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Physiology of genotypic differences in wheat grain weight among elite cultivars of contrasting grain number under contrasting N conditions or exposed to heat

Jinwook Kim

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Doctoral Thesis

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Jinwook Kim

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Director/a
Roxana Savin
Gustavo A. Slafer

Tutor/a
Roxana Savin

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Abstract

Generally, wheat yield can be divided into two main components; grain number per m^2 (GN) and average grain weight (AGW). Yield is commonly strongly associated with GN, but there is also a common negative relationship between AGW and GN. As this may represent a penalty in achievable yield gains should grain growth be limited by the source strength, it will be important to elucidate whether the reduction in AGW in response to increases in GN represents a case of source-limitation, and to recognize whether AGW in elite germplasm is limited by resource availability to fill the grains. We selected and studied under contrasting nitrogen conditions a group of modern wheat cultivars to quantify, and to analyze the causes behind, the relationship between GN and AGW. GN and AGW showed a noticeable difference in phenotypic plasticity in response to the contrasting nitrogen condition. Across the three field studies, GN and yield showed to be very plastic, while AGW was extremely conservative. The proportion of distal grains (i.e. grains in 10^{th} percentile), that are constitutively smaller than those that are proximal (i.e. grains in 90^{th} percentile), was increased when GN increased, indicating that the negative relationship was at least in part due to a non-competitive cause. However, proximal grains were also affected as the consequence of increased GN, indicating the size of all grain classes was affected when GN increased. To explore this more specific negative relationship, source-sink manipulations were imposed. Overall, AGW did not respond to de-graining, while defoliation resulted in small but consistent decreases in AGW. Further supporting these results, GN was dramatically increased in response to halving the competition during stem elongation, while AGW was only slightly reduced. This result indicates that the trade-off between AGW and GN across elite material must have been mainly due to differences in the intrinsic potential weight of the grains. Also, even though grain growth was dominantly sink-limited, current cultivars are approaching to experience some degree of source-limitation to grain growth.

Further increasing wheat productivity is more challenging due to rising temperatures. Heat stress negatively affects both main components; GN and AGW of wheat. Wheat genotypes differ in the relevance of each of these components in determining yield as well as in the tolerance to heat. However, it is not clear (i) whether the compensation (cultivar with higher GN having lower AGW) would reflect competition between growing grains, and (ii) if the sensitivity to heat would be related to the relevance of the component in each cultivar and affected by an eventual source limitation (should grains compete for resources in cultivars with high GN and lower AGW). Therefore, two contrasting genotypes regarding GN and AGW were selected from the previous study and analysed (i) in detail the causes for their differences in AGW and (ii) their GN and AGW responses to heat stress imposed in the field at both pre- and post-anthesis using portable chambers with transparent polyethylene and to different source-sink ratios. Again, the reduced AGW of the cultivar with more GN was not due to a competitive mechanism as there was no evidence of lack of assimilates during grain filling. It was related to an increased proportion of grains with constitutively low weight potential, but again heavier grains were also affected by increased GN, indicating the trade-off between AGW and GN would be mainly due to differences in the intrinsic potential weight of the grains. Therefore, in this Thesis, a new hypothesis of non-competitive mechanism to explain the trade-off between GN and AGW was suggested. When GN increases, the reduction of AGW was not due to solely an increased proportion of smaller grains, but due to potential grain weight *per se* of each genotype together with an increase in the proportion of constitutively small grains.

Exposing wheat cultivars to pre- and post-anthesis heat stress resulted in yield penalties associated with the components mainly being determined at those stages, GN and AGW,

respectively, but yield was more sensitive to pre- than to post-anthesis heat stress, consistently across both locations and genotypes. In this Thesis, pre-anthesis heat stress was imposed at booting stage (DC.4.3), and it did not affect the number of spikes per m^2, and therefore the penalty on GN operated through affecting spike fertility, indicating that the reduction GN (and final yield) caused by heat stress is mainly mediated through grain abortion. The penalty of AGW by post-anthesis heat stress (DC.7.5) did not result from competition for resources as (i) heavier grains were reduced similarly to, or more than small grains by the heat stress, and (ii) grain weight did not clearly respond to the increase in assimilate availability, indicating that the reduction of AGW caused by heat stress was not related to source strength changes. Therefore, the reduction of AGW caused by heat stress at post-anthesis might have been simply due to a direct effect reducing PGW *per se*. When transient heatwaves were imposed twice at both pre- and post-anthesis, the reduction of AGW caused by that post-anthesis heat stress was diminished compared with the effect of the same post-anthesis heat treatment imposed solely, indicating that the effect of transient heatwaves was not additive during the whole growing period of wheat, but to some extent, there was sort of a priming effect of an earlier heat stress on the magnitude of the effect of a succeeding heat stress. It seemed that the plasticity of a yield component in response to heat stress was inversely related to the relevance of that component for yield determination in unheated conditions in the first season (Chapter IV), but the effect of post-anthesis heat stress effect on AGW was virtually equal to that on GN by pre-anthesis heat stress in the second season (Chapter V), showing AGW was apparently starting to explore an incipient source-limitation. It was found a trend for the cultivar constitutively producing more grains having less sensitivity of GN to pre-anthesis heat stress, while the cultivar constitutively having heavier grains having less sensitivity of AGW to post-anthesis heat stress in the first season. However, this trend was not confirmed in the second season in which heat treatments might have affected both GN and AGW simultaneously through direct and indirect mechanisms (i.e. reducing availability of assimilates).

All in all, considering all experiments carried out it is suggested that breeders may need to consider not only further improvements in sink-strength (through either higher GN or higher PGW) but also simultaneously in source-strength during grain filling. This is because it was found in the different approaches that modern cultivars may still be sink-limited during grain filling but close to experience some degree of source limitation (in general AGW not responding to de-graining but being reduced in response to defoliations).

Resumen

Generalmente, el rendimiento de trigo se puede dividir en dos componentes principales: número de grano por m^2 (GN) y peso medio de grano (AGW). El rendimiento suele estar fuertemente asociado con GN, pero también existe una relación negativa entre AGW y GN. Como esto puede representar una penalización en las ganancias de rendimiento alcanzables si el crecimiento del grano está limitado por la fuerza de la fuente, es importante dilucidar si la reducción en AGW en respuesta a aumentos en GN representa un caso de limitación por fuente, y reconocer si AGW en germoplasma de élite está limitado por la disponibilidad de recursos para llenar los granos. En esta tesis hemos seleccionado y estudiado bajo condiciones de nitrógeno contrastantes un grupo de cultivares de trigo modernos para cuantificar y analizar las causas detrás de la relación entre GN y AGW. GN y AGW mostraron una diferencia notable en la plasticidad fenotípica en respuesta a la condición de nitrógeno contrastante. En los tres años experimentales de campo, GN y rendimiento mostraron ser muy plásticos, mientras que AGW fue extremadamente conservador. La proporción de granos distales (es decir, granos en el percentil 10), que son constitutivamente más pequeños que los que son proximales (es decir, granos en el percentil 90), se incrementó cuando GN aumentó, lo que indica que la relación negativa se debió, al menos en parte, a causas no competitivas. Sin embargo, los granos proximales también se vieron afectados como consecuencia del aumento de GN, lo que indica que el tamaño de todas las clases de granos se vio afectado cuando aumentó el GN. Para explorar esta relación negativa de forma más específica, se impusieron manipulaciones fuente-sumidero. En general, AGW no respondió al desgranado, mientras que la defoliación resultó en una disminución de AGW. Respaldando aún más que el GN aumentó drásticamente en respuesta a los recursos adicionales disponibles para los cultivares cuando la competencia se redujo a la mitad durante el inicio de elongación del tallo, mientras que el AGW se redujo solo ligeramente. Este resultado indica que la compensación entre AGW y GN a través del material de élite debe haberse debido principalmente a diferencias en el peso potencial intrínseco de los granos. Además, a pesar de que el crecimiento del grano fue predominantemente limitado por el sumidero, los cultivares actuales se están acercando a experimentar algún grado de limitación de fuente para el crecimiento del grano y se sugiere que los mejoradores pueden necesitar también considerar mejoras en el tamaño del sumidero en el futuro.

Incrementar aún más la productividad del trigo es un gran desafío debido al aumento de las temperaturas. El estrés por alta temperatura afecta negativamente a ambos componentes principales; GN y AGW en el cultivo de trigo. Los genotipos de trigo difieren en la relevancia de cada uno de estos componentes para determinar el rendimiento, así como en la tolerancia a altas temperaturas. Sin embargo, no está claro (i) si la compensación (cultivar con mayor GN y menor AGW) reflejaría la competencia entre granos en crecimiento, y (ii) si la sensibilidad a las altas temperaturas estaría relacionada con la relevancia del componente en cada cultivar y afectados por una eventual limitación de la fuente (en caso de que los granos compitan por los recursos en cultivares con un GN alto y un AGW más bajo). Por lo tanto, seleccioné dos genotipos contrastantes con respecto a GN y AGW y analicé (i) en detalle las causas de sus diferencias en AGW y (ii) sus respuestas de GN y AGW al estrés por altas temperaturas impuesto en el campo tanto pre- como post-antesis utilizando cámaras portátiles con polietileno transparente y con diferentes relaciones fuente-sumidero. Nuevamente, el AGW reducido del cultivar con más GN no se debió a un mecanismo competitivo ya que no hubo evidencia de falta de asimilados durante el llenado del grano. Se relacionó con una mayor proporción de granos con un peso potencial constitutivamente bajo, pero nuevamente los granos más pesados también se vieron afectados por un aumento de GN, lo que indica que la compensación entre AGW y GN se debería

principalmente a diferencias en el peso potencial intrínseco de los granos. Por lo tanto, en esta Tesis, una nueva hipótesis de mecanismo no competitivo para explicar la compensación entre GN y AGW fue sugerida. Cuando GN aumenta, la reducción de AGW no se debe únicamente a una mayor proporción de granos más pequeños, sino al peso potencial de grano per se de cada genotipo junto con un aumento en la proporción de granos constitutivamente pequeños.

La exposición de los cultivares de trigo a estrés por altas temperaturas en pre- y post-antesis resultó en penalizaciones de rendimiento asociadas con los componentes que se determinaron principalmente en esas etapas, GN y AGW, respectivamente, pero el rendimiento fue más sensible al estrés en pre- que en post-antesis, consistentemente en ambos sitios y genotipos. En esta Tesis, el estrés por altas temperaturas en pre-antesis se impuso en el estado de bota (DC.4.3), y no afectó el número de espigas por m^2, por lo que la penalización sobre GN operó afectando la fertilidad de espiga, lo que indica que la reducción de GN (y rendimiento final) causado por el estrés por altas temperaturas está mediado principalmente por el aborto de los granos. La penalización de AGW por estrés por altas temperaturas post-antesis (DC.7.5) no resultó de la competencia por los recursos, ya que (i) los granos más pesados se redujeron de manera similar o más que los granos pequeños por el estrés térmico, y (ii) el peso del grano no respondió de modo claro al aumento en la disponibilidad de asimilados, lo que indica que la reducción de AGW causada por el estrés térmico no estuvo relacionada con los cambios en la fuerza de la fuente. Por lo tanto, la reducción de AGW causada por el estrés térmico en post-antesis podría haber sido simplemente debido a un efecto directo sobre el PGW *per se*. Cuando se impusieron olas de calor transitorias tanto en pre- como en post-antesis, la reducción de AGW causada por el estrés térmico en post-antesis fue menor que con el mismo tratamiento térmico post-antesis impuesto únicamente, indicando que el efecto de las olas de calor no fue aditivo durante todo el período de crecimiento del trigo: hasta cierto punto, hubo una especie de efecto “priming” de un estrés térmico anterior sobre la magnitud del efecto de un estrés térmico subsiguiente.

Parecía que la plasticidad de un componente de rendimiento en respuesta al estrés térmico estuvo inversamente relacionada con la relevancia de ese componente para la determinación del rendimiento en condiciones sin estrés en el primer año experimental (Capítulo IV), pero el efecto del estrés por calor post-antesis en AGW fue virtualmente igual al de GN por estrés térmico en pre-antesis en la segundo año experimental (Capítulo V), lo que muestra que AGW aparentemente estaba comenzando a explorar una limitación de fuente incipiente. Se evidenció una tendencia por la cual el cultivar que produce constitutivamente más granos presentaba menor sensibilidad de GN al estrés térmico en pre-antesis, mientras que el cultivar tuvo granos más pesados de manera constitutiva tuvo menos sensibilidad de AGW al estrés térmico en post-antesis en el primer año experimental. No obstante, esta tendencia no fue confirmada en el segundo año experimental, en el que los tratamientos térmicos podrían haber afectado tanto el GN como el AGW a través de mecanismos directos e indirectos (es decir, reduciendo la disponibilidad de asimilados) simultáneamente.

En general, considerando todos los experimentos llevados a cabo, se sugiere que los mejoradores pueden necesitar considerar no solo mejoras adicionales en la fuerza del sumidero (ya sea a través de un GN más alto o un PGW más alto) sino también simultáneamente en la fuerza de la fuente durante el llenado del grano. Esto se debe a que se encontró en los diferentes enfoques llevados a cabo en esta Tesis, que los cultivares modernos aún pueden estar limitados por el sumidero durante el llenado del grano, pero cerca de experimentar algún grado de limitación de la fuente (en general, el AGW no responde al desgranado pero se reduce en respuesta a las defoliaciones).

Resum

Generalment, el rendiment de blat es pot dividir en dos components principals: nombre de grans per m^2 (GN) i pes mitjà del gra (AGW). El rendiment sol estar fortament associat amb GN, però també hi ha una relació negativa entre AGW i GN. Com que això pot representar una penalització en els guanys de rendiment assolibles si el creixement del gra està limitat per la força de la font, és important dilucidar si la reducció en AGW en resposta a augments en GN representa un cas de limitació per font, i reconèixer si AGW a germoplasma d'elit està limitat per la disponibilitat de recursos per omplir els grans. En aquesta tesi hem seleccionat i estudiat sota condicions contrastants de nitrogen un grup de cultivars de blat moderns per quantificar i analitzar les causes darrere de la relació entre GN i AGW. GN i AGW van mostrar una diferència notable en la plasticitat fenotípica en resposta a la condició de nitrogen contrastant.
Als tres anys experimentals de camp, GN i el rendiment van mostrar ser molt plàstics, mentre que AGW va ser extremadament conservador. La proporció de grans distals (és a dir, grans al percentil 10), que són constitutivament més petits que els que són proximals (és a dir, grans al percentil 90), es va incrementar quan GN va augmentar, la qual cosa indica que la relació negativa es va deure, almenys en part, a causes no competitives. No obstant això, els grans proximals també es van veure afectats com a conseqüència de l'augment de GN, la qual cosa indica que la mida de totes les classes de grans es va veure afectada quan va augmentar el GN. Per explorar aquesta relació negativa de forma més específica, es van imposar manipulacions font-embornal. En general, AGW no va respondre al desgranat, mentre que la defoliació va resultar en una disminució d'AGW, donant suport encara més que el GN va augmentar dràsticament en resposta als recursos addicionals disponibles per als cultivars quan la competència es va reduir a la meitat durant l'inici d'elongació de la tija, mentre que l'AGW es va reduir només lleugerament. Aquest resultat indica que la compensació entre AGW i GN a través del material d'elit és deguda principalment a diferències en el pes potencial intrínsec dels grans. A més a més, malgrat que el creixement del gra va ser predominantment limitat per l'embornal, els cultivars actuals s'estan acostant a experimentar algun grau de limitació de font per al creixement del gra i se suggereix que els milloradors poden necessitar també considerar millores en la mida de l'embornal en el futur.

Incrementar encara més la productivitat del blat és un gran desafiament a causa de l'augment de les temperatures. L'estrès per alta temperatura afecta negativament els dos components principals; GN i AGW al cultiu de blat. Els genotips de blat difereixen en la rellevància de cadascun d'aquests components per determinar-ne el rendiment, així com en la tolerància a altes temperatures. No obstant això, no és clar (i) si la compensació (cultivar amb major GN i menor AGW) reflectiria la competència entre grans en creixement, i (ii) si la sensibilitat a les altes temperatures estaria relacionada amb la rellevància del component a cada cultivar i afectats per una eventual limitació de la font (en el cas que els grans competeixin pels recursos en cultivars amb un GN alt i un AGW més baix). Per tant, vaig seleccionar dos genotips contrastants respecte a GN i AGW i vaig analitzar (i) en detall les causes de les seves diferències en AGW i (ii) les seves respostes de GN i AGW a l'estrès per altes temperatures imposades al camp tant pre- com a post-antesi utilitzant càmeres portàtils amb polietilè transparent i amb diferents relacions font-embornal. Novament, l'AGW reduït del cultivar amb més GN no es va deure a un mecanisme competitiu ja que no hi va haver evidència de manca d'assimilats durant l'ompliment del gra. Es va relacionar amb una proporció més gran de grans amb un pes potencial constitutivament baix, però novament els grans més pesats també es van veure afectats per un augment de GN, la qual cosa indica que la compensació entre AGW i GN es deuria principalment a diferències en el pes potencial intrínsec dels grans. Per tant, en aquesta Tesi, una nova hipòtesi de mecanisme no

competitiu per explicar la compensació entre GN i AGW va ser suggerida. Quan GN augmenta, la reducció d'AGW no es deu únicament a una proporció més gran de grans més petits, sinó al pes potencial de gra *per se* de cada genotip juntament amb un augment en la proporció de grans constitutivament petits.

L'exposició dels cultivars de blat a estrès per altes temperatures en pre- i post-antesi va resultar en penalitzacions de rendiment associades amb els components que es van determinar principalment en aquestes etapes, GN i AGW, respectivament, però el rendiment va ser més sensible a l'estrès en pre- que en post-antesi, consistentment en ambdós llocs i genotips. En aquesta Tesi, l'estrès per altes temperatures en pre-antesi es va imposar a l'estat de bota (DC.4.3), i no va afectar el nombre d'espigues per m^2, per la qual cosa la penalització sobre GN va operar afectant la fertilitat d'espiga, la qual cosa indica que la reducció de GN (i rendiment final) causat per l'estrès per altes temperatures està intervingut principalment per l'avortament dels grans. La penalització d'AGW per estrès per altes temperatures post-antesi (DC.7.5) no va resultar de la competència pels recursos, ja que (i) els grans més pesats es van reduir de manera similar o més que els grans petits per l'estrès tèrmic , i (ii) el pes del gra no va respondre de manera clara a l'augment en la disponibilitat d'assimilats, la qual cosa indica que la reducció d'AGW causada per l'estrès tèrmic no va estar relacionada amb els canvis a la força de la font. Per tant, la reducció de AGW causada per l'estrès tèrmic en post-antesi podria haver estat deguda simplement a un efecte directe sobre el PGW *per se*. Quan es van imposar onades de calor transitòries tant en pre- com en post-antesi, la reducció d'AGW causada per l'estrès tèrmic en post-antesi va ser menor que amb el mateix tractament tèrmic post-antesi imposat únicament, indicant que l'efecte de les onades de calor no va ser additiu durant tot el període de creixement del blat, fins a cert punt, va haver-hi una mena d'efecte "priming" d'un estrès tèrmic anterior sobre la magnitud de l'efecte d'un estrès tèrmic subsegüent.

Semblava que la plasticitat d'un component de rendiment en resposta a l'estrès tèrmic va estar inversament relacionada amb la rellevància d'aquest component per a la determinació del rendiment en condicions sense estrès el primer any experimental (Capítol IV), però l'efecte de l'estrès per calor post-antesi a AGW va ser virtualment igual al de GN per estrès tèrmic en pre-antesi al segon any experimental (Capítol V), la qual cosa mostra que AGW aparentment estava començant a explorar una limitació de font incipient. Es va evidenciar una tendència per la qual el cultivar que produeix constitutivament més grans presentava menor sensibilitat de GN a l'estrès tèrmic en pre-antesi, mentre que el cultivar va tenir grans més pesats de manera constitutiva va tenir menys sensibilitat d'AGW a l'estrès tèrmic en post-antesi el primer any experimental. No obstant això, aquesta tendència no va ser confirmada el segon any experimental, en què els tractaments tèrmics podrien haver afectat tant el GN com l'AGW a través de mecanismes directes i indirectes (és a dir, reduint la disponibilitat d'assimilats) simultàniament.

En general, considerant tots els experiments duts a terme, se suggereix que els milloradors poden necessitar considerar no només millores addicionals a la força de l'embornal (ja sigui a través d'un GN més alt o un PGW més alt) sinó també simultàniament a la força de la font durant l'ompliment del gra. Això és perquè es va trobar en els diferents enfocaments duts a terme en aquesta Tesi, que els cultivars moderns encara poden estar limitats per l'embornal durant l'ompliment del gra, però prop d'experimentar algun grau de limitació de la font (en general, l'AGW no respon al desgranat però es redueix en resposta a les defoliacions).

Chapter I

General Introduction

Chapter I: General Introduction

1.1. Relevance of wheat

Wheat (*Triticum aestivum L.*) is a unique crop, as the grains contain gluten proteins capable of forming an elastic dough. These gluten proteins are indispensable to produce various foods around the world. At the global scale, wheat is crucial to ensure food security (e.g. Reynolds et al., 2012), as it is the most widely grown crops (over c. 220 Mha, FAOSTAT, 2019). It provides ca. 20% of nutrients (e.g. calories and protein) to the human diet worldwide (Hawkesford et al., 2013; Khan and Shewry, 2009; Shewry and Hey, 2015). Particularly in Europe, for the last two decades, wheat accounts for 12% (c. 26Mha) and 21% (c. 140Mt) of global production area and quantity respectively (FAOSTAT, 2019), being clearly and by far the main crop grown in the EU (Eurostat 2019).

The Green revolution brought forth a quantum leap of wheat yield (Lumpkin, 2015), and since then we had experienced breakthrough yield improvements. Wheat yield has increased linearly since the Green Revolution, but for the last decades, the improvement has been reduced c. 50% compared with the onset of the Green Revolution in both the world and EU (Fig.1.1A). In Spain, yield improvement was less reduced than in global or EU, but the variability was increased c. 3 times more than before during the last two decades (Fig.1.1B).

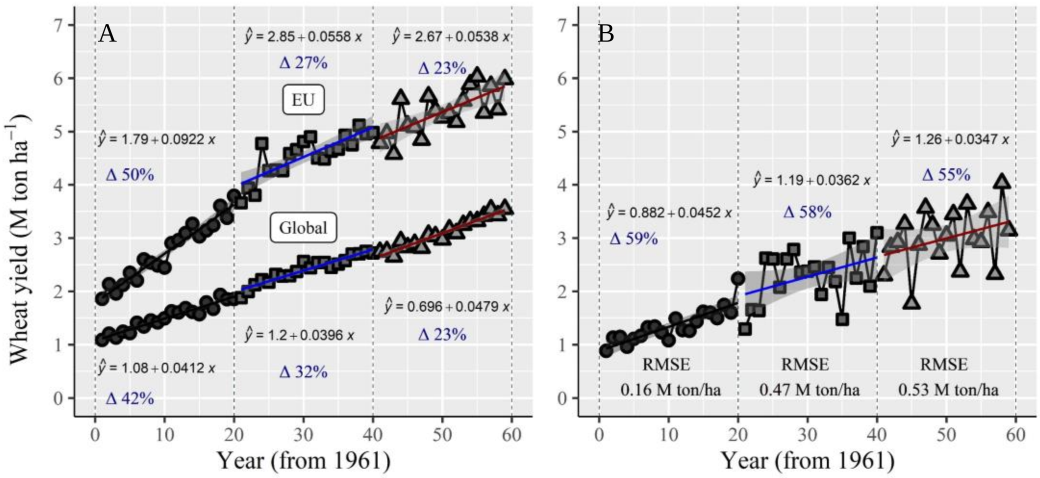

Fig.1.1. Average wheat yield from 1961 to 2019 in global and EU (A) and Spain (B). Data were obtained from FAO (https://www.fao.org/faostat/en/#data). Solid lines on points were fitted data from 1961 to 2019 with bi-linear according to two decades. Dashed lines represent the interval of two decades. Yield improvement (Δ) was presented in each two decades.

This may be indicating that due to increased temperature, wheat yield has been losing stability. Currently, wheat yields are growing at a much lower rate than those that characterised the green revolution: on average the rate of yield gains has been less than 1% (Ray et al., 2013).

As most of the land suitable for cropping is already in use (Connor and Mínguez, 2013), and wheat production would be more restricted due to un-expandable and unpredictable farming conditions. The genetic gain in wheat would be more constrained by diminishing returns due to narrowed response to selection and/or high-input farming systems, resulting in yield stagnation (Ray et al., 2012). In this context, many studies estimate that global wheat production need to increase c. 2.4% per year (Ray et al., 2013), and should be increased up to 50% by 2050 to fulfill the demand of human population (Ray et al., 2013, 2012). Therefore, it would be crucial to increase wheat yield through improving genetic and environmental effects on wheat performances in response to various environmental conditions, and understanding crop-physiological mechanisms beyond wheat yield will be more important than ever as the genomic approach to elucidate yield potential will be less efficient when environmental conditions have fluctuated.

1.2. Grain yield and its major components

Wheat yield is an extremely complex trait, and there is a growing consensus that using an approach more analytical, based on understanding crop-physiological processes determining crop yield, would contribute to increase the current rates of genetic gains (e.g., Fischer, 2007; Slafer, 2003; Araus et al., 2008). It is well known that grain number per m^2 (GN) and averaged grain weight (AGW) are the two major yield components of wheat (Frederick and Bauer, 1999; Slafer et al., 2021). Even though AGW is also important to wheat yield, most of the studies showed that wheat yield is more strongly associated with GN as the major wheat yield component (Savin and Slafer, 1991; Borrás et al., 2004), showing the greater source of variation (López-Bellido et al., 2005; Marti and Slafer, 2014; Cossani and Sadras, 2019), while AGW is a relatively stable trait, showing higher heritability (Sadras and Slafer, 2012). Wheat yield more strongly responds to GN (Slafer et al. 2014) and therefore GN is the main driving force to improve wheat yield rather than AGW (Richards, 1996; González et al., 2005). However, despite of less plasticity than GN, AGW is also an important source of variation in grain yield (Calderini et al., 1999; 2001) to improve yield and resilience of yield against environmental stresses. In addition, there is usually a negative relationship between GN and AGW (Slafer et al., 2021) and understanding the nature of that relationship could open room to alternative hypothesis for further raising yield. Indeed in most breeding programmes this negative relationship became evident: selection of genotypes with more grains brought about reductions in AGW (Calderini et al., 1999; Cartelle et al., 2006)

The negative relationship between GN and AGW could be interpreted as reflecting competitive or non-competitive mechanisms. For some crops, the trade-off may well represent an increased competition for resources among growing grains, like in rapeseed (Labra et al., 2017) or maize (Borrás et al., 2004) , but see also (Ordóñez et al., 2018). If the availability of assimilates is a critical factor to determine grain yield during grain filling (i.e. source limitation), the competitive mechanism must explain the negative relationship between GN and AGW. Thus, when GN increases, more grains will compete for limited resources during grain filling, reducing AGW and particularly the size of the weaker grains in the population of grains of the crop (in competitive relationships the weaker competitors would be more affected). Actually, this concept is what is most widely accepted in the literature. However, Slafer et al. (2014) indicated that negative relationship between GN and AGW in wheat do not usually represent a true trade-off (both due to environmental and genotypic effects). Non-competitive mechanisms for the negative relationship have been studied to explain the non-linear trade-off between GN and AGW. For instance, the reduction of AGW may be the result of increased proportion of smaller grains, particularly in distal positions within spikelets, contributing to decreased AGW independently of any change in competition for assimilates among grains. (Slafer and Savin, 1994; Miralles and Slafer, 1995; Acreche and Slafer, 2006).

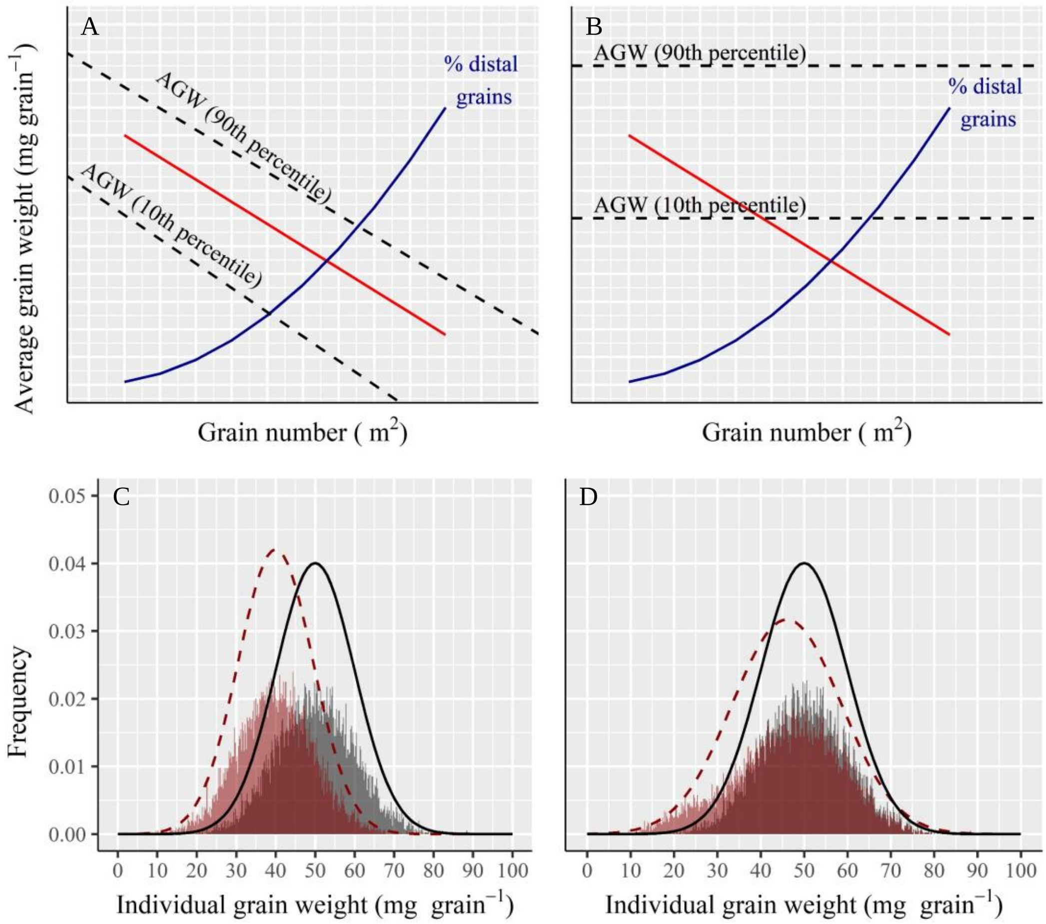

Fig. 1.2. The interpretation of competitive and non-competitive mechanisms regarding the trade-off between grain number and average grain weight. Competitive case to explain a proportion of smaller grains increases, and all grain size decreases when grain number increases (A, C); and non-competitive case to explain a proportion of smaller grains increases, but grain size does not decrease (B, D). In top panels, the red line indicates the negative relationship between GN and AGW; and the blue curve line indicates the increase of % smaller grains; and the dotted line indicates the change of 10^{th} and 90^{th} percentile's AGW when grain number increases. In bottom panels, curve lines stand for the normal distribution adjusted to the frequencies observed. Red dotted line indicates a genotype with more grain numbers than other genotypes (black solid line).

