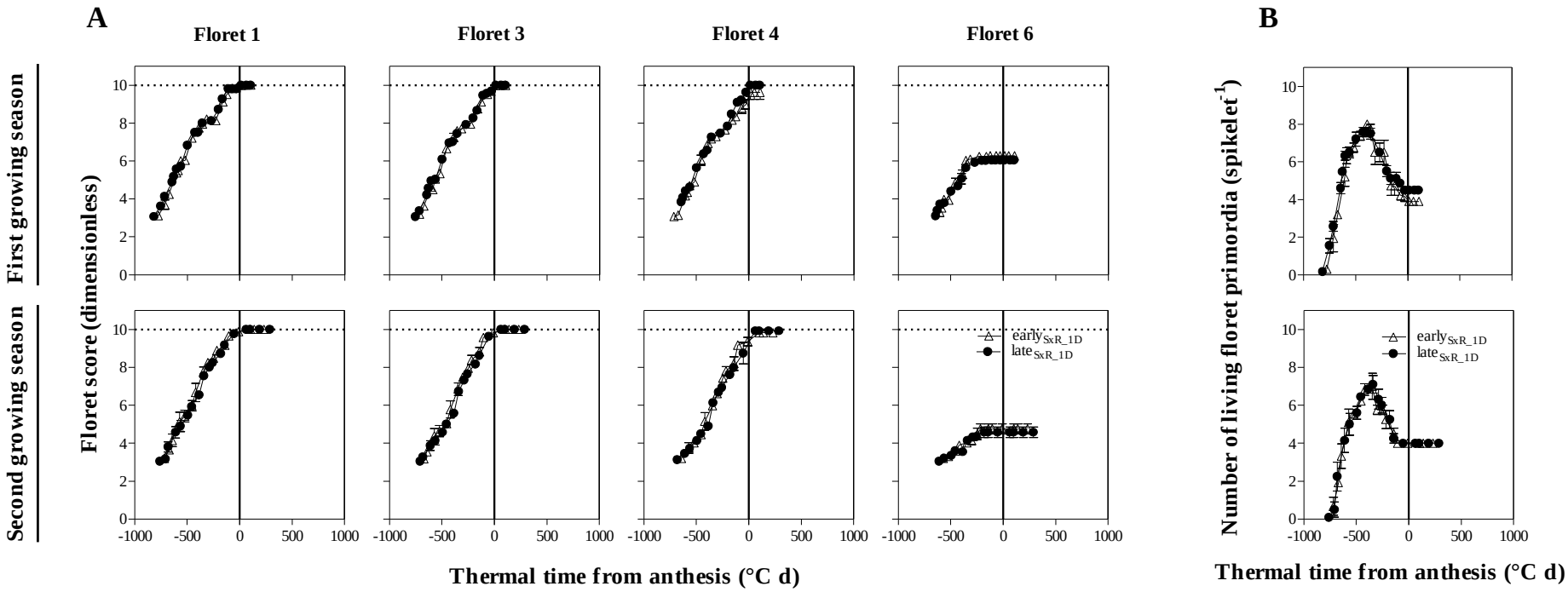

Figure 5.7. Dynamics of the floret development of F1, F3, F4 and F6 in central spikelets of the main-shoot (A) and the number of living floret primordia (B) through thermal time from anthesis in the *Eps* NILs carrying either the late (closed circles) or the early allele (open triangles) from the SxR in 1D during the first (*top panels*) and the second growing seasons (*bottom panels*). Each data-point is the average of all replicates and within each replicate the value was the average of 3 plants, bars stands for the standard error of the means.

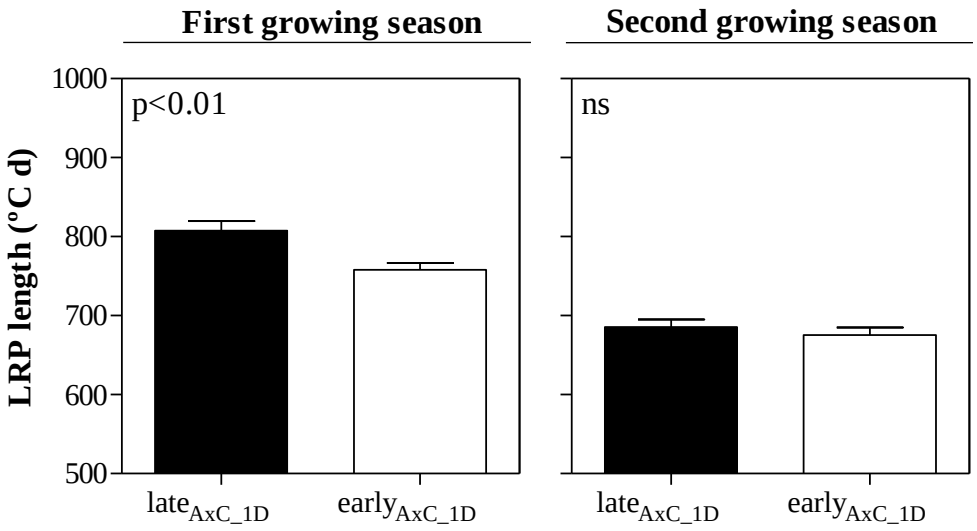

Figure 5.8. Late reproductive phase (LRP) length in the *Eps* NILs AxC 1D late and early variant in both growing seasons. Error bars stand for the standard error of the means. The level of significance (p-value) resulted from the comparison between the NILs is indicated inside each panel.

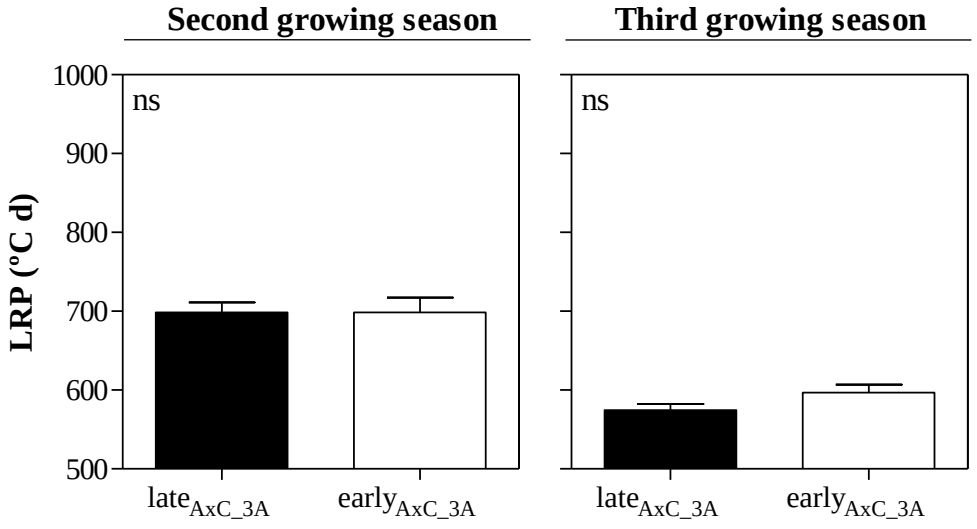

Figure 5.9. Late reproductive phase (LRP) length in the *Eps* NILs AxC 3A late and early variant in both growing seasons. Error bars stand for the standard error of the means. The level of significance (p-value) resulted from the comparison between the NILs is indicated inside each panel.

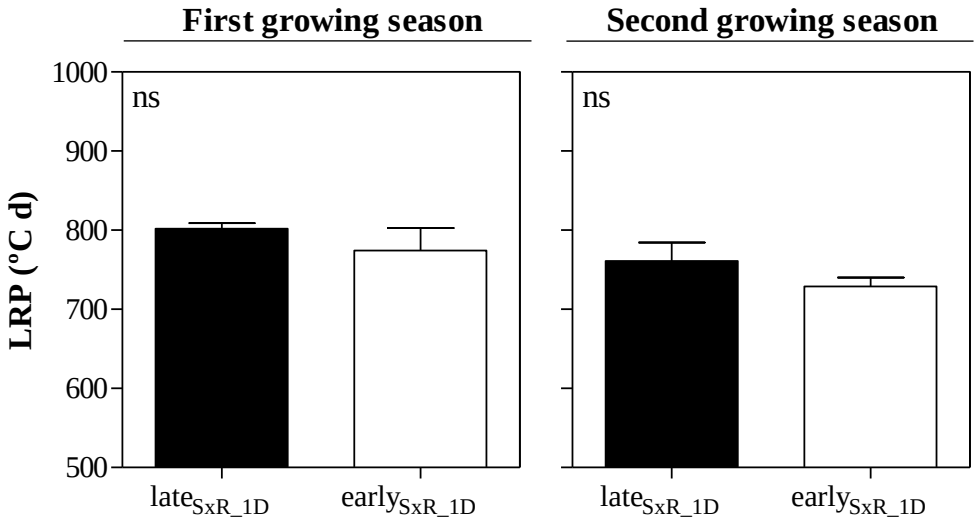

Figure 5.10. Late reproductive phase (LRP) length in the *Eps* NILs SxR 1D late and early variant in both growing seasons. Error bars stand for the standard error of the means. The level of significance (p-value) resulted from the comparison between the NILs is indicated inside each panel.

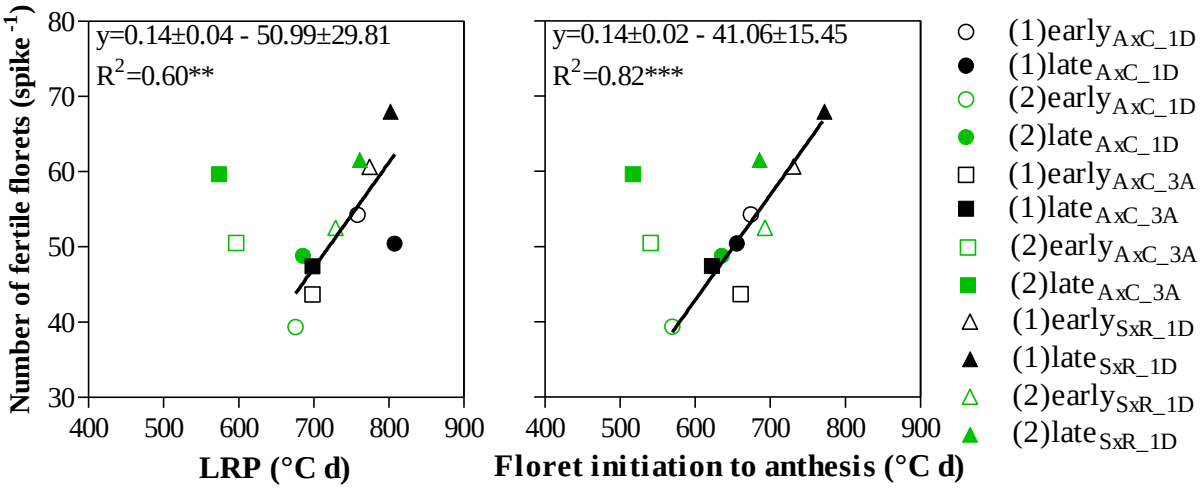

Figure 5.11. Number of fertile florets per spike against the LRP (left panel) and the floret intiation to anthesis phase (right panel) among the *Eps* NILs early and late from the AxC_1D (circles), AxC_3A (squares) and SxR_1D (triangles) during the first (black) and second (green) growing seasons. The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

4. Discussion

Although exceptions can be found (Slafer and Rawson, 1994), particularly in diploid wheat under controlled conditions (Lewis *et al*., 2008; Bullrich, *et al*., 2002; Appendino and Slafer, 2003), the *Eps* genes normally have relatively small effects on developmental attributes and are useful to fine-tuning adaptation. The number of grains per m^2 depends on the number of spikes and spike fertility. The former component is normally critical for coarse regulations (large changes in grain number due to genetic or

environmental factors) whilst spike fertility is the component of grain number more likely responding as a fine regulator to more subtle changes in grain number (Slafer *et al*., 2014). As *Eps* effects on developmental processes are relatively small, expectedly they did not include major changes in dynamics of tillering and tiller mortality (Ochagavía *et al*., 2016). Thus, effects on the number of fertile florets had to operate, if existing, on the number of fertile florets per spike, through effects on floret development.

Coinciding with most literature (Lewis, *et al*., 2008; Bullrich *et al*., 2002) the *Eps* alleles had only minor effects on time to anthesis under field conditions, which was slightly less in NILs with *Eps*-early than in those with *Eps*-late alleles (Ochagavía *et al*., 2016). Most literature on the effects of these alleles have been focused exclusively on the duration of the whole cycle (see above), assuming most commonly that any difference seen in time to heading would be either reflecting differences in duration of vegetative phases or the consequence of affecting all pre-anthesis phases equally (see Slafer, 1996). However, there are not solid evidences to support these assumptions. In a previous study it was shown that there is genotypic variation (comparing cultivars) that were not restricted to particular phases (Slafer and Rawson, 1995); the results in this chapter further shows that *Eps* alleles on chromosome 1D affected the late reproductive phase, while those in chromosome 3A did the opposite. This proves that quantifying in detail the phenological differences between NILs of particular *Eps* genes it might be possible to fine-tuning not only the time to anthesis but also the partitioning of time among pre-anthesis phases.

Even though results were not always consistent between growing seasons, in general it seemed that there tended to be a subtle effect of *Eps* genes on the number of fertile florets at anthesis: the NILs with the *Eps*-late alleles did always produce more or similar number of fertile florets per spike, and when this happened, the increased spike fertility was apparent in most spikelets, likely through reducing the rate of floret mortality (the main determinant of floret survival) immediately before flowering. This is in line with most literature in that whenever a treatment increases spike fertility it does so most frequently through floret survival (e.g. Siddique *et al*., 1989; Miralles *et al*., 1998). There are evolutionary reasons for this to be so, as it will be discussed in the last Chapter of the thesis on General Discussion. The mechanism by which the late alleles seemed to have decreased floret mortality was by allowing a slightly longer developmental period of labile florets: whilst these florets (e.g. Floret 4 in central spikelets) developed insufficiently to reach the stage of fertile florets and were induced to a late mortality in NILs with the *Eps*-early allele, when the late allele was introgressed that late mortality of labile florets was prevented. In many cases the same effect on extending the developmental phase of particular floret primordia was also seen in more distal positions which would have never reach the stage of fertile florets, suggesting that the effect of floret development is generic (not linked to particular florets).

Thus, as the bibliography reported weak effect of the *Eps* genes on the developmental phases length (Bullrich *et al*., 2002; Slafer *et al*., 2015) it was almost expected to find only slight, and not strongly consistent, differences in detailed traits analyzed in this

Chapter. The outcome is that *Eps* genes still might be useful to fine-tune time to flowering and it might also be helpful for slightly manipulating in spike fertility.
It has been shown that the effects of *Eps* genes may also depend on the temperature (Appendino and Slafer, 2003) and the results from this work may support that finding as the inconsistency found between growing seasons would be related to changes in temperatures. Therefore, experiments which included selected *Eps* NILs across different temperatures were set to find out a possible interaction with temperature on developmental processes. These experiments are presented in the following Chapter VI.

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6. Annex 5: Supplementary data

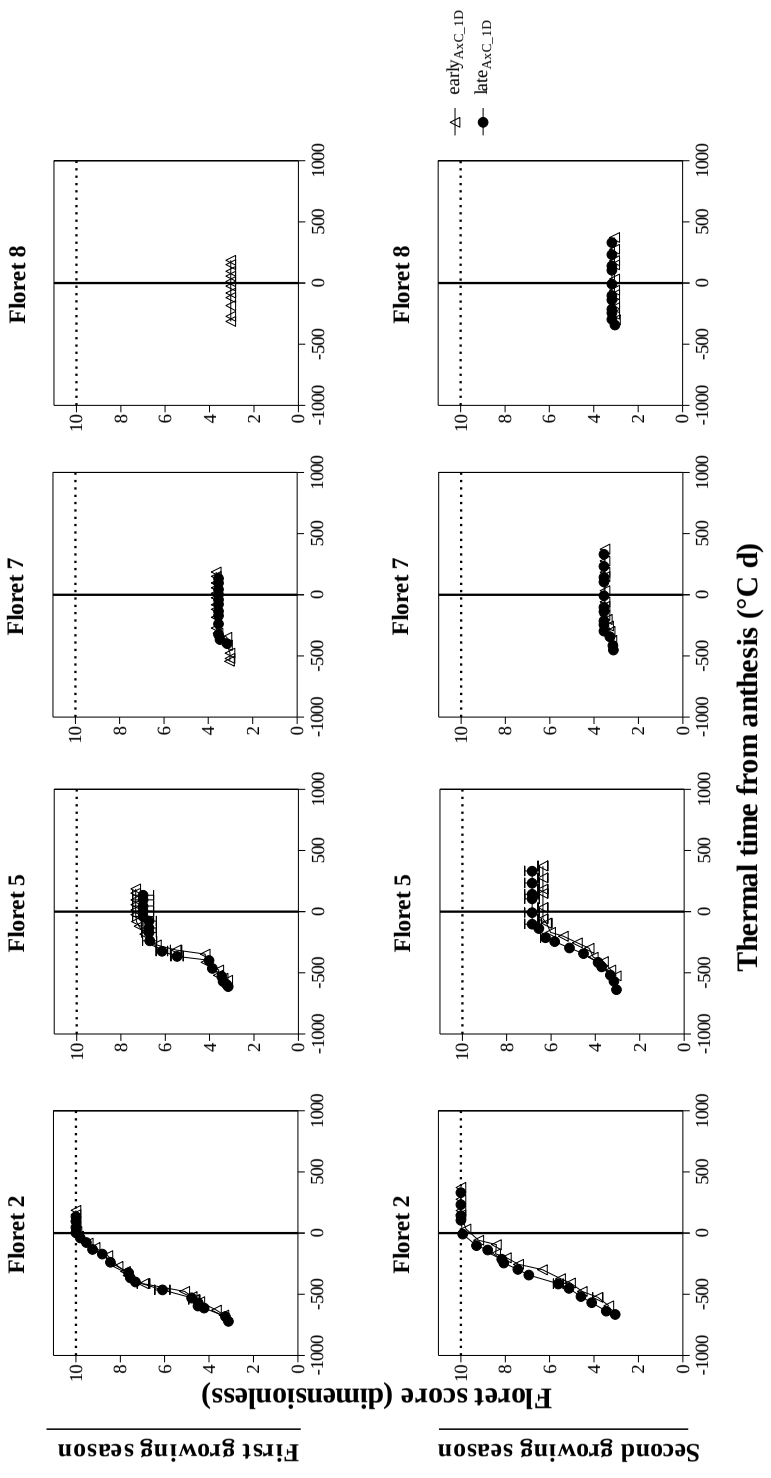

Figure A5.1. Dynamics of the floret development of F2, F5, F7 and F8 in central spikelets of the main-shoot through thermal time from anthesis in the *Eps* NILs carrying either the *Eps* late (closed circles) or the early allele (open triangles) from the AxC in 1D during the first (*top panels*) and the second growing seasons (*bottom panels*). Each data-point is the average of all replicates and within each replicate the value was the average of 3 plants, bars stands for the standard error of the means.

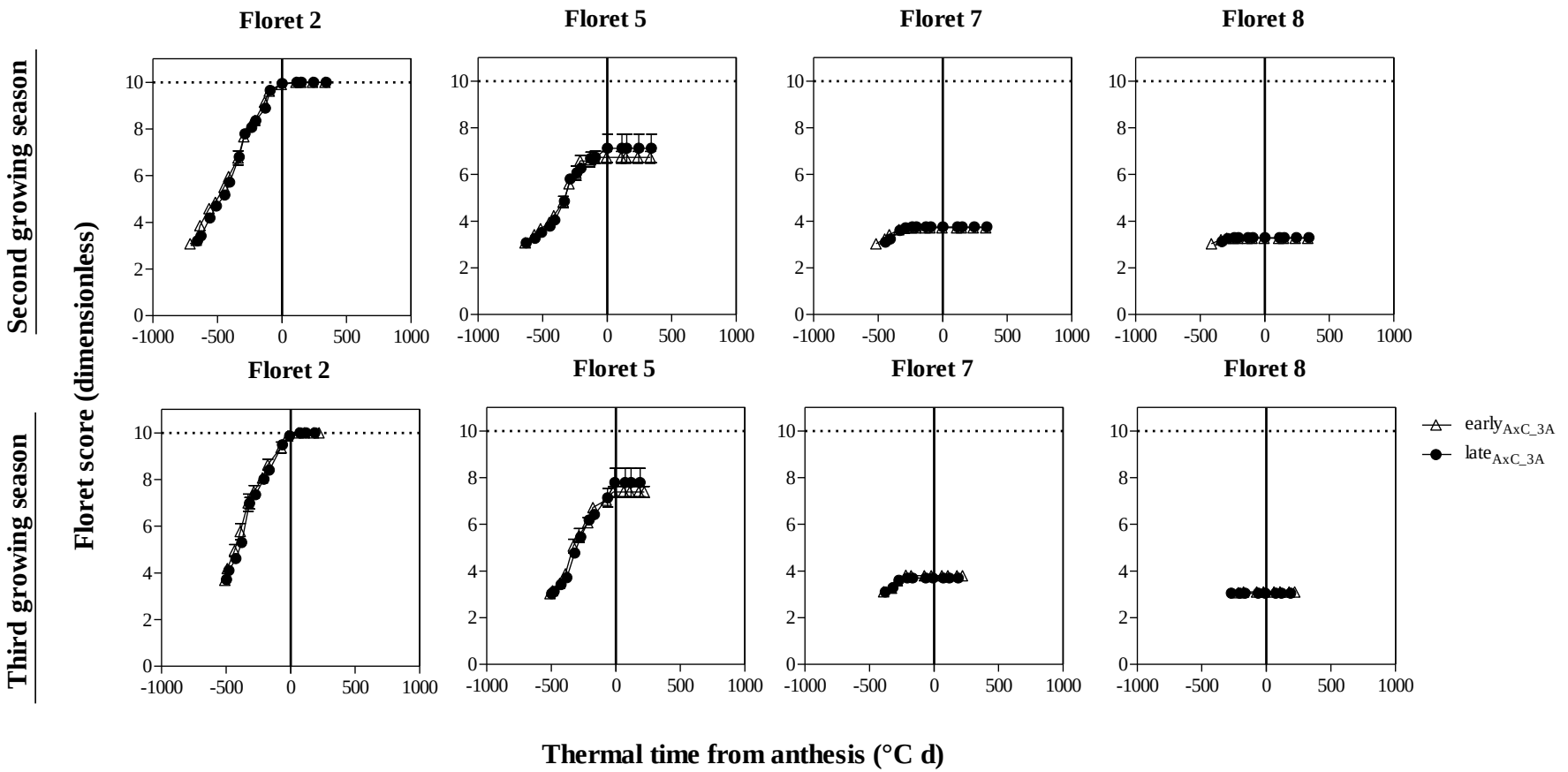

Figure A5.2. Dynamics of the floret development of F2, F5, F6, F7 and F8 in central spikelets of the main-shoot through thermal time from anthesis in the *Eps* NILs carrying either the *Eps* late (closed circles) or the early allele (open triangles) from the AxC in 3A during the first (*top panels*) and the second growing seasons (*bottom panels*). Each data-point is the average of all replicates and within each replicate the value was the average of 3 plants, bars stands for the standard error of the means.

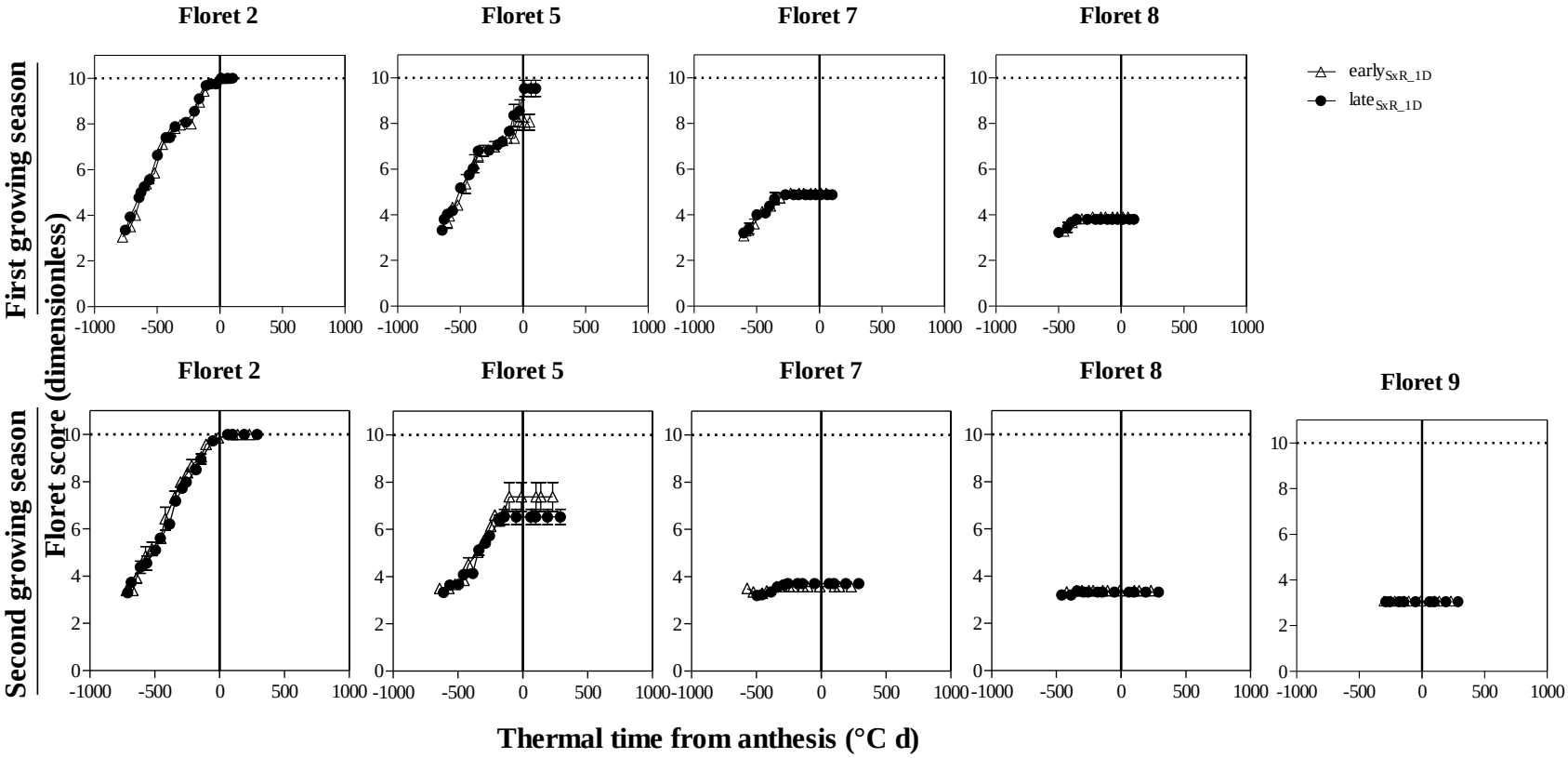

Figure A5.3. Dynamics of the floret development of F2, F5, F7, F8 and F9 in central spikelets of the main-shoot through thermal time from anthesis in the *Eps* NILs carrying either the *Eps* late (closed circles) or the early allele (open triangles) from the SxR in 1D during the first (*top panels*) and the second growing seasons (*bottom panels*). Each data-point is the average of all replicates and within each replicate the value was the average of 3 plants, bars stands for the standard error of the means.

Chapter VI: Physiological determinants of fertile florets as affected by Eps genes x temperature

1. Introduction

Wheat adaptability to a wide range of environmental conditions becomes necessary in front the variable scenarios due to climate change effects. Major genes controlling adaptability involve *Ppd*, *Vrn* and *Eps*. The latter, normally with smaller effects, are quite relevant in regions where the crop has been already reasonably well adapted as they allow for fine-tuning adaptation (Slafer, 2012; Gomez *et al*., 2014). Early flowering was reported in *Triticum aestivum* by Zikhali *et al*., (2015) due to a deletion of the chromosomal region including ELF3 linked to the earliness *per se* locus *Eps*-D1. In addition, the *Eps-A^{m}1* allele from cultivated *T. monococcum* ssp. *Monococcum*, colinear to *Eps*-D1 (Alvarez *et al*., 2016 and references quoted therein), was associated with delayed heading time and increased number of spikelets per spike relative to the allele from wild *T. monococcum* ssp. (Lewis e*t al*., 2008)

Even though the trait is expected to produce an effect in wheat (and other crops) phenology independent of the environment (and that is why it is named "*per se*"), the effect of this locus was larger when plants were grown at 16 than at 23°C suggesting a role of temperature on the modulation of the effects of this gene (Bullrich *et al*., 2002; Appendino and Slafer 2003; Lewis *et al*., 2008), which may be not only quantitative but also qualitative (changing the ranking of earliness depending on the range of growing temperatures explored; Slafer, 1996). Moreover, the *Eps-3A^m* loci were also reported to determine the number of spikelets and regulate flowering time, responses whose magnitude depended on growing temperature (Gawroński *et al*., 2014).

The dynamics of floret generation/degeneration and consequently on the number of fertile florets are the critical processes between the determination of spikelet number and grains per spike and, to the best of my knowledge, the interaction of these genes with the growing temperature on floret number determination has not been studied yet.

Results previously described in Chapter V revealed the importance of few *Eps* gene to fine-tuning flowering time with effects on floret fertility that were small but relevant. Some differences in responses between two different growing seasons highlighted the more than likely dependence of these *Eps* genes on environmental conditions to determine their effects (quantitatively and qualitatively) on the determination of fertile florets. As these effects are expected to produce a difference between genotypes after requirements of vernalisation were satisfied and photoperiod was long, the main environmental factor likely responsible for the interaction shown with the growing season (Chapter V) is the temperature.

The main aim of this Chapter was to analyse the effect of the *Eps* genes across a wide range of temperatures on the number of fertile florets at anthesis as result of the effects on the floret development patterns. For this purpose I conducted a series of controlled-conditions experiments with vernalised plants under long photoperiod with selected NILs for *Eps* under contrasting temperatures.