Quintero et al. (2018) suggested three possibilities to explain the negative relationship between GN and AGW; (i) the lack of resources per growing grain, (ii) increased proportion (%) of smaller grains with lower potential grain weight (PGW), and (iii) the interaction of (i) and (ii). In this context, we hypothesized two possible scenarios concerning the competitive and non-competitive relationship between GN and AGW. The first scenario is that when GN increases, the proportion (%) of smaller grains is increased, and all grains (both smaller and bigger size) will be decreased (Fig 1.2A), contributing to decreasing AGW (Fig 1.2C) as a sign of competition among growing grains. The second scenario is that even though grain number increases, grain weight (both smaller and bigger size) is not changed (Fig 1.2B), indicating the actual reduction of grain weight is mainly due to the increased proportion (%) of smaller grains, not due to competition (Fig 1.2D).

However, in the second scenario, even though it is not due to competition among growing grains, grain weight could be also decreased if PGW of a genotype with more GN is intrinsically smaller than a genotype with less GN. Ovary growth of fertile floret around pre-anthesis is important to determine the potential grain weight (Calderini et al., 1999), and when GN is more generated within limited space of spikelets, ovary growth might be restricted, resulting in less endosperm cell division during post-anthesis (Calderini et al., 2001; González et al., 2014), and eventually less grain weight due to lower PGW *per se*.

1.3. Source-sink balance during grain filling

To further elucidate the actual causes of the negative relationship between GN and AGW, understanding the response of grain size to manipulations of the source-sink strengths is relevant. Generally, source strength is regarded as the assimilate supply from green tissues (leaves, stem, spike) including actual photosynthesis and translocation from remobilisation from stems to grains, and also partitioning of assimilates, while sinks strength is the ability of the grains to accumulate assimilates from sources (Asseng et al., 2017). Wardlaw (1990) suggested that sink strength might be increased when grain size becomes larger due to more unloading area (membrane) where carbon is partitioned from source, but the growth rate of grains (i.e. sink activity) rather than grain size is more relevant to grain yield. In wheat, many studies frequently demonstrated that yield is sink-limited during grain filling (Jenner, 1979; Savin and Slafer, 1991; Slafer and Savin, 1994; Miralles and Slafer, 1995; Richards, 1996; Kruk et al., 1997; Borra and Slafer, 2004; Calderini et al., 2006); even in environments characterised by stressful conditions (Cartelle et al., 2006; Pedro et al., 2011; Serrago et al., 2013). However, there are also some studies disagreeing with the previous reports, and examples in the literature with grain weight responding to source–sink manipulations during grain filling can be also found in wheat (Bremner and Rawson, 1978; Fischer and HilleRisLambers, 1978; Sandaña et al., 2009). Part of the differences could be related to the timing at which the treatments were imposed. For instance, in the experiments made by Fischer and HilleRisLambers, (1978), the source–sink manipulation treatments were imposed at anthesis. Therefore, it is possible that the responses found in these experiments could be related to increases in grain weight potential (PGW), more than due to alleviation of an eventual source limitation during grain filling (Serrago et al., 2013). In the last years, several studies investigated the effects of pre-anthesis environments affecting PGW (Slafer et al., 2021). In the first studies, the effect of pre-anthesis conditions on AGW of wheat has been ascribed to carpel/ovary weight at anthesis (Calderini and Reynolds, 2000; Calderini et al., 2001; Xie et al., 2015). Hasan et al., (2011) showed that carpel weight, grain length, and stabilised water content (water content at the water plateau) were key drivers of PGW in wheat. More recently, Calderini et al., (2021) described other processes and traits (increasing α-expansins by gene expression) that are possibly relevant in determining PWG in weight from booting to grain setting in wheat.

To physiologically identify whether wheat is source- or sink-limited during grain filling, analysing biomass at both pre-anthesis and maturity (Fig. 1.3A), or correlation between AGW and PGW (Fig. 1.3B) can be suggested. Post-anthesis growth per grain (PAGG) is calculated as the biomass (g/m^2) difference between at pre-anthesis and at maturity per final GN (m^2), and it is defined as the potential capacity of a crop to fill the grain. If PAGG is greater than AGW, it indicates that the potential capacity of a crop to fill the grain is greater than its potential yield (Fig. 1.3A, Case I). Then, adequate assimilates might be translocated to other tissues, not only to grain. During grain filling, carbon accumulation into the grains is the main priority in wheat, and therefore if assimilates move to other tissues at this growing phase, it means that resources for grain filling exceed the sink capacity of grains (Richards, 1996; Borras and Slafer, 2004; Cartelle et al., 2006; Serrago et al., 2013) as a sign of sink-limitation during grain filling. However, although the potential capacity of a crop to fill the grain is less than its potential grain weight per grain (Fig. 1.3A, Case III), it might not be directly relevant to source limitation as reserved carbohydrates on stems at pre-anthesis can be remobilised to grains (Serrago et al., 2013; Talukder et al., 2013) up to 50% (Gent, 1994; Blum, 1998; Borrás et al., 2004). This is the reason why wheat hardly becomes source-limited. On the contrary, if the potential capacity of a crop to fill the grain is the same as the final grain weight (Fig. 1.3A, Case II), it means all resources moved to the growing grains except for the loss in respiration, indicating still a clear sink limitation as the water-soluble carbohydrates, that are available to fill the grains would have not been used. Only when PAGG is substantially less than AGW (say c. 50%, see above) it could be stated that grain growth is under clear source limitation.

Also, source or sink limitation could be identified by PGW of each genotype. Generally, PGW is determined by sink manipulation (i.e. de-graining), and under the de-graining condition, theoretically the amount of assimilates available to support growth of the grains doubles (Cartelle et al., 2006), and grain weight achieved in that condition is considered to be the PGW. In correlation between AGW and PGW, if all data lie on 1:1 ratio line (Fig. 1.3B), it indicates source strength (i.e. assimilate supply) might not be a critical factor to determine final yield as there are already adequate or excess assimilates to support the restrictive growing of the grains during grain filling. In this case, intrinsic capacity of grains to accumulate adequate assimilates would be more important,

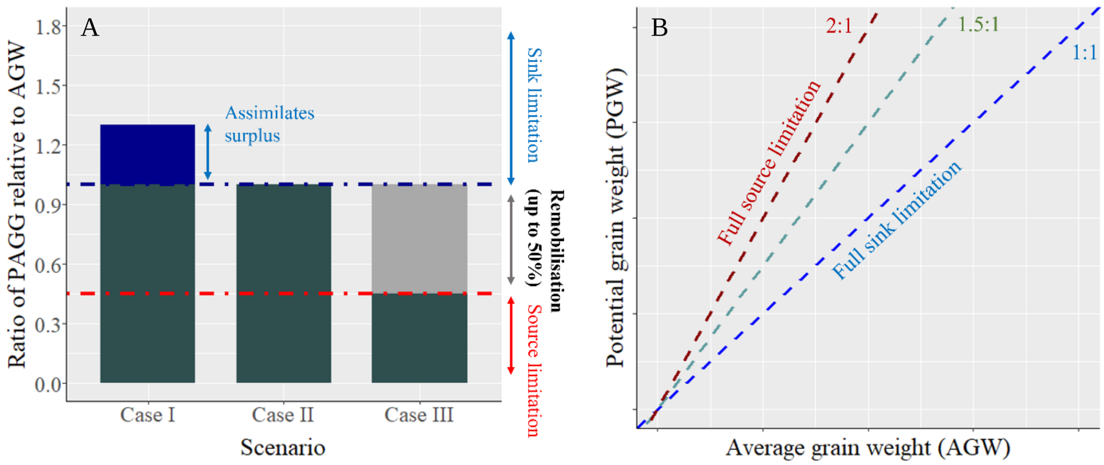

Fig. 1.3. Scenarios to determine source or sink limitation in wheat during grain filling. The ratio of post-anthesis growth per grain (PAGG) relative to Average grain weight (AGW) (A) and the correlation between AGW and Potential grain weight (PGW) (B). In panel A, blue dotted line indicates a full source-sink balance when AGW was equal to PAGG; and red dotted line indicates up to 50% contribution of pre-anthesis carbon reserves from stem to final grain weight. Above blue dotted line indicates adequate assimilates (i.e. sink limitation), and below the red dotted line indicates lack of assimilates (i.e. source limitation). In panel B, dotted lines indicate the ratio of 100% 150% 200% between PGW and AGW respectively.

indicating wheat is sink-limited during grain filling. On the other hand, if all data lie on 2:1 ratio line, it implies that in the control final grain weight was severely source-limited during grain filling, as doubling the amount of resources per grain doubled the size reached by the grains (Acreche and Slafer, 2009). In case data lie in between these two lines, it would represent different degrees of source-limitation, and likely when in between the 1:1 and 1.5:1 lines, yield during grain filling would be subjected to a sort of co-limitation.

Many studies suggested that periodically the degree to which high-yielding modern elite wheat cultivars are becoming source-limited must be evaluated (Calderini et al., 2006) as modern wheat cultivars might be also limited by the source due to the consistent breeding selection based on GN (Kruk et al., 1997; Álvaro et al., 2008). It is possible that modern elite wheat cultivars would be facing in the future some source-limitation due to the consequence of breeding for increasing GN. Therefore, three different hypotheses could be suggested (Fig. 1.4). In general, source and/or sink manipulation is generally suggested as a method to resolve whether the change of grain weight in response to adjusting source-sink ratio is the result of a competitive or non-competitive process during grain filling (Serrago et al., 2013). Even though both source and sink strength are manipulated during grain filling, if grain weight does not change, it would be the case of a strong sink-limitation in grains (Fig. 1.4A), but on the contrary, if grain weight significantly responds to the change of assimilate availability in both source and sink manipulation (Fig. 1.4B), it would be the case of strong source limitation during grain filling. However, in case grain weight is not responsive to more supplied assimilates, but significantly responsive to less assimilates (Fig. 1.4C), this case would be co-limited, indicating that wheat yield

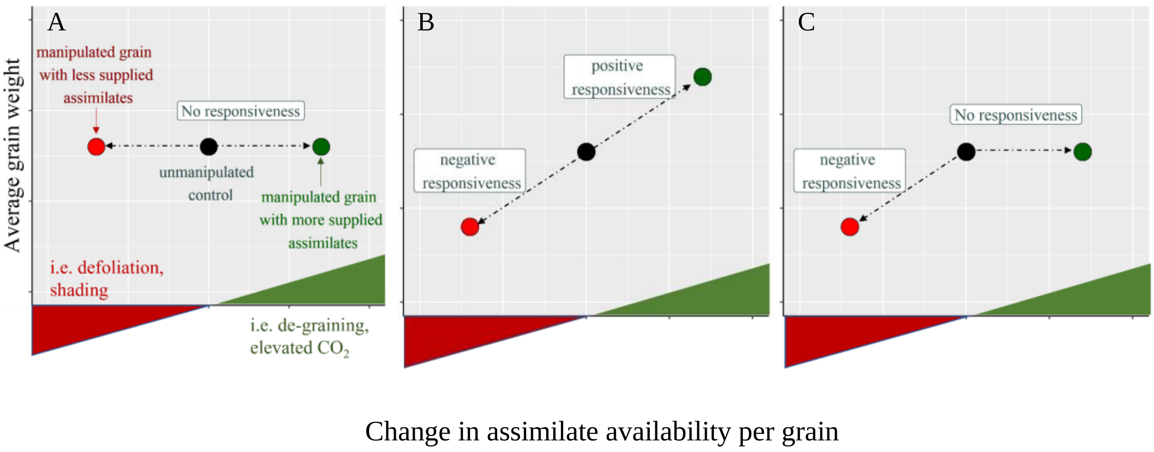

Fig. 1.4. Hypothesis to identify whether modern wheat cultivars are source or sink or co-limited by both during the effective period of grain filling. Each case of full sink limitation (A), source limitation (B), and close to co-limitation (C). Red color in the bottom line in panels indicates the degree of decreased assimilate availability, manipulating source strength, and green color indicates the degree of increased assimilate availability, manipulating sink strength. Control grain is non-manipulated (having intrinsic assimilate availability).

is still sink-limited during grain filling, but just on the limit to start experiencing some degree of source limitation.

It is well known that if severe environmental stresses were imposed on grain filling of wheat, yield is actually affected during grain filling. Savin and Nicolas (1996) indicated that when environmental stresses (i.e. heat and drought) were imposed at post-anthesis, it reduced c 30% of individual grain weight. But again the stresses may have affected the intrinsic growing capacity of grains. For instance, when Slafer and Miralles (1992), imposed heat stress during grain filling, grain weight was reduced, but that reduction was not reversed by increasing the availability of resources for grain growth, indicating that the intrinsic capacity of grains might be a more critical factor to determine grain yield responses to stress during grain filling.

1.4. Plasticity between GN and AGW

Phenotypic plasticity can be understood as the ability of a genotype to alter its phenotype in response to environmental changes (Aspinwall et al., 2015), and seems rather relevant to adapt crops to climatic change (Nicotra et al., 2010) as phenotypic plasticity can be explained as various mechanisms in terms of genetic variance, fitness, stress tolerance or carbon acquisition in response to environmental variation with beneficial phenotypic changes (DeWitt and Scheiner, 2004; Fordyce, 2006). Indeed, phenotypic plasticity seems decisive in evolutionary terms under heterogeneous conditions (Price et al., 2003), as higher plasticity might improve plant adaptation to prevailing climatic conditions (Chevin et al., 2013). In other organisms, it has been observed that heat tolerance may be related to lower phenotypic plasticity in response to heat stress (Kelly et al., 2017). But although ecological theory favors this idea, empirical evidence on field crops are virtually inexistent. In an agronomic context, lower plasticity would confer resilience, but would also reduce responsiveness to improvements in growing conditions (e.g. more adequate management). Then, it may be a potential conflict between the degree of plasticity required to exploit yield potential and that for being resilient to abiotic stresses. However, it is only a "potential" conflict because the mechanisms involved in the plasticity to changes in availability of resources (most of what management does) and in signals (such as temperature, and particularly heat waves) might well be different and it could be potentially possible to combine high plasticity in response to resources (required for yield potential) with low plasticity in response to signals (likely required for resilience); and particularly if extreme events such as heat waves are considered. This has not been studied in field crops yet. The use of the concept of phenotypic plasticity has been used recently to understand better the complex relationships between yield components based on evolutionary and breeding constraints (Peltonen-Sainio et al., 2011; Sadras and Rebetzke, 2013). Slafer et al. (2014) recently proposed a framework to analyse the genetic control and environmental modulation of yield components of wheat depending on whether coarse or fine regulations of yield were expected, based on previous ideas on the likely differences in plasticity of yield components (Sadras and Slafer, 2012). Therefore, in this Thesis, the possible causes of the changes, trade-off and plasticity between GN and AGW will be study through (i) wheat genotypes with contrasting GN and AWG, (ii) different environmental effects such as amount of resources (through nitrogen availability) or signals (short periods of heat waves).

1.5. Objectives

The general aim of this Thesis is to analyse and characterise quantitatively the phenotypic plasticity of yield and of their physiological determinants of wheat elite material selected for differing in yield determination components. For this purpose, the objective is divided into two main studies that include understanding the trade-off and plasticity between GN and AWG under different source-sink ratios with:

I. different nitrogen availability (**Chapter III**)

II. in response to transient heat waves at pre- or post-anthesis (**Chapter IV**).

III. elucidate whether enhanced resource availability at pre-anthesis brings about more resilience in grain filling in response to a transient heatwave, and also to identify whether wheat yield components can show some degree of acclimation in response to a transient heatwave (**Chapter V**).

The first hypothesis was that trade-off between GN and AGW would be

- mainly due to non-competition mechanisms and it would be more relevant to the intrinsic weight potential of grains.

The second hypothesis was that transient heat waves would

- reduce GN and AGW with different magnitude at each growing period,
- mostly affect sink strength rather than source strength during grain filling as the trade-off between main yield components would not be relevant to assimilates availability.
- affect yield components with more effect on genotypes with less efficiency in determining that component (i.e. a cultivar more efficient in setting grains would be less sensitive to heat in pre-anthesis, when GN is being determined; whilst a cultivar with more efficiency in determining AGW would be less sensitive to heat in post-anthesis)
- not be additive when wheat was exposed to heat waves at early growing stage, and it might be able to provide sort of priming effect on grains when imposed at early stage of development.

The responses will be studied through analyzing:

- yield and yield components
- weight of individual grains of the population of grains in the crop and studying the frequency of distributions of sizes determining average grain weight.
- weight of particular grains located at specific positions of the spikes characterised for contrasting grain size potential (both across and within spikelets).

1.6. Outline of the Thesis

The present doctoral Thesis is divided into seven chapters. These chapters include a general introduction and the main objectives (this Chapter I), followed by general procedures with the methodology used in most experiments throughout the experimental chapters of the thesis (Chapter II) reported in each of the three experimental chapters (Chapters III, IV, V) and a final chapter of general discussion and conclusions (Chapter VI). In addition, there are two Annexes (I and II) that report details of experimental procedures in the different experimental Chapters.

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Chapter II

General procedure

2. Chapter II: General procedure

2.1. General conditions and treatments

Seven field experiments were carried out in this Thesis, from 2015/2016 to 2019/2020 growing seasons. Experiments I, II, III, IV and VI were carried out at within Bell-lloc d'Urgell (41.64ºN, 0.79ºE), and experiments V and VII were carried out in the premises of the School of Agronomy, University of Lleida (41.63ºN, 0.60ºE). Both locations within Catalonia, North-East Spain.

- **Experiments I, II and III (Chapter III)** were performed during 2015/2016 to 2017/2018 growing season nearby Bell-lloc d'Urgell. Twenty modern elite wheat cultivars (*Triticum aestivum L.*) were sown in 2015/2016 growing season (Exp. I), and five modern elite wheat cultivars were chosen from Exp. I to sow in the following two experiments 2016/2017 and 2017/2018 growing seasons (Exps. II and III). The nitrogen (N) treatments consisted in two contrasting N amounts (N_0 = unfertilised, N_1= 200 kg·N ha^{-1}) broadcasted as urea (46-0-0) just before the onset of stem elongation stage; DC3.0 (Zadoks et al., 1974) in all three experiments (Table 2.1). In addition, in experiments II and III, three source- sink manipulation treatments (unmanipulated control, defoliation, de-graining) were performed.

- **Experiments IV (Chapter IV)** were performed during 2018/2019 growing season nearby Bell-lloc d'Urgell and Lleida (ETSEA campus), respectively. Treatments consisted in two wheat genotypes (chosen from Exps. II and III), pre- and post-anthesis heat stress and the same three source-sink manipulations during the effective period of grain filling than in Exps II and III, under the contrasting heat treatments (Table 2.1). The two cultivars were selected as showing contrasting yield components; Pistolo (low grain number per m^{-2}, GN but high average grain weight, AGW) and Sublim (high GN and low AGW). Heat wave treatments consisted of an unheated and two heated treatments of 7 effective days each (one starting at DC4.3 -early booting-, and the other one starting at 15 days after anthesis –c. DC7.5). An effective day of heat treatment was considered as a fully/mostly sunny (as the heat is produced through a "glasshouse effect" requiring incoming radiation); i.e. very cloudy/rainy days, in which temperature was virtually not increased was disregarded when counting the number of days under treatment (in each timing of heat there was only one non-effective day both locations). Heat treatments were imposed by installing, over the designated plots, wood structures of 1.5 m height above the whole plot covered with transparent polyethylene film (Elía et al., 2018). These portable tents are a valuable resource to study heat effects in field conditions, particularly when electric power is not available, quantifying reliably the effects with negligible confounded effects (see **Annex 1**: **Are portable polyethylene tents reliable for imposing heat treatments in field-grown wheat?)**. Temperature sensors (connected to dataloggers EM5b Decagon Devices) were set up inside and outside the structures, at the height of the top of the canopy, to hourly record the temperature during the imposition of the heat treatments.

Table. 2.1. Details of the seven experiments sown with different genotypes and treatments in NE Spain from 2015-16 to 2019-2020.

Growing season	EXP	Locations	Plot size [sowing density m^{-2}]	Treatments			
				Genotypes	N application (kg N ha^{-1})	Source-sink manipulation	Heat stress
2015/2016	I	Bell-lloc	1.2m*4.0m [400 plants]	Acorazado, Aficion, Alabanza, Algido, ArthurNick, Avelino, Bologna, Diamento, Falado, Ingenio, Kilopondio, Marcopolo, Pistolo, Rebelde, Rimbaud, Sar32, Solehio, Star, Sublim, Tocayo	$N_0 = 0$ $N_1 = 200$	*N/A*	*N/A*
2016/2017	II	Bell-lloc	1.2m*4.0m [400 plants]	Avelino, Ingenio, Marcopolo, Pistolo, Sublim	$N_0 = 0$ $N_1 = 200$	**[DAA[+] 10]** Unmanipulated, Defoliation[a], De-graining[b]	*N/A*
2017/2018	III	Bell-lloc	1.2m*6.0m [380 plants]	Avelino, Ingenio, Marcopolo, Pistolo, Sublim	$N_0 = 0$ $N_1 = 200$	**[DAA 10]** Unmanipulated, Defoliation, De-graining	*N/A*
2018/2019	IV	Bell-lloc	1.2m*4.0m [400 plants]	Pistolo, Sublim	$N_1 = 200$	**[DAA 14]** Unmanipulated, Defoliation, De-graining	pre-AN HT[c] post-AN HT
2018/2019	V	Lleida	1.2m*4.0m [400 plants]	Pistolo, Sublim	$N_1 = 200$	**[DAA 14]** Unmanipulated, Defoliation, De-graining	pre-AN HT post-AN HT
2019/2020	VI	Bell-lloc	1.2m*4.0m [380 plants]	Pistolo, Sublim	$N_1 = 200$	**[DC 3.0]** Unthinning, Thinning **[DAA 14]** Unmanipulated, Defoliation, De-graining	pre-AN HT post-AN HT
2019/2020	VII	Lleida	1.2m*4.0m [380 plants]	Pistolo, Sublim	$N_1 = 200$	**[DAA 14]** Unmanipulated, Defoliation, De-graining	pre-AN HT post-AN HT Double HT

[+] *DAA: Days after anthesisa Flag leaf and second leaf were removed per plant.*
[b] *All grains from half spike were removed per plant.*
[c] *Pre-AN HT: Heat stress imposed at pre-anthesis; Post-AN HT: Heat stress imposed at post-anthesis; Double heat HT: Heat treatment twice at both pre- and post-anthesis*

■ **Experiments VI (Chapter V)** were performed during 2019/2020 growing season nearby Bell-lloc d'Urgell and Lleida (ETSEA campus). Treatments consisted in (i) the same two wheat genotypes from the previous experiments, (ii) pre- and post-anthesis heat stress and (iii) the same three source-sink manipulations during the effective period of grain filling under the contrasting heat treatments (Table 2.1). In addition, to increase source strength at pre-anthesis, a thinning treatment at stem elongation stage; DC3.0. was performed. The thinning consisted in removing alternate rows in the assigned plots. Heat wave treatments were performed with the same portable tents as Experiments IV and V, but the duration of the treatments was assigned a similar head load, i.e. refers to hourly temperature difference between heat and control temperatures (ºC h; accumulated heat, Wardlaw et al., 2002) at both pre- and post- anthesis. In addition, at Lleida, a third heat treatment (double heat wave) was imposed at both periods (DC 4.3 and DC 7.5), in order to elucidate whether the effect of heat stress is additive during the growing period, or if its magnitude may be pre-empted by previous heat stress (bringing about some degree of acclimation due to a sort of priming).

All experiments in each growing season were irrigated and fertilised according to the particular experimental design in Exps I, II and III and, with only 200 kg N ha^{-1} in Exps. IV and V. Experiment plots were kept always free of weeds, insect pests, and disease by applying the recommended chemical measures.

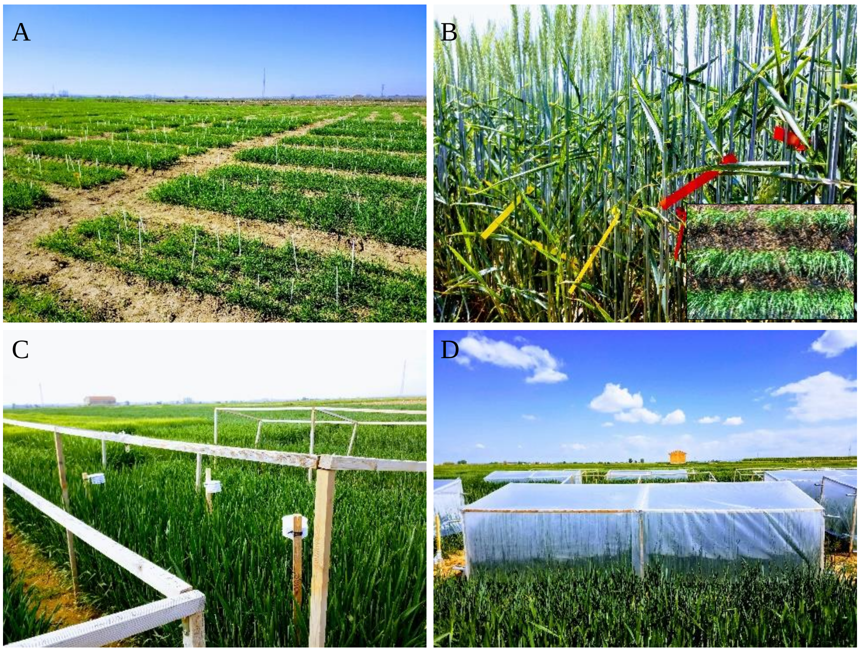

Fig. 2.1. Illustration of field experiments carried out on this Thesis. Overview of the experimental field (A), source-sink manipulation; defoliation (yellow tag), de-graining (red tag) and thinning (insert) (B), and the structure of transient heat treatment (C) and the treatment of heat stress at different growing stages (D).

2.2. Measurement and analysis

2.2.1. Above ground biomass, partitioning and yield

Total biomass was obtained by sampling 1m-linear of a central row (previously labeled to have the exact plant density with seedlings uniformly distributed). The sample was cut at the soil level for lab processing, at anthesis (DC65) and at physiological maturity (DC 95). At maturity, plants with source-sink manipulated (three defoliated, three de-grained, and three unmanipulated controls) were separately harvested to analyse the change of grain weight in response to assimilate availability. Plants sampled at both 1m-linear and source-sink manipulated were (i) divided into main shoots and tillers respectively, and then further divided into spikes and rest of the above-ground biomass, (ii) oven-dried for at least 48 hours at 65 ºC, and (iii) weighed. At maturity, the spikes were threshed, the number of grains counted and, after being oven-dried again, weighed from which we determined total grain and chaff weights.

2.2.2. Individual grain weights

All grains from the maturity sample were scanned by MARViN ProLine seed analyzer (MARViTECH GmbH, Germany). The digital image scanning provided morphometric measurements (width, length, and area) for each individual grain. In order to be able to convert the measured area of each grain to individual weight, a single model equation to trustworthily convert grain linear dimensions to weight for wheat grains was generated. For this purpose, a set of data to establish linear relationships between dry weight and either length, width, or area of individual grains of different wheat cultivars grown in different experiments were used to select a calibrated model to be used to predict grain weight from 2-D images, and then validated the calibrated model with independent data (including using cultivars and environmental factors not considered in model calibration) (see **Annex II**: **Weight of individual wheat grains estimated from high-throughput digital images of grain area).** Through this equation**,** I was able to determine not only AGW for each particular treatment combination, but also analysed the dry weight distribution of the individual grains composing that AGW (allowing to determine whether any effects detected in AGW were due to effects on all grains or in a particular size fraction of the population).

2.2.3. Grain mapping process

In the nine plants per experimental unit reserved for detailed analysis of the responses to source-sink manipulations, I determined not only the responses of AGW but also separated and weighed individually each of the grains of three specific spikelet positions in the spikes (basal, central, and apical spikelets); weighing them with a 0.01 mg precision balance (VWR International, SMG 2285Di-ION, Italy). Individual grains within each of these spikelets were named according to their position with respect to the rachis from G_1 (the most proximal to the rachis), through G_2 (the second most proximal to the rachis) to G_n (the most distal grain in that spikelet, which could have been G_2, G_3, G_4, or G_5, depending on the specific spikelet and treatment, as described in detail by Acreche and Slafer (2006).

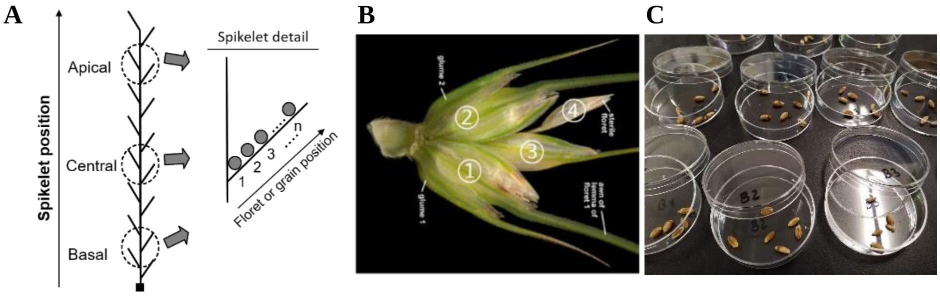

Fig. 2.2. Illustration of grain mapping process. General scheme of grain mapping from Miralles and Slafer, (1995) (A), Grain position within a spikelet (Image source: Iowa State Univ.) (B), and grain separation per different position of grains within a spikelet, and of spikelet in a spike (C).

2.2.4. Source-sink manipulations

For source-sink manipulation treatments, 9 independent uniform plants (having similar canopy height, flowering time, number of spikelets) from central rows of each main plot were selected, and their main shoots labeled at anthesis (DC 65; and randomly 3 of them were conducted by each source-sink manipulation (unmanipulated, defoliation and de-graining) after 10 (Exps II, III) or 14 (Exps IV, V, VI, VII) days after anthesis.