2. Materials and methods

2.1 General description

Different experiments were carried out under controlled conditions in order to test the effect of *Eps* genes at a wide range of temperatures. These experiments were carried out in chambers (GER-1400 ESP, Radiber, S.A) at the University of Lleida (UdL) and in controlled environment room (CER) at the John Innes Centre (JIC).
Before starting the experiments, seedlings were subjected to a vernalisation pre-treatment (to minimise any interference from vernalisation responsiveness). Vernalisation was carried out in a cool room with the seeds directly germinating in the pots used in the experiment (i.e. there was no transplanting of seedlings to pots after the vernalisation pre-treatment avoiding any stress from transplanting plants, but simply a transfer of pots from the vernalisation room to the growth chamber). Seeds were sown directly in pots (200 or 400 cm^3, at the UdL and JIC, respectively) filled with a mixture of 30% peat and 70% soil (UdL) or a cereal mix compost (JIC) at a rate of a single seed per pot. All experiments were fertilised (N, P, K) and irrigated so that there were no nutrient or water limitations to growth. We sowed pots in excess (50-100% more pots than those required for the experiment, see below) in order to be able to select the required number of pots of each NIL for each temperature condition that were equally developed at the onset of the experiment (after 49 days of vernalisation pre-treatment). Pots were sown at a 1 cm depth, irrigated to warrant seed imbibition and left one day at room temperature to trigger the germination process. Then all pots were moved to a cool room at 4°C in dark conditions and maintained in that room during 49 days to satisfy vernalisation requirements.
In all the experiments (both sites and all temperatures) photoperiod was set under long conditions (18 h). Radiation was 110 μmol m^{-2} s^{-1} of photosynthetically active radiation at the plant level in the UdL chambers, whilst radiation was almost thrice that value in chambers at JIC. Within each chamber all pots were rotated once a week during the whole duration of the experiments and when samples were taken (see below) pots were re-arranged to maintain the structure “close to a canopy” throughout.
In both locations plants grew normally within the chambers (Fig. 6.1).

2.2 Treatments

Treatments consisted of the factorial combination of *Eps* NILs and temperatures. The *Eps* NILs selected resulted from the cross of Spark and Rialto (chromosome 1D) (Table 6.1). I selected two pairs of NILs (4 genotypes) for the experiment but as the differences of the two pairs were only their source of seed, all differences were only ascribed to the effect of that of the specific *Eps* gene, the effects were analyzed as the average in the variables between the 2 lines.

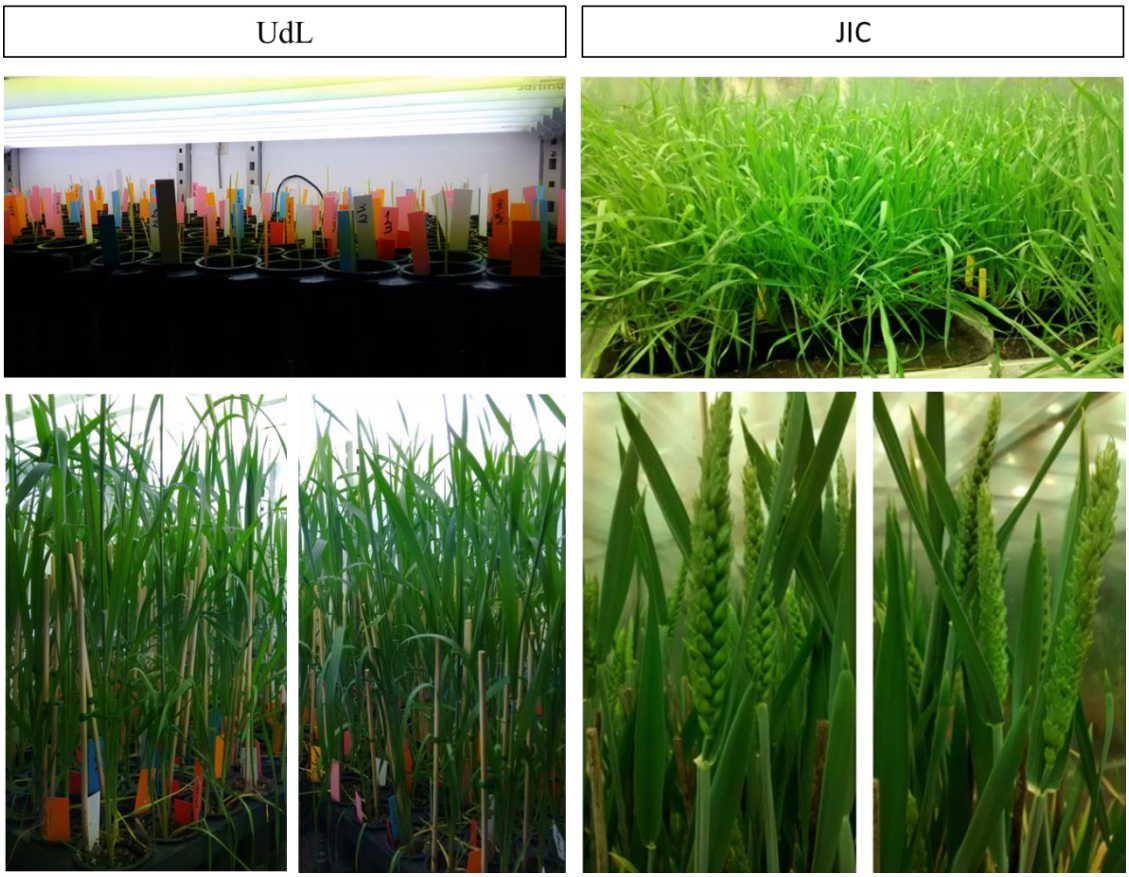

Figure 6.1. Image of plants growing in growth chambers at both UdL (*left*) and JIC (*right*).

Table 6.1. Plant material used in the experiments consisting of *Eps* NILs resulted from the Spark x Rialto cross differing in in early or late alleles in chromosome 1D.

Parentals	Background	Donor	Allele	Code
Spark x Rialto	Rialto	Rialto	late	$late_{SxR\_1D}$
Spark x Rialto	Rialto	Spark	early	$early_{SxR\_1D}$

The temperatures tested at UdL were 6, 9, 15, 21 and 24ºC and those tested at JIC were 12 and 18ºC.

In the UdL experiments 55 pots per genotype (all in all 220 pots) while in JIC experiments 63 pots per genotype (all in all 252) pots were arranged in a complete randomized design with three replicates at each temperature regime. Therefore, within each chamber, genotypes were arranged in three replicates with 18 (UdL) or 21 (JIC) pots per genotype each. These pots were used for dissection throughout development (see below), leaving always at least 3 pots per replicate for the determination of fertile florets at anthesis.

2.3 Measurements and analysis

From the onset of stem elongation onwards, three plants from each genotype, 1 pot per replicate (3 pots per genotype and 6 pots per *Eps* allele), were sampled frequently (the actual frequency depended on the speed of development exhibited by the plants, in turn

depending on the temperature regimes). Floret development dynamics were followed in basal (the fifth position counting from the base of the spike), in central (exactly the central position in each spike, when even spikelet positions was the central minus one) and apical (one positions below the terminal spikelet) spikelets. The number of floret primordia was counted and the Waddington scale (Waddington *et al*., 1983) was used to determine the stage of development of each primordium analyzed, as explained in Chapter II. At anthesis, at least 3 plants per genotype and replicate (all in all at each temperature regime 9 pots per genotype and 18 pots per *Eps* allele) were sampled. In these plants the number of fertile florets in each spike was counted, plants were separated into stems, leaves and spike and biomass and its partitioning determined.
The results from each experiment (UdL and JIC) were analyzed separately because even though the main developmental background factors (vernalisation, photoperiod) were the same, some other conditions such as radiation and soil were different.
In order to illustrate floret development dynamics some particular florets positions were chosen and presented in this Chapter, the rest of the cases are presented in Annex 6 of the present Thesis.

3. Results

3.1 Abnormalities in development under extreme temperatures

Growing the isogenic lines under long photoperiod and at temperatures of 6, 21 or 24°C resulted in patterns of plant development with different types of abnormalities.
Plants growing under 6°C expectedly showed a slow developmental rate until heading; but the delay from then on was much longer than expected from the developmental rates at intermediate temperatures. Even though the experiment at this lowest temperature was continued for a rather long time (to maximize the likelihood of observing anthesis) and that the external aspect of the spikes seemed morphologically normal, the florets within the spikelets did not show a normal morphology and the anthers lost their color and the florets seemed to be aborted before pollination. In addition, apical spikelets started to lose colour before flowering (Fig. 6.2 A, B).
At 21°C plants showed high senescence levels from early stages of the developmental phases (Fig. 6.2 C) although there was no water limitation affecting growth. Some NILs carrying the *Eps*-early allele reached more advanced phenological stages than those with the *Eps*-late, although none of them presented a normal appearance (Fig. 6.2 D). In the case of the experiment at 24°C the appearance was even worst with higher levels of senescence affecting all the genotypes and when the spikes were removed from the shoots they presented a very abnormal development with low number of spikelets and very different morphology and colour among spikelets positions (Fig. 6.2 E, F).

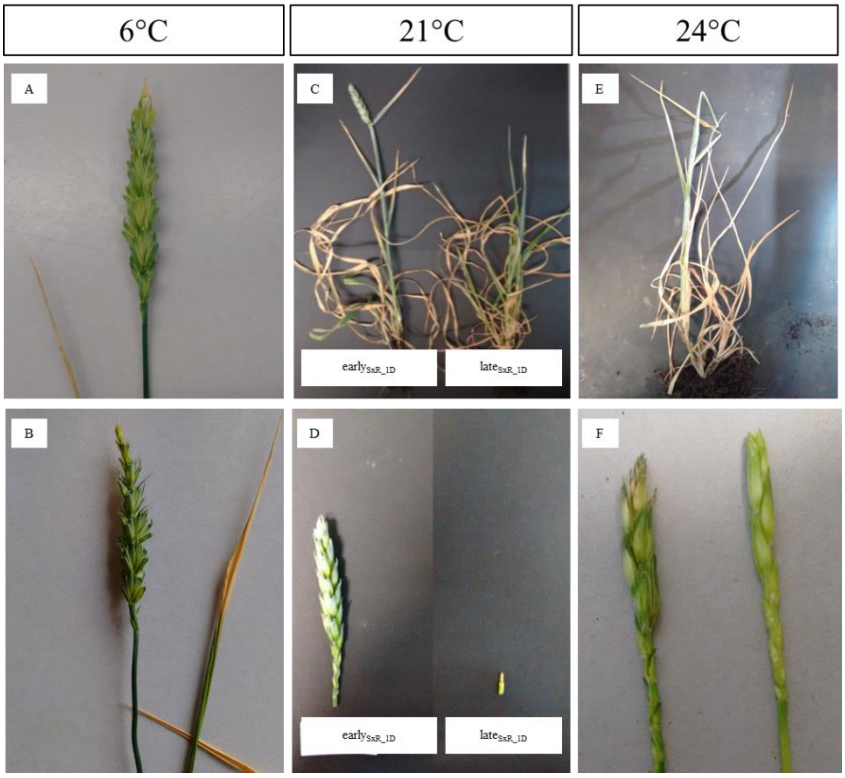

Figure 6.2. External view of the spikes of *Eps* NILs plants growing in growth chambers under 6° C (A, B). External view of *Eps* NILs plants carrying either early or late variant (C) and their spikes/apex view (D) under 21°C. External (E) and spikes view (F) of *Eps* NILs plants growing under 24°C.

Due to the abnormalities in development under the extreme temperatures analyzed in the present study (6, 21 and 24ºC), which always affected the floret developmental phase, the actions of, and interactions between, *Eps* alleles and temperatures on fertile florets and their determinants will be focused in the intermediate thermal regimes (9-18ºC) which did not impair normal development until flowering and the establishment of a particular level of spike fertility.

3.2 Number of fertile florets at anthesis

In the experiments carried out in both locations an interaction of *Eps* allele and temperature was, at least, apparent. In the UdL, the difference in number of fertile florets between NILs was clearer at 9 than at 15°C (Fig. 6.3). In JIC the number of fertile florets per plants was virtually the same for both NILs when growing at 12°C (Fig. 6.4 left panels), while NILs differed significantly in fertile florets per plant when growing at 18°C (Fig. 6.4, top-right panel) with large effect on tiller spikes (Fig. 6.4, bottom-right panel). But, rather relevantly, the nature of the interactions in both locations was opposite: while in UdL the *Eps*-early alleles tended to have more fertile florets than the NILs with the *Eps*-late alleles at the lowest temperature, in JIC the *Eps*-late alleles induced an increase in number of fertile florets at the highest temperature (Figs. 6.3 and 6.4).

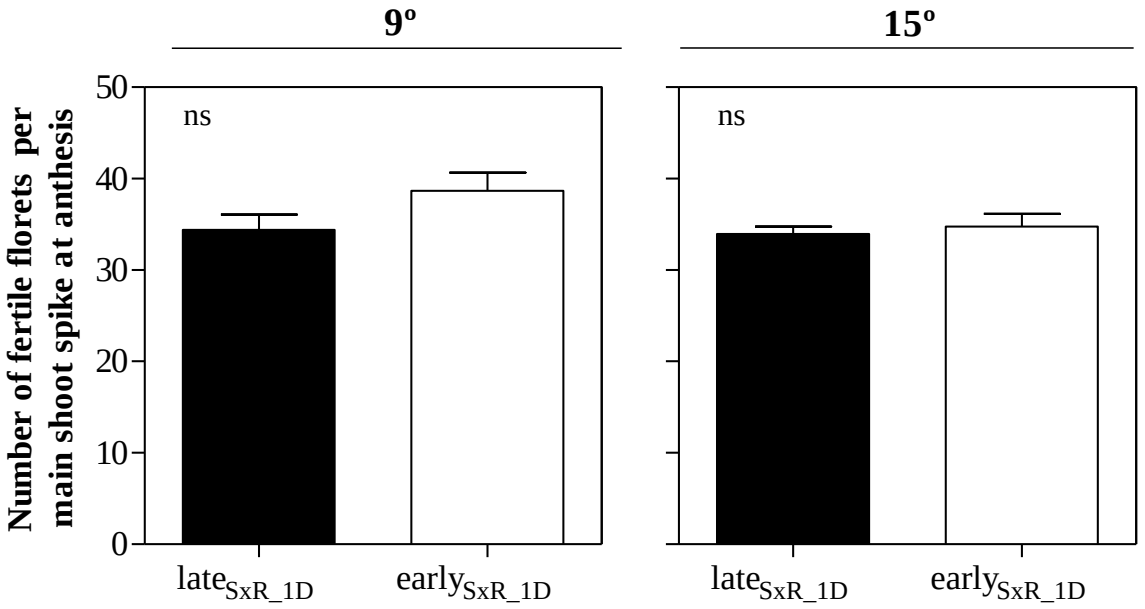

Figure 6.3. Number of fertile florets per main-shoot spike at anthesis between the *Eps* NILs carrying either the late or early variant at 9ºC (left panel) and 15ºC (right panel). Error bars stand for the standard error of the means. P-value resulted from t-test is shown inside each panel. The level of significance (p-value) is indicated inside each panel.

Indeed, if I considered the four temperatures together a more clear evidence of the temperature effect on the impact of this *Eps* gene on the final outcome of floret development emerges. As the background growing conditions (beyond photoperiod and pre-vernalisation treatments that were uniform in both locations) were different I cannot compare fertile florets across locations but I can analyze the effect of *Eps* alleles (differences between contrasting NILs) across the conditions. I have done so by calculating the difference in number of fertile florets between NILs carrying the late *Eps* allele and those carrying the early allele, both in absolute (fertile florets) and relative (proportion) terms, against the growing temperature (Fig. 6.5). The impact of the growing temperature on the effect of the *Eps* alleles on the number of fertile florets was noticeable and not only in quantitative but also in qualitative terms. Quantitatively, it was clear a positive trend, largely linear, of the difference with the growing temperatures (i.e. the higher the temperature condition of growth the larger the difference in favor of the NILs with the late *Eps* alleles; Fig. 6.5). The qualitative impact of temperature on the effects of *Eps* alleles on fertile florets is evidenced by the fact that the relationship explored both positive and negative values of the difference in fertile florets (Fig. 6.5); when negative -at the lowest temperature analyzed- it implies that, compared with the NILs carrying the *Eps*-early alleles, the *Eps*-late alleles reduced the number of fertile florets and when positive -at the highest temperature analyzed- it reflects that these alleles increased the number of fertile florets per spike and per plant. At the intermediate temperatures the differences were negligible (Fig. 6.5).

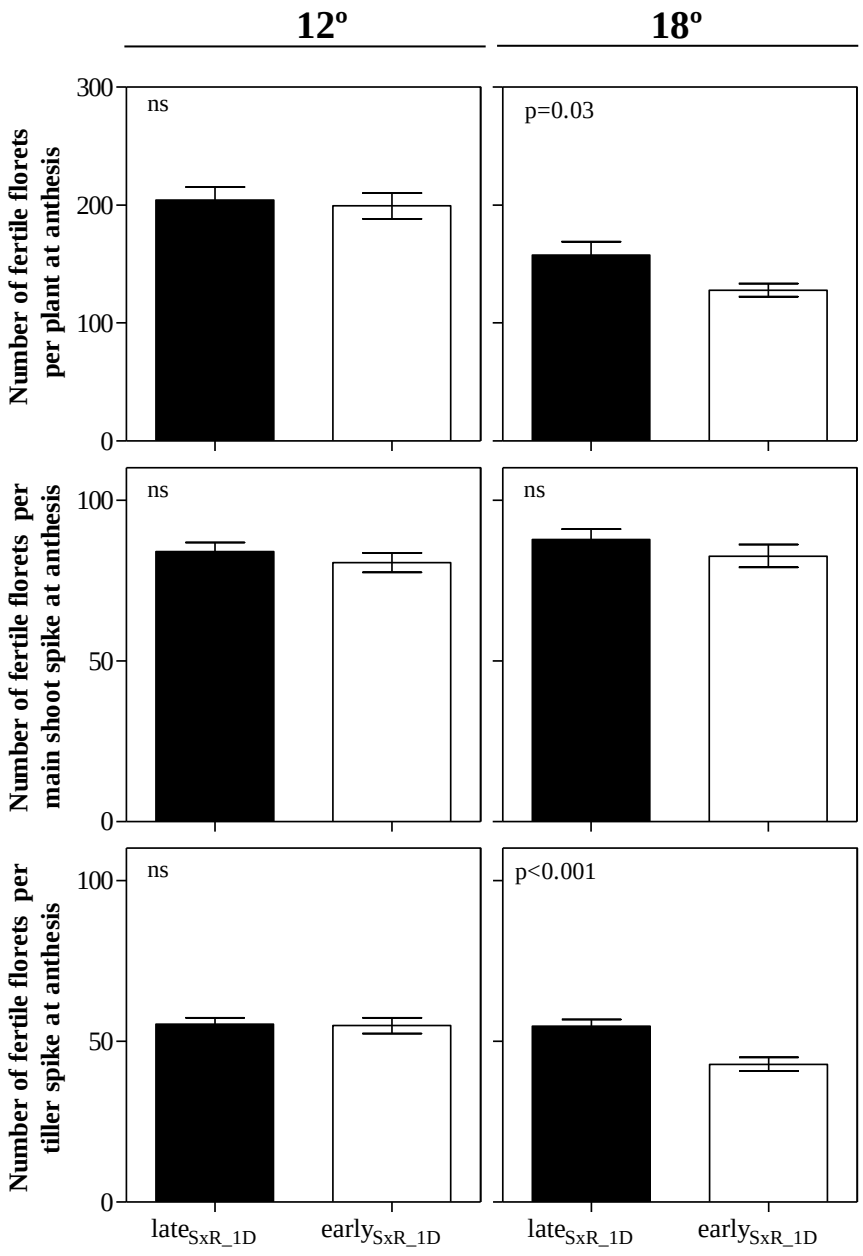

Figure 6.4. Number of fertile florets per plant (top panels), main-shoot spike (middle panels) and per tiller spike (bottom panels) at anthesis between the *Eps* NILs carrying either the late or early variant at 12ºC (left panels) and 18ºC (right panels). Error bars stand for the standard error of the means. P-value resulted from t-test is shown inside each panel. The level of significance (p-value) is indicated inside each panel.

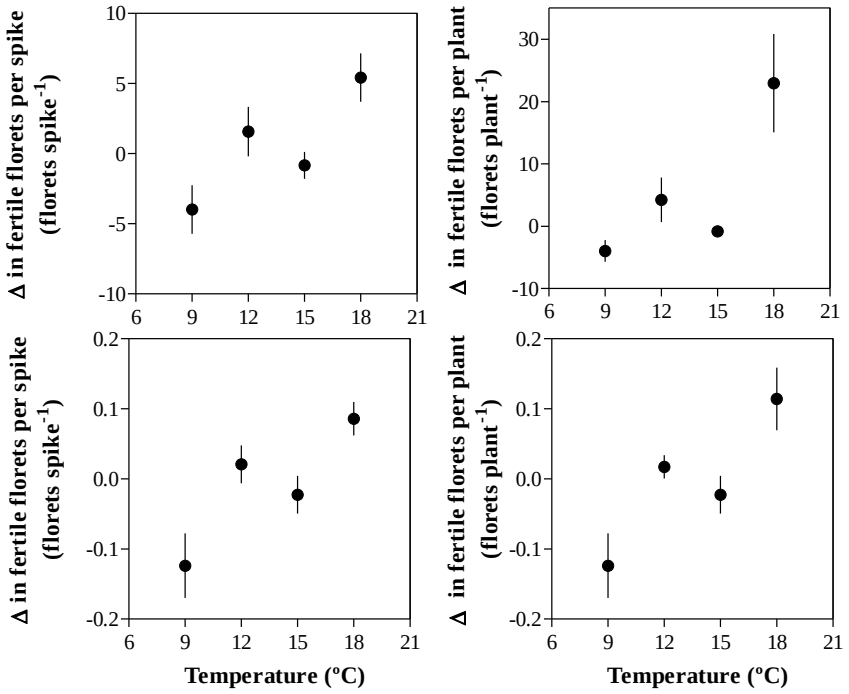

Figure 6.5. Differences in the number of fertile florets per spike (*left panels*) and per plant (*right panels*) at anthesis between the NILs carrying the *Eps*-late and the *Eps*-early plotted against the thermal regime of the experiment within the range of temperatures in which abnormal developmental processes were not observed; both in absolute (*top panels*) and relative values (*bottom panels*). Each data-point is the average of all the plants of all replicates (18) and the segment in each datapoint stands for the standard error of the means.

3.3 Mapping fertile florets

In the experiments carried out at the University of Lleida, the trend to increase the number of fertile florets per spike due to the action of the *Eps*-early alleles at the lowest temperature (9°C) was clear in the bottom half of the spike, in which the difference was significant in a number of spikelets (Fig. 6.6, left panel). At 15ºC, where the differences in florets per spike were negligible, there was no clear differences between NILs with *Eps*-late and -early alleles at any of the spikelets (Fig. 6.6, left panel), though there seemed to have been a slight trend for the NILs with the late allele to increase fertile florets in the bottom half of the spike and *vice-versa* in the top half. Regarding the experiments conducted at the John Innes Centre, although the differences between contrasting NILs at 12ºC were negligible in florets per spike, the NILs with *Eps*-late alleles tended to show more fertile florets in many spikelets than the NILs with *Eps*-early alleles, particularly in tiller spikes and in the apical positions of main-shoot spikes (Fig. 6.7, left panels). When grown under warmer conditions (18°C) differences in favor of the NILs with late alleles were more consistent and larger, particularly in the tiller spikes (Fig. 6.7, right panels).

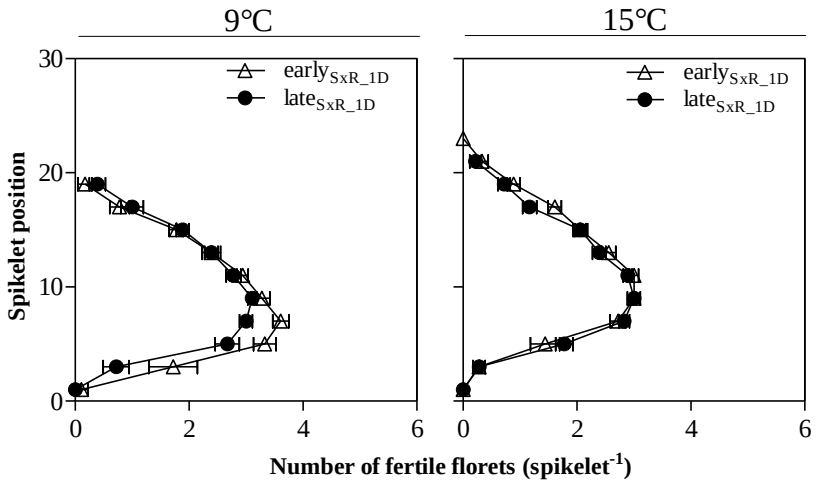

Figure 6.6. Number of fertile florets in each spikelet position on the main-shoot spike at 9ºC (left panel) and 15ºC (right panel) between *Eps* NILs carrying the late (closed circles) or early variant (open triangles). Each data-point is the average of all the plants of all replicates (18) and the segment in each datapoint stands for the standard error of the means.

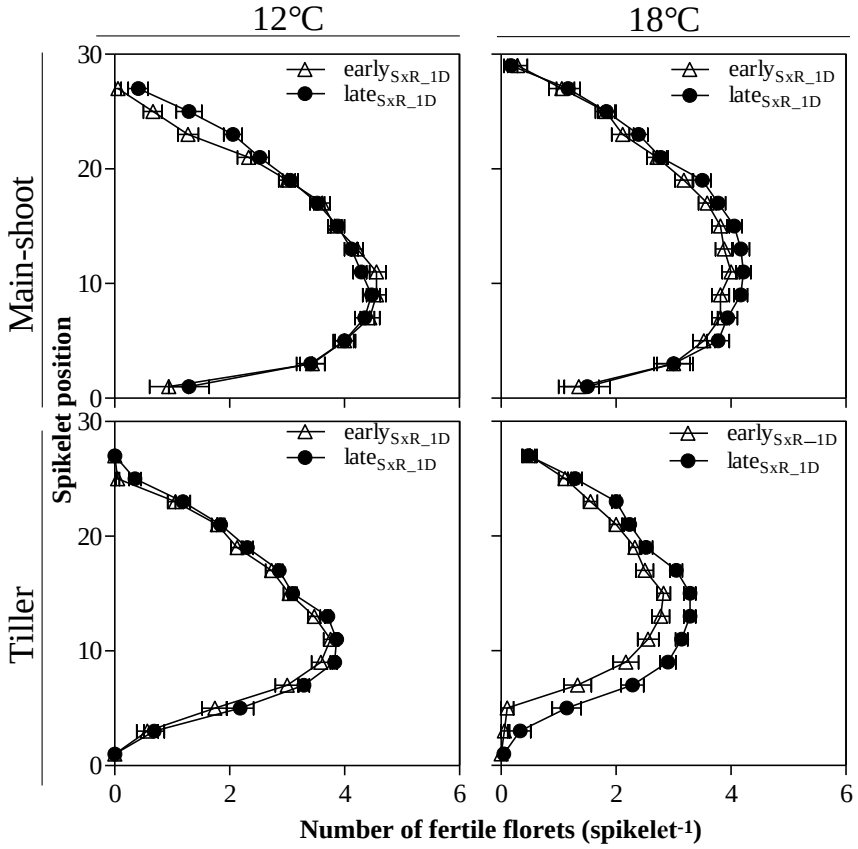

Figure 6.7. Number of fertile florets in each spikelet position on the main-shoot spike (top panels) and tiller spike (bottom panels) at 12ºC (left panels) and 18ºC (right panels) between *Eps* NILs carrying the late (closed circles) or early variant (open triangles). Each data-point is the average of all the plants of all replicates (18) and the segment in each datapoint stands for the standard error of the means.

3.4 Dynamics of development of individual floret primordia and of living florets

At 9°C floret development dynamics between NILs of the different florets (F1, F3, F4) and from different spikelet positions (apical, central and basal) did not show noticeable differences (Fig. 6.8A and Fig. A6.1) except of F1 in the apical positions which was fertile in all the plants with the *Eps*-late allele and only in the 78% of the those with the *Eps*-early allele (Fig. 6.8A, top-left panel); and the F3 in the basal position which was always fertile in the early type but only in a 67% in the late ones (Fig. 6.8A, bottom-middle panel). In addition, the *Eps*-early lines tended to present advanced stages of development of F4: in central spikelets 25% of the plants reached the fertile stage while this occurred only the 8% of the plants with *Eps*-late; in the basal positions 33% of the plants of the early variant reached the fertile stage while none of the late variant reached that stage (Fig. 6.8A middle, bottom-left panels). Moreover, in any spikelet position the length of the floret development phase, the maximum number of floret primordia developed and the final number at anthesis differed between NILs (Fig. 6.8B) despite the difference in the basal spikelets in which seemed that the *Eps*-early presented higher number of fertile floret at anthesis than the late one (Fig. 6.8B, bottom panel) which was consistent with the slight difference seen in the mapping in these positions. At 15°C almost no differences were seen on the floret development between the *Eps*-early and late (Fig. 6.9A and A6.2) with a slightly difference in the F3 in the central positions which was always fertile in the early variant and in the 91% of the plants in the late variant (Fig. 6.9A, middle-middle panel) and in the F2 in the basal position (A6.2) the 75% of the late variant reached the fertile stage while only the 46% of the early one. No differences were clear on the number of living floret primordia dynamics at 15°C (Fig. 6.9B), without differences in the length of the floret development phase or in the maximum and final floret number.

At 12°C differences were seen only in the F1 in the apical positions which was always fertile in the late variant while only in the 67% of the early plants (Fig. 6.10A top-left panel) and even more the 25% of the plants of the late variant presented the F2 fertile (A6.3, top-left panel) which was reflected in the final number of floret primordia dynamics (6.10B, top panel) despite no other major differences were seen in either of the parameters of the floret dynamics in any of the three spikelet positions analyzed (6.10 A, B and A6.3). At 18°C main differences between NILs carrying either the *Eps*-late or early alleles were in the central positions where F4 of all the late plants reached the fertile stage (and even 8% of the plants presented a F5 fertile as well, Fig A6.4) while only the 85% of the plants with the early variant presented F4 reaching the fertile stage (Fig. 6.11A, middle-left panel). In addition, in basal spikelets, F4 was fertile in 83% of the late plants while this was so in just 53% of the early ones (Fig. 6.11A, bottom-right, panel). At 18°C the NILs did not differ in the floret development phase length in any of the spikelet positions (Fig. 6.11B). Carrying the *Eps*-late allele developed higher maximum number of floret primordia than the early one in the apical and central positions (Fig. 6.11B, top and middle panels) and tended to present higher final number of fertile florets in all the positions (Fig. 6.11B).