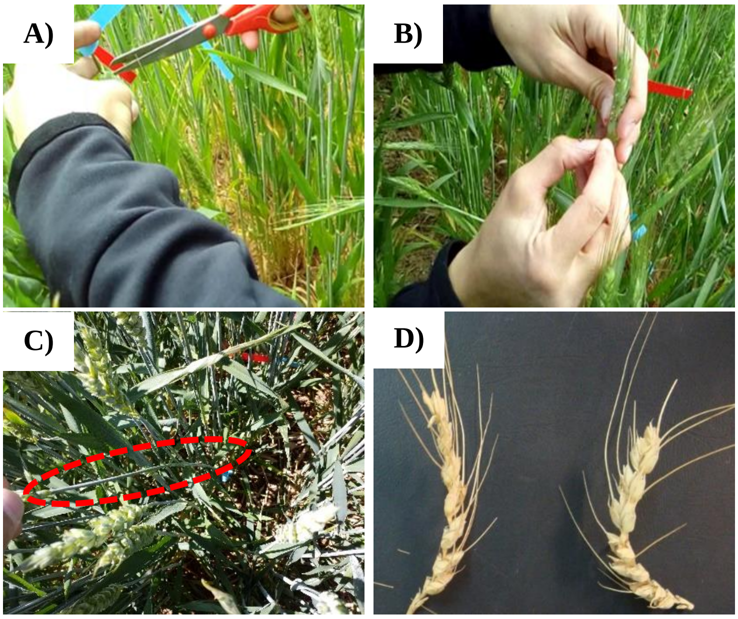

Fig. 2.3. Illustration of source-sink manipulation at post-anthesis. The process of source manipulation (A, C) and sink manipulation (B, D). The red circle highlights a stem without first and second leaves after defoliation.

AGW at main stem well-represented AGW of the whole plant, and therefore only in main stem, source and sink strength were altered. Sink size was modified by removing half of the spikelets and defoliation consisted in removing the laminae of the two top leaves for reducing source-strength per grain following the methodology of Sanchez-Bragado et al. (2020). Thinning treatment was performed by removing alternate rows in the plots at stem elongation stage to increase source strength at pre-anthesis.

2.3. References

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Chapter III

Physiological bases for the trade-off between grain weight and grain number across modern wheats under contrasting soil N conditions

3. Chapter III: Physiological bases for the trade-off between grain weight and grain number across modern wheats under contrasting soil N conditions

3.1. Introduction

In the past decades, the introgression of *Rht1* and *Rht2* alleles into the standard height (tall) wheat resulted in semi-dwarf wheat genotypes, and as the result of dwarfing genes, wheat yield was improved through better biomass partitioning (Slafer, 2003; Fischer, 2007; Álvaro et al., 2008), increasing grain number and harvest index (Siddique et al., 1989; Slafer and Andrade, 1993; González et al., 2005), but not grain weight. It became then clear that individual grain weight was less responsive to the change of assimilate availability, and it was concluded that wheat grain yield during grain filling is mostly sink-limited (Slafer and Savin, 1994; Borrás et al., 2004; Serrago et al., 2013; Slafer et al., 2015) and therefore, increasing sink size (chiefly grain number) would increase the yield potential (Fischer, 2007; Reynolds et al., 2007; Slafer et al., 2015). As the result of such a breeding effort with increasing grain number per m^2 (GN) with slight reductions of average grain weight (AGW), the modern elite wheat genotypes had a greater GN per spike than the old genotypes (Cartelle et al., 2006), improving wheat yield for the last decades. However, further increasing GN might less improve grain yield if the source-sink balance would have been modified due to the consistent increase in sink-strength with no increases in source-strength. For example, biomass partitioning to increase spike dry weigh (SDW) seems not a major alternative to further increases yield in most breeding programmes (Slafer, 2003), and due to the current breeding strategy of increasing GN, some degree of source-limitation might have appeared (Kruk et al., 1997; Calderini et al., 2006; Álvaro et al., 2008). Also, greater GN with proportionally smaller grains (i.e. in distal positions) (Slafer and Savin, 1994; Calderini and Reynolds, 2000; Acreche and Slafer, 2006) would have negative effects on seedling vigour, resistance against environmental stresses, and final grain quality such as a lower nutrient concentration in grains (Calderini and Ortiz-Monasterio, 2003; Lizana et al., 2010). Therefore, future breeding programs, may need to explore alternatives to increasing grain size (though increasing grain size potential or through alleviating any source-limitation that might have been produced) to keep improving grain yield (Lizana et al., 2010).

The concept that yield is most frequently sink-limited during grain filling, does not get along well with the fact that a negative relationship between AGW and GN is commonly found when genotypic or management factors increase GN. As the critical period to determine GN and AGW was minimally overlapping during wheat growth (Slafer et al., 2014), this negative relationship might not be involved in feedback process (Slafer, 2003; Acreche and Slafer, 2006); i.e. changes in grain growth during the effective period of grain filling cannot be compensated by adjustments in GN. Generally, this negative relationship has been interpreted as reflecting a competitive mechanism (i.e. a true trade-off between GN and AGW), though non-competitive alternatives, representing only an apparent trade-off are possible (Slafer et al., 2014). Ascertaining whether the negative relationship driven by genotypic factors is due to competition or not would be critical to justify (or otherwise) that further increasing GN might be still a good strategy (Foulkes et al., 2011; Serrago et al., 2013).

Furthermore, whether or not growth of grains of particular wheats are mainly sink-limited during grain filling may depend on the background environmental condition. For example, Quintero et al. (2018) analysed GxE interaction concerning the trade-off between GN and AGW using various elite CIMMYT varieties in both Mexico and Chile. In Mexico, GN was strongly related to AGW showing the negative relationship, and wheat cultivars showed a lack of association between grain yield and GN due to a strong trade-off between GN and AGW, whereas, in Chile, the trade-off between GN and AGW was seemingly more apparent than real, showing a positive association between grain yield and GN. These results indirectly imply that perhaps the physiological bases for a negative relationship between AGW and GN when comparing genotypes might depend on the background environmental condition. If so, perhaps in regions with lower chances of successfully exploiting increases in GN, attempting to increase AGW might be a better breeding strategy. In this context, it would be a key point to quantify the magnitude of source or sink limitation in modern elite wheat cultivars, and plasticity of wheat yield components in response to environmental conditions to understand the putative traits related to GN and AGW in modern, elite genotypes of wheat. Therefore, the main objective of this research was to elucidate whether the reduction in AGW in response to genetic increases in GN represents a case of source-limitation under low or high solid fertility conditions.

3.2. Materials and Methods

I carried out field experiments over three consecutive years, from the 2015/2016 to the 2017/2018 growing seasons, at Bell-lloc d'Urgell (41.64ºN, 0.79ºE) in Catalonia, North-East Spain. The experiments were sown within the optimal sowing period for the region (Table 1) at a high density (400, 400 and 380 viable seeds m^{-2}, respectively). In order to minimise experimental error through maximising uniformity of the plot when sampling, soon after seedling emergence and well before the onset of tillering, I labelled in each plot few areas of 1 m of a row (excluding borders), that were designated to be sampled at maturity. In those sampling areas, as well as in the rows surrounding them, I thinned manually the rows leaving 50 seedlings uniformly distributed both within the row and in size (i.e. leaving seedlings that emerged simultaneously), representing a stand density of 250 plants per m^2 across all three experiments. The soil type in Bell-lloc was a typic Calcixerept fine-loamy over sandy skeletal, mixed and thermic (SSS, 1999).

All experiments were designed comprising a factorial combination of a number of genotypes and two contrasting soil nitrogen levels, being these combinations of genotypes x N assigned to main plots. In the last two experiments the [fewer] cultivars x N treatments were further combined with three levels of source-sink manipulations assigned to subplots (Table 1).

Table 3.1. Description of the experimental treatments for the experiments carried out over three growing seasons.

Growing seasons	Plot size [Sowing date]	Treatments		
		Genotype [# of genotypes]	**N application**	**Source-sink manipulation** [+]
2015/ 2016	1.2 m x 4.0 m [20/11/2015]	Acorazado, Aficion, Alabanza, Algido, ArthurNick, Avelino, Bologna, Diamento, Falado, Ingenio, Kilopondio, Marcopolo, Pistolo, Rebelde, Rimbaud, Sar32, Solehio, Star, Sublim, Tocayo [20]	N_0 = unfertilised N_1= 200 Kg·N ha^{-1}	Unmanipulated
2016/ 2017	1.2 m x 4.0 m [16/11/2016]	Avelino, Ingenio, Marcopolo, Pistolo, Sublim [5]	N_0 = unfertilised N_1= 200 Kg·N ha^{-1}	Unmanipulated, Defoliated [a], De-grained [b]
2017/ 2018	1.2 m x 6.0 m [17/11/2017]	Avelino, Ingenio, Marcopolo, Pistolo, Sublim [5]	N_0 = unfertilised N_1= 200 Kg·N ha^{-1}	Unmanipulated, Defoliated, De-grained

[+] *These treatments were imposed 10 days after anthesis.*
[a] *Flag leaf and second leaf were removed,* [b] *All grains from half spike were removed (through removing all spikelets on one side of the spike).*

In the first growing season (2015/2016), we grew 20 modern commercial cultivars, representing the main pool of elite wheats used by breeders in crosses when aiming to further increase yield, under two contrasting levels of soil N availability (N_0 = unfertilised, N_1= fertilised with 200 Kg·N ha^{-1}, applied just before the onset of stem elongation; stage DC3.0 in the scale of Zadoks et al., 1974) (Table 3.1).

In the two following growing seasons a selection of 5 of these cultivars was grown under the same contrasting N conditions but adding post-anthesis source-sink manipulation treatments. For the latter treatments, 9 independent uniform plants (having similar plant height, flowering time, and spike size) from central rows of each main plot were selected and their main shoots labelled at anthesis, and 3 of them were assigned to each source-sink manipulation: an unmanipulated control, a defoliated and a de-grained treatment imposed 10 days after anthesis.

For the defoliation treatment, I removed either the laminae of the two top leaves (the flag and second leaves; reducing the source-strength per grain through defoliation), while the de-graining consisted in removing all spikelets on one side of the spike (reducing sink-strength by halving the number of growing grains during the effective period of grain filling while not altering the distribution of different grains sizes of the spike, as described in detail in Sanchez-Bragado et al. 2020).

The other three plants were left unmanipulated as controls for this particular treatment. The plot size was 1.2 m wide (6 rows 0.20m apart) and 4 m long in the first two seasons and 6 m long in 2017/2018 season.

At physiological maturity, I sampled a 1 m of a central row (that had been previously labelled to have the exact plant density with seedlings uniformly distributed; see above), cutting at the soil level for lab processing. In addition, at maturity I did also sample the plants representing the three different post-anthesis source-sink treatments in each experimental unit.

Plants were divided into spikes and rest of the above-ground biomass, (ii) oven-dried for 48 hours at 65ºC, and (iii) weighed. The spikes were then threshed, the grains counted and, after being oven-dried again, weighed. Then all grains were scanned in a MARViN ProLine seed analyser (MARViTECH GmbH, Germany). This digital image scanning provided morphometric measurements (width, length, and area) for each individual grain, and we converted the measured area of each grain to individual weight of each grain using a validated equation (Kim et al., 2021; see Annex II of this Thesis). Thus, I determined not only AGW for each particular treatment combination, but also analysed the dry weight distribution of the individual grains composing that AGW (allowing to determine whether any effects detected in AGW were due to effects on all grains or only on a particular size fraction of the population).

In the nine plants per experimental unit reserved for detailed analysis of the responses to source-sink manipulations, I determined not only the responses of AGW but also separated and weighed individually each of the grains of three specific spikelet positions in the spikes (basal, central, and apical spikelets); weighing them with a 0.01 mg precision balance (VWR International, SMG 2285Di-ION, Italy).

Agronomic components were analysed by ANOVA and the effect of source-sink alteration was analysed by split-plot using JMP software (*version 14, SAS Institute Inc., NC*) to elucidate the effects of treatments, genotypes, and their interaction. Multiple comparisons of studied variables were made using LSD test. The normal distribution of individual grains was graphically represented using RStudio (*RStudio Team 2020*). The significance level of α=0.05 was used in all statistical models.

3.3. Results

3.3.1. Genotypic variation in, and relationship between, GN and AGW under the contrasting N conditions

Despite that all genotypes were modern and well adapted cultivars, there was a relevant degree of genotypic variation at either of the two soil N levels for yield (Fig. 3.1A) and for its both major components: GN (Fig. 3.1B) and AGW (Fig. 3.1C). GN and yield were highly responsive to the level of nitrogen availability (55.0 and 58.7%, respectively averaging across all cultivars), while AGW did virtually not respond (avg. 2.2% across all cultivars) (Fig. 3.1).

Consequently, overall sources of variation, yield was very strongly related to GN (Fig. 3.2A) whilst the relationship with AGW was not significant (Fig. 3.2B). Naturally, the soil N availability was a major driver for these relationships. Focusing on the genotypic differences within each N level, the relationships were still stronger with GN (r = 0.64 $_{p<0.01}$ and 0.61 $_{p<0.01}$ for N_0 and N_1, respectively) than with AGW, but the latter were not negligible anymore (r = 0.40 $_{p<0.10}$ and 0.45 $_{p<0.05}$ for N_0 and N_1, respectively).

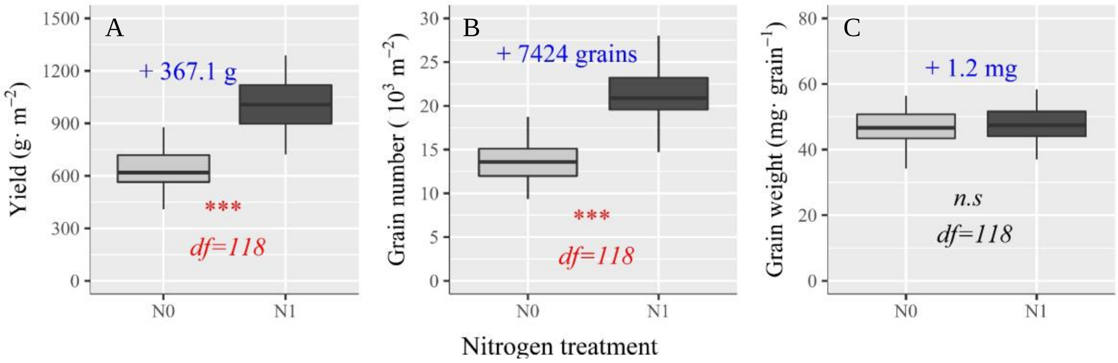

Figure 3.1. Boxplots of yield (A) and its two major components, GN (B) and AGW (C) representing the degree of variation across the 20 cultivars at each level of soil N availability; N_0 (unfertilised) and N_1 (fertilised with 200 kg N ha^{-1}). The difference value between N_0 and N_1 was inserted in each panel. The significance of the N effect (*** p<0.001; *n.s.* = not significant) is indicated inset each panel.

When comparing within each N level, there was a negative relationship between AGW and GN (Fig. 3.3A). To infer whether this trade-off reflects an increased competition among growing grains in cultivars with larger number of grains or a constitutive genotypic difference, I analysed whether the differences in AGW were related to differences in either all grain size classes or only a particular size class.

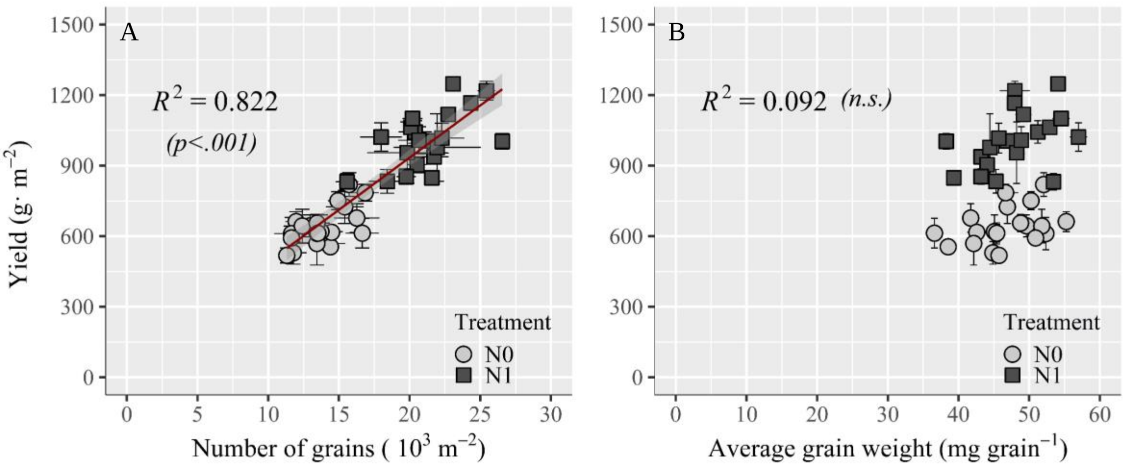

Figure 3.2. Relationship between yield and either GN (A) or AGW (B) across the 20 cultivars at each level of soil N availability; N_0 (unfertilised) and N_1 (fertilised with 200 kg N ha^{-1}). Inset are the coefficients of determination for the linear regression for the whole dataset. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001; *n.s.* = not significant).

It seemed clear that genotypic differences in AGW were related to the differences in the size of their lightest (Fig. 3.3B) and heaviest (Fig. 3.3C) groups of grains.

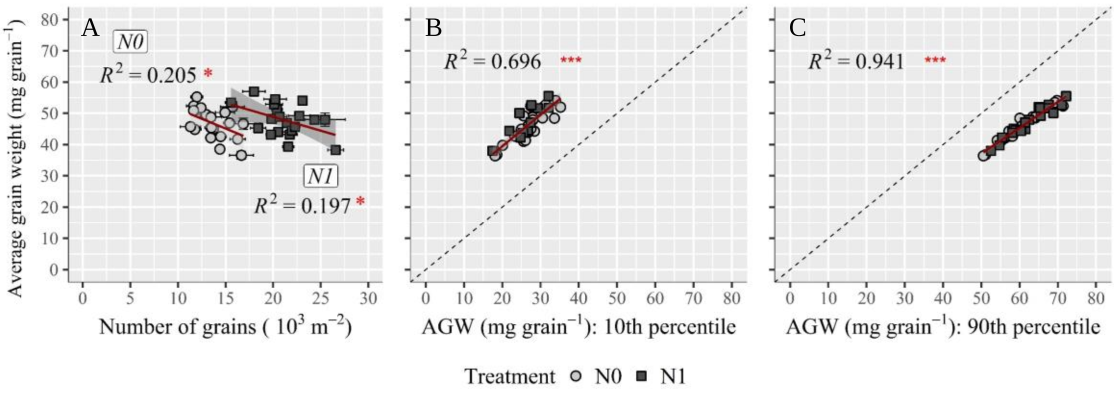

Figure 3.3. The negative relationship between GN and AGW across 20 cultivars (A); and correlation between overall AGW (y-axis) and AGW of 10th (B) and 90th (C) percentile (x-axis) of each cultivar in 2015-2016 growing season under N_0 (0 kg N ha^{-1}) and N_1 (200 kg N ha^{-1}) condition. Dashed lines in panels B and C are the 1:1 ratio where actual average grain weight and 10th or 90th percentile's average weight are equal. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001).

Moreover, when the distribution of the individual grain weights was compared between cultivars with the extreme values of AGW (i.e. Bologna for N_0 and Rebelde for N_1 as the cultivars with lowest AGW, and Ingenio as that with largest AGW for both N_0 and N_1), it seemed clear that the genotypic differences were due not only to differences in the size of the lightest grains, but also to the differences in size of the heaviest grains, likely representing the potential grain size (Fig. 3.4A,B). Thus, when both 10th and 90th percentile of grain weight of each of these cultivars were compared, that with the lowest AGW had also significantly smaller grains corresponding to both of these percentiles under both N_0 (Fig. 3.4C) and N_1 (Fig. 3.4D) conditions than the cultivar with the greatest AGW.

The fact that all grains are smaller in the cultivar with lower AGW may imply that differences are due to competition among grains or constitutively related to genotypic differences in potential grain weight. To resolve the issue is relevant as it implies whether or not breeding shall direct efforts to increase post-anthesis source strength or should keep increasing post-anthesis sink strength further. For that purpose, it is necessary to carry out more detailed studies which in turn requires narrowing down the cultivars studied. I then selected 5 of the cultivars tested in this first experiment and combined them not only with two N levels but also with three source-sink manipulations imposed at the onset of grain filling. The cultivars selected were those exhibiting a clear negative relationship between AGW and GN while grouped within the cultivars with higher AGW for each level of GN, in order to maximise the likelihood of identifying source limitation for grain growth should it be a real factor. These cultivars were Avelino, Ingenio, MarcoPolo, Pistolo, and Sublim (Fig. 3.5).

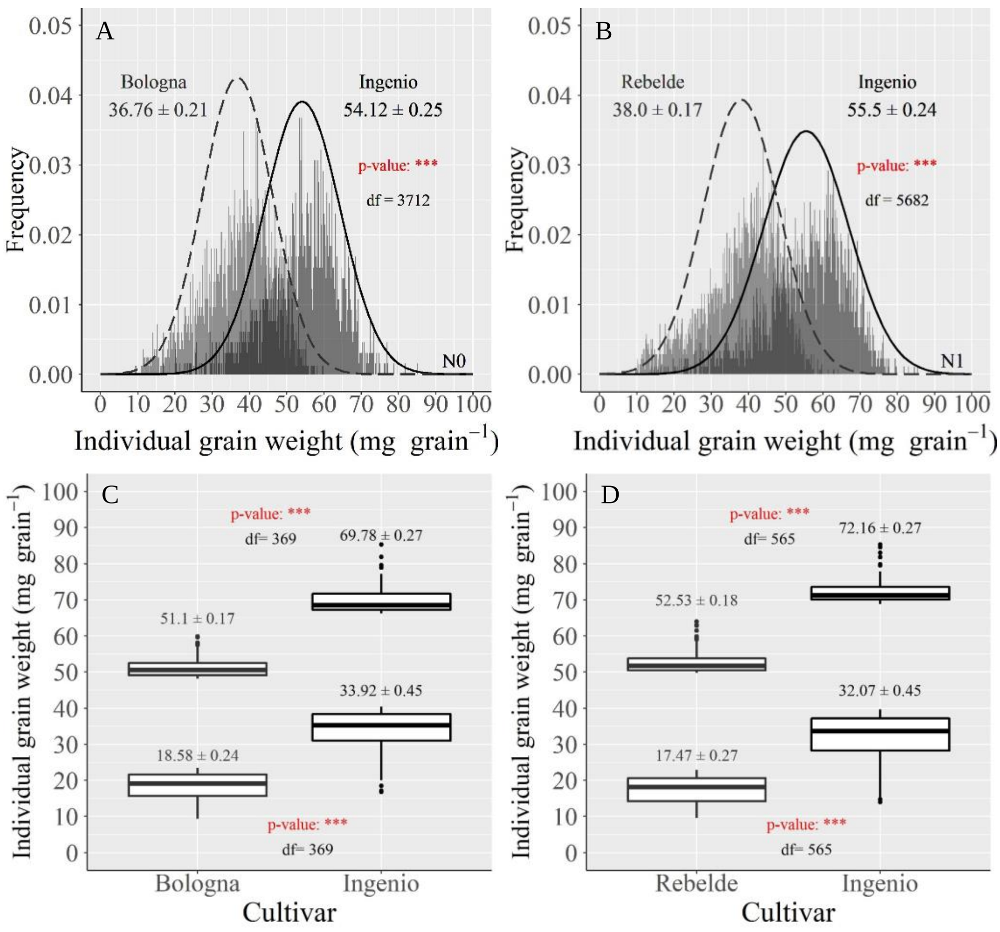

Figure 3.4. The frequency distribution of cultivar with lowest AGW (dotted line) and greatest AGW (plain line) in N_0 (A) and N_1 (B) conditions; and boxplots of both 10th and 90th percentiles of the two cultivars in N_0 (C) and N_1 (D) conditions. In panel A and B, dotted lines indicate Bologna and Rebelde which are the cultivar with lowest AGW in N_0 and N_1 conditions respectively; and black solid line indicates Ingenio which is a cultivar with the greatest AGW in both N_0 and N_1 conditions. In panel C and D, boxplots in bottom indicate 10th grain weight of 10th percentile, while boxplots on top indicate grain weight of 90th percentile.

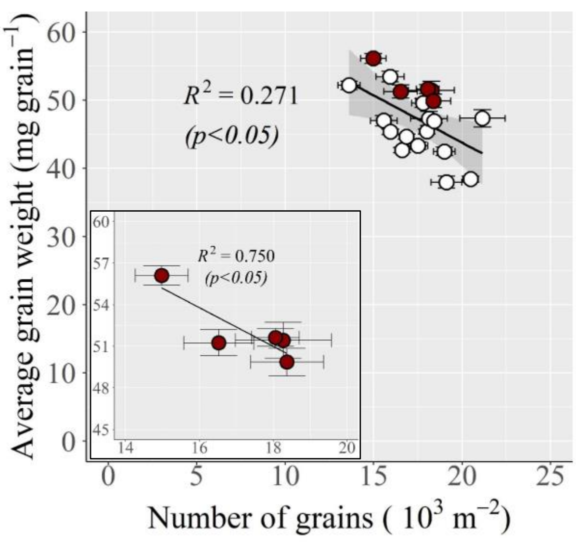

Figure 3.5. The negative relationship between GN and AGW across 20 cultivars averaged across the two N levels, highlighting the five cultivars selected for the more detailed studies in the following two growing seasons (Red colour in the main figure and illustrated with a more detailed scale in the inset). Lines fitted by linear regression.

3.3.2. Plasticity of GN and AGW under the contrasting N conditions across 5 cultivars (2016-2017 and 2017-2018 season)

In both seasons, again GN and grain yield were highly responsive (58.0 and 55.3%, respectively averaging across five cultivars), while AGW did not respond (avg. -1.1% across five cultivars) to the nitrogen condition, as I found in the 2015-2016 growing season. Nitrogen was the main source of variation in GN and grain yield, while genotype was the main source variation in AGW. I analysed data with the average of two nitrogen conditions (N_0+N_1) with two growing seasons (2016-2017 and 2017-2018) to focus on the genotypic variation. In line with expectations when the cultivars were selected, there was a negative correlation between GN and AGW across all cultivars (Fig. 3.6A). Ingenio and Pistolo showed the greatest AGW but the lowest GN, while Sublim and MarcoPolo showed the greatest GN but the lowest AGW (Fig. 3.6B,C).

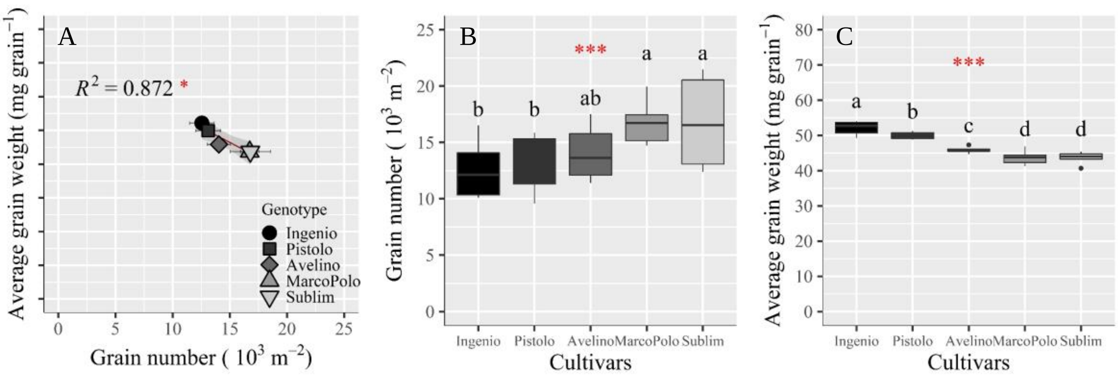

Figure 3.6. Negative relationship between grain number (GN) and average grain weight (AGW) across all genotypes (A); and the difference of GN (B) and AGW (C) among genotypes. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001). Data were averaged from 2016-2017 and 2017-2018 growing season with two nitrogen conditions.