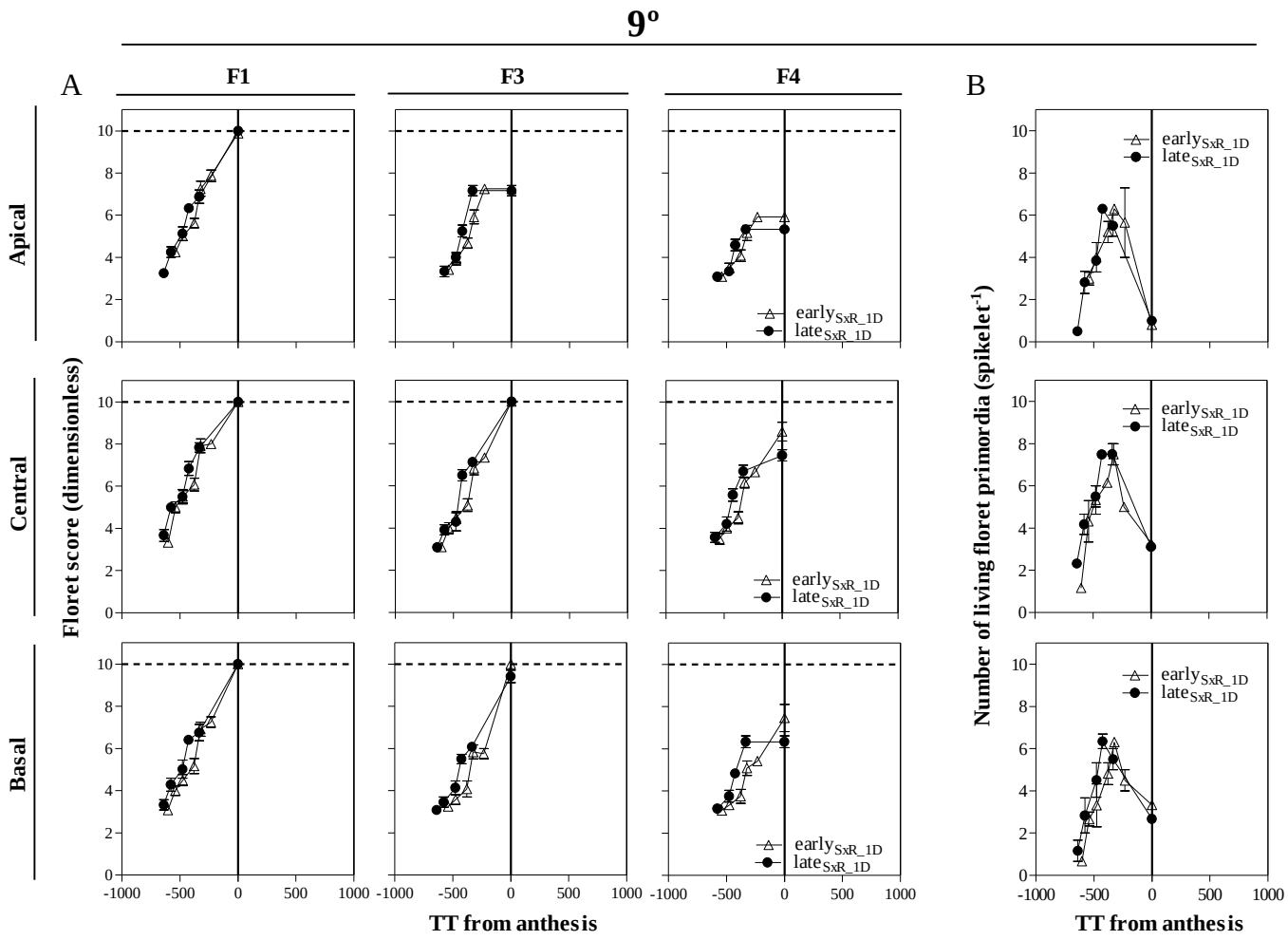

Figure 6.8. Dynamics of the floret development of floret F1, F3 and F4 (A) and the number of living floret primordia (B) through thermal time from anthesis in the apical (top panels), central (middle panels) and basal (bottom panels) spikelets between NILs carrying either the *Eps*-late (closed circles) or early variant (open triangles) growing at 9º. Each data-point is the average of 2 plants per 3 replicates and the segment in each datapoint stands for the standard error of the means.

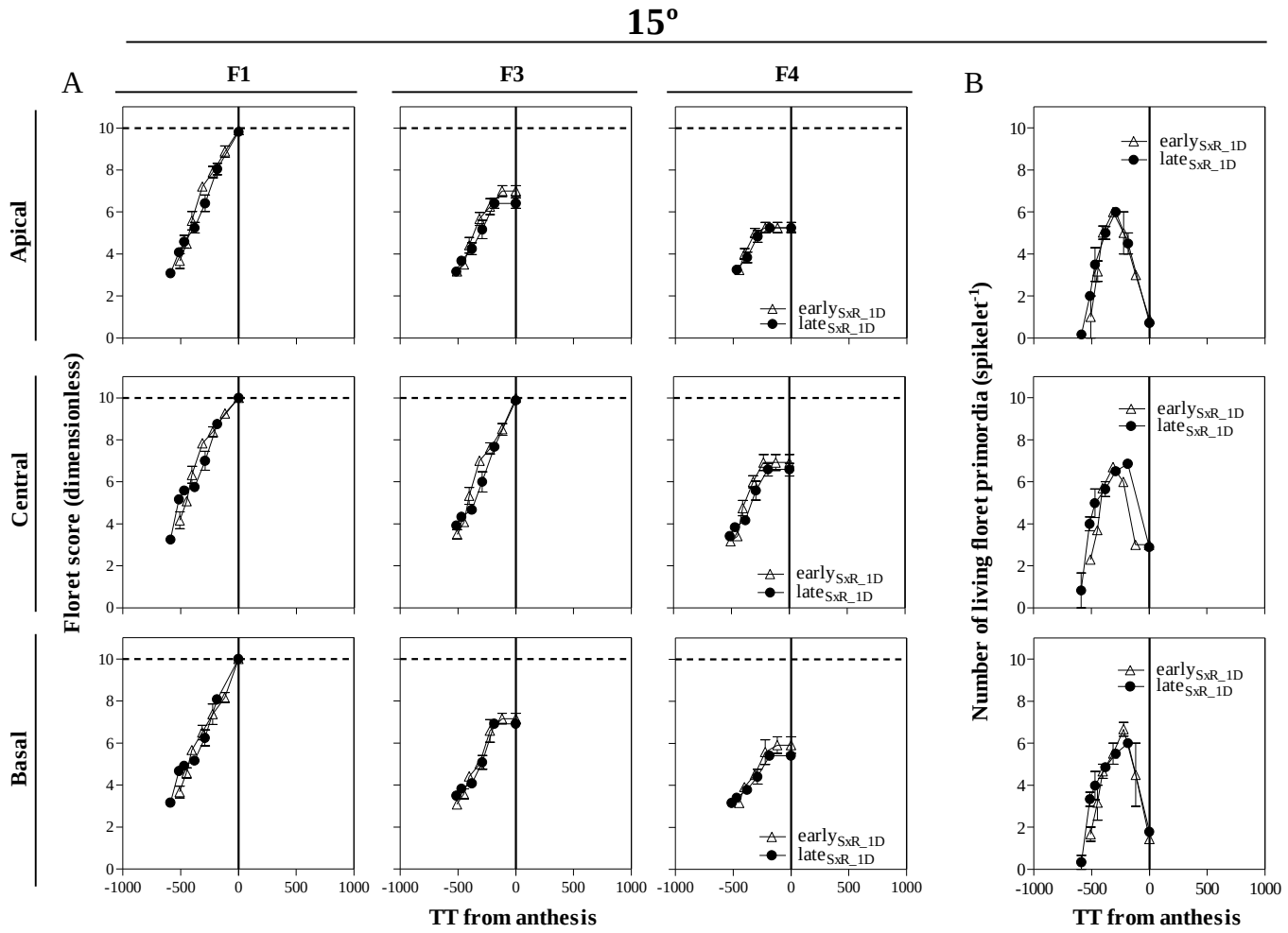

Figure 6.9. Dynamics of the floret development of floret F1, F3 and F4 (A) and the number of living floret primordia (B) through thermal time from anthesis in the apical (top panels), central (middle panels) and basal (bottom panels) spikelets between NILs carrying either the *Eps*-late (closed circles) or early variant (open triangles) growing at 15º. Each data-point is the average of 2 plants per 3 replicates and the segment in each datapoint stands for the standard error of the means.

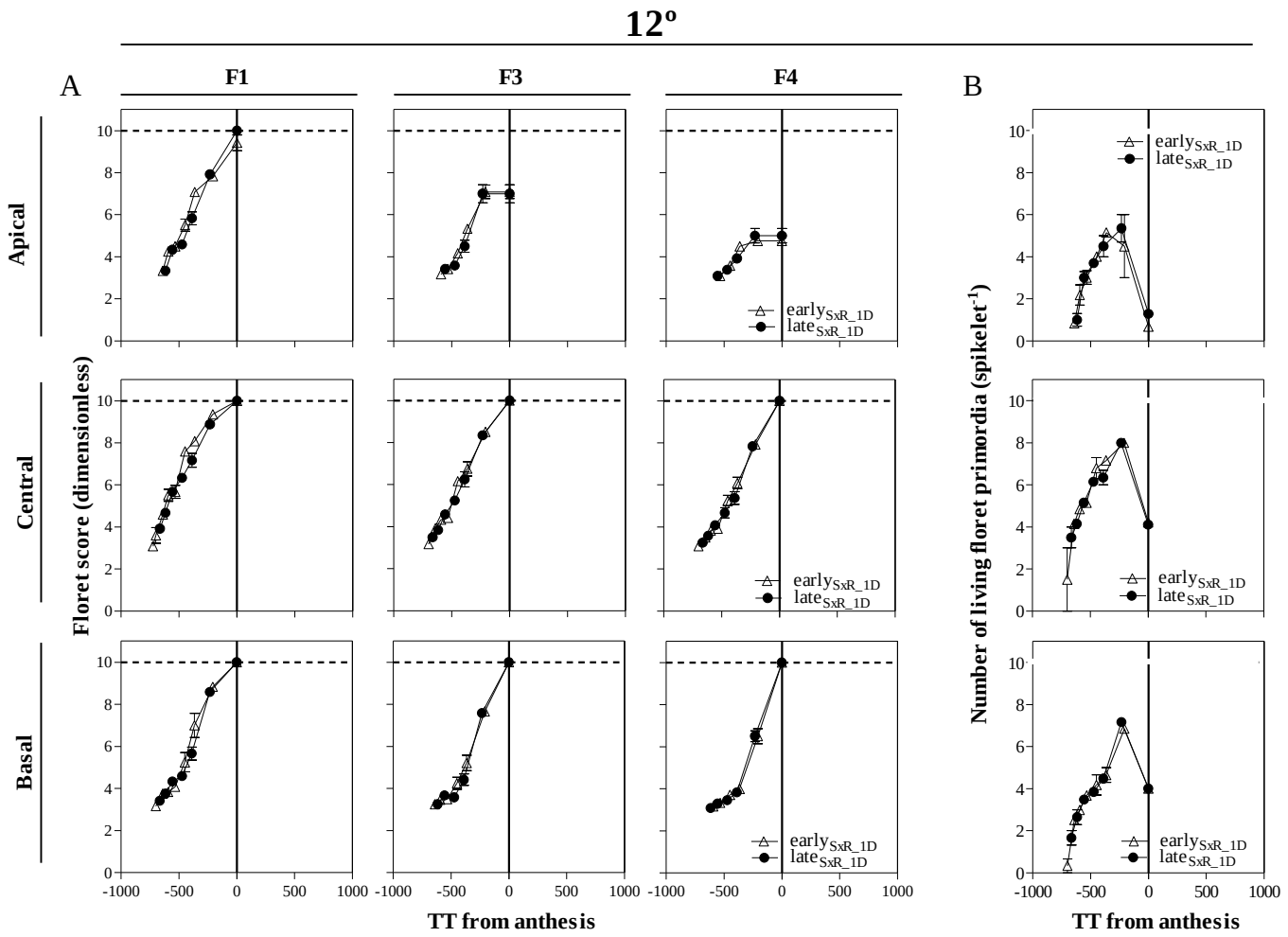

Figure 6.10. Dynamics of the floret development of floret F1, F3 and F4 (A) and the number of living floret primordia (B) through thermal time from anthesis in the apical (top panels), central (middle panels) and basal (bottom panels) spikelets between NILs carrying either the *Eps*-late (closed circles) or early variant (open triangles) growing at 12º. Each data-point is the average of 2 plants per 3 replicates and the segment in each datapoint stands for the standard error of the means.

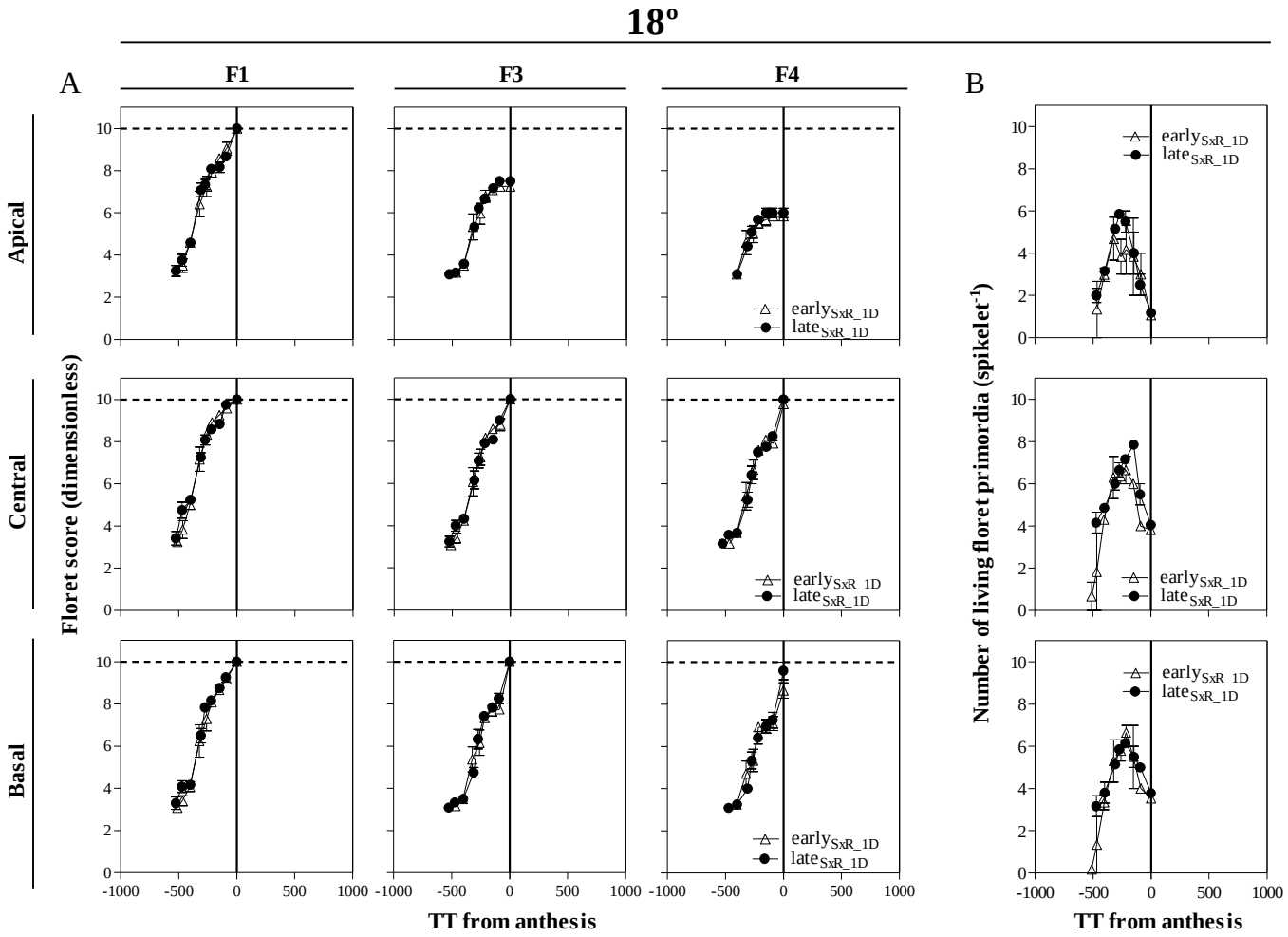

Figure 6.11. Dynamics of the floret development of floret F1, F3 and F4 (A) and the number of living floret primordia (B) through thermal time from anthesis in the apical (top panels), central (middle panels) and basal (bottom panels) spikelets between NILs carrying either the *Eps*-late (closed circles) or early variant (open triangles) growing at 18º. Each data-point is the average of 2 plants per 3 replicates and the segment in each datapoint stands for the standard error of the means.

Floret development was followed in the main-shoot spikes; however, as it was seen in the spike mapping, important differences were seen at 18°C in the tillers spikes. Even we did not measured on them due to the time-consuming it was shown that they presented lower number of fertile florets than the main-shoots (Fig. 6.4) they should present shorter floret development phase length (due to the own tiller development dynamics) in comparison to what was seen in the main-shoots and a higher differences between the *Eps*-early and late in the final number of fertile florets per spikelet.

3.5 Number of fertile florets and spike dry weight at anthesis

Strong positive relationship was found between the number of fertile florets and the spike dry weight (SDW) at anthesis (Fig. 6.12, left panel); which in turn were related to differences in growth more than in partitioning (Fig. 6.12, right panel).

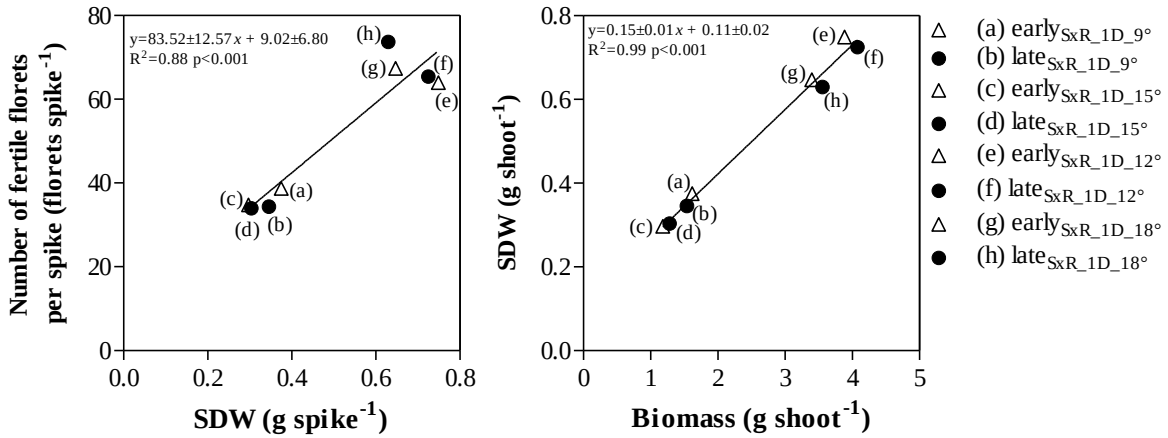

Figure 6.12. Relationship between the number of fertile florets per spike at anthesis and the spike dry weight (SDW, *left panel*) and relationship between the SDW and the biomass (*right* panel) for the NILs carrying either the *Eps*-late or early variant growing at 9, 12, 15 and 18° C. Each data-point is the average of all the plants of all replicates (18). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

However, much of the relationships were driven by the differential growing conditions (mainly between JIC and UdL). Within each of the growth chambers, the difference in spike fertility, when existed, were not well explained by the effect of the alleles on spike dry weight: in fact in the chamber at 9ºC (where the *Eps*-early alleles increased the number of fertile florets) the early lines produced an average of 103.6 fertile florets per gram of spike at anthesis, slightly higher than the 100.9 florets g_{spike}^{-1} of the late lines; whilst in the chamber at 18ºC (in which the difference in favor of the *Eps*-late lines was maximized) the late lines produced a clearly higher average number of fertile florets per gram of spike at anthesis than the of the early lines (123.8 and 113.1 florets g_{spike}^{-1}, respectively).

4. Discussion

Understanding the effect of *Eps* genes x temperature interaction on the setting of the spike fertility level resulted in a complex challenge mainly due to the effects reported of these genes were unsurprisingly weak (Chapter 5), as expected for genes useful for fine-tuning plant development (Griffiths *et al*., 2009). Notwithstanding the risks, we found in line with previous results focused on phasic development (Appendino and Slafer 2003) that the effects of the *Eps* genes analyzed on fertile florets were different depending on the temperature. And remarkably, the impact of the thermal regime of growth seemed even qualitative: depending on the growing temperature the particular *Eps* alleles may have increased or decreased (or unaffected) the number of fertile florets per spike and per plant.

In addition, abnormal development at 6, 21 and 24ºC was seen among the NILs indicating the importance of the temperature and the *Eps* x temperature interaction. The abnormality seen at 6°C was related to the latest developmental phases: development seemed not to continue to the following developmental stage and the flowering did never happen, this may suggest an increment of the base temperature along the plant developmental phases, which is in line with previous reports (e.g. Slafer and Savin, 1991), and the base temperature could be around 6°C near anthesis stage. Regarding to the abnormal development seen at 21 and 24°C it may be due to that these temperatures might have been higher than the optimum temperature for normal development. Moreover, at 21°C different developmental pattern was seen between carrying early or late variant while the early reached advanced stages of development the late one remained in the very early reproductive stages, which may indicate a different optimum temperature for each one of the *Eps* variant.

Main results showed that the effect of carrying either the *Eps*-late or early variant, an important tool to fine-tuning flowering time, depends on the temperature and could be regulating the number of fertile florets through survival mechanisms. The influence of these *Eps* alleles on the survival of fertile florets seemed largely independent to changes in the amount of assimilates partitioned to the spikes. When analyzing rather large genetic effects on biomass partitioning to reproductive structures (and dry matter allocation to the juvenile spikes immediately before anthesis) due to comparisons between old or modern cultivars or tall vs dwarf wheats floret survival at anthesis linked was clearly linked to the proportion of assimilates partitioned to the spikes (Slafer and Andrade, 1993; Miralles *et al*., 1998). In the case analyzed in this study, the genetic effects were much more subtle and the effect, when occurred, seemed to have been more related to the efficiency with which spike dry weight was used to set a particular level of spike fertility. This is commensurate with the differences in spike fertility between elite lines of wheat, where differences are also much weaker than when comparing extremely different materials, in which fruiting efficiency seemed much more relevant than spike dry matter to explain genetic differences in spike fertility (Elia *et al*., 2016).

5. References

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6. Annex 6: Supplementary data

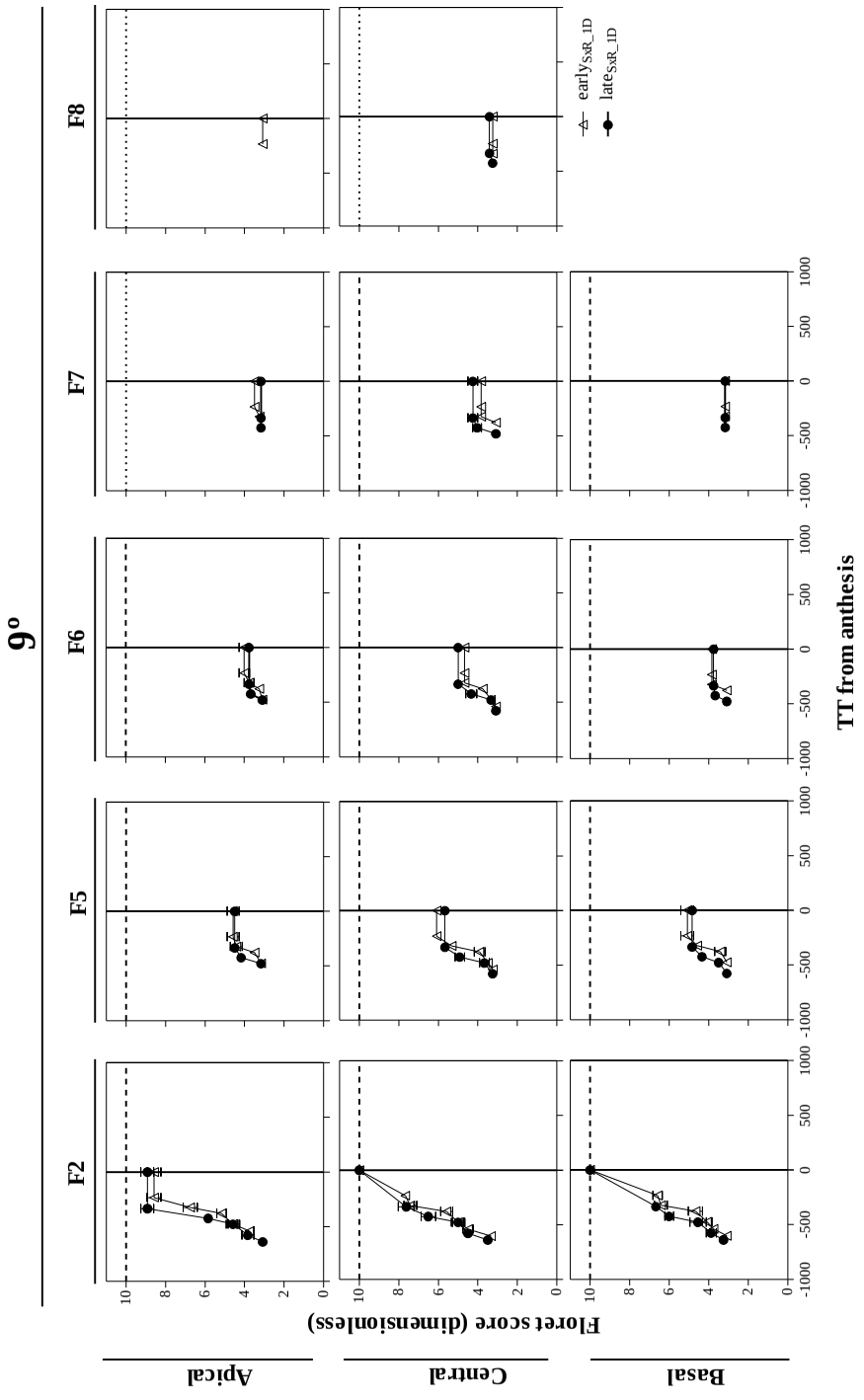

Figure A6.1. Dynamics of the floret development of floret F2, F5, F6, F7 and F8 through thermal time from anthesis in the apical (top panels), central (middle panels) and basal (bottom panels) spikelets between NILs carrying either the *Eps*-late (closed circles) or early variant (open triangles) growing at 9º. Each data-point is the average of 2 plants per 3 replicates and the segment in each datapoint stands for the standard error of the means.

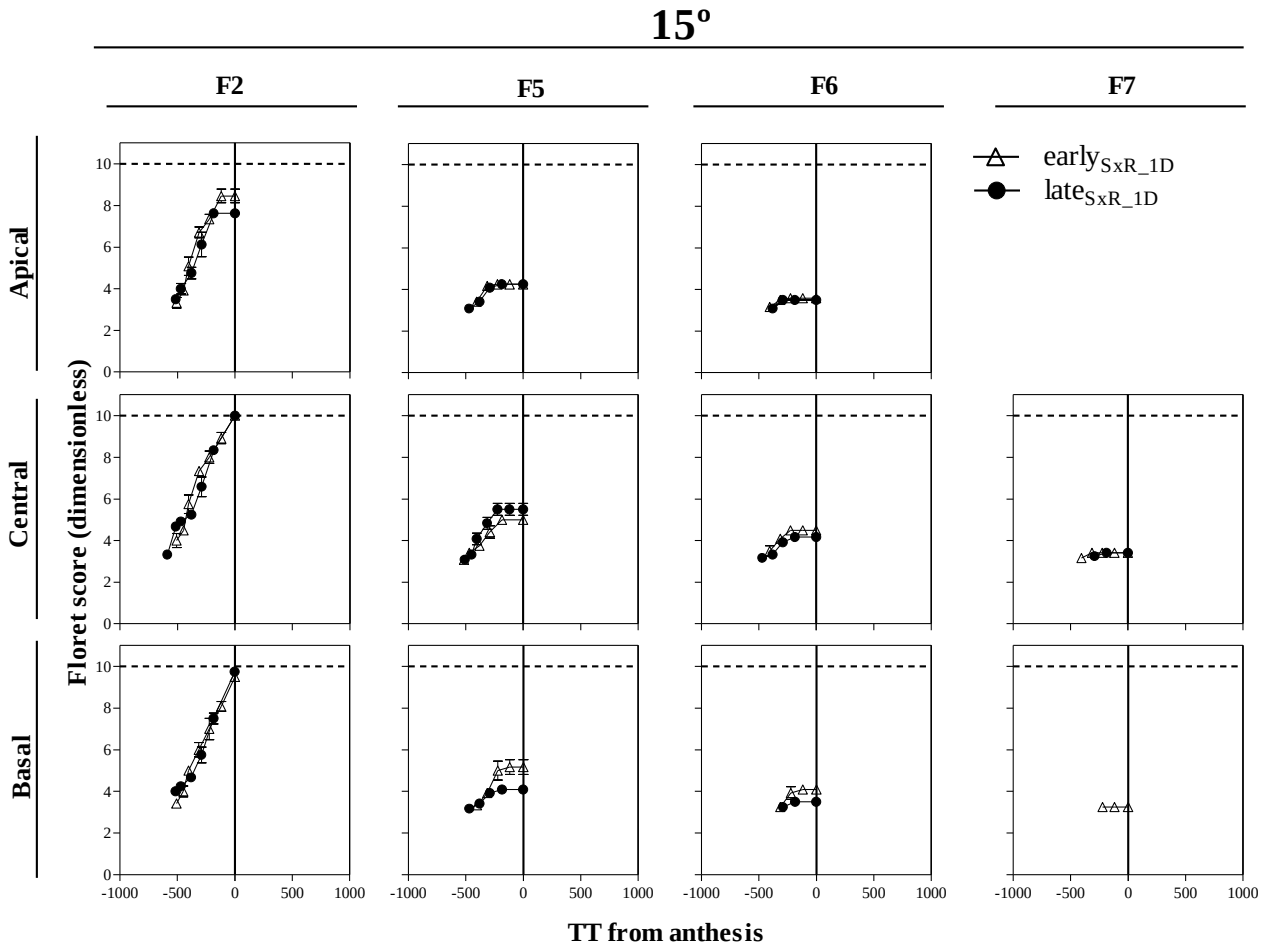

Figure A6.2. Dynamics of the floret development of floret F2, F5, F6 and F7 through thermal time from anthesis in the apical (top panels), central (middle panels) and basal (bottom panels) spikelets between NILs carrying either the *Eps*-late (closed circles) or early variant (open triangles) growing at 15º. Each data-point is the average of 3 plants per replicate, bars stands for the standard error of the means. Each data-point is the average of 2 plants per 3 replicates and the segment in each datapoint stands for the standard error of the means.