To elucidate this negative relationship between GN and AGW is whether due to competition among growing grains or not, firstly I compared the weights of the individual grains that compose the AGW. Ingenio showed the greatest AGW among five cultivars, and therefore Ingenio was compared with the other cultivars to analysed the distribution of grain weight. The difference of AGW between Ingenio and Sublim seemed that all grain sizes were reduced, indicating that both heavier and lighter grains become smaller in Sublim (Fig. 3.7D). This result was also similar with Avelino (Fig. 3.7B) and MarcoPolo (Fig. 3.7C), but in Pistolo, the difference of AGW was minor and it seemed that only few smaller grains increased, compared with Ingenio (Fig. 3.7A). To elucidate whether or not cultivars with more GN contains more distal grains in spikes, contributing to reduction of AGW, GN within each spikelet was measured. It seemed that GN was increased in most spikelets in cultivars with more GN, not only in spikelets located at extreme positions (basal or apical) within a spike (Fig. 3.7E). Also, to identify both heavier and/or lighter grains were actually reduced in cultivars with more GN, 10^{th} and

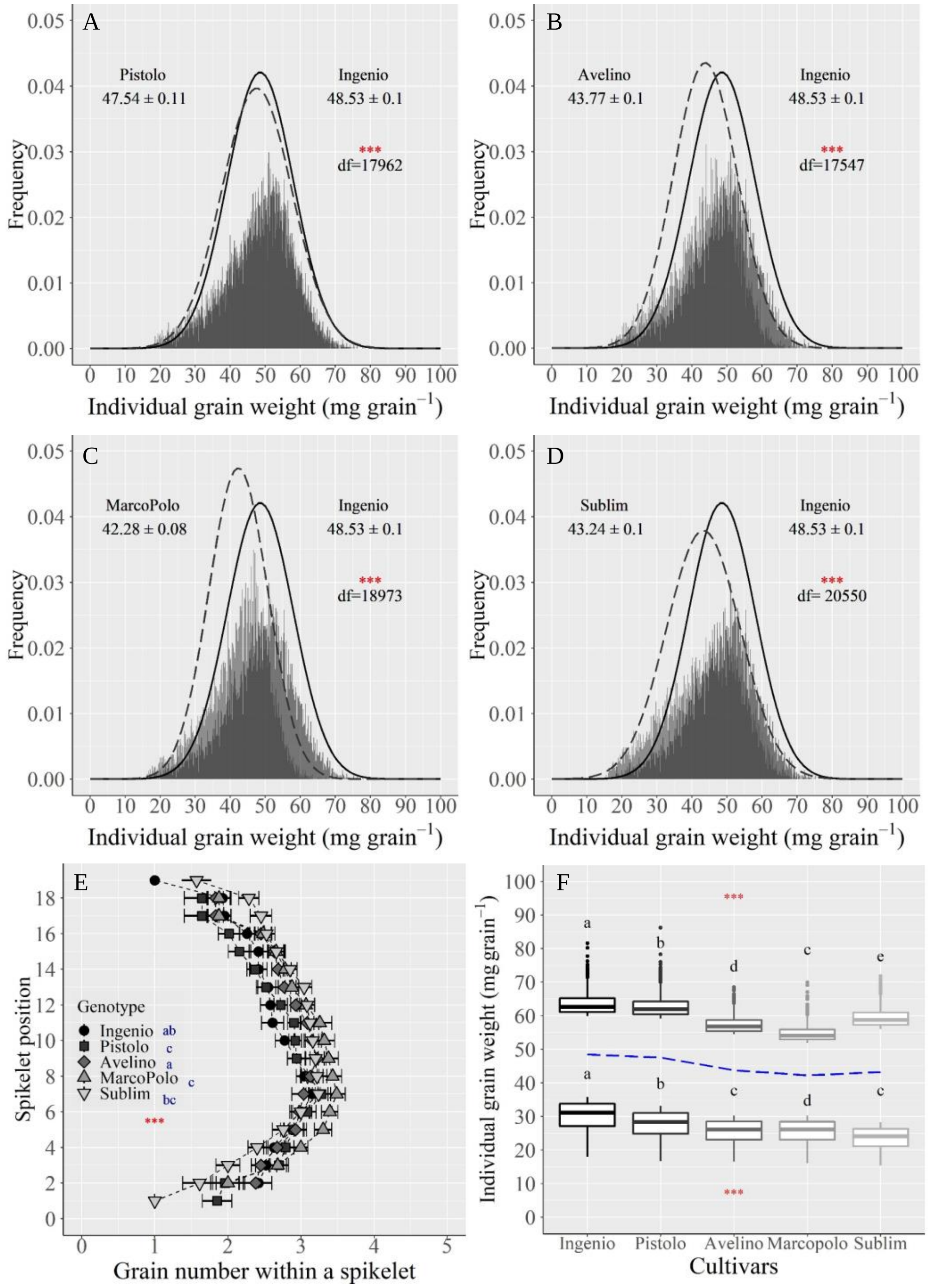

Figure 3.7. The frequency distribution between Ingenio and Pistolo (A), Avelino (B), MarcoPolo (C) and Sublim (D); and grain number within a spikelet position per spike (E); and difference of AGW at 10th and 90th percentile among cultivars (F). In panel A-D, plain line indicates Ingenio as control (heaviest average grain weight) and dotted line indicates each other cultivars to compare with Ingenio. In panel F, dotted blue line indicates the change of average grain weight when GN increased across cultivars; and top and bottom boxplots indicate 90th and 10th percentile of grains respectively. Asterisks indicates the significance of the effect (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Data were averaged from 2016-2017 and 2017-2018 growing season with two nitrogen conditions.

90th percentile of grain weight per cultivar were selected. All grains at both 10th and 90th percentile became significantly smaller in cultivars with more GN (Fig. 3.7F).

3.3.3. Responsiveness of grain weight to the change of assimilate availability during grain filling.

To determine whether the negative relationship between the two main yield components of wheat was due to competition for resources among growing grains or not, source-sink manipulation treatments were imposed during grain-filling. When assimilates were less supplied to grains (by defoliation) during grain filling, it reduced final grain weight among all cultivars, while more supplied assimilates (by de-graining) increased only in MarcoPolo and Sublim (Fig. 3.8A). However, in both cultivars, the magnitude of the increase was very minor in response to a rather severe treatment. Potential grain weight per each cultivar was regarded as grain weight achieved in the de-grained treatment: the greater the AGW in the de-grained spikes the higher the potential grain weight of the cultivar. Ingenio had the greatest potential grain weight, while Sublim and MarcoPolo had the lowest potential grain weight. (Fig. 3.8B insert). All data points were closed to a 1:1 ratio line (as differences were mostly not significant and when significant trivial in magnitude) (Fig. 3.8B). This result indicates that the massive increase in supply of assimilates per grain virtually did not affect final grain weight during grain filling.

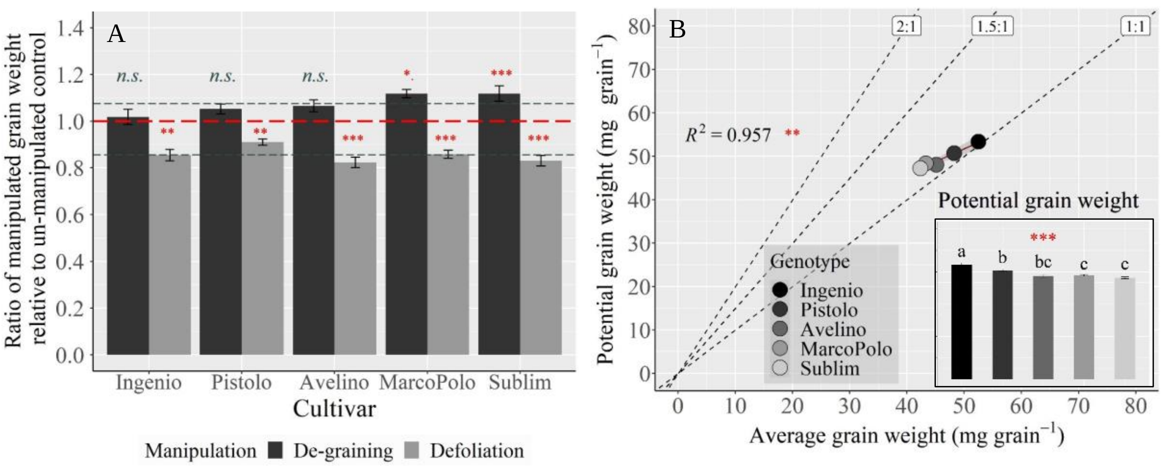

Figure 3.8. Relative value (%) of manipulated grains to control grains (un-manipulated) in response to source and sink manipulation across five cultivars (A); and the correlation between AGW and potential grain weight (B). In panel A, red dotted line indicates that grain weight of manipulated grains and control grains are the same (full sink limitation), and block dotted lines indicated average grain weight by defoliation and de-graining respectively. In panel B, dotted lines indicate the ratio of 100% 150% 200% between PGW and AGW respectively; and the bar graph inside the box indicates the difference of potential grain weight among cultivars. Asterisks indicates the significance of the effect (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; *n.s.* = not significant). Data were averaged from 2016-2017 and 2017-2018 growing season with two nitrogen conditions.

Therefore, the reduction of AGW when GN increases across cultivars did not represent competition among growing grains. The trade-off would have been mainly due to genotypic differences in intrinsic potential grain weight.

However, the reduction of assimilates during grain filling actually reduced final grain weight, which means that modern elite wheat cultivars even though still showing sink limitation for grain growth, they might be approaching to some degree of source limitation that might become relevant if further increases in GN are not followed up with similar increases in source-strength.

3.4. Discussion

3.4.1. Plasticity of GN and AGW

GN and AGW showed a noticeable difference in phenotypic plasticity (in response to the contrasting nitrogen condition). Across the three field studies, GN and yield showed to be very plastic, while AGW was extremely conservative. This is commensurate with most literature evidencing a very strong positive relationship between yield and GN in response to N (Fischer et al., 1993; Gaju et al., 2011). This is likely reflecting that (i) yield is strongly source-limited during the critical period for GN determination but mostly sink-limited during grain filling (Slafer and Savin, 2006), and (ii) that is why the critical period of yield determination is when GN is determined (Slafer, 2003; Borrás et al., 2004).

But the relevance of particular components in determining yield is not independent from the source of variation. Whilst GN was much more strongly affected by two contrasting soil nitrogen availability levels (F-ratio: 412.16) than by genotypic variation among modern and well adapted cultivars (F-ratio: 4.87), the genotypic effect was quite relevant for AGW (F-ratio: 59.47) in the 2015-2016 growing season in which many genotypes were grown. This main effect affecting GN and AGW was consistent among five genotypes of the following two seasons. The fact that in response to resource availability, AGW is a conservative (rather stable across most management practices) trait, while GN is far more plastic has been recurrently established (Peltonen-Sainio et al., 2007; Sadras, 2007; Sadras and Slafer, 2012; Sadras and Rebetzke, 2013; Slafer et al., 2014).

Although evidently less plastic than GN, AGW showed a significant degree of variation among elite genotypes, that was negatively related to the GN of the cultivars. Even though the effect of N availability on increasing GN might not have brought about competitive reductions in AGW (as N availability does also increase source-strength; actually improving yield through alleviating the level of source-limitation for GN determination), the nature of the relationship between AGW and GN might be different among modern cultivars. Indeed, differences in AGW may were relevant to explain part of the genotypic differences in yield, something that (i) is not evident when analysing wider levels of genotypic variation (e.g. when comparing semidwarf *vs* tall or modern *vs* old genotypes; Acreche, et al., 2008; Rebetzke et al., 2012), and (ii) is hidden when analysing all sources of variation together being the environmental factors normally of much stronger effect (Slafer et al., 2014). However, considering all elite lines, this variation may be relevant for suggesting AGW as a possible trait for further raising yield, if the genotypic differences are due to differences in their potential size rather than reflecting a consequence of increased sharing of limited assimilates to fill the grains when GN is increased.

In the present study of the causes for the genotypically-driven negative relationship, I observed that the reduction in AGW was not due to an increased proportion of small grains as when GN increased, discarding the most plausible explanation for such negative relationship not involving competition among grains (Miralles and Slafer, 1995; Acreche and Slafer, 2006). In the present study, cultivars with lower AGW not only had more grains of smaller size but also exhibited a clear difference in size of their largest grains (here the 90^{th} decile, or 10% heaviest grains of each cultivar): in general, all grain size classes were smaller in genotypes with more GN than inn those with less GN. This may be compatible with either (i) an increased competition among grains during grain filling when GN was increased genotypically, or (ii) that cultivars constitutively differed in AGW (they differ in potential grain size and then differences in AGW may well be independent of the level of competition among growing grains). This can be further clarified studying the responses of grain size to the manipulation of source-sink balances during the effective period of grain growth.

3.4.2. Grain weight in response to source-sink treatments.

The degree of limitation of grain growth by the source- or sink-strength was analysed through responses of AGW to defoliation and de-graining during the effective period of grain filling. The imposed source-sink manipulations proved that the trade-off between AGW and GN when comparing contrasting genotypes (all modern and well adapted) was not chiefly due to competition among grains. Increasing massively the assimilates supplied per grain (by c. 100%, through removing c. 50% of the grains to be filled) did not significantly affect, or only marginally increased, AGW. This result revealed that wheat yield in these experiments was mainly sink-limited during grain filling. And the trade-off between AGW and GN was hardly due to competition among growing grains for limited resources. Furthermore, data-points felt close to the 1:1 ratio line when AGW was related to PGW, , indicating the actual weight of the grains was virtually the same that the potential, which is only compatible with a scenario of lack of noticeable scarcity of assimilates to fill the grains , which is in line with previous studies (e.g. Serrago et al., 2013 and references quoted therein). Therefore, the trade-off between AGW and GN across elite material must have been mainly due to differences in the intrinsic potential weight of the grains.

This result suggests AGW as a possible trait for further raising yield when environmental variability increases. Particularly, if the genotypic differences of AGW are mainly due to PGW, not due to competition, increasing PGW would be a good strategy to cope with environmental variability. Sink strength is defined as the combination between sink size and sink activity (White et al., 2016), and this result suggests that increasing sink activity (PGW) would be an option to raise yield as good as increasing GN. Said that, I also found some degree of responsiveness to defoliation at the onset of grain filling. This means that even though grain growth was dominantly sink-limited, these current cultivars are approaching to experience some degree of source-limitation to grain growth and breeders may need to also consider improvements in sink size in the future.

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Chapter IV

Quantifying pre- and post-anthesis heat on the number and weight of the wheat grains of cultivars with contrasting balance of these major yield components

4. Chapter IV: Quantifying pre- and post-anthesis heat on the number and weight of the wheat grains of cultivars with contrasting balance of these major yield components

4.1. Introduction

The element of climate change that is predicted with more certainty is that temperatures will be sensibly increased during the wheat growing season (Asseng *et al.*, 2011; Challinor et al., 2014). Indeed, many regions are already experiencing warming of more than 1.5°C above the pre-industrial era (Allen et al., 2018). Two decades ago, several studies indicated that wheat yield decreased c. 2.5 (Stone *et al.*, 1995) to 4.0 % (Islam and Wardlawb, 1978; Wardlaw and Wrigley, 1994) with every °C increases, and more recently Asseng *et al.* (2015) and Zhao et al., (2017) estimated that wheat production would be globally decreased by 6% with every °C increases. Therefore, maintaining wheat production might be more difficult due to consistently rising temperatures (Asseng *et al.*, 2017). As temperature continually increases, transient heatwave on crops will also be more frequent (e.g. Meehl and Tebaldi, 2004; Perkins et al., 2012; Seneviratne et al., 2014; Rivelli et al., 2021), and have a negative impact on crop yield (Hatfield and Prueger, 2015; Slafer and Savin, 2018; Moore et al., 2021).

This negative effect on wheat yield must be related to that produced on its components. Elía *et al.* (2018) imposed transient heat stress on high-yielding, modern well-adapted wheat cultivars, and both major components (grain number per m^2, GN and average grain weight, AGW) were negatively affected. However, it is not clear yet whether these effects of heat stress are direct (affecting the development of florets and/or capacity of grains to grow) or indirect (through reducing growth and thus imposing a source restriction to the determination of yield components).

Generally, the sequence of wheat yield generation is from the production of fertile florets, grain set, and grain growth (Slafer *et al.*, 2014), and it means the determinant periods of GN and AGW would only be slightly overlapped (Slafer, 2003; Acreche and Slafer, 2006; Calderini et al., 2021). GN is largely determined during stem elongation between initiation of terminal spikelet and few days after anthesis (Siddique et al., 1989; Savin and Slafer, 1991) through floret development, pollination, and grain setting, while grain weight is realised during the effective period of grain filling occurring from 1-2 weeks after anthesis to physiological maturity (Slafer et al., 2021). Although final grain weight seems related to the potential size, the grain may have determined through carpel size at anthesis and number of endosperm cells and maximum water content during the lag phase between anthesis and the onset of effective grain filling (Calderini et al., 2021; Slafer et al., 2021), definitively temperature conditions during grain filling affect noticeably final grain weight (Sofield et al., 1977 and a myriad of papers confirming this thereafter).

The critical period to determine yield is when grain number is determined (Slafer, 2003), and due to sensitivity of floret development in response to the availability of assimilates

(Ferrante *et al.*, 2013; Pérez-Gianmarco *et al.*, 2019), GN is strongly source-limited during pre-anthesis, suggesting that its reduction due to heat stress in pre-anthesis may well be an indirect effect (i.e. heat affects plant growth and less growth would be responsible for increased floret mortality). But floret development itself is also highly sensitive to temperature for which direct effects cannot be discarded. Grain growth occurs during post-anthesis. It is known that under heat stress, leaf senescence is accelerated, reducing photosynthetic activities (Al-Khatib and Paulsen, 1984; Harding et al., 1990; Shah and Paulsen, 2003; Zhao et al., 2007; Bergkamp et al., 2018), and carbohydrates for grain growth are mostly supplied through the actual post-anthesis photosynthetic activity (Kobza and Edwards, 1987) complemented by remobilisation of water-soluble carbohydrates stored before the onset of grain filling (Pheloung and Siddique, 1991; Blum, 1998; Talukder et al., 2013). However, it has been known since many years ago that the effect of heat stress may well be independent of limiting the supply of assimilates through directly affecting the capacity of the grains to grow (Wardlaw et al., 1980; Slafer and Miralles, 1992; Porter and Gawith, 1999). In addition to enzymatic inhibitions (Bhullar and Jenner, 1985; Jenner, 1994; Zhao *et al.*, 2008), heat stress reduces the duration of grain filling (Sofield et al., 1977; Wheeler et al., 1996; Yin et al., 2009), not compensated by the accelerated rate of growth (Wardlaw et al., 1980; Tewolde *et al.*, 2006; Rezaei *et al.*, 2015). Most studies have elucidated such a negative impact on wheat yield estimating the effects on AGW. However, analysing the weight of individual grains will provide more clear evidence to understand whether the effect of heat stress on grain growth would be direct or indirect (see Annex II**).**

In addition, genotypes may differ on whether the impacts of heat on these components are direct or indirect as well as on the magnitude of their sensitivities. At least in part, the different sensitivity may be related to the importance of GN and AGW in determining yield. Generally, GN shows large plasticity, while AGW is far less plastic (Peltonen-Sainio et al., 2007; Sadras, 2007; Sadras and Slafer, 2012), coinciding with the fact that genotypic differences in wheat yield are expectedly based on differences in GN than in AGW (Álvaro et al., 2008; Slafer et al., 2015). However, to the best of our knowledge, no studies have yet compared the sensitivities of GN and AGW to heatwaves to elucidate whether genotypic differences in responsiveness to transient heatwaves would be at least partly related to the relevance of the two major yield components. In this context, it would be relevant to study the responses to transient heat treatments of modern, high-yielding cultivars with contrasting relevance of GN and AGW to determine their yield.

In this study, I quantified pre- and post-anthesis heat stress effects on GN and AGW. For the latter, I elucidated whether the effects were direct, indirect or both through (i) analysing not only the response of AGW but also that of each individual grain of the crops, and (ii) determining the responses of grain weight to an increased availability of assimilates (due to de-graining) during the effective period of grain filling under the contrasting heat treatments.

4.2. Materials and Methods

Field experiments were carried out in 2018-2019 at two locations: Bell-lloc d'Urgell (41.64ºN, 0.79ºE) and Lleida (41.63ºN, 0.60ºE) in Catalonia, North-East Spain. Experiments were sown on 20 December 2018 at Bell-lloc and 14 January 2019 at Lleida, with a sowing density of 400 viable seeds m^{-2}. In order to minimise experimental error through maximising uniformity of the plot when sampling, soon after seedling emergence and well before the onset of tillering, I labeled in each plot few areas of 1 m of a row (excluding borders), that were designated to be sampled later at anthesis and maturity. In those sampling areas, as well as in the rows surrounding them, I thinned manually the rows leaving 60 seedlings uniformly distributed both within the row and in size (i.e. leaving seedlings that emerged simultaneously), representing a stand density to 300 plants per m^2. The soil type at Bell-lloc was a typic Calcixerept, fine-loamy over sandy skeletal, mixed and thermic (SSS, 1999), and soil type at Lleida was a typic Xerofluvent, fine loamy, mixed (calcareous), thermic (SSS, 1999). The weather condition was similar in the two locations (Fig. 4.1).

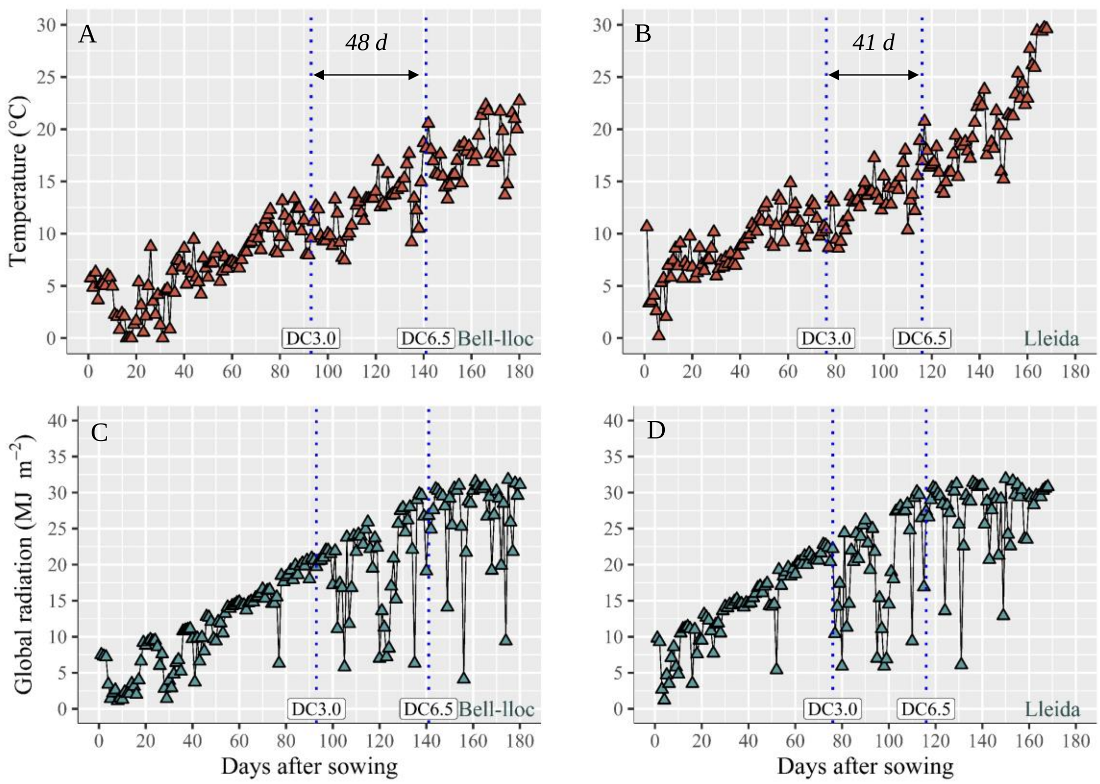

Figure 4.1. Daily average temperature (A, B) and global radiation (C, D) over the growing season in both locations: Bell-lloc (A, C) and Lleida (B, D). The dates of the onset of stem elongation (DC3.0) and anthesis (DC6.5), averaged across both cultivars are indicated with dashed lines and the duration of the stem elongation period in each of the two experiments explicitly indicated.

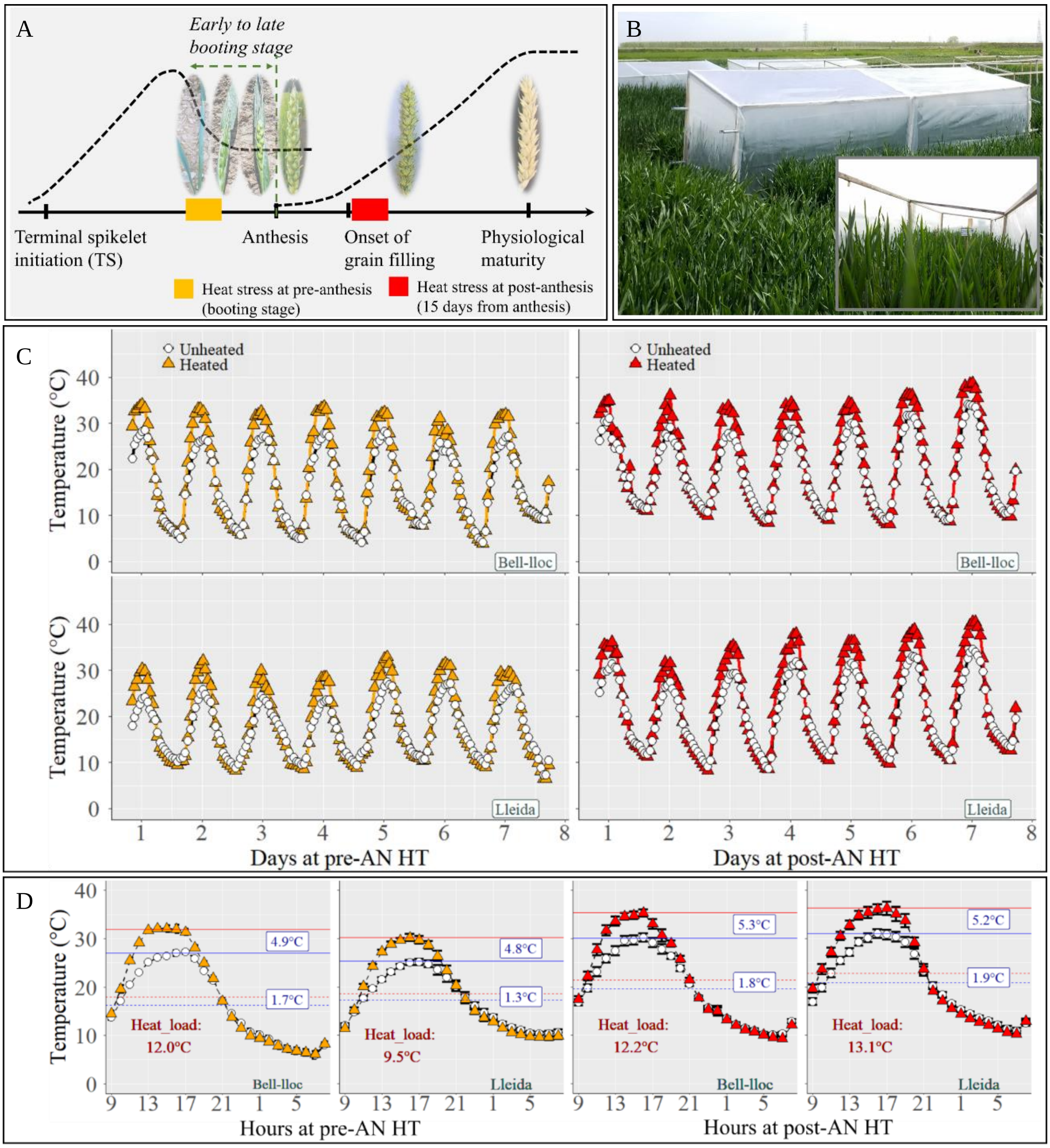

Figure 4.2. Description of the elevated canopy air temperature treatments. **Panel A**: timing when treatments were imposed during the wheat growing stage, illustrating the dynamics of both floret initiation and mortality (highlighting that the heat was imposed during floret mortality) and grain growth (highlighting that the heat was imposed after the onset of effective grain filling). **Panel B**: partial field view of transient heat treatment (HT) with portable polyethylene tents, including a detailed view from inside. **Panel C**: hourly dynamics of temperature for the unheated control (open circles) and high-temperature treatments (triangles) for the 7 effective days imposed at either booting or the onset of grain filling (pre- and post-anthesis (AN), respectively) at both experimental locations. **Panel D**: details of hourly temperature for an average day of treatment, indicating the average maximum (solid lines) and mean daily temperatures (dashed lines) for the heated and unheated treatments (upper and lower lines, respectively; figures included stand for the differences between them). The difference in heat load (i.e. additional degrees of average temperature accumulated over the heat treatment duration respect to the unheated plots) was also included.

Treatments consisted of the factorial combination of two elite genotypes, three heat waves and two source-sink manipulations (control and de-graining).

Genotypes were two commercial well-adapted and high-yielding wheat cultivars, Pistolo and Sublim. They were selected as in previous studies they consistently showed contrasting relevance of GN and AGW to determine potential yield (i.e. under high-yielding growing conditions, Sublim consistently had more but lighter grains than Pistolo, but both yielded similarly; see Annex I**)**. Heat wave treatments consisted of an unheated and two heated treatments of 7 effective days each (one starting at DC4.3 -early booting-, and the other one starting at 15 days after anthesis –c. DC7.5; Fig. 4.2A).

Heat treatments were imposed by installing, over the designated plots, wood structures of 1.5 m height above the whole plot covered with transparent polyethylene film (Fig. 4.2B). These portable tents are a valuable resource to study heat effects in field conditions, particularly when electric power is not available, quantifying reliably the effects with negligible confounded effects (see Annex I). Temperature sensors (connected to dataloggers EM5b Decagon Devices) were set up inside and outside the structures, at the height of the top of the canopy, to hourly record the temperature during the imposition of the heat treatments. In the heat-treated plots, the temperature gradually increased, inside the portable tents during daylight, thus increasing maximum daily temperatures at the height of the spikes but unaffected the minimum (Fig. 4.2C, D). It is relevant to note that the overall heat treatment was negligible when considering the increase in average temperature with respect to the control for the whole stem elongation or grain filling periods (within which the treatments were imposed; Fig. 4.2A). In each effective day of treatment, the average temperature increased only 1-2ºC (Fig. 4.2D) and the treatment was imposed only on c. fourth-fifth of the corresponding phenological phase (i.e. mean temperature during either the stem elongation phase when spike fertility is determined, or the grain filling phase, when grains do grow, increased only by c. 0.3-0.5ºC).

For source-sink manipulation treatments, 9 independent uniform plants (having similar canopy height, flowering time, and spike size) from central rows of each main plot were selected and their main shoots labeled at anthesis, and 3 of them were assigned to each source-sink manipulation. For that purpose, 14 days after anthesis, I removed either the laminae of the two top leaves (the flag and second leaves; reducing through defoliation the source-strength per grain) or all spikelets on one side of the spike (reducing sink-strength by halving the number of growing grains during the effective period of grain filling while not altering the distribution of different grains sizes of the spike, as described in detail in Sanchez-Bragado et al. 2020). The other three plants were left unmanipulated as controls for this particular treatment.

Agronomic components were analysed by ANOVA and the effect of source-sink alteration was analysed by split-plot using JMP software (*version 14, SAS Institute Inc., NC*) to elucidate the effects of treatments, genotypes, and their interaction. Multiple comparisons of studied variables were made using LSD test. The normal distribution of individual grains was graphically represented using RStudio (*RStudio Team 2020*). The significance level of α=0.05 was used in all statistical models.

4.3. Results

4.3.1. Genotypic differences in the controls

Even though both experiments were irrigated, fertilised and protected against biotic stresses, yield and the number of grains were greater in Bell-lloc than in Lleida (Table 4.1), likely due to the differences in the duration of the critical period for yield determination (Fig. 4.1). Although the patterns of temperature and solar radiation were similar (and both experiments were irrigated, neglecting the relevance of eventual differences in rainfall), sowing was delayed to January in Lleida, and the critical period for yield determination, between terminal spikelet and anthesis, was one week shorter than in Bell-lloc.

As expected, when selecting Pistolo and Sublim for this study, both cultivars had similar yields at each of the two locations, though Sublim had a slightly greater yield than Pistolo in Lleida. However, the expected differences in GN and AGW between genotypes (i.e. Pistolo had always less but heavier grains than Sublim) were evidenced consistently across both locations (Table 4.1).

Table 4.1. Yield and yield components (number of grains per unit land area and average weight of these grains, GN and AGW, respectively) of the two genotypes grown in the control plots (unheated and unmanipulated regarding source-sink treatments) at the two locations of the study.