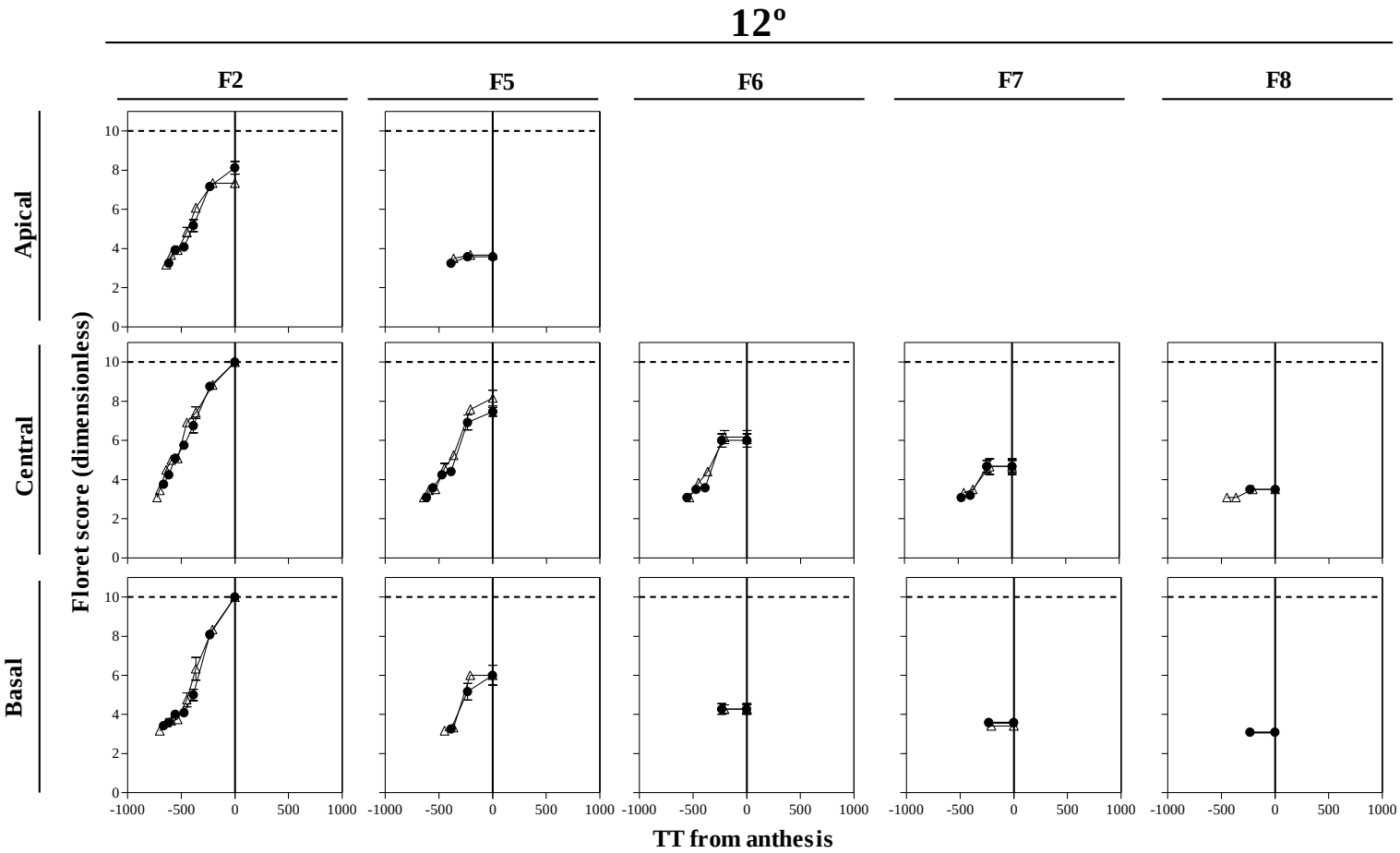

Figure A6.3. Dynamics of the floret development of floret F2, F5, F6, F7 and F8 through thermal time from anthesis in the apical (top panels), central (middle panels) and basal (bottom panels) spikelets between NILs carrying either the *Eps*-late (closed circles) or early variant (open triangles) growing at 12º. Each data-point is the average of 2 plants per 3 replicates and the segment in each datapoint stands for the standard error of the means.

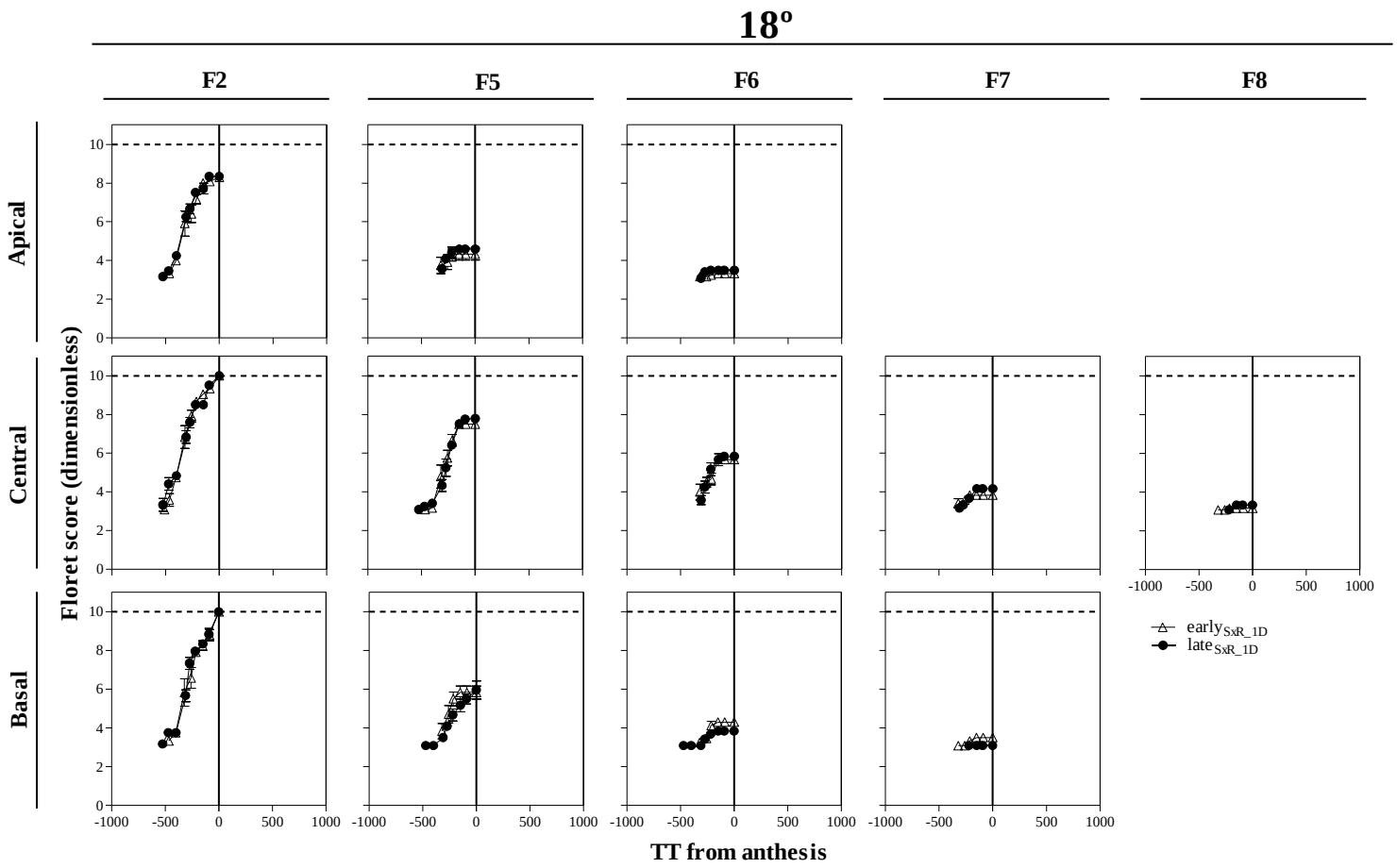

Figure A6.4. Dynamics of the floret development of floret F2, F5, F6, F7 and F8 through thermal time from anthesis in the apical (top panels), central (middle panels) and basal (bottom panels) spikelets between NILs carrying either the *Eps*-late (closed circles) or early variant (open triangles) growing at 18º. Each data-point is the average of 2 plants per 3 replicates and the segment in each datapoint stands for the standard error of the means.

Chapter VII: General discussion

1. Briefly recapping context

Breeders have always tried to fine-tune flowering time to avoid stresses affecting grain yield. The main avenue to achieve this aim is manipulating the developmental controlling genes: *Ppd*, *Eps*, *Vrn*. In order to be able to predict to what degree such changes in development would affect yield we need to understand how these genes affect major yield components. When breeders try to improve yield (or other complex traits) they are reluctant to use wide crosses, to avoid introgressing agronomically poor traits, and normally restrict the crosses to elite material (Rasmusson, 1996), mainly current commercial cultivars. So that they may pyramid well adapted traits. Thus, determining the range of genotypic variation in critical traits within elite material is of paramount importance.

In wheat grain number is related to the number of fertile florets (this is self-evident in cleistogamous plants; but even in self-incompatible species this would be true if pollen availability were not scarce). As grain weight is much more conservative than grain number (which is then far more plastic; Sadras, 2007), to understand how genetic factors related to development may affect yield we need to understand how they affect the generation of fertile florets, as the number of fertile florets determines the number of grains (Kirby, 1988; Slafer and Andrade, 1993; Miralles *et al*., 1998, 2000). Very few works focused on understanding the effects of developmental changes on the floret generation/degeneration process.

The advantage that the number of fertile florets is indeterminate (which is a biological basis for its plasticity) becomes an important tool for breeding. Variability in the number of fertile florets were reported due to both differences in genetic constitution (Miralles *et al*., 1998) and responses to environmental factors, including resources and signals (Sibony and Pinthus, 1988; Ferrante *et al*., 2010; Ugarte *et al*., 2010; Miralles, *et al*., 2000; González, *et al*., 2003*a*; González, *et al*., 2003*b*; González, *et al*., 2005*a*; González *et al*., 2005*b*; Ghiglione *et al*., 2008; Serrago, *et al*., 2008). In all these cases a great plasticity of the major developmental components determining the number of fertile florets was exhibited; commensurate with the large plasticity of grain number (Sadras, 2007; Sadras and Slafer, 2012). These major components are the duration of the phase of floret generation/degeneration, the maximum number of floret priomordia initiated (in response to the floret generation dynamics) and the rate of floret survival (in response to the floret mortality dynamics).

This Thesis was focused on the study of the mechanisms behind the setting of the spike fertility due to the variation in the number of fertile florets at anthesis related to the different floret development dynamics due to genetic variation in wheat. For this purpose, I considered both unspecific sources of variation by comparing modern cultivars (whose characterization would provide relevant information for breeding programs interested in using in their crosses parents with particular dynamics), and specific effects of particular alleles through the exploitation of near isogenic lines for *Ppd* and *Eps* genes (whose characterization would provide relevant information on the effects of these particular genes on the developmental processes determining spike fertility). Furthermore I also analyzed the interaction *Eps* x temperature on floret

development and the consequent number of fertile florets. It has been hypothesized in this Thesis that (i) the length of the floret development phase would be affected by genetic variability among elite genotypes and reduced by the introgression of either *Ppd*-insensitivity or *Eps*-early alleles, (ii) changes in the duration of this phase would bring about parallel changes in the final number of fertile florets at anthesis, due to (iii) affecting the floret primordia generation/degeneration dynamics. In addition, based on the emerging evidences that earliness *per se* would be likely influenced by temperature it was also inferred that the effects the *Eps* alleles on the determination of fertile florets in wheat would be dependent upon the temperature in which plant growth took place.
In this final chapter I avoided repeating the results described in the experimental chapters and after making explicit the singularity of the approach (a major reason for this sort of studies have not been carried out frequently), I have mainly focused in integrating, when possible, some results across chapters, in summarizing the outcomes of testing of the main hypotheses (explicitly mentioned, or implicit in objectives, of previous chapters), in highlighting the contributions to knowledge achieved, and in offering ideas on future research derived from the work presented herein; before finishing with revisiting major conclusions.

2. Singularity of the approach

This Thesis was based on research focused on accurate, though rather low-throughput, detailed analyzes of the main physiological determinants of the fertile florets among elite cultivars and as affected by NILs for *Ppd* and *Eps* genes (and the effects of the latter under a wide range of temperatures). Although there exists many reports using similar populations they focused mainly on effects on phenology and some of them on grain yield components (particularly, number of grains); very few concentrated on the effects on floret fertility, a major determinant of the number of grains; and even less was it described the effects on floret primordia development dynamics, in turn the physiological bases of the final number of fertile florets. In addition, these very few reports only showed differences in the floret development dynamics basing their results on differences in stages of floret development at particular sampling timings rather than doing dissections systematically for each particular genotype (and environmental condition) (Guo *et al*., 2016); and in the cases in which dynamics were properly followed far less treatments were analyzed (Miralles *et al*,. 1998; González *et al*., 2005*a*; Ferrante *et al*., 2013*a*). Thus, the present Thesis is quite unique in terms of offering results that, to the best of my knowledge, were never published using such a valuable (though terribly time-consuming) approach considering: different genetic background, the effect of the *Ppd* and *Eps* genes in the same background but considering not only a relative high number of alleles source also for the *Eps* two different chromosomes and the effects under different temperatures giving a wide range of very detailed analyzed of floret development patterns. Furthermore almost all experiments were carried out in the field with a normal crop structure which, although reducing the accuracy of the results, produces results that are expected to be much more extrapolated to real agronomic conditions. In the experiments testing the effect of *Eps* x

temperature controlled conditions were needed as accurate control of temperatures in the field are not possible and this was the unique exception in which conclusions were not reached with field plots.

3. Integrating main results across chapters

Across the different experimental chapters, I characterized in detail the dynamics of floret primordia initiation and degeneration and the consequent effect of these processes on the number of fertile florets in a set of modern cultivars of hexaploid wheat and in NILs for *Ppd* and *Eps* genes.

Overall sources of genetic variation (both unspecific when compared modern cultivars and specific when comparing NILs), changes in the number of fertile florets at anthesis was mainly due to differences in floret survival and, at least in part, in the length of the floret development phase.

This is reflected in the Fig. 7.1 in which my results from modern cultivars and *Ppd*+Paragon and *Eps* NILs from all growing seasons were analyzed together. Similar pathway seemed to be involved in the effects on floret survival due to genetic differences among modern cultivars, differences in *Ppd* insensitivity and earliness *per se* in comparison to the effects of different environmental conditions such as resources availability (Ferrante *et al.*, 2013*b*).