Location	Genotype	Yield ($g\ m^{-2}$)	GN (m^{-2})	AGW (mg)
Bell-lloc	Pistolo	713.8[a]	15391[b]	46.3[a]
	Sublim	705.9[a]	17342[a]	41.3[c]
Lleida	Pistolo	498.6[b]	11590[c]	43.0[b]
	Sublim	580.9[ab]	14093[bc]	41.1[c]

Different letters indicate that the difference between genotypes and/or locations for that variable was statistically significant ($p<0.05$)

Genotypic differences in AGW seemed clearly constitutive, rather than reflecting a compensation due to different levels of post-anthesis competition for resources among growing grains related to the genotypic differences in GN. Two independent sources of evidence support this statement. Firstly, differences were evident across the whole population of grains rather than limited to the relatively smaller grains (Fig. 4.3A), indeed the difference between genotypes was similar for the deciles of the heavier and lighter grains of each genotype (Fig. 4.3B). Secondly, it seems grains of both cultivars would have not been growing with the scarcity of resources that would be necessary to establish a competition among them: (i) the growth of the crop during post-anthesis was practically equal to or greater than that required to fill the grains (Fig. 4.3C), and (ii) grain weight did not respond to de-graining imposed at the onset of grain filling (Fig. 4.3D), something expected to occur should grain growth be limited by the strength of the source.

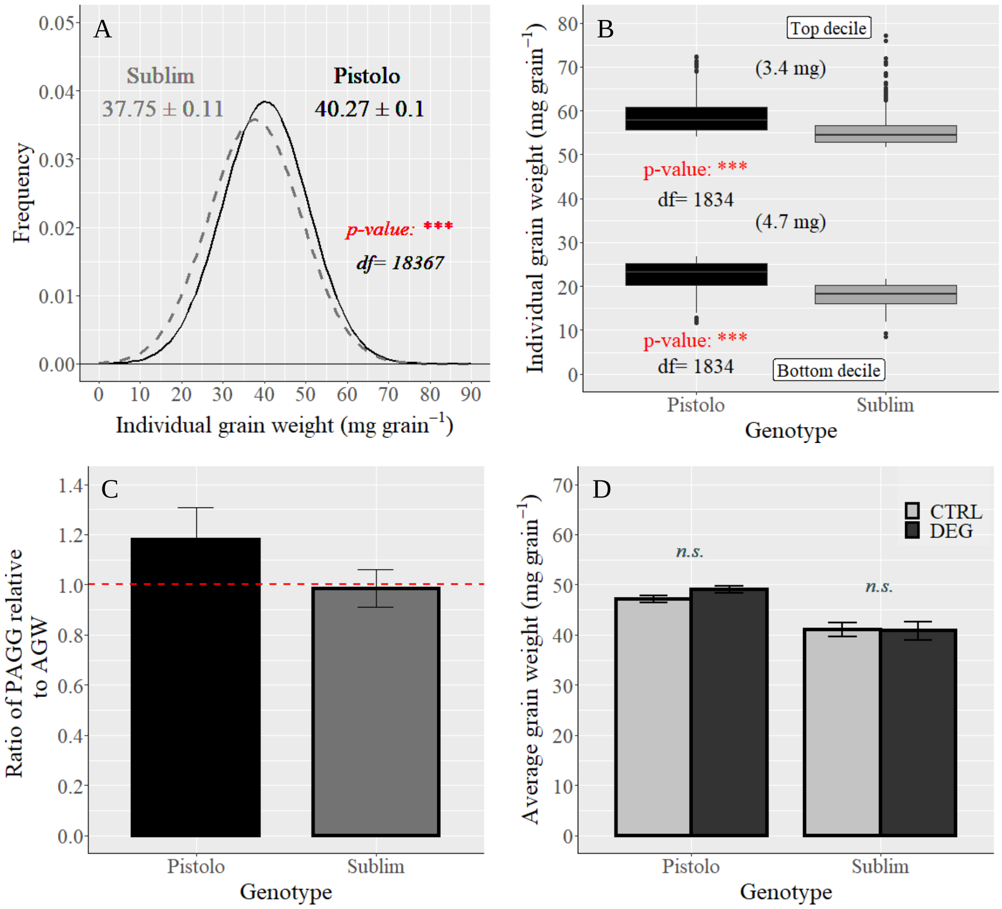

Figure. 4.3. Panel A: Frequency distribution of the weight of individual grains for Pistolo (plain line) and Sublim (dashed line). Means (± standard error) derived from the distribution, as well the significance level of the difference and the degrees of freedom are inset. **Panel B**: Boxplots of the individual weight of grains corresponding to the top and bottom deciles of the two genotypes. The difference in average individual grain weight between the two genotypes are also shown between parentheses for each of the two deciles considered. **Panel C**: Ratio of the post-anthesis growth per grain (PAGG) and AGW for each of the genotypes. PAGG is the ratio between total biomass accumulated from anthesis to maturity and the number of grains. **Panel D**: Grain weight in the unmanipulated control (CTRL) and de-grained (DEG) plants. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001; *n.s.* = not significant). Data for panels A and B were pooled for both locations, while those in panels C and D are averaged across them.

4.3.2. GN and AGW response to transient heat stress

Heat wave treatments affected significantly both GN (F-ratio = 10.84; p<0.001) and AGW (F-ratio = 11.10; p<0.001). On the other hand, interactions between heat treatments and locations were clearly not significant (F-ratios for GN and AGW were 0.02 and 1.71, p>0.20, respectively). Therefore, I averaged across the two locations the heat treatments effects (value in the heated treatment relative to that in the control) for both yield components on each of these genotypes.

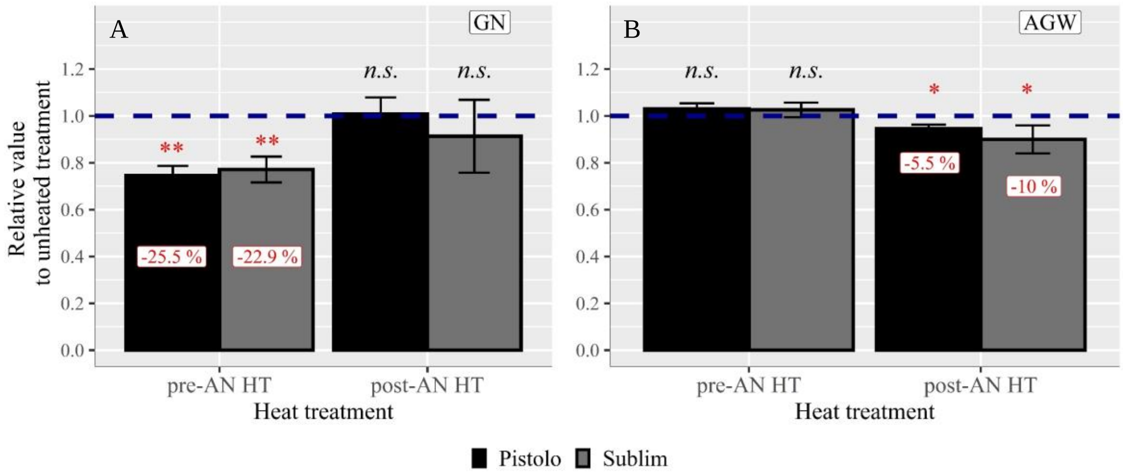

Figure 4.4. Effect of the heat treatments (imposed in pre- and post-anthesis) considering the ratio of the trait between the heated and unheated treatments for the number of grains per unit land area (GN, A) and the average grain weight (AGW, B), with the dashed line represents the unheated treatment. Whenever the effects were statistically significant, I included inset the corresponding bar the actual reduction in percentage of the unheated control. Segments stand for the SEM. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001; *n.s.* = not significant). Data were averaged across the locations.

GN was significantly reduced by pre-AN HT, while the post-AN HT did not reduce GN significantly (Fig. 4.4A). The opposite was true for AGW. Post-AN HT reduced it significantly, but the treatment imposed before anthesis did not have a significant effect (Fig. 4.4B). It is noteworthy (i) that GN was more sensitive to pre-AN HT than AGW to post-AN HT (more than 20% reduction in GN, while the reduction in AGW was 5-10%; Fig. 4.4) even though the treatment was stronger in post- than in pre-anthesis at least in one of the two experiments (Fig. 4.2D), and (ii) that the differences in sensitivity between the two contrasting genotypes were only slight, with a trend for the genotype with constitutively greater GN and lower AGW to have less penalty in GN and more penalty in AGW than the cultivar with constitutively greater AGW and lower GN.

4.3.3. Causes for the reduction in GN

Perhaps because the pre-AN HT was imposed at the last fraction of the stem elongation phase, the number of spikes per m^2 was insensitive, while GN per spike was significantly reduced (Fig. 4.5A). The effect was more noticeable in the cultivar with constitutively less but bigger grains (Pistolo) than in the cultivar more efficiently setting grains though constitutively smaller (Sublim).

Most fertile florets set a normal grain in the unheated conditions, with Sublim producing more fertile florets and grains per spike than Pistolo, even though Sublim had some (c. 19%) grain abortion (Fig. 4.5B top). The pre-AN HT treatment produced only a minor increase in floret primordia death, being the number of fertile florets only slightly less in

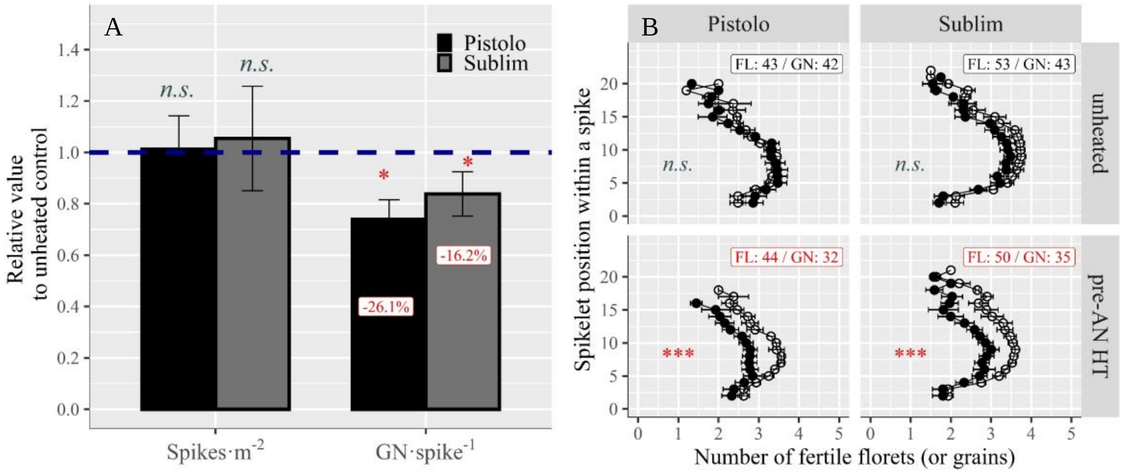

Figure 4.5. Effect of the pre-AN HT treatment on the number of spikes per m^2 and on number of grains per spike considering for each trait the ratio between the heated and unheated treatments (A); and the numbers of fertile florets at anthesis (open symbols) and grains at maturity (closed symbols) for the different spikelets of the spikes in the unheated controls and in the pre-AN HT treatment (B). In panel A, dashed line represents the unheated treatment Figures inset stand for the relative reduction in GN per spike (A) and for the number of fertile florets (FL) or grains (GN) per spike(B). Segments stand for the SEM. Asterisks indicate the significance of the effect (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; *n.s.* = not significant). Data were averaged across both locations.

the heated than in the unheated control for both cultivars (Fig. 4.5B). However, grain abortion increased in all spikelet positions (Fig. 4.5B, cf. top vs bottom panels), suggesting that even though the transient heat treatment did not increase noticeably floret death, it did increase the lability of the fertile florets to set a grain.

4.3.4. Causes for the reduction in AGW

Even though AGW was not limited by the availability of resources in the unheated control condition in any of the two genotypes (see above), post-AN HT may have affected grain growth (i) indirectly through affecting senescence and post-anthesis growth, and/or (ii) directly reducing the capacity of the grains to grow. To understand which physiological mechanism contributed to the reduction in grain weight, I firstly analysed how post-AN HT affected the distributions of individual grain sizes and later analysed whether the penalty imposed by this stress could be reverted by de-graining at the onset of the effective grain growth.

Post-AN HT reduced the average size of the grains, again more clearly in the cultivar characterised for a larger GN and lower AGW (Sublim) than in Pistolo (Fig. 4.6A, B), and the reduction in AGW was a consequence of reducing the size of all grain classes, although the effect was more noticeable in the larger than in the smaller grains (Fig. 4.6C, D). Although not proving it, this result is compatible with the hypothesis that temperature would have affected more the capacity of the grains to grow than exacerbating the competition for resources among the grains growing under the heat wave.

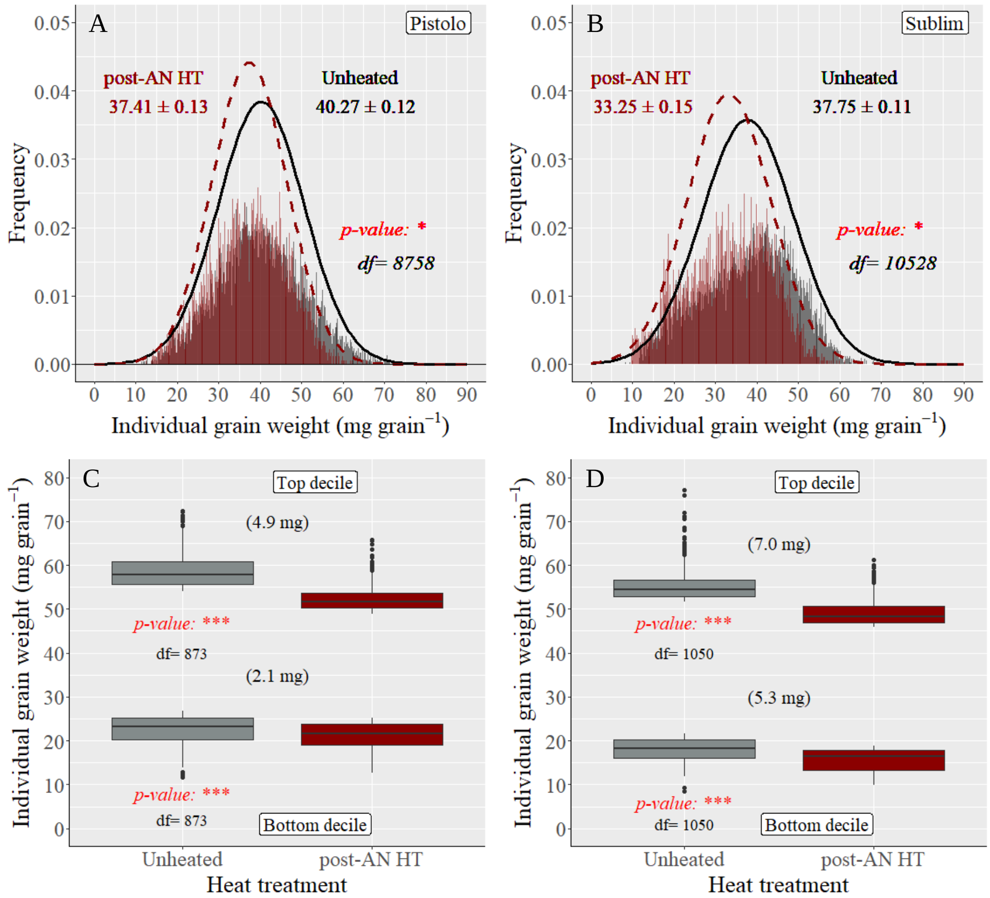

Figure 4.6. Frequency distribution of individual grain weights as affected by the post-AN heat treatment (dashed lines) compared to the unheated control (plain line) (A, B), and boxplots of the individual weight of grains corresponding to the top and bottom deciles in the unheated controls and in the post-AN heat treatments (C, D), for Pistolo (A, C) and Sublim (B, D). The difference in average individual grain weight between the unheated controls and in the post-AN heat treatments are also shown between parentheses for each of the two deciles considered. Asterisks indicate the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001). Data were pooled across both locations.

Further supporting that hypothesis, the potential capacity of the crop to fill the grains (i.e post-anthesis growth per grain; PAGG) seemed adequate to allow grain growth to proceed without restrictions imposed by the strength of the source (Fig. 4.7). Although the pre-AN heat treatment did actually impaired growth from anthesis to maturity more than GN, even in these cases chances are that the deficit in growth per grain would have been covered by remobilisation of reserves (that would be compatible with the fact that this heat treatment did not reduce AGW).

Therefore, post-AN HT would have then reduced AGW significantly through a direct effect, as post-anthesis growth would have been enough to fill the grains (and there would have been reserves not, or only marginally, used in Pistolo and Sublim, respectively; Fig. 4.7).

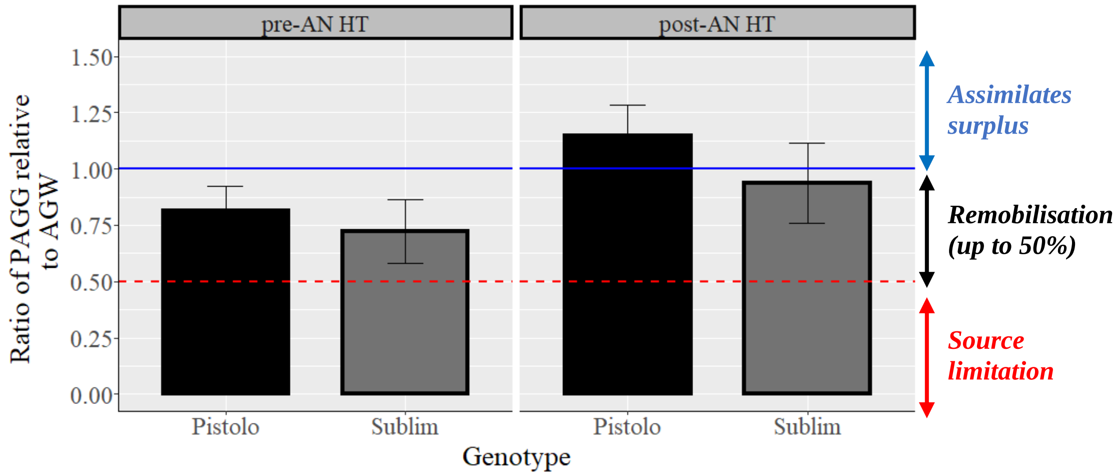

Figure. 4.7. Ratio of the post-anthesis growth per grain (PAGG) to AGW for each of the genotypes when they were subjected to pre- or post-AN HT. On the right, there is a rough indication of the possible situation, with an excess of assimilates produced by the crop after anthesis with respect to the demands of the growing grains (ratios > 1), a range of values compatible with sink limitation as remobilisation of water-soluble carbohydrates may complement actual crop growth as a source of carbohydrates to fill the grains, and a low range of values most likely indicating clear source limitation for grain growth. The limit between the two latter was set at 50% (dotted line) as there are evidence in the literature indicating that up to 50% of grain growth may be contributed by reserves (Blum, 1998; Gent, 1994; Serrago et al., 2013). Data were averaged across both locations.

Furthermore, I estimated potential grain weight as the value achieved in the treatment that was de-grained at the onset of the effective period of grain growth. When plotted potential vs actual grain weight (PGW vs AGW), Pistolo had greater PGW than Sublim (Fig. 4.8 inset), further supporting that the difference in grain size between these cultivars is constitutive. Also, data-points for the unheated or heated treatments are all around the 1:1 ratio line (Fig. 4.8), implying, again, that the reduction of AGW produced by post-AN HT might not be due to a competitive cause, but to have reduced PGW *per se*.

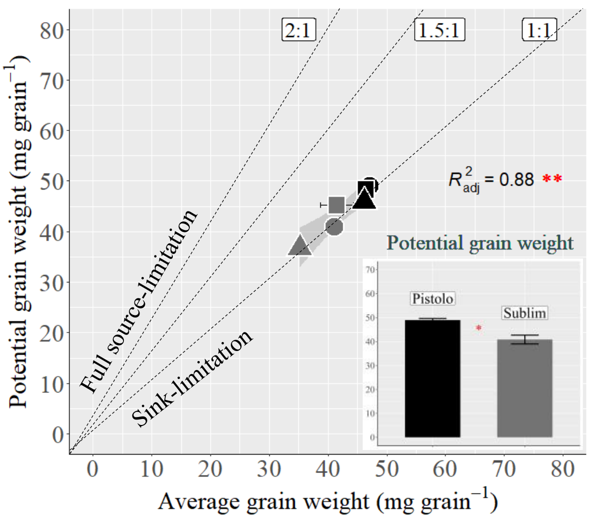

Figure. 4.8. Relationship between potential and actual average grain weight for Pistolo (black) and Sublim (grey) grown under unheated (circle), pre-AN (square) and post-AN (triangle) heated conditions respectively. Each dotted line indicates 1:1, 1.5:1 and 2:1 ratio between PGW and AGW. The bar graph inset is a mean comparison of potential grain weight between Pistolo and Sublim. Asterisks indicate the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001). Data were averaged across both locations.

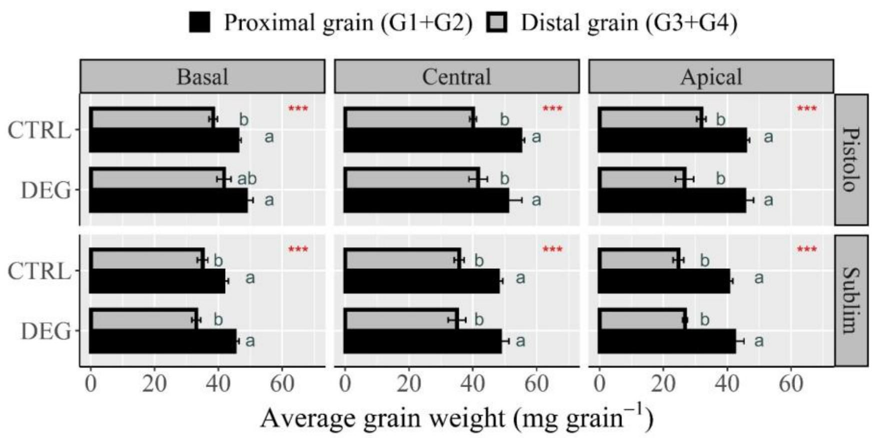

Figure. 4.9. Grain weight at particular spikelet positions (basal, central and apical) under the heat stress at post-anthesis. Grain weight of proximal grain is the average of 1st and 2nd grains closely located to rachis, and grain weight of distal grain is the average of 3rd and 4th grains located far from rachis within each particular spikelet. CTRL means AGW of unheated/un-manipulated and DEG means de-graining treatment at 14 days from anthesis. Asterisks indicate the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001). Data were averaged across both locations.

Moreover, AGW and PWG were highly related to each other (Fig. 4.8), further supporting that the differences in AGW (due to genotypes x heat treatments) were constitutively determined before grain growth took place.

Finally, I considered the responses to de-graining of very specific and equivalent grain positions (i.e. first + second –proximal- and third + fourth –distal- positions from the rachis of apical, central and basal spikelets) under the post-AN HT. I found no improvements in final grain weight of any of the many grain categories analysed in detail (Fig 4.9).

4.4. Discussion

4.4.1. Genotypic difference in yield components

GN and final yield were lower in Lleida than in Bell-lloc, while AGW was similar (Table 4.1). The most likely reason for the difference is that the experiment in Lleida was sown substantially later (due to rainfall events at the time scheduled for sowing). This delayed sowing did not affect significantly the date of anthesis but delayed the onset of stem elongation, resulting in a shortened duration of the stem elongation period, which is of critical relevance for determining wheat yield (Slafer, 2003; Fischer, 2011), and increased period of stem elongation could increase grain number (Miralles and Slafer, 2007), as a consequence of increasing the number of fertile florets at anthesis (Pérez-Gianmarco et al., 2019). Indeed, in experimental conditions in which the duration of stem elongation was shortened, the number of grains was concomitantly reduced (e.g. González et al., 2005).

Differences in GN and AGW between genotypes were consistently shown across both locations. Pistolo had always less grains but heavier than Sublim, which resulted in a sort of trade-off between major yield components when comparing the contrasting cultivars. However, this trade-off was seemingly independent of any different level of competition among growing grains between the cultivar with low- or high-GN. The differences were instead clearly constitutive as not only there was no scarcity of resources to satisfy the demands of the growing grains in both cultivars, but also because there was not either an increased proportion of potentially small grains as a consequence of increasing GN (which has been pointed out as a major driving force for the AGW-GN trade-off in absence of competition among the grains during grain filling; Slafer and Savin, 1994; Miralles and Slafer, 1995; Calderini and Reynolds, 2000; Acreche and Slafer, 2006). In the case of these two cultivars, all grain classes (collectively determining AGW) were heavier in Pistolo than in Sublim. Previous studies usually analyzed the proportion of potentially small grains, counting few grains located at distal positions of spikelet in a spike and/or grains from rachis within a spikelet. It might not represent the whole spikes due to a small sample size. In this study, the sample size (c. 18,000 grains) was much greater than previous studies, and I analyzed as a population of individual grain weight and elucidated that all grains in Sublim were smaller than in Pistolo across all grain size classess. Grains did not grow more in response to an increased availability of assimilates during grain filling (due to de-graining). This is not surprising as it has been most commonly found that yield is strongly sink-limited during grain filling in wheat (e.g.; Borrás et al., 2004; Reynolds et al., 2021); whilst it is source-limited during the stem elongation phase (Slafer et al., 2021) when grain number is determined.

4.4.2. Heat effect on yield and its components

The heat treatments were imposed in field plots with increases in temperature that are well within what could be occurring naturally. Both elements are critical for reaching sound conclusions that can be trustworthily applicable to real crops. Most studies focusing on the effects of heat are carried out with isolated plants grown in pots under controlled conditions, normally imposing very high temperatures. Results from experiments on isolated individuals and even more if grown under controlled conditions may prevent extrapolating conclusions to real crops (Passioura, 2010; Sadras and Richards, 2014; Pedró et al., 2012). Although our approach using portable tents of transparent polyethylene is not perfect either, it has been shown to be highly reliable (Elía et al., 2018; see Annex I).

Agreeing with previous studies (Calderini et al., 1999; Porter and Gawith, 1999; Stone and Nicolas, 1995; Zhao et al., 2007), I found that heat affected yield in both cultivars. Exposing the crops to pre- and post-AN HT resulted in yield penalties associated with the components mainly being determined at those stages, GN and AGW, respectively. What has not received much attention before was whether the pre- or post-AN HT would be more damaging and whether cultivars with contrasting relevance of GN and AGW in yield determination would differ in sensitivity to these heat stresses. I found that yield was more sensitive to pre- than to post-AN HT, consistently across both locations and genotypes. Therefore, GN was more markedly reduced by pre-AN HT than AGW by post-AN HT. Thus, the well-known plasticity differences between these two major yield components to changes in resource availability (e.g. Peltonen-Sainio et al., 2007; Sadras, 2007; Gambín and Borrás, 2010; Sadras and Slafer, 2012; Ferrante et al., 2017) does also apply to when they are affected by heat waves. Although it had been already demonstrated for maize that yield was far more sensitive to heat waves around silking than during grain filling (e.g. Rattalino Edreira and Otegui, 2013; Ordóñez et al., 2015), these conclusions could not be straightforwardly extrapolated to wheat due to the enormous differences in floral biology between the two cereals.

The differential sensitivity of GN to pre-AN HT and AGW to post-AN HT might be related to the fact that GN determination is highly sensitive to resources, and therefore the effect of pre-AN HT might be both direct (affecting floret development *per se*) and indirect (affecting source strength). In our study, as pre-AN HT was imposed at booting, it did not affect the number of spikes per m^2 (determined slightly earlier than floret number; Slafer et al., 2021), and the penalty on GN operated through affecting spike fertility. This reduction of GN per spike was mainly mediated through grain abortion. The most likely mechanisms might have been that although the heat in these cases did not reduce the survival of floret primordia, it might have reduced the growth of the ovaries, resulting in fertile florets more prone to abortion (Guo et al., 2016). The reduction of AGW caused by post-AN HT was not related to source strength changes. Should it be so, the smallest grains would have been more affected than the largest grains (as the small grains are constitutively less competitive and located in positions more distal to the rachis). On the other hand, in this study, heavier grains were reduced similarly to, or more than small grains. Indeed, it was clear that the heat produced a penalty in potential grain weight. Therefore, the reduction of AGW caused by heat stress at post-anthesis might have been simply due to a direct effect reducing PGW *per se*. The fact that de-graining did not produce any reduction in the penalty imposed by heat does reinforce this conclusion.

Although the genotypic differences in sensitivities of GN to pre-AN HT and AGW to post-AN HT were not clear, there seemed to be a trend for the cultivar constitutively producing more grains having less sensitivity of GN, while the cultivar constitutively having heavier grains having less sensitivity of AGW to post-AN HT. It seemed that the plasticity of a yield component in response to heat stress was inversely related to the relevance of that component for yield determination in unheated conditions. Should this result be confirmed (and more clearly shown) in future studies, it may open an opportunity for breeders to select for improved sink-strength through either higher GN or higher PGW depending on what of these two timings of heat would be targeted to.

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Chapter V

Physiological bases of grain number and weight response to heat waves in contrasting wheat cultivars under field conditions

5. Chapter V: Physiological bases of grain number and weight response to heat waves in contrasting wheat cultivars under field conditions

5.1. Introduction

In the previous chapter, it was evidenced that significant yield penalties were produced by heat waves, even when the heat load produced was relatively small (Chapter 4). It was also determined that

(i) the penalties were due to the detrimental effect on grain number per m^2 (GN) when the heat wave was imposed pre-anthesis (pre-AN HT), or on average grain weight (AGW) when the heat wave was imposed post-anthesis (post-AN HT);
(ii) the sensitivity of GN was larger than that of AGW;
(iii) the sensitivity of these components to heat seemed (there was only a trend) influenced by each genotype's better ability to produce one component out of two yield components, questioning that genotype producing more GN has more resilience of GN to heat stress at pre-anthesis and *vice-versa* for AGW.

Although, to the best of our knowledge, the difference in sensitivity to heat waves between GN and AGW were never shown before for wheat, that result was suitable with the fact that wheat yield is more strongly associated with GN than with AGW (Slafer and Savin, 1994; Borrás et al., 2004), with GN showing greater plasticity (Sadras, 2007; Peltonen-Sainio et al., 2011; Marti and Slafer, 2014; Cossani and Sadras, 2019), while AGW is a relatively stable trait, showing higher heritability (Sadras and Slafer, 2012).

Part of the differences in plasticity between these two components, that may be the cause for the higher sensitivity of GN to heat, reflects that GN responds strongly to source-sink manipulations during pre-AN (when this component is determined, Fischer, 2011) while AGW seems rather insensitive to changes in availability resources to fill the grains, being grain filling strongly sink limited (e.g. Borrás et al., 2004; Serrago et al., 2013). Combining heat waves with changes in source-strength during pre-anthesis could help further understanding if the higher sensitivity of GN is due to the heat stress (HT) affecting not only directly the fertility of florets or abortion of grains, but also through affecting the availability of resources (should it be a reduction in the penalty imposed by heat when resources are increased at the same time).

Another issue (that to the best of our knowledge has not been considered so far regarding the effect of heat waves) is whether the effect is additive during the growing period, or if its magnitude may be pre-empted by previous heat stress (bringing about some degree of acclimation due to a sort of priming). For other stresses acclimation has been shown to occur, and even for heat it has been shown that the exposure to other stresses like N stress produced a sort of general acclimation mitigating the magnitude of the heat wave (Slafer and Savin, 2018; Giménez et al., 2021), although this acclimation was not reported in other cases (e.g. Cossani and Sadras, 2019). I am not aware of studies analysing whether the expected penalty in AGW produced by post-AH HT would be mitigated if the crop was already exposed to a previous HT condition (even less under field conditions). This is relevant as the frequency of HT events is increasing with climate change and the cases in which the same crop would be exposed to more than one heat wave event would also increase.

In this study, I quantified the effects of pre- and post-AN HT on GN and AGW on the same two cultivars used in the previous study (Chapter 4) but adding (i) a double treatment at both stages (pre- + post-AN HT) in one of the two locations (Lleida), and (ii) combined the pre- and post-AN HT treatments with an increase in source strength at pre-anthesis through removing alternate rows in the plots at terminal spikelet in the experiment carried out in Bell-lloc (a "thinning" treatment). In both experiments, source strength per grain during the effective period of grain filling was either decreased (through a defoliation removing) or increased (de-graining through halving the spikes) in combination with each of the other treatments.

5.2. Materials and Methods

5.2.1. Experimental setup

Field experiments were carried out in the 2019-2020 growing season at two locations: Bell-lloc d'Urgell (41.64ºN, 0.79ºE) and Lleida (41.63ºN, 0.60ºE) in Catalonia, North-East Spain. Experiments were sown on 28 November 2019 at Bell-lloc and 25 November 2019 at Lleida, with a sowing density of 360 viable seeds m^{-2}. After seedling emergence (before the onset of tillering), to minimise experimental error through maintaining uniformity of the plot for sampling, I labeled in each plot few areas of 1 m of a row (excluding borders), that were designated to be sampled later at anthesis and maturity. In those sampling areas, as well as in the rows surrounding them, I thinned manually the rows leaving 40 and 60 seedlings, uniformly distributed both within the row and in size, representing a stand density to 200 and 300 plants per m^2 in Bell-lloc and Lleida, respectively.

Experiments were well irrigated to minimise water stresses, and herbicides, insecticides and fungicides were applied as required to avoid biotic interferences. All plots were fertilised with 200 Kg N ha^{-1} broadcasted just before the onset of stem elongation; stage DC3.0 in the scale of Zadoks et al. (1974). The soil type at Bell-lloc was a typic Calcixerept, fine-loamy over sandy skeletal, mixed and thermic (SSS, 1999), and soil type at Lleida was a typic Xerofluvent, fine loamy, mixed (calcareous), thermic (SSS, 1999). The weather condition was similar in the two locations (Fig. 5.1).

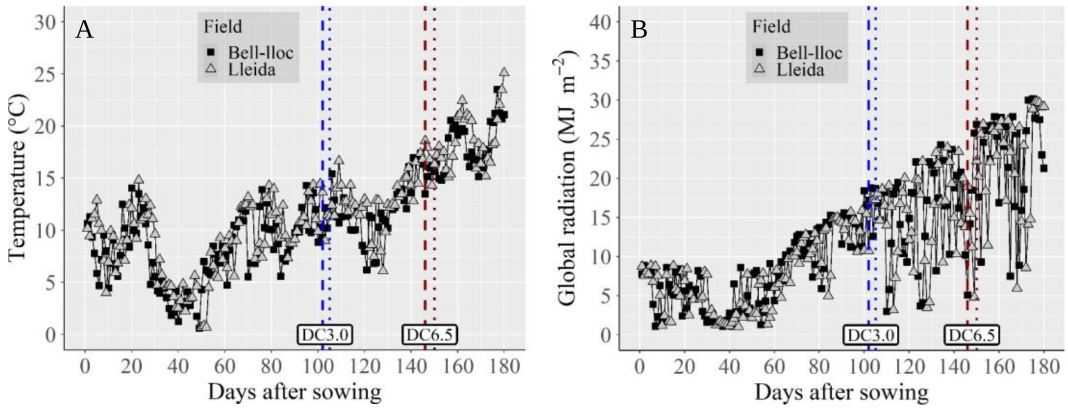

Figure 5.1. Daily average temperature (A) and global radiation (B) over the growing season in both locations; Bell-lloc and Lleida. The dates of the onset of stem elongation (DC3.1) and anthesis (DC6.5) of each location, averaged across both cultivars, are indicated with dashed (Bell-lloc) and dotted (Lleida) lines.

5.2.2. Treatments

Treatments in both experiments consisted of the factorial combination of the same two elite genotypes used in the previous study, three heat waves and three source-sink manipulations (with additional treatments factorially combined with these ones in each of the two experiments: a pre-anthesis "thinning" treatment in Bell lloc, and a double HT treatment in Lleida).

The details of the treatments applied in both experiments are offered in the description of the previous study (Chapter 4). To recap briefly, I grew two well-adapted and high-yielding wheat cultivars with contrasting relevance of GN and AGW (i.e. Sublim with more but lighter grains and Pistolo with less but heavier grains; Kim et al., 2021b; Chapter 4). Heat wave treatments, imposed by installing portable tents of transparent polyethylene with temperature sensors inside (see Kim et al., 2021b, and figure 4.2B in Chapter 4), consisted of an unheated, pre-anthesis heat stress (starting at DC4.3 -early booting), and post-anthesis heat stress (starting at 15 days after anthesis –c. DC7.5; Fig. 5.2A). These treatments are mentioned from now on as pre-anthesis and post-anthesis heat (pre-AN HT and post-AN HT, respectively). Temperature inside the tents gradually increased during daylight in relation to the radiation levels, and the average temperature increased c. 3-4 ºC (Fig. 5.2D) for 9-11 days (duration was modified to obtain a similar heat load on all treatments). The post-AN source sink treatments consisted of an unmanipulated control, a de-graining, and a defoliation. For that purpose, 9 independent uniform plants (having similar canopy height, flowering time, and spike size) from central rows of each main plot were selected and their main shoots labeled at anthesis, and 3 of them were assigned to each source-sink manipulation. Fourteen days after anthesis, I removed either the laminae of the two top leaves (the flag and second leaves; reducing through defoliation the source-strength per grain), or all spikelets on one side of the spike, increasing source-strength per grain by halving the number of growing grains, not altering the distribution of different grains sizes of the spike (Chapter 4 and Sanchez-Bragado et al. 2020).

In each of the two experiments, I added one extra treatment, in factorial combination with the abovementioned combinations of genotypes x HT x post-AN source-sink manipulations: a treatment increasing source strength during stem elongation (when GN is being determined) in Bell-lloc, and a double HT treatment combining the pre- and post-AN HT treatments in Lleida. For increasing source strength during GN determination in Bell-lloc, alternate rows in the assigned plots were removed at the onset of stem elongation (DC3.0), so that remaining plants were growing a much greater availability of resources per plant than in the unmanipulated plots (Fig. 5.2B). In Lleida, I combined the pre- and post-AN HT treatments with a third HT treatment combining both of them (Fig. 5.2C).

Plot size was 1.2 m wide (6 rows 0.20 m apart) and 4 m long. The experiments were arranged as split plot design with 36 and 32 plots in Bell-lloc and Lleida, respectively. Main plots (arranged in a randomised complete block design with three replications in Bell-lloc and four in Lleida) were assigned to the genotype x HT combinations in Lleida and the genotype x HT x thinning combinations in Bell-lloc. Sub-plots consisted of the post-AN source-sink manipulations.

5.2.3. Sampling and measurements

At physiological maturity, I sampled a 1m-linear of a central row (that was previously labelled to have the exact plant density with seedlings uniformly distributed; see above), cutting at the soil level for lab processing. In addition, at maturity I did also sample the plants in which I manipulated source-sink strengths in each experimental unit (three defoliated, three de-grained, and three unmanipulated controls).

Plants sampled were (i) divided into spikes and rest of the above-ground biomass, (ii) oven-dried for 48 hours at 65ºC, and (iii) weighed. the spikes were then threshed, the number of grains counted and, after being oven-dried again, weighed from which I determined total grain and chaff weights. Then all grains were scanned in a MARViN ProLine seed analyser (MARViTECH GmbH, Germany), from which I estimated the dry weight of each individual grain (Kim et al., 2021a). Thus, I determined not only AGW for each particular treatment combination, but also analysed the dry weight distribution of the individual grains composing that AGW (allowing to determine whether any effects detected in AGW were due to effects on all grains or only on a particular size fraction of the population).

In the nine plants per experimental unit reserved for detailed analysis of the responses to post-AN source-sink manipulations, I determined not only the responses of AGW but also separated and weighed individually each of the grains of three specific spikelet positions in the spikes (basal, central, and apical spikelets); weighing them with a 0.01 mg precision balance (VWR International, SMG 2285Di-ION, Italy).

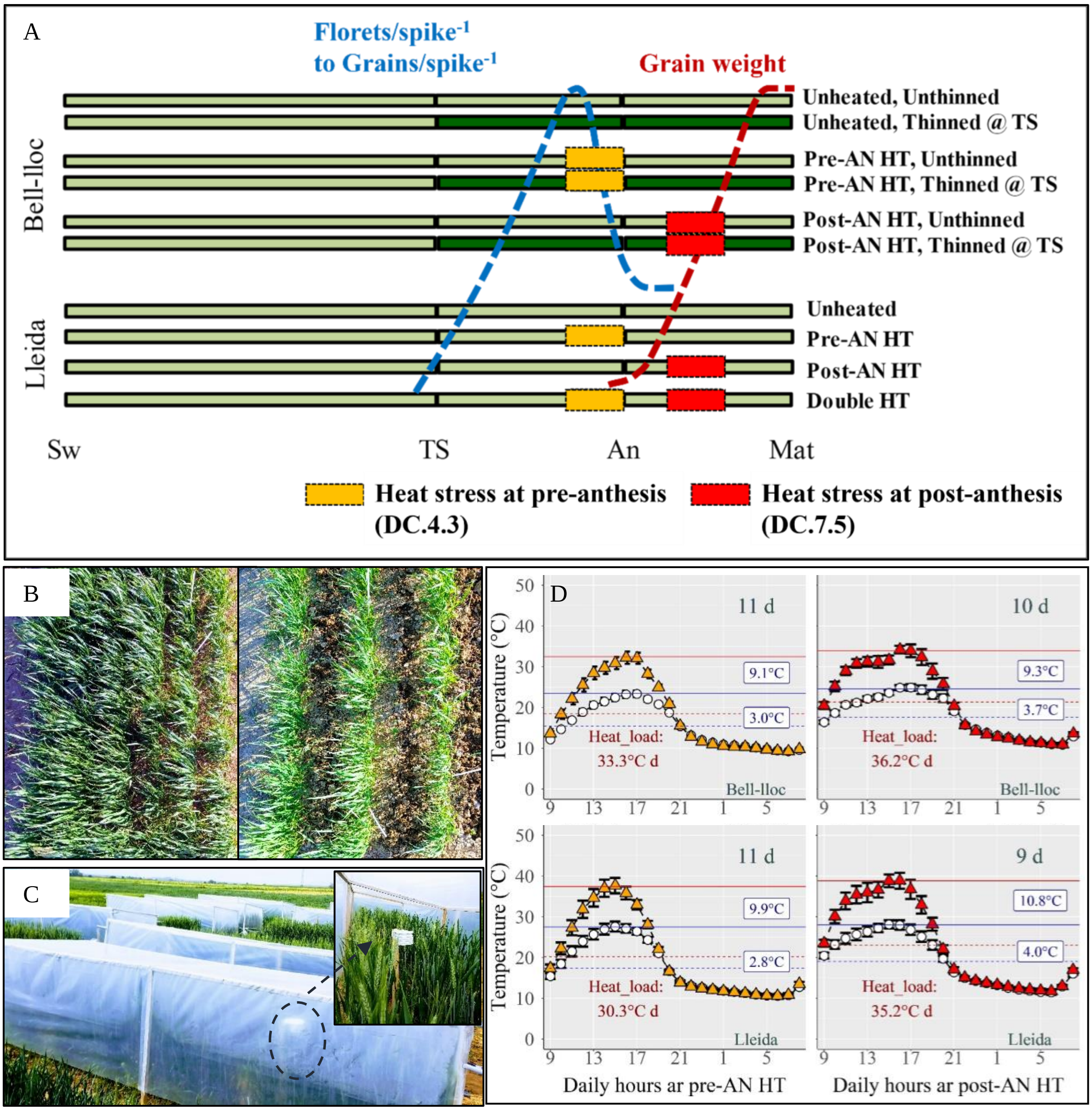

Figure 5.2. Description of the elevated canopy air temperature and thinning treatments. **Panel A**: timing when thinning and heating treatments were imposed, illustrating the dynamics of both floret initiation and mortality (highlighting that the heat was imposed during floret mortality) and grain growth (highlighting that the heat was imposed after the onset of effective grain filling). **Panel B**: Top view of an unthinned plot (left) and a thinned one (right) soon after the treatments were imposed at DC3.0. **Panel C**: partial field view showing the portable tents used to impose the transient heat treatment (HT), including inset a detailed view from inside. **Panel D**: details of hourly temperature for an average day of treatment in pre-AN (left) and post-AN (right) in Bell-lloc (top) and Lleida (bottom), indicating the average maximum (solid lines) and mean daily temperatures (dashed lines) for the heated and unheated treatments (upper and lower lines, respectively; figures included stand for the differences between them). The difference in heat load (i.e. additional degrees of average temperature accumulated over the heat treatment duration respect to the unheated plots) and treated days were also included.

Individual grains within each of these spikelets were named according to their position with respect to the rachis, as described by Acreche and Slafer (2006): from G_1 (the most proximal to the rachis), through G_2 (the second most proximal to the rachis) to G_n (the most distal grain in that spikelet, which could have been G_2, G_3, G_4, or G_5, depending on the specific spikelet and treatment.

Agronomic components were analysed by ANOVA and the effect of source-sink alteration was analysed by split-plot using JMP software (*version 14, SAS Institute Inc., NC*) to elucidate the effects of treatments, genotypes, and their interaction. Multiple comparisons of studied variables were made using LSD test. The normal distribution of individual grains was graphically represented using RStudio (*RStudio Team 2020*). The significance level of α=0.05 was used in all statistical models.

5.3. Results

5.3.1. Yield and yield components of the two contrasting cultivars under unthinning (NT) and thinning (T) treatments

Consistently with what was expected when originally selected for this study and with results observed in the previous experiments reported in Chapter 4, in the unheated condition of both experiments, Pistolo yield was similar to that of Sublim but constructed with less GN and greater AGW (Table 5.1).

GN was dramatically increased in response to the extra resources available to the plants when the competition was halved during stem elongation, while AGW was only slightly reduced (Table 5.1). Although these effects were evident in the two genotypes, the magnitude of the thinning effect was much larger in Sublim (the cultivar more efficient in setting grains) both in absolute (Table 5.1) and relative terms (Fig. 5.3A). The increased grains per m^2 in response to the thinning treatment was due to increased number of fertile

Table 5.1. Aerial biomass (TDW), yield and major yield components of Pistolo and Sublim grown under unheated conditions in Bell-lloc and Lleida in unmanipulated plots (not thinned, NT) as well as under conditions in which half of the rows were removed at the onset of stem elongation (thinned, T). Data corresponding to T plots were estimated assuming the values that would have the crops if the extra resources were available for the unmanipulated stand structure.

Location	Genotype	TH	TDW (g/m^2)	Yield (g/m^2)	GN (m^2)	AGW (mg)
Bell-lloc	Pistolo	NT	2235.9±63.9^{b}	1026.9±36.4^{b}	22953±497de	44.7±1.3^{a}
		T	2986.3±224.5^{a}	1379.1±105.2^{a}	31360±2181^{b}	43.9±0.3^{a}
	Sublim	NT	1869.5±113.1^{c}	900.9±71.3^{b}	26026±1140^{cd}	34.5±1.5^{b}
		T	3080.5±74.6^{a}	1578.5±66.8^{a}	50028±2050^{a}	31.6±0.7^{b}
Lleida	Pistolo		1931.6±54.0^{bc}	901.6±42.4^{b}	20480±728^{e}	44.0±0.7^{a}
	Sublim		2030.0±87.2^{bc}	978.3±24.3^{b}	28267±808^{bc}	34.7±1.4^{b}

Different letters indicate that the difference among genotypes, thinning treatment and locations for that variable was statistically significant (p<0.05).

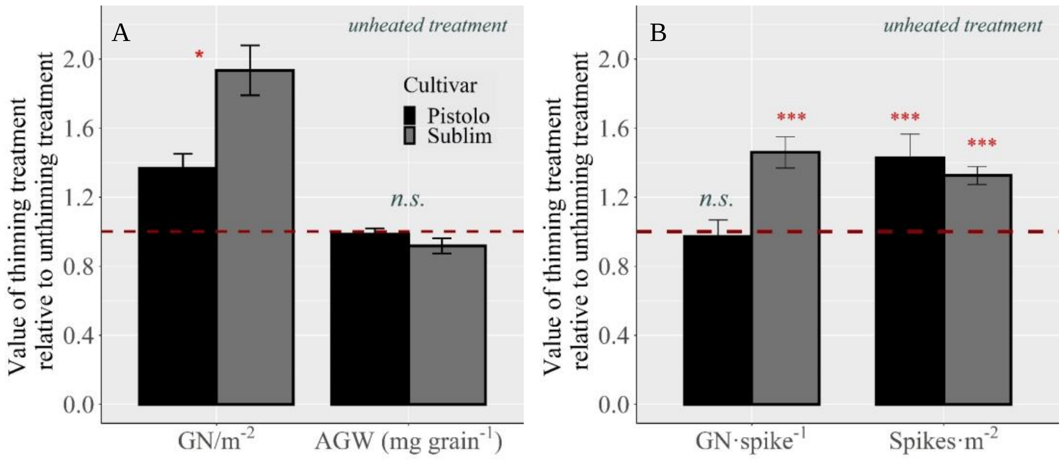

Figure 5.3. Yield components under the thinning treatment relative to unthinning treatment for grain number per m2 and average grain weight (A); and grain number per spike and spike number per m2 (B) in Bell-lloc. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001; *n.s.* = not significant).

tillers (more spikes per m^2) in both cultivars, as well as for an increased number of grains per spike only in Sublim (Fig. 5.3B). The small differences in AGW produced by thinning the plots at DC3.0 (Table 5.1) seemed to reflect that grain weight did not highly respond to increased resource availability during the stem elongation period that dramatically increased GN in any of the two genotypes. This result concurs with the hypothesis that grain growth in these genotypes is mainly sink-limited.

To determine the nature of the apparent trade-off between GN and AGW when comparing the two genotypes, I analysed again (as in the previous study) the weights of the grains corresponding to the extreme deciles of grain sizes in each case, highlighting whether or not the difference in AGW was reflecting changes in small but not in large grains (and small grains would be more frequently represented in a cultivar with larger GN).

Grain weight in Sublim was smaller than in Pistolo consistently across all grain size classes in both locations (Fig. 5.4). Thus, both lighter (bottom decile) and heavier (top decile) grains were clearly and significantly heavier in Pistolo than in Sublim (Fig. 5.4C,D), indicating that the difference in average grain size between the cultivars was constitutive, affecting similarly to all grain sizes. Therefore, the difference between the genotypes in AGW would reflect differences in potential weight of grains. This hypothesis is further supported by the fact that thinning the plots at DC3.0 increased GN dramatically, while AGW was only slightly reduced and mainly in the cultivar with larger response of GN to the thinning treatment that would have increased the proportion of small grains, but not affecting the size of the grains in any of the grain size classes.

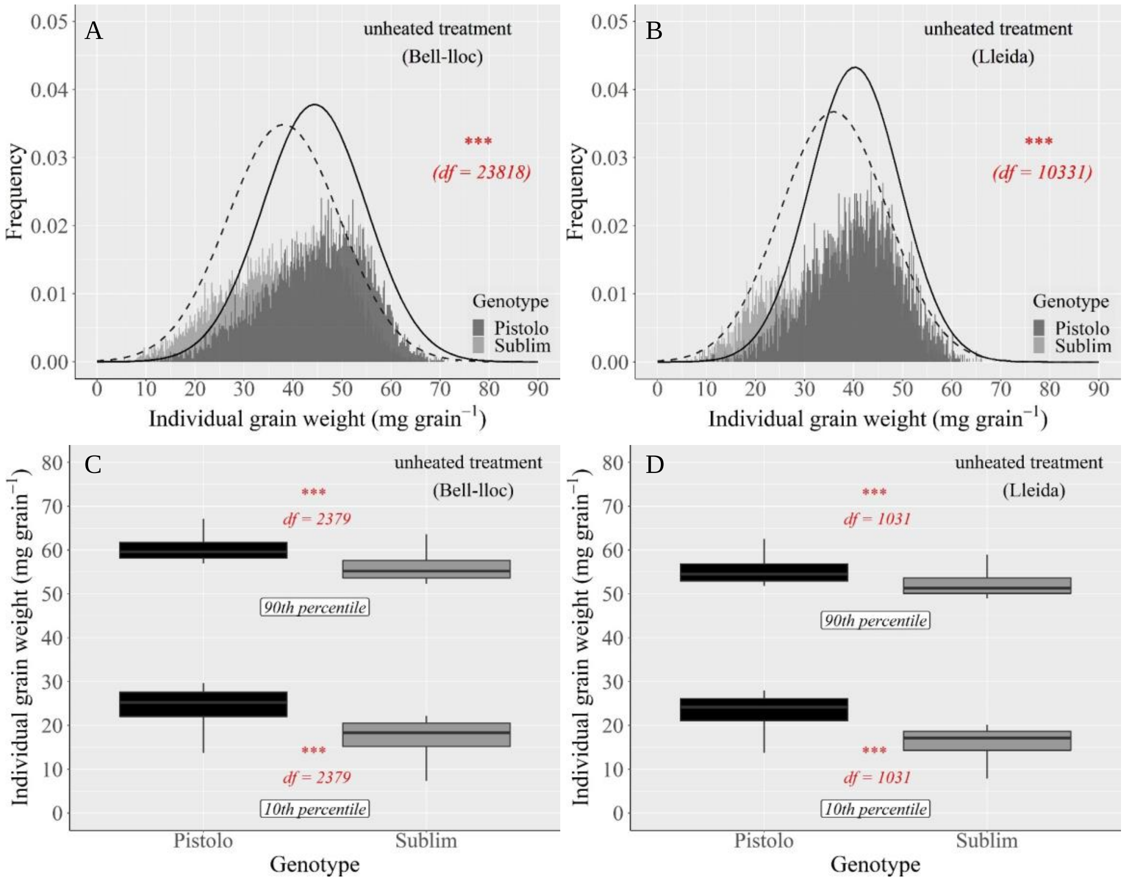

Figure 5.4. Panel A,B: Frequency distribution of the weight of individual grains for Pistolo (plain line) and Sublim (dashed line). **Panel C,D**: Boxplots of the individual weight of grains corresponding to the top and bottom deciles of the two genotypes. In both panels, the significance level of the difference and the degrees of freedom are inset. In panel A, data was averaged between thinning and no thinning treatment per genotype. All data correspond to the unheated controls. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001).

5.3.2. Yield components in response to transient heat stress

In Bell-lloc, transient heat stress affected two main yield components in wheat, but according to the stage when heat stress was imposed. Pre-AN HT affected GN but not AGW (Fig. 5.5A,B), while the latter was affected by post-AN HT that did not significantly affect GN (Fig 5.5C,D).

In Lleida, transient heat stress also affected the main yield components in relation to the timing of stress imposition. Pre-AN HT diminished GN in both genotypes, while post-AN HT did not affect GN significantly in any of the two genotypes (Fig. 5.6A), while negatively affecting AGW (Fig. 5.6B). Pre-AN HT did increase AGW (the effect was statistically significant only in Pistolo) as an indirect effect of having decreased GN (Fig. 5.6B).

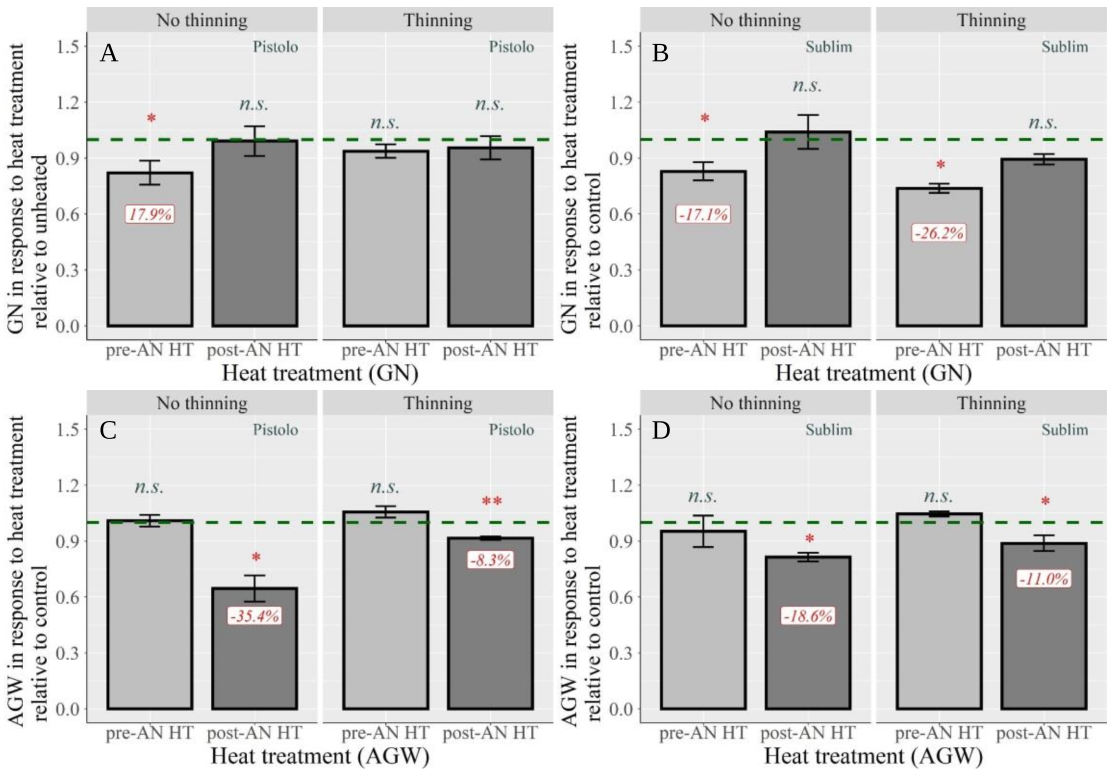

Figure 5.5. Effect of the heat treatments (imposed in pre- and post-anthesis) at Bell-lloc, considering the ratio of the trait between the heated and unheated treatments for the number of grains per unit land area (GN, A and B) and the average grain weight (AGW, C and D), with the dashed line represents the unheated treatment in Pistolo (left panel) and Sublim (right panel). Thinning treatment was divided into a panel. Whenever the effects were statistically significant, I included inset the corresponding bar the actual reduction in percentage of the unheated control. Segments stand for the SEM. Asterisks indicates the significance of the effect (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; *n.s.* = not significant).

Unlike what I found in the previous study, GN was not more sensitive to pre-AN HT than AGW to post-AN HT. It actually seemed the other way around particularly in Sublim (Fig. 5.6). However, I must keep in mind that even though I aimed to have similar levels of stress in both timings, the HT treatments were more severe in post- than in pre-AN (Fig. 5.2D). When the relative loss per unit of heat load was calculated, the sensitivity of both components was similar (pre-AN HT decreased GN by 0.72±0.13% [ºC d]$^{-1}$, while post-AN HT decreased AGW by 0.82±0.10 % [ºC d]$^{-1}$, averaged across genotypes and locations). Also, Sublim with more GN did show a consistently higher resilience for GN against pre-AN HT than Pistolo (Fig. 5.6A), but Pistolo with greater AGW did not show this component to be more resilient against post-AN HT than Sublim (Fig. 5.6B). The double HT treatment evidenced that when the crop was exposed at a pre-AN HT the following exposure to a post-AN HT was less damaging on the AGW than when the exposure was limited to the post-AN HT only (Fig. 5.7).

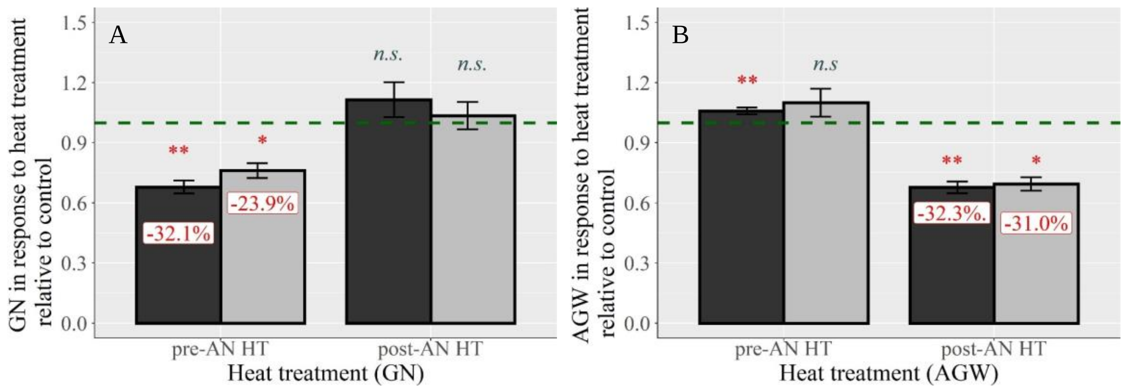

Figure 5.6. Effect of the heat treatments (imposed in pre- and post-anthesis) at Lleida, considering the ratio of the trait between the heated and unheated treatments for the number of grains per unit land area (GN, A) and the average grain weight (AGW, B), with the dashed line represents the unheated treatment in Pistolo (black bar) and Sublim (grey bar). Segments stand for the SEM. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001; *n.s.* = not significant).