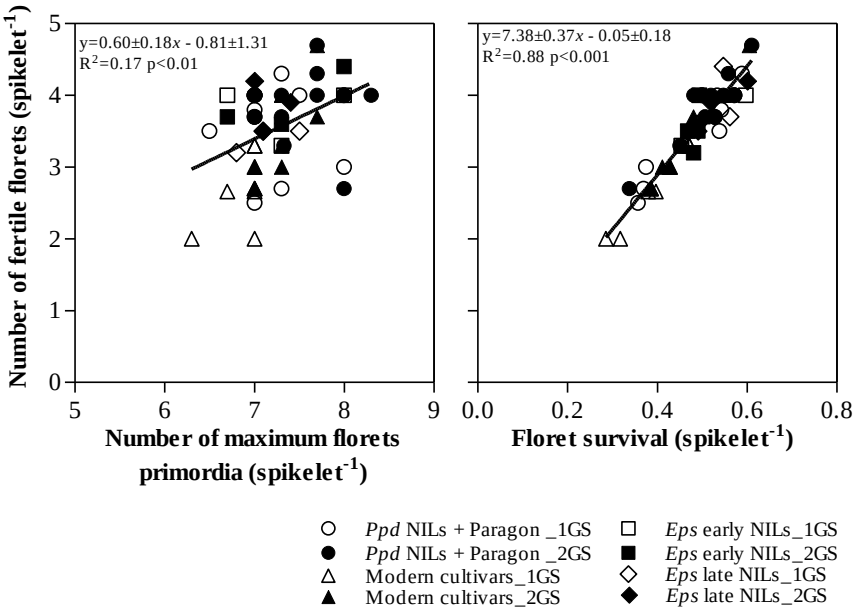

Figure 7.1. Relationship between the number of fertile florets in the central spikelet and the number of maximum floret primordia achieved (*right panel*) and the floret survival (*left panel*) among modern cultivars and *Ppd* NILs+Paragon and *Eps* NILs during all growing seasons. The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

In this report the improvement of floret survival was associated to better growing conditions (nitrogen availability) which is compatible with what had been described earlier by González *et al.*, (2005*a*) about the relationship between the fate of floret

primordia and the spike growth as affected by photoperiod treatments during stem elongation. Similarly, but based on differences due to a genetic factor, Miralles *et al.* (1998) showed higher rates of spike growth before anthesis and higher floret survival due to the effects of introgressing *Rht* genes. This has been integrated in a later discussion by Sadras and Slafer (2012) about the adjustment of the number of florets that become fertile to the availability of the resources during the few weeks preceding anthesis when the juvenile spikes, where florets are developing, do grow. Theory for this is based on the fact that a virtually unlimited number of floret primordia can be initiated while their size (dry matter) is negligible, as building up these microscopic structures would imply negligible energy costs providing an exceptional resource of organs potentially able to grow if resources were available at the time the requirements stop being negligible. This is an essential evolutionary strategy for crops (and plants in general) whose yield (or the amount of offspring in natural conditions) is heavily source-limited at the critical time for defining the number of grains (or of offspring) immediately before anthesis in the case of wheat, even when they become mostly sink-limited during grain filling (Reynolds *et al*., 2005; Slafer and Savin, 2006). Thus, when primordia structures start to grow (which is essential if development proceeds normally) the availability of resources become more limiting and the fate of the primordia is defined triggering the floret survival mechanism González *et al.,* (2011) and Ferrante *et al*., (2013*b*) associated the onset of floret death to the resources availability, even though it had been proposed earlier that the onset of floret death would be a pure developmental feature (depending on the degree of development of the oldest floret primordia; Bancal, 2008 and 2009). Results from both different modern cultivars and *Ppd* NILs in the present Thesis further reinforced the fact that the onset of floret death would not be triggered by the stage of development of the most proximal floret of central spikelets (Fig. 7.2), as the stage of floret 1 from central spikelets ranged between *c.* 5 to more than *c.* 9 when the maximum of floret primordia is achieved (onset of floret death).

Once floret mortality is triggered, the rate of floret mortality, largely determining the final rate of floret survival (the proportion of floret primordia initiated that produced a fertile floret) has also been related to flux of assimilates to the spikes from the onset of floret death to anthesis (see above), but when the effects on development are subtle (e.g. comparing modern well adapted cultivars, or NILs for *Eps* genes) differences in spike dry matter allocation are not clear and there is still possibilities for differences in survival due to slightly longer mortality periods, increasing the likelihood of a labile primordia to continue developing normally to become a fertile floret. When this happens, the improved fertility is associated to improvements in fruiting efficiency, and this seemed to have been the mechanism acting in the processes analyzed in the present Thesis, chiefly in Chapters III, V and VI.

In addition, there existed genetic variation in synchrony of floret initiation and in the duration of the period from the onset of floret initiation to anthesis (Chapters III and IV). Floral synchrony has been shown to affect the levels of abortion in maize, increasing the synchrony of pollination improving the number of grains set (Cárcova *et al*., 2000), but to the best of my knowledge, it had never been tested whether the synchrony in primordia development might be regulating the rate of floret mortality (associated to a sort of apical dominance process). In this Thesis I developed a model to estimate the degree of synchrony in development of different floret primordia and it seemed to have influenced positively in both floret survival and number of fertile florets at least in some cases (Chapter III).

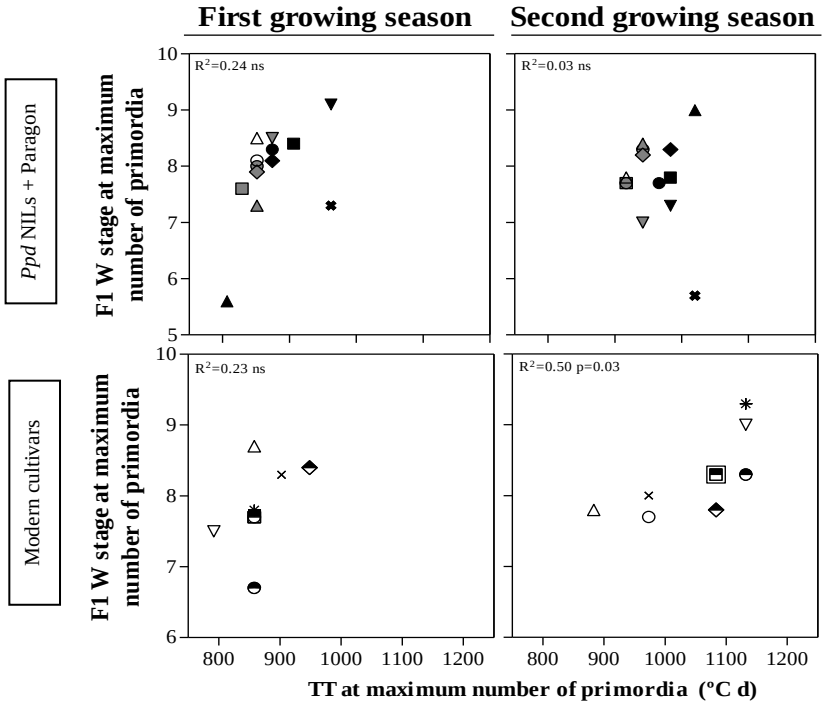

Figure 7.2. Relationship between Waddington stage of F1 at maximum number of primordia achieved against thermal time at maximum number of primordia achieved among *Ppd* NILs+ Paragon (*top panels*) and modern cultivars (*bottom panels*) during the first (*left panels*) and the second growing seasons (*right panels*). The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

4. Appraising the outcome of testing of the hypotheses

The global hypothesis of the Thesis implied that the **number of fertile florets at anthesis resulted from the floret generation/degeneration dynamics would be reduced as the duration of the phase of floret development was reduced** by genetic differences between modern cultivars or by the introgression of *Ppd*-insensitivity alleles or *Eps*-early alleles. Results of most genotypes from the different experimental chapters indicated that **the hypothesis was accepted**. In particular, is important to notice that the amount of variation seen in the number of fertile florets and in the phase duration among the genotypes analyzed depended strongly on the different backgrounds in the elite cultivars and in the particular *Ppd* and *Eps* genes and in, most cases, in the background growing conditions.

To contrast the general hypothesis different specific objectives were outlined and to avoid inconsistencies in this Chapter, I transformed them to specific hypothesis, simply by making explicit the implicit hypotheses behind the objectives:

i) **(a) number of fertile florets at anthesis would be variable among modern cultivars due to difference in the dynamics of floret initiation and degeneration associated to variability in the maximum floret primordia developed or in the floret survival**. The results of the Chapter III let us **partially accept the hypothesis** due to even differences in the number of fertile florets were well-explained for differences in the floret generation/degeneration dynamics were related to different floret survival.

(b) **synchrony in floret primordia development would explain the differences among cultivars in the maximum, final floret primordia or in the floret survival**. I failed to explain differences among modern cultivars in the maximum, final floret primordia and the floret survival due to only the synchrony of the floret primordia development, then **this particular hypothesis was rejected**.

ii) (a) **photoperiod insensitivity would reduce the number of fertile florets at anthesis due to reducing the floret development phase which would cause a reduction in the maximum and final number of fertile florets and a reduction in the floret survival**. **This hypothesis again was partially accepted**, the insensitivity reduced the number of fertile florets at anthesis and presented a shorter floret development phase although the reduction was more linked to the floret survival rather than the maximum number of floret primordia developed

(b) **photoperiod effects would be stronger as the number of insensitivity alleles increase and particularly the stronger effects would involve the insensitivity in D genome**. No clear pattern was found regarding the doses of *Ppd* alleles considering all the variation sources. Although a trend was reported that increasing the number of insensitive alleles would in average increase the magnitude of the effect, the individual NILs showed contrasting effects evidencing that even when the dose may have a role this is not dominant. Also it was not identified a particular stronger effects of D genome across the different NILs considered. **This hypothesis was rejected**.

iii) ***Eps* early alleles would affect the number of fertile florets at anthesis through reducing the floret development phase due to a reduction in either the maximum floret primordia developed or floret survival**. Even though the effects were subtle and depended on the background and

particular *Eps* genes considered, results of the Chapter V allows the **hypothesis be in general accepted**.

iv) **the strength *Eps* effects on the number of fertile florets and the floret development dynamics and phase length would depend on the growing temperature**. The **hypothesis was accepted** and the results of Chapter VI showed not only the quantitative effects hypothesised but also much stronger becoming at extreme temperatures tested (which were not extreme temperature strictly talking) and also qualitative effects.

5. Main contributions to knowledge

In the experimental work done in the context of the present Thesis I made contributions to knowledge ranging from original, completely novel (as far as I am aware), through confirmations of issues that were analyzed earlier by others but that could not be considered as established knowledge yet (i.e. consolidating with the results reported emerging knowledge), to confirmation of things already known, reasonably well established in the literature.
I did not aim to produce a comprehensive list of these types of contributions. I did rather aim to highlight below the existence of the three types of contributions, mentioning only an example of each.

i) Original contributions to knowledge

The analysis of the floret appearance rate and its reciprocal the "floret plastochron" (average time interval between the initiation of two consecutive floret primordia) was never published before and, more relevant, the use of this rate to assess the degree of synchrony (the shorter this plastochron the more synchronous the development of florets developed across time). The results are not comparable with anything reported, but establishing a parallelism with the organogenesis of other organs, they are different from what was reported for leaf and spikelet phyllochron, where the effects of *Ppd* alleles altering the rate of phasic development (changing the duration of the phase; González *et al.*, 2002; Whitechurch and Slafer, 2001; 2002; Foulkes *et al.*, 2004) did not noticeably affect the rate of organogenesis.
Furthermore, I used this assessment of synchrony in floret development to test whether it might have been responsible for differences in floret fertility, again something never done before and therefore a completely novel contribution from this Thesis. As discussed in Chapters III and IV there must have existed a compensation between the rate of floret appearance and the length of the phase, as faster rates did not show necessary higher number of primordia reached and on the other hand the reduction in the floret development phase did not cause necessarily a reduction in the maximum number of floret primordia developed.

ii) Consolidating emerging new knowledge

As illustrated in Fig. 7.2, joining my results from the different experimental Chapters (III, IV and V) the number of fertile florets was associated more to the variability in the

floret survival rather than in the maximum floret primordia developed. This confirms for a wider range of conditions (as it were never proven for modern commercial cultivars nor for the action of particular *Ppd* or *Eps* genes) which was previously described by Ferrante *et al*. (2013*a*); González-Navarro *et al*. (2015) and Guo, *et al.* (2016).

iii) Confirming established knowledge

Considering globally the results shown, most of them were consistent with all the previous reports about differences in the developmental phases length due to: i) genetic differences, although slightly among modern cultivars (Elia *et al.*, 2016), ii) different photoperiod environments (Miralles *et al.*, 2000; González *et al.*, 2003*b*) or due to iii) the effects of *Eps* alleles (Lewis *et al.*, 2008) and their interaction with the temperature (Bullrich *et al*., 2002; Appendino and Slafer 2003; Lewis *et al*., 2008). In addition, results were consistent with the effects described on the number of fertile florets due to genotypic and), environmental effects (e.g. Sibony and Pinthus, 1988; Miralles *et al*., 1998; Miralles, *et al*., 2000; González, *et al*., 2003*a*; González, *et al*., 2003*b*; González, *et al*., 2005*a*; González *et al*., 2005*b*; Ghiglione *et al*., 2008; Serrago, *et al*., 2008; Ferrante *et al*., 2010).

6. Future research

As always, the achievements of a research process open room for further research in many directions. Just to illustrate some of them, further studies may focus on:

- The results of the *Ppd* effects not being consistent completely across growing seasons suggest that it might be interesting to analyze and quantify photoperiod x temperature interactions identifying the developmental phases most strongly affected by this interaction, if it were significant, including how the parameters of response (daylength thresholds and sensitivity) are affected. As the two growing seasons explored very similar daylengths, the main cause of the inconsistency in sensitivity would be temperature. Determining the existence, characterizing the response models and quantifying the effects would be empirically relevant in the context of temperature increments.

  The concept developed of synchrony of floret development should be further explored and exploited, firstly challenging to what degree the synchrony may be altered by (environmental and genetic) manipulations, and secondly by analyzing with these sources of variation recognized to what degree the changes in synchrony may be responsible for differences in rate of floret mortality.

- Combine different treatments using the modern cultivars under photoperiod treatments and the *Ppd* NILs as well with different resources availability such as nitrogen, water-soluble carbohydrates or spikelet-trimming in order to analyze if there exist compensation between the duration of the developmental phases and the assimilates availability and how the synchrony of floret primordia

appearance, the maximum floret primordia developed and the floret survival are affected and how these affect the number of fertile florets.

7. Major Conclusions

- Modern cultivars well adapted from the Mediterranean region presented differences in the number of fertile florets due to a combination of differences in floret development phase length, the rate of floret appearance and the floret survival. Advantageous cultivars (those possessing contrasting attributes contributing to improved spike fertility) could be taken into account for strategic crosses in future breeding.

- Photoperiod insensitivity did affect spike fertility. Carrying the photoperiod insensitivity reduced the number of fertile florets due to a combination of effects on floret development phase length, the rate of floret appearance and the floret survival.

- There was not a clear pattern of the strength effects depending of the genome from which the insensitivity came from and it was not seen a clear doses response.

- Earliness *per se* alleles did show slight effects on spike fertility under natural conditions, even slightly trends were seen depending on the different genetic background and the specific *Eps* gene.

- Importantly, both qualitative and quantitative temperature effects on the *Eps* action were identified. The qualitative effects would be revealing that threshold temperatures would not be really extremes (and therefore easily found in realistic field growing conditions), and there was apparently a difference between NILs carrying the early or late allele in optimum temperature.

- Floret development and the number of living floret primordia dynamics are useful tools to compare treatments and for reflect the nature behind the differences in the number of fertile florets at anthesis.

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