This result indicates that the effect of double heat stress at post-anthesis on wheat was not additive to AGW (Fig. 5.7), exhibiting a sort of priming effect.

Transient heat stress at post-anthesis reduced not only AGW, but also the weight of all grains across the whole population of grain size classes in Pistolo (Fig. 5.8A,C), and Sublm (Fig. 5.8B,D in both locations); thus, the difference in size was significant not only for AGW, but also for the weight of the individual grains belonging to the extreme deciles of the distributions (Fig. 5.8). This indicates that transient heat stress during grain filling directly affected the capacity of the grains to grow. The priming effect when the post-AN HT was pre-empted by the exposure to pre-AN HT, was also evident in all grain classes (Fig. 5.9).

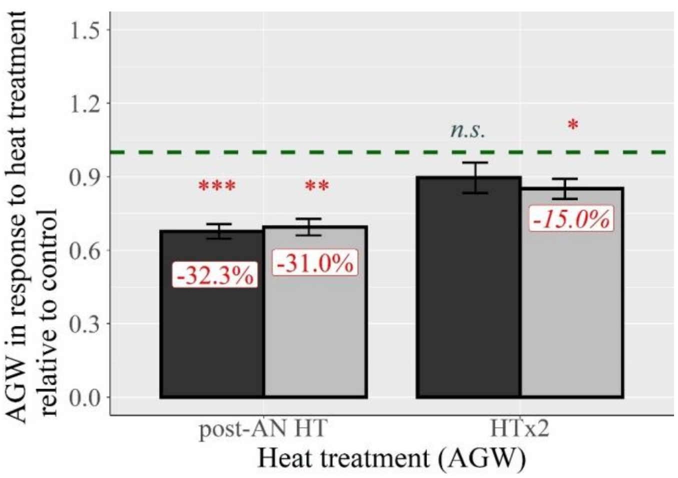

Figure 5.7. Effect of the double heat treatments (HTx2) compared with post-AN HT on the average grain weight (AGW), considering the ratio of the trait between the heated and unheated treatments, with the dashed line representing the unheated treatment in Pistolo (black bar) and Sublim (grey bar). Segments stand for the SEM. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001; *n.s.* = not significant).

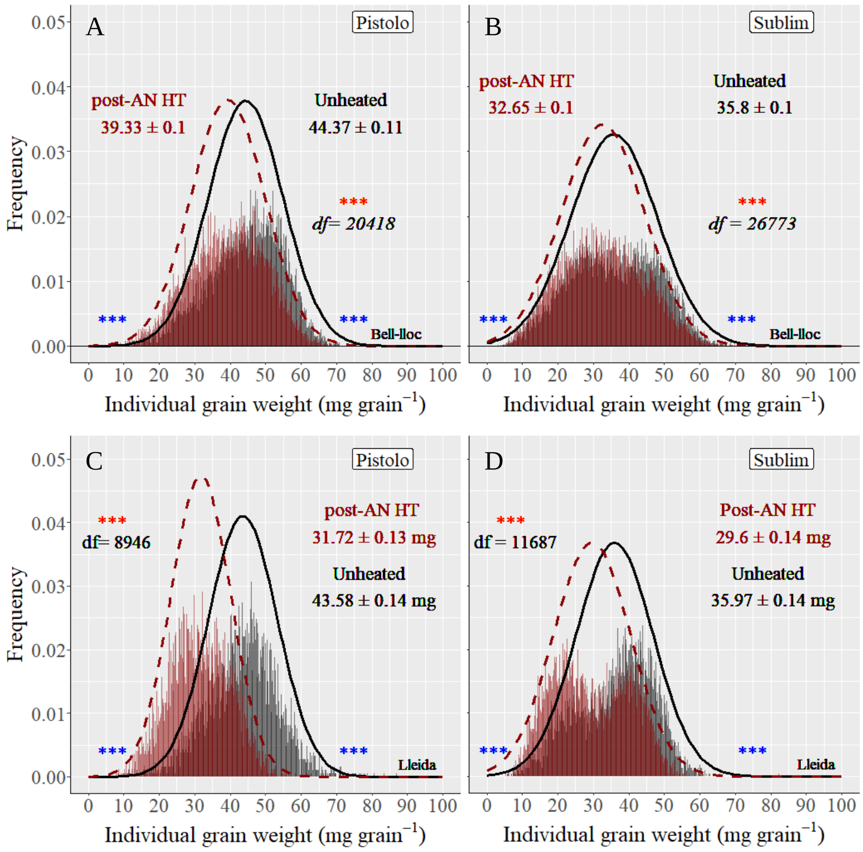

Figure 5.8. The frequency distribution of grain weights at both under control (solid line) and the post-anthesis heat stress treatments (dotted line) in Pistolo (A, C) and Sublim (B, D) at Bell-lloc (top panel) and Lleida (bottom panel). The symbol *** at the edge of the frequency indicates the significance (p<.001; heat stress reduced AGW at each certain percentile). In Bell-lloc, data was pooled between thinning and no thinning treatment per genotype, and therefore degree of freedom is much greater than in Lleida. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001).

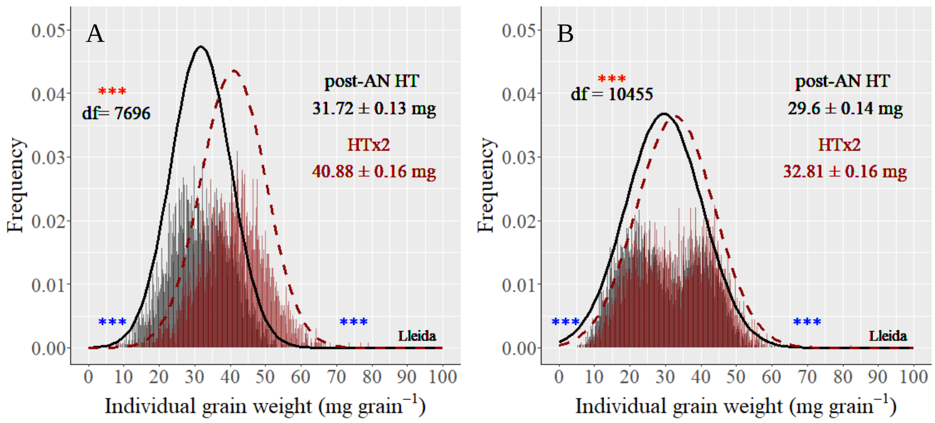

Figure 5.9. The frequency distribution of grain weights at both under post-AN HT (solid line) and the double heat treatment (HTx2) (dotted line) to Pistolo (A) and Sublim (B) in Lleida. The symbol *** at the edge of the frequency indicates the significance (p<.001; heat stress reduced AGW at each certain percentile). Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001).

5.3.3. Grain weight in response to the change of assimilate availability.

To further study the physiological nature of the trade-off between GN and AGW when comparing the cultivars, and the causes for the effects of post-AN HT on growing grains, I manipulated source and sink strength at the onset of grain filling. When assimilate availability was increased (virtually doubled) by de-graining at unheated condition, the change of grain weight of particular position of grains was minor in Pistolo (Fig. 5.10A,C) and Sublim (Fig. 5.10B,D) at both locations, showing all data points are close to 1:1 ratio line. This result indicates that wheat grains in both cultivars were dominantly sink-limited during grain filling, confirming results of the previous study (Chapter 4) that genotypic differences in AGW were due to the differences in potential grain weight (PGW). In our study, PGW (grain weight in the de-grained treatment) was analysed from the whole spikes, not only particular grain positions in spikelets, to reduce the variation. Pistolo had greater PGW (44.3 – 45.6 mg) than Sublim (35.3 – 37.7 mg) at both locations (Fig. 5.10, figures inset the de-grained panels).

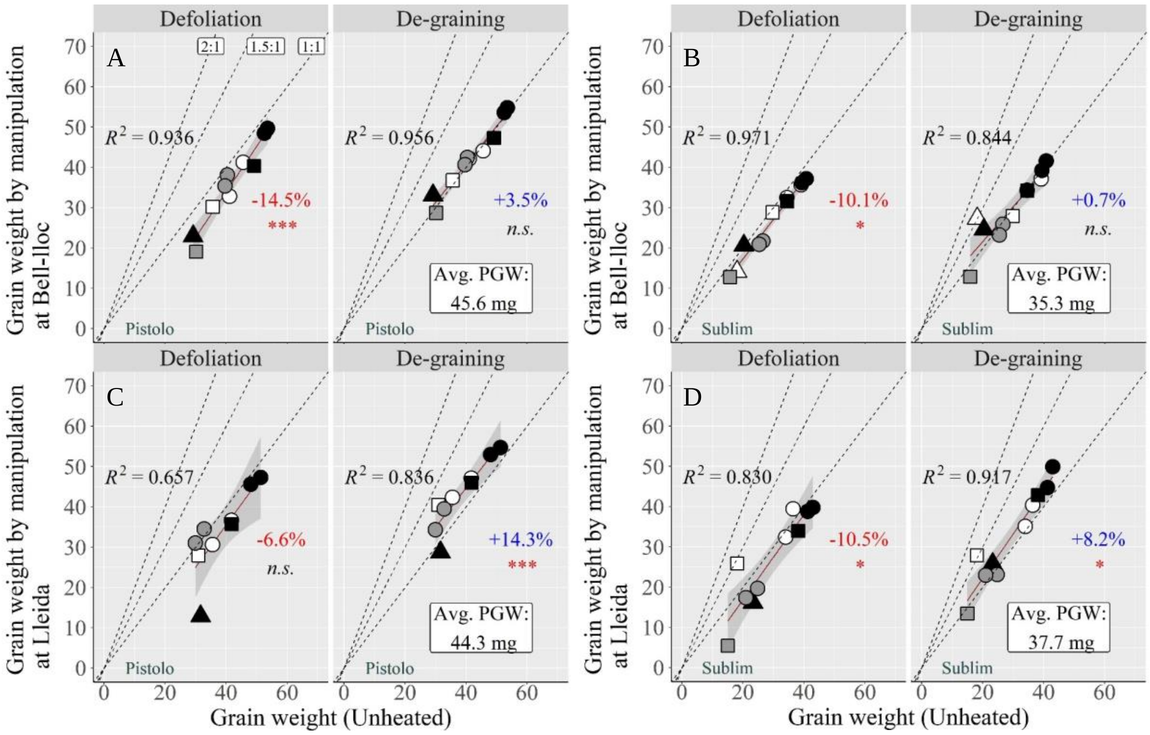

Figure 5.10. Correlation between grain weight in manipulated spikes (either defoliated or de-grained; left and right half of each panel) and grain weight in the unmanipulated control spikes for grains of particular positions from the rachis (circle is for proximal, 1st and 2nd, grains; square for the 3rd grain, and triangle is for the 4th grain) within basal (open symbols), central (closed symbols),and apical spikelets (grey symbols) in Pistolo (A,C) and Sublim (B,D) at Bell-lloc (top panels) and Lleida (bottom panels). The average responsiveness (%) to defoliation or de-graining (with its statistical significance), and the potential grain weight (PGW) of each genotype in each of the two experiments were presented inset. In Bell-lloc, data was averaged across the thinning treatments. All data correspond to the unheated controls.

Therefore, the apparent trade-off between AGW and GN when comparing the two cultivars mainly resulted from each cultivar's intrinsic PGW rather than due to a true compensation linked to their differences in GN. Defoliation seemed to have slightly decreased the weight of the grains (Pistolo: 6.6 – 14.5%, Sublim: 10.1 – 10.5%) at both locations, having been the effect stronger than in the opposite response to de-graining (in 3 out of the 4 cases). Defoliation significantly reduced grain weight compared with unmanipulated control (except Pistolo in Lleida but the reduction was 6.6 %). For de-graining, grain weight was not changed at Bell-lloc, while increased at Lleida (though marginally compared to the magnitude of the treatment, doubling the availability of resources per grain). This indicates that even though the growth of the grains were dominantly sink-limited, they would not have been far from experiencing a certain degree of source-limitation in Bell-lloc and in Lleida, indicating there would have been an incipient degree of source limitation.

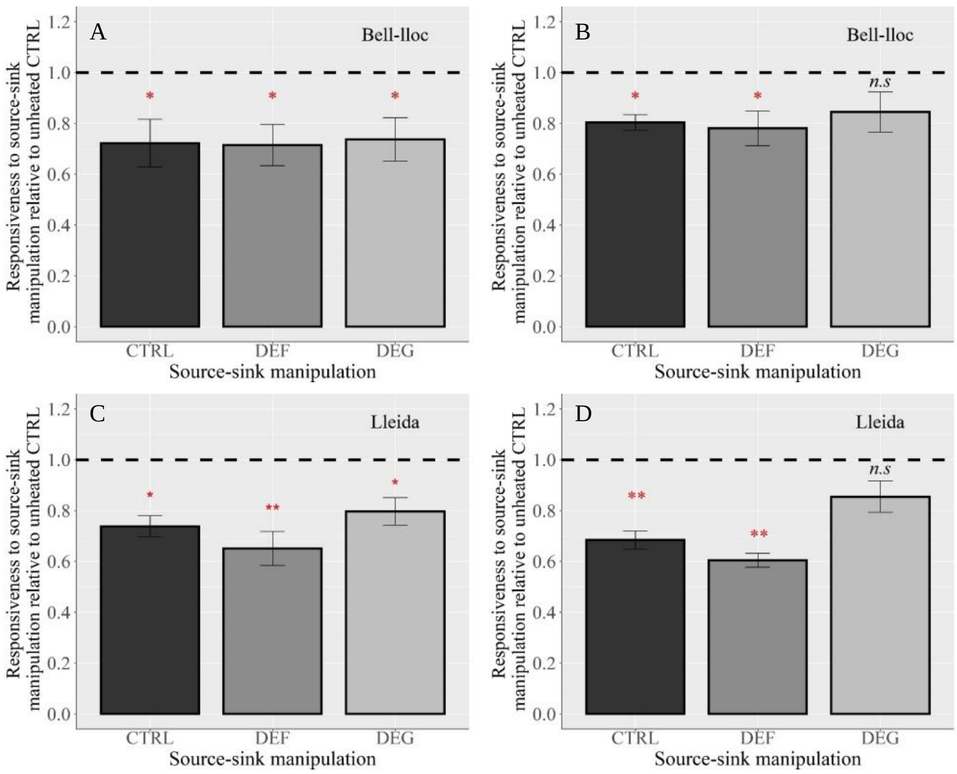

Figure. 5.11. Grain weight responsiveness to source-sink manipulation (CTRL: control, DEF: Defoliation, DEG: De-graining) under the post-anthesis heat stress in Pistolo (A,C) and Subim (B,D) at Bell-lloc (top panel) and Lleida (bottom panel). The dotted line indicates the un-heated control. In Bell-lloc, data was averaged between thinning and no thinning treatment per genotype. Segments stand for the SEM. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001; *n.s.* = not significant).

Regarding the post-AN HT effects on AGW, when more assimilates per grain were made available through de-graining, the effect of post-AN HT reducing grain weight was not reversed neither in Pistolo (Fig. 5.11A) nor in Sublim (Fig. 5.11B) in Bell-lloc, where the defoliation did not worsen the effect of post-AN HT neither. On the other hand, in Lleida the magnitude of reduction produced by post-AN HT was relatively alleviated by de-graining and slightly worsened by defoliation (Fig. 5.11C,D). This indicates that in Lleida at least perhaps part of the effect of post-AN HT on AGW would have been related to the effect of HT on the availability of resources for grain growth (particularly for Sublim) as well as to have affected the capacity of the grains to grow. This is in line with the abovementioned results for the unheated condition in Lleida (Fig. 5.10C,D).

5.4. Discussion

5.4.1. Genotypic difference in yield components

Differences in GN and AGW between these two genotypes were consistently shown across both locations: Sublim had always lower AGW, but higher GN than Pistolo, in agreement with what I observed in previous studies (Chapters 3 and 4). At least in part, the difference could be ascribed to the fact that Pistolo has awned spikes, while spikes in Sublim are awnless, as the presence of awns would reduce GN due to an increased infertility of distal florets (Sanchez-Bragado et al., 2020). This pattern implies a sort of trade-off between two yield components. Although the most popular interpretation of such trade-off is that when a genotype sets more grains, there is an increased competition for resources by the growing grains resulting in a reduction of AGW (Fischer and HilleRisLambers, 1978), other non-competitive alternatives are also possible considering the increased proportion of grains that are constitutively smaller (Miralles and Slafer, 1995; Acreche and Slafer 2006) or through directly affecting the potential size of all grains (Slafer et al., 2015).

In the present study, it seemed clear that such a trade-off was not either due to scarcity of resources to satisfy the demands of the growing grains or an increased proportion of potentially small grains as a consequence of increasing GN. The change of AGW in response to increased (virtually doubling the) assimilate supply was not significant in Bell-lloc, and even though it was significant in Lleida, the responsiveness was rather minor (8.2 – 14.3%). Moreover, when comparing individual grain weight in two genotypes, all grains were heavier in Pistolo than in Sublim, indicating the differences of AGW between two genotypes are constitutive, in agreement with results of the previous study (Chapter 4). Other studies had suggested an increased proportion of potentially small grains as a major driving force for the AGW-GN trade-off (Miralles and Slafer, 1995; Acreche and Slafer, 2006). In the present study, I analysed individual grain weights based on a sample size much greater than what has been customary in previous studies. Thus, in addition of considering the final AGW, I analyzed the individual weight of the population of grains determining that grains of Sublim were smaller than in Pistolo across all grain size classes. This discards the possibility that the trade-off was due to the increased proportion of smaller grains (e.g. more grains in distal positions), as heavier grains (i.e. 90th percentile of AGW) were also lighter in Sublim than in Pistolo.

Therefore, the difference in AGW between Pistolo and Sublim was explained by their differences in PGW *per se* of each genotype. Although our results supported that wheat yield is sink-limited during the effective period of grain filling (Slafer, 2003; Cartelle et al., 2006; Serrago et al., 2013), it seemed that these modern high-yielding cultivars are close to reach a sort of co-limitation. The reason for this assertion is that I observed at least a trend to decrease AGW when assimilate availability was decreased (by defoliation) during grain filling. Due to the current breeding strategies to consistently increase sink strength (Acreche and Slafer, 2009), the degree of source limitation could be increased in modern wheat cultivars (Kruk et al., 1997; Álvaro et al., 2008). Therefore, it would be necessary to evaluate the degree of source limitation in high-yielding wheats periodically (Calderini et al., 2006).

5.4.2. Responsiveness to increased assimilate availability at pre-anthesis

When the availability of resources per plant during the critical periods of grain number and grain weight determination was virtually doubled through thinning half of the rows at the onset of stem elongation (Fig. 5.2B), GN was dramatically increased in both genotypes, but the plasticity of GN in response was much greater in Sublim than in Pistolo, as in the former both spike number per m^2 and grain number per spike increased in response to thinning, while in Pistolo only the number of spikes per m^2 increased. Whether this different plasticity is also related to the difference in presence of awns requires further experimentation, but it seemed clear that the cultivar more effectively setting grains in the control condition was more responsive to an increased availability of resources. AGW did not respond to the extra resource availability. Only Sublim showed some reduction of AGW, while Pistolo did not respond at all. The reduction of AGW in Sublim would be due to increased GN (mostly distal grains) in a spike.

5.4.3. Effect of transient heat waves on GN and AGW

The effect of heat waves on major yield components was different depending on the timing. GN was affected by pre-AN HT, while AGW was affected by post-AN HT at both cultivars as already found in the previous study (Chapter 4). Even though the transient heat wave produced a virtually negligible effect on average temperature over the whole growing period, it strongly affected the two main yield components (each one depending on the timing of the stress), indicating yield penalties are more relevant from increased frequency of heat waves than what would be expected from a higher mean temperatures representing an overall equivalent heat load (Slafer and Savin, 2018; Elía et al., 2018). This reaffirms the need to study the effects of heat waves directly and prevents to make extrapolations from studies modifying the mean temperature over the whole (or large part of the) growing season.

Unlike what I observed in the previous study, post-AN HT effect on AGW was virtually equal to that on GN by pre-AN HT (while in the experiments presented in Chapter 4, the sensitivity of GN was larger than that of AGW). At least in part the difference between both seasons may be that in the first season (Chapter 4), there was a clear sink limitation for grain filling whilst in the second season (the present study), AGW was apparently starting to explore an incipient source-limitation. Then in this second season, heat treatments might have affected both GN and AGW through simultaneously through direct and indirect mechanisms (i.e. reducing availability of assimilates). In Lleida, de-graining treatment at post-AN HT slightly reversed heat effect, particularly in Sublim. This suggestion is further supported by the thinning treatment. When the extra resources were available during the stem elongation period, damage of heat effect was also reversed (for Pistolo, 35.4% to 8.3%; for Sublim, 18.6% to 11.0%). In Chapter 4, I found a trend for the cultivar constitutively producing more grains having less sensitivity of GN to pre-AN HT, while the cultivar constitutively having heavier grains having less sensitivity of AGW to post-AN HT. In the present study, I did not identify these differences in plasticity to heat waves that clearly.

5.4.4. Priming effect on AGW as enhanced adaptability

When transient heatwaves were imposed twice at both pre- and post-anthesis, the reduction of AGW caused by that post-AN HT was diminished compared with the effect of the same post-AN HT treatment imposed solely. It means that the effect of transient heatwaves was not additive during the whole growing period of wheat, but to some extent, there was sort of a priming effect of an earlier HT on the magnitude of the effect of a succeeding HT. Previous studies indicated that heat priming effect at pre-anthesis in wheat might improve heat tolerance at post-anthesis (Wang et al., 2014; Mendanha et al., 2018), but heat priming treatment was mostly conducted in the controlled-conditions, and it is not realistic in actual fields. In our study, heat stress at pre-anthesis was imposed in actual fields at early booting stage; DC4.3 and 15 days from anthesis; DC7.5, supporting the idea of a priming effect. Treatment effect at small interval section of growing period could not be generalised to conclude that heat priming effect at pre-anthesis could enhance resilience against heat stress at post-anthesis. Therefore, the effect of heat priming at different growing stages should be more studied.

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Chapter VI

General discussion

6. Chapter VI. General discussion

6.1. Why this research? Brief context

Wheat yield remarkably increased since the Green Revolution as a huge breakthrough of global agriculture during the 20th century (Borojevic et al., 2005; Hazell, 2014; Ray et al., 2012; Asseng et al., 2017 and references quoted therein). The *Rht1* and *Rht2* alleles shortened the length of internodes, and therefore partitioning of assimilates to the growing juvenile spikes was enhanced during pre-anthesis (Slafer and Andrade, 1993; Slafer, 2003; Quintero et al., 2018). This brought about less mortality of floret primordia, improving the numbers of fertile florets and grains (Miralles et al., 1998; Foulkes et al., 2011). This process then boosted sink strength during grain filling, mainly through increasing grain number per m^2 (GN) (Richards, 1996; Fischer, 2007) and harvest index (Austin et al., 1980; Siddique et al., 1989). Indeed, wheat breeders have successfully increased grain number but at the same time produced most frequently reductions of average grain weight (AGW), representing a compensation or trade-off between components (Cartelle et al., 2006). Beyond the physiological bases of the green revolution and of the successful yield gains achieved in the past, it seems also relevant identifying physiological traits that might be relevant for future breeding. When aiming to improve yield (or any complex agronomic attribute) breeders tend to restrict the crosses to elite x elite material. In this context and for prospective analysis, it seems relevant to establish the physiological bases for the differences in AGW in relation to GN among elite materials. Ascertaining the causes for the frequently reported trade-off between AGW and GN restricting the analysis to elite material is therefore relevant. Although AGW is realised during the effective period of grain filling, there are indications it may actually be positively related to potential grain weight (PGW), which represents the intrinsic capacity of grains to accumulate dry matter (Bremner and Rawson, 1978). As breeding has been consistently increasing sink-strength, it would be critical to periodically evaluate the degree of source-limitation regarding high-yielding modern elite wheat cultivars to elucidate the physiological causes of the trade-off between AGW and GN, responding to whether it is due to competition (source limitation) or non-competition (sink limitation). According to this, the breeding strategy and farming practice may be different. Also, it would be important to elucidate how much magnitude of plasticity of GN and AGW is possibly relevant to assimilate availability (resources) and high temperature heat stress (signals) to understand possible physiological responses of wheat to future environmental scenarios.

In this context, I conducted 5 seasons consecutive field research and elucidated source and sink strength in response to resources and signals. In Chapter III, I elucidated plasticity of GN and AGW in response to the contrasting nitrogen condition (resources), and plasticity of GN and AGW in response transient heatwaves (signals) in Chapter IV and V.

6.2. Integration of the main results

In this chapter, I have mainly focused on (i) highlighting main results (though avoiding lengthy repetitions of what has been given in previous chapters), (ii) integrating them across different experimental chapters, (iii) summarising the outcomes of testing of the main hypothesis, and finally (iv) the contributions of the knowledge achieved.

6.2.1. Plasticity of GN and AGW in response to resource availability

In 2015-2016 growing season, I analysed 20 different cultivars to elucidate genotypic variation in response to nitrogen level for yield and yield components. GN and yield were highly responsive (55.0 and 58.7%, respectively across all cultivars), while AGW did not clearly respond (avg. 2.2% across all cultivars) to the nitrogen condition (Fig. 6.1).

The plasticity of yield to nitrogen condition in each cultivar was strongly related to plasticity of GN, even showing similar variation (see boxplots in Fig.6.1). Therefore, the main source of variation in yield is more relevant to GN rather than AGW. When yield increased c. 367.1g averaging across all cultivars to increased N condition, GN increased c. 7,424 grains, while c. 1.2mg increased in AGW (Fig. 3.1 in Chapter III). Also, when responsiveness of yield to nitrogen condition was compared with and GN and AGW, responsiveness between GN and yield showed clear linearity (R^2=0.891) (Fig. 6.2A), while no relationship was shown between responsiveness between AGW and yield (Fig. 6.2B).

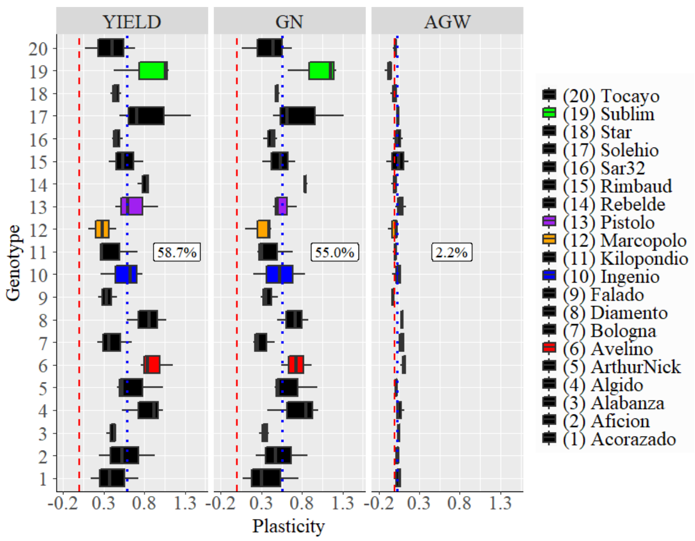

Figure 6.1. Boxplot of plasticity in yield, GN and AGW to nitrogen condition across 20 cultivars. Yield, GN and AGW was divided into sub-panels in a panel. The name of 20 cultivars and its corresponding number was presented to the legend (right). Five cultivars for the following two seasons were colored. Red dashed line indicates no changes of cultivars in response to nitrogen condition, and blue dotted line indicates the average of plasticity across all cultivars (average value is also inserted in each sub-panel).

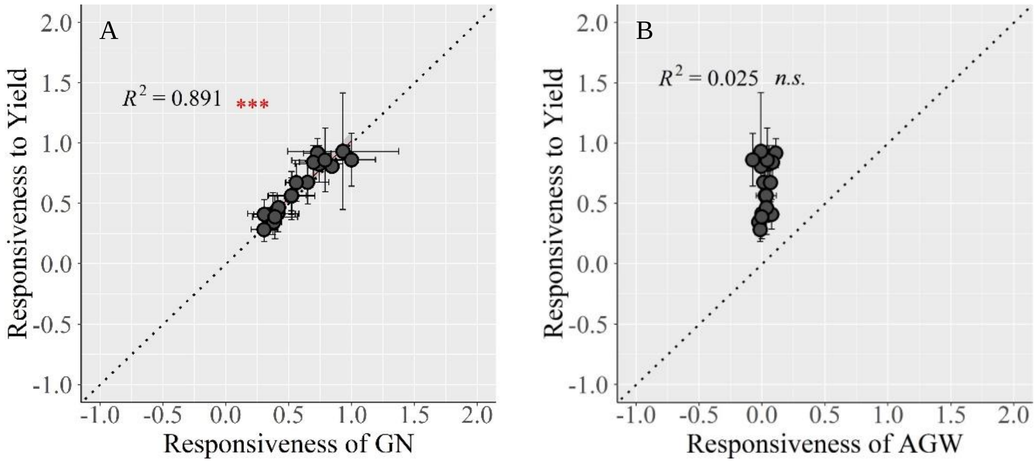

Figure 6.2. Correlation between responsiveness of yield and GN (A) and AGW (B) in 2015-2016 growing season. Dashed lines in each panel are the 1:1 ratio where responsiveness of each yield component and yield is equal. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001; *n.s.* = not significant).

However, even though AGW was a minor source of variation for final yield, there was a common negative relationship between GN and AGW driven by genotypic differences (Fig. 3.3A in Chapter III).

Obviously, AGW did not clearly respond to increased resource availability. I analyse the change of AGW in response to nitrogen condition (Chapter III); and increased assimilate availability at stem elongation (Chapter V). When the distribution of the individual grain weights was compared between N_0 and N_1; and thinned and un-thinned treatment, all grains were hardly affected by increased GN due to increased nitrogen availability (Fig. 6.3A) and assimilate availability (Fig. 6.3B)

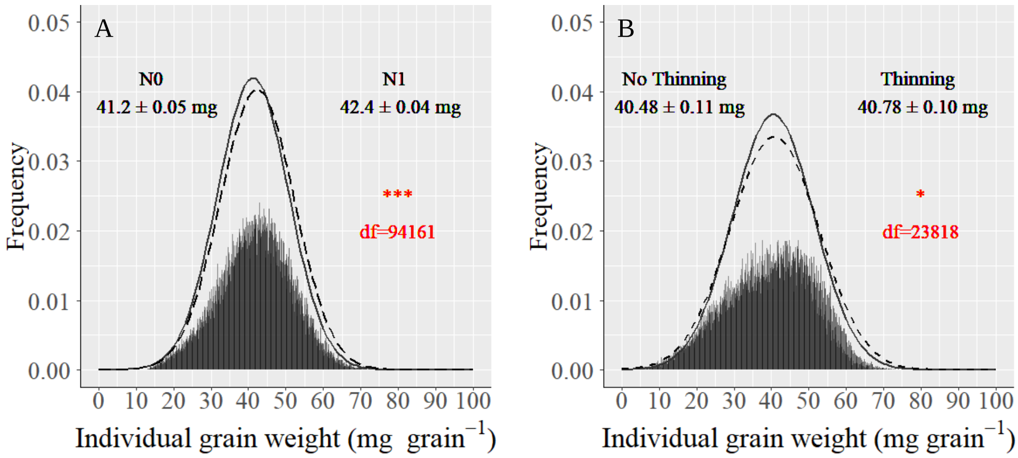

Figure 6.3. The frequency distribution of cultivars to nitrogen condition (A) and thinning treatment (B). In panel A and B, dotted curve lines for N_1 condition and thinned treatment; and solid curve lines for N_0 condition and un-thinned treatment respectively. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001). In panel A, 20 cultivars were pooled and in panel B, 2 cultivars were pooled.

Enhanced nitrogen and assimilate availability increased GN by increasing both grain number per spike and spike number per m^2, but increased GN was more related to number of spikes (i.e. increased fertile tillers) than grain number per spike (i.e. increased distal grains) (Fig. 6.4).

Therefore, to elucidate whether the trade-off between GN and AGW reflects an increased competition among growing grains in genotypes with more GN or a constitutive genotypic difference, I narrowed down 5 cultivars out of 20 cultivars for the following two seasons (2016-2017 and 2017-2018 growing season), and the distribution of the individual grain weights was compared across all cultivars (Fig. 6.5).

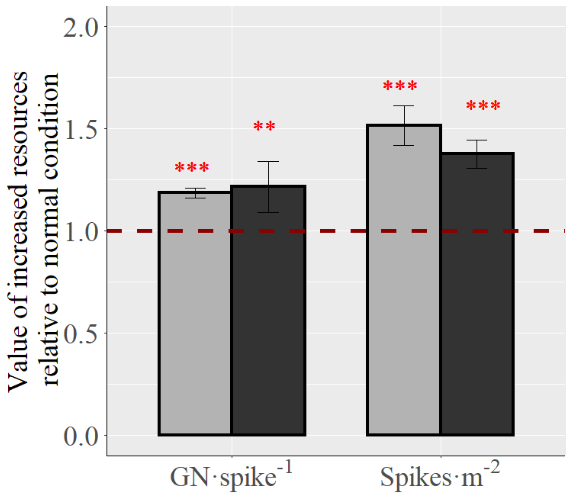

Figure 6.4. Yield components under the enhanced resource availability relative to normal condition (control) for grain number per spike (A) and spike number per m^2 (B). Gray and black bar indicate nitrogen treatment (Chapter III) and thinning treatment (Chapter V) respectively. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001).

The difference of AGW among cultivars seemed that all grain sizes were reduced as the consequence of increased GN, indicating that both heavier and lighter grains become smaller when GN increases.

To determine whether the negative relationship between the two main yield components of wheat truly reflects competition for resources among growing grains or not, source-sink manipulation was conducted during grain-filling. Wheat cultivars were mostly sink-limited during grain filling as there was no (or there was a very minor) change of AGW by de-graining, showing data-points for all genotypes are around the 1:1 ratio line (Fig. 6.6).

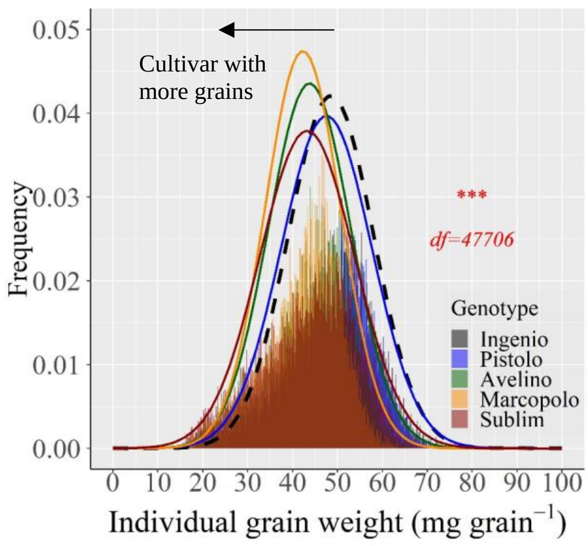

Figure 6.5. The frequency distribution of 5 different cultivars. Dotted curve line indicates Ingenio having greatest AGW, and solid curve lines are 4 other cultivars. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001). Two seasons and two nitrogen conditions (N_0, N_1) were pooled.

This result implies that the reduction of AGW as the consequence of increased GN might not be due to competitive cause. If de-grained AGW is regarded as potential grain weight (PGW), the trade-off between two main yield components might be mainly due to the intrinsic potential grain weight *per se*. Further supporting was that when plotted potential vs actual grain weight (PGW vs AGW), it showed greater R^2, indicating current AGW fully represents its intrinsic potential weight. However, there was some reduction of AGW (c.14.4%) by defoliation during grain filling (Fig. 6.6), indicating that those modern elite wheat cultivars do not show a strong sink limitation, and some degrees of source limitation might be possible, particularly in cultivars with lower potential grain weight.

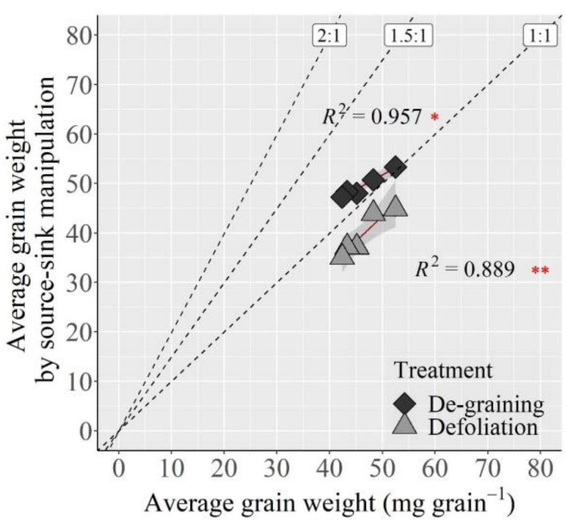

Figure 6.6. Correlation between AGW and manipulated AGW by each source or sink treatment in five different cultivars. Each dotted lines indicate 1:1, 1.5:1, and 2:1 ratio between manipulated AGW and control AGW respectively. Two seasons (2016-2017 and 2017-2018 growing seasons) and two nitrogen conditions (N_0, N_1) were averaged. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001).

6.2.2. Plasticity of GN and AGW in response to transient heatwaves

GN was significantly reduced by pre-anthesis heat stress (pre-AN HT), while AGW was mostly reduced by post-anthesis heat stress (post-AN HT) (Fig. 6.7), while heat stress at pre- and post-anthesis did not significantly affect AGW and GN respectively (Fig. 4.4A,B in Chapter IV).

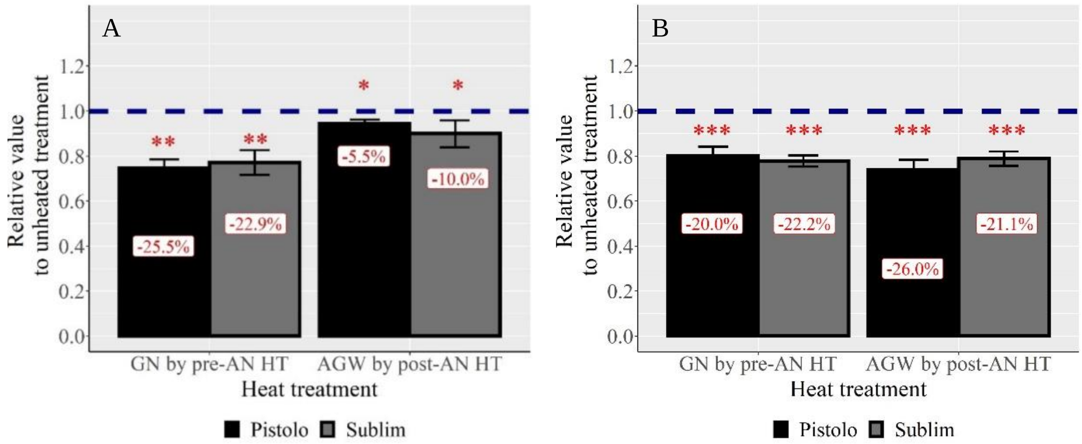

Figure 6.7. Effect of the heat treatment of each critical period of GN (pre-AN HT) and AGW (post-AN HT) considering the ratio of the trait between the heated and unheated treatments in 2018-2019 growing season (A; Chapter IV) and 2019-2020 growing season (B; Chapter V). Dashed line represents the unheated treatment. The inset is the corresponding bar the actual reduction in percentage of the unheated control. Segments stand for the SEM. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001). In panel A, two locations (Bell-lloc and Lleida) were averaged; and in panel B, two thinning treatments (thinned and un-thinned) with two locations (Bell-lloc and Lleida) were averaged.

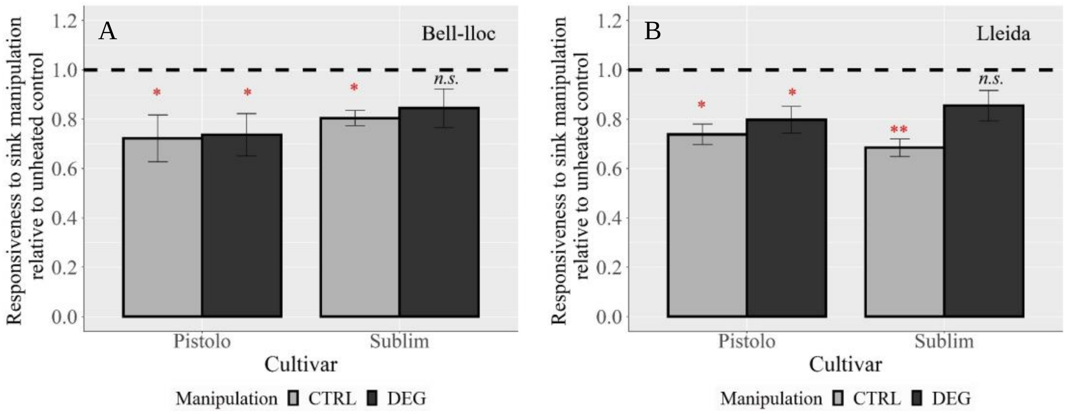

Figure 6.8. Grain weight responsiveness to sink manipulation (CTRL: control, DEG: De-graining) under the post-anthesis heat stress at Bell-lloc (A) and Lleida (B) in 2019-2020 growing season. The dotted line indicates the un-heated control. Segments stand for the SEM. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001; *n.s.* = not significant). In panel A, data was averaged between thinning and no thinning treatment per genotype.

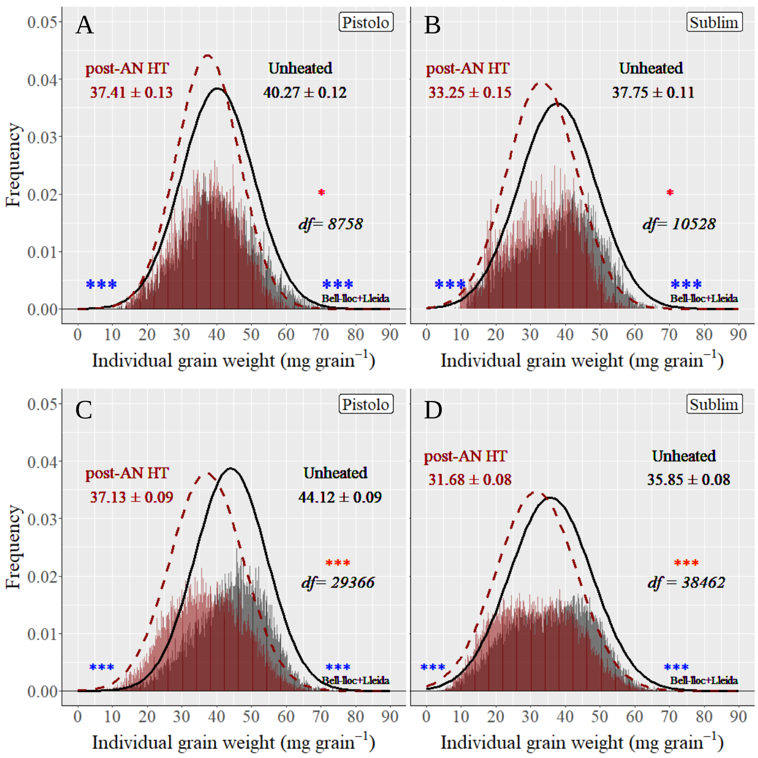

Figure 6.9. The frequency distribution of grain weights at both under control (solid black line) and the post-anthesis heat stress treatments (dashed red line) in 2018-2019 (top panels) and 2019-2020 growing season (bottom panel). The symbol *** at the edge of the frequency indicates the significance (p<.001; heat stress reduced AGW at each certain percentile). In top panels, data was pooled between thinning and no thinning treatment with two locations; and in bottom panels, data was pooled between two locations. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001).

GN was more sensitive to pre-AN HT than AGW to post-AN HT in 2018-2019 growing season (c. more than 20% reduction in GN, while c. 5-10% in AGW; Fig. 6.7A), but the sensitivity of each yield component to different stage of heat stress was similar in the next season (Fig. 6.7B). This result indicates that AGW might start to explore an incipient source-limitation as in this second season, indicating that heat treatments might have affected both GN and AGW simultaneously through direct and indirect mechanisms (i.e. reducing availability of assimilates).

This suggestion is further supported by de-graining treatment in 2019-2020 growing season. De-graining treatment at post-AN HT slightly reversed heat effect at both locations (Fig. 6.8A,B).

When comparing distribution of individual grain weight, both lighter and heavier grains were affected by post-AN HT in both seasons (Fig. 6.9), which might suggest that transient heatwaves affected both source and sink strength. Transient heatwaves might affect heavier grains, and lighter grains might be more affected by transient heatwaves, resulting in reduced source strength.

Although transient heatwaves might have reduced source or sink strength, when wheat was exposed at a pre-AN HT at Lleida in 2019-2020 growing season, the following exposure to a post-AN HT was less damaging on the AGW than when the exposure was limited to the post-AN HT only (Fig. 6.10).

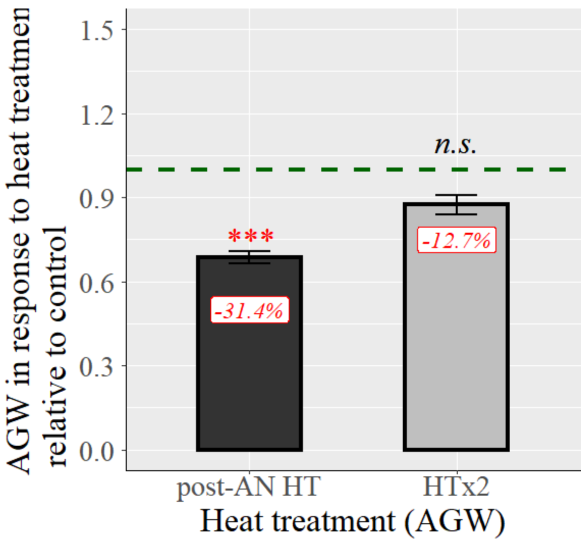

Figure 6.10. Effect of the double heat treatments (HTx2) compared with post-AN HT on the average grain weight (AGW), considering the ratio of the trait between the heated and unheated treatments, with the dashed line representing the unheated treatment with inset of the corresponding bar the actual reduction in percentage of the unheated control. Segments stand for the SEM. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001; *n.s.* = not significant). Data was averaged between two genotypes (Pistolo and Sublim).

6.3. Verifying the scenario of competitive and non-competitive mechanisms

In Chapter I, I suggested two scenarios to interpret competitive and non-competitive mechanisms to understand the trade-off between GN and AGW. The first scenario was that when GN increases, all grains (both smaller and bigger size) are decreased (Fig 1.2A), contributing to decreasing AGW (Fig 1.2C) as a sign of competition among growing grains. The second scenario was that even though grain number increases, grain weight (both smaller and bigger size) is not changed (Fig 1.2B), indicating the actual reduction of grain weight is mainly due to the increased proportion (%) of smaller grains, not due to competition (Fig 1.2D).

To verify those scenarios, I combined all data of GN and AGW for the 5 growing seasons and calculated the ratio of each genotype per treatment in GN, AGW, 10th and 90th percentile's AGW to the average value of the trait. Obviously, it showed the negative relationship between GN and AGW of two genotypes (Fig. 6.11B).

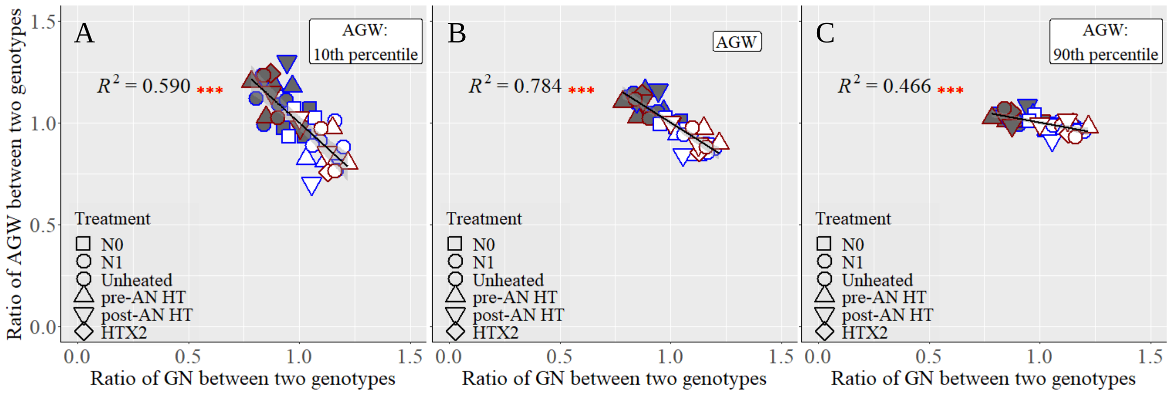

Figure 6.11. Correlation between ratio of GN and 10th percentile's AGW (A); grand AGW (B); 90th percentile's AGW (C) in Pistolo (grey color) and Sublim (white color). Blue and red border indicate Bell-lloc and Lleida respectively. Asterisks indicates the significance of the effect (* p<0.05; ** p<0.01; *** p<0.001).

Moreover, in both group of lighter (Fig. 6.11A; 10th percentile) and heavier group (Fig. 6.11C; 90th percentile), it also showed the trade-off between GN and AGW, but the magnitude of trade-off was much greater in 10th percentile than in 90th percentile. It showed clear differences between Pistolo and Sublim in GN, AGW, and both 10th and 90th percentile's AGW (Fig. 6.12), indicating Pistolo has always greater AGW in all grain size classes with less GN than Sublim.

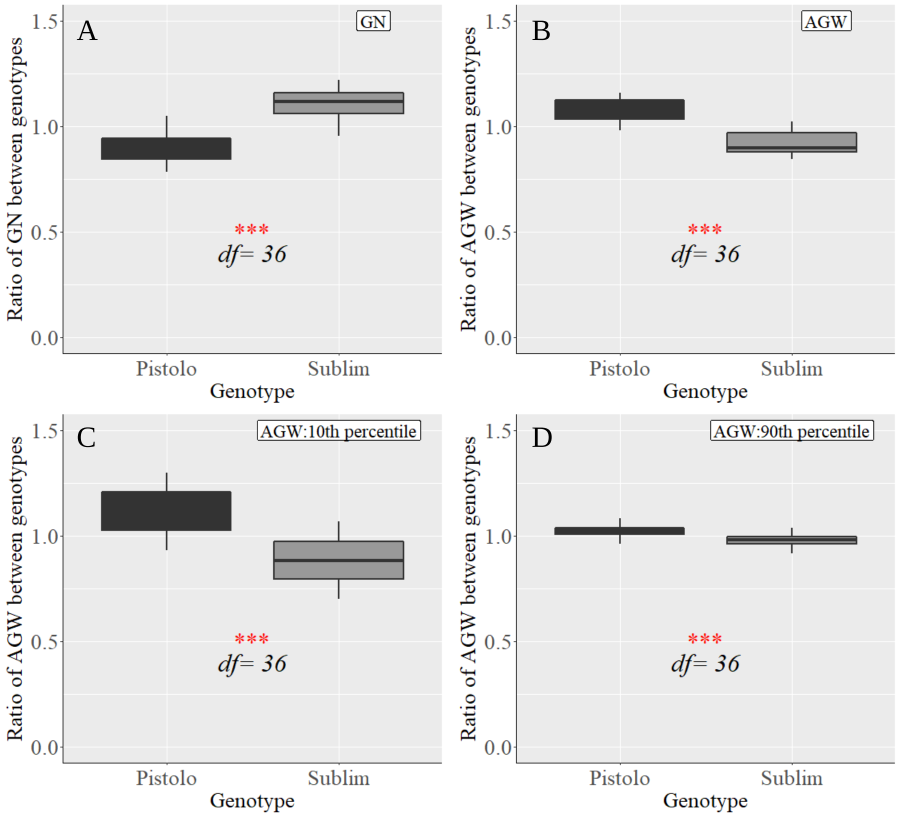

Figure 6.12. Boxplots between Pistolo and Sublim for GN (A), AGW (B), 10th percentile's AGW (C) and 90th percentile's AGW (D). Asterisks indicates the significance of the effect (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

The analysis of all data from all experiments suggests that our non-competitive scenario should be amended. In the scenario of non-competition (Fig. 1.2B,D), I assumed that when GN increases, both lighter and heavier grains would not be changed, and the reduction of AGW due to increased GN would be mainly due to an increased proportion of smaller grains in agreement with that many studies have indicated (but mainly working with more diverse genotypes, like with NILs for semi dwarfism). However, in this thesis, I suggest that the trade-off between GN and AGW is mainly due to potential grain weight *per se* rather than an increased proportion of smaller grains. Therefore, the non-competitive scenario should be changed as Fig. 6.13A. When GN increases, both lighter and heavier grains will be reduced (Fig. 6.13B), but it is not due to competition, but due to potential grain weight of each genotype together with an increase in the proportion of constitutively small grains making the slope of the relationship for the 10th percentile much steeper than that for the 90th percentile.

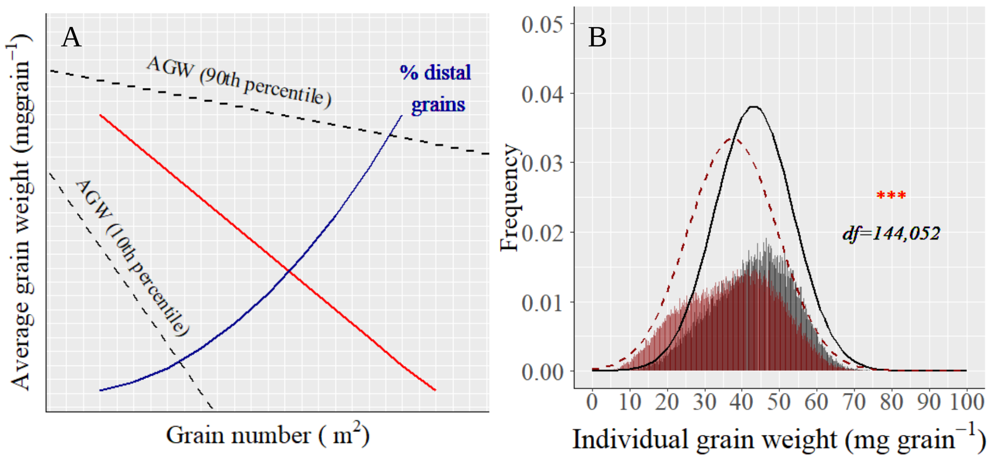

Figure 6.13. The new interpretation of non-competitive mechanisms regarding the trade-off between grain number and average grain weight (A), and the frequency distribution of individual grain weight between two genotypes (B). Asterisks indicates the significance of the effect (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). In Panel B, red dotted curve line indicates Sublim (genotype with more GN) and black solid curve line indicates Pistolo (genotype with less GN), and data from 5 growing seasons with all experiments were pooled.

6.4. Testing the hypothesis

The hypotheses offered in Chapter I were verified through independent experiments (Chapter III – V) along this Thesis.

Hypothesis I: Trade-off between GN and AGW would be mainly due to non-competition mechanisms, and it would be more relevant to the intrinsic weight potential of grains.

This hypothesis was accepted as there were no evidence of source-limitation during grain filling in modern elite wheat cultivars tested under contrasting nitrogen condition (Fig. 6.6). Therefore, the trade-off between GN and AGW across genotypes resulted from potential grain weight *per se.*

Hypothesis II: Transient heat waves would reduce GN and AGW with different magnitude at each growing period.

This hypothesis was accepted as the penalty of each component caused by heat waves were different at each growing period. GN was mainly affected by heat waves at pre-anthesis, while heat waves at post anthesis affected more AGW (Fig. 6.7). Our hypothesis was that there would be a trend for the cultivar constitutively producing more grains having less sensitivity of GN to pre-AN HT, while the cultivar constitutively having heavier grains having less sensitivity of AGW to post-AN HT. However, in two consecutive growing seasons, I did not that clearly identify these differences in plasticity regarding each yield components to heat waves.

Hypothesis III: Transient heat waves would mostly affect sink strength rather than source strength during grain filling as the trade-off between main yield components would not be relevant to assimilates availability.

This hypothesis was accepted as transient heat stress reduced both lighter and heavier grains (Fig. 6.9). If heat stress affected source strength, lighter grains would be more affected due to lack of resources, while heavier grains would show some resilience against reduced resources. To support this hypothesis, when assimilate availability was increased under heat stress, grain weight was less responsive in Pistolo and Sublim at both locations (Fig. 6.8). If heat stress affected source strength, when more assimilates were supplied, grain weight should be responsive to the increased assimilate availability. Therefore, transient heat waves mostly affected sink strength rather than source strength during grain filling. However, in Sublim, it showed AGW was slightly reverted by de-graining at both locations compared with Pistolo (Fig. 6.8A,B). This result indicates that AGW may apparently be starting to explore an incipient source-limitation, particularly in Sublim.

Hypothesis IV: Transient heat waves would affect yield components with more effect on genotypes with less efficiency in determining that component (i.e. a cultivar more efficient in setting grains would be less sensitive to heat in pre-anthesis, when GN is being determined; whilst a cultivar with more efficiency in determining AGW would be less sensitive to heat in post-anthesis).

This hypothesis was rejected. Although I found results in line with the hypothesis in Chapter 4, in the following experimental season (Chapter 5) both cultivars responded similarly regardless of their efficiency in determining a particular component.

Hypothesis V: Transient heat waves would not be additive when wheat was exposed to heat waves at early growing stage, and it might be able to provide sort of priming effect on grains when imposed at early stage of development.

This hypothesis was accepted as when heat was exposed at early heat stress before anthesis and again during grain filling, it showed some resilience against heat stress at post-anthesis, showing less reduction of AGW than the single heat stress imposed 15 days after anthesis (Fig. 6.10).

6.5. Main contribution of knowledge

Through testing the hypotheses with the experiments carried out along the research of this Thesis, several contributions of knowledge were made.

First, elite modern wheat cultivars are still sink-limited during grain filling, though there might be evolving a certain degree of source-limitation, indicating that future wheat cultivars will become co-limited unless breeders find ways to increase source strength while further increasing sink strength (Fig. 1.4C; hypothesis of co-limitation). This is based on the fact that even though increasing the availability of assimilates per grain did hardly increase AGW, reductions in availability of assimilates per grain diminished AGW. Therefore, future breeding strategy should be increase both sink and source strength of wheat to improve yield.

Second, when increasing sink strength, it would be as important to increase potential grain weight (sink activity) as grain number (sink size). Actual trade-off between GN and AGW was mainly due to potential grain weight *per se* rather than an increased proportion of smaller grains (though the latter also contributed partially). This result shows novelty as to date most studies indicated the trade-off is due to an increased grains having lower grain potential. Our approach to elucidate the trade-off, combining responses of individual grains (and considering many thousands of individual grains in each sample) with alterations of source-sink balances was novel.

Third, transient heatwaves mainly affected sink strength rather than source strength. The sensitivity of heat stress was different in pre- and post-anthesis, but the main yield penalty was in general driven by heat stress at pre-anthesis, and I discussed for the first time on the nature (direct and/or indirect) heat effect to setting grains and to growing grains.

Fourth, I demonstrated under field conditions that there would be a priming produced by an earlier heat event on the magnitude of the later heat event. Slafer and Savin (2018) indicated that nitrogen stress at pre-anthesis could bring about sort of priming effect to mitigate yield penalty caused by heat stress. Therefore, when the frequency of heat stress increases, it would be important to take this priming when assigning expected penalties due to heat.

6.6. Major conclusions of the Thesis

- GN and AGW presented differences in phenotypic plasticity (in response to the contrasting nitrogen condition). Across the three field studies involving N availabilities, GN and yield showed to be very plastic, while AGW was extremely conservative.
- However, whilst GN was mostly much more strongly affected by availability levels (two contrasting soil nitrogen effect) than by genotypic effect variation among modern and well adapted cultivars, the genotypic effect was more quite relevant to for AGW.
- In the present study, cultivars with lower AGW not only had more grains of smaller size, but also exhibited a clear difference in size of their largest grains (here the 90^{th} decile, or 10% heaviest grains of each cultivar): heavier grains were also affected. All grain size classes were smaller in genotypes with more GN than genotype in those with less GN.
- Even though grain growth was dominantly sink-limited, the elite cultivars which I analysed in this Thesis seemed approaching to experience some degree of source-limitation to grain growth and breeders may need to also consider improvements in source strength together with further raising GN or PGW.

- Exposing the crops to heat stress at pre- and post-anthesis resulted in yield penalties, but yield was more sensitive to pre-anthesis heat stress than to post-anthesis, consistently across both locations and genotypes (i.e. GN was more markedly reduced by pre-anthesis heat stress; pre-AN HT than AGW by heat stress at post-anthesis; post-AN HT).
- The reduction of AGW caused by post-AN HT was in general not related to source strength changes, but due to a direct effect reducing potential grain weight *per se*.
- There seemed to be a trend (clear in one season but not in the other one) for the cultivar constitutively producing more grains having less sensitivity of GN to pre-anthesis heat stress, while the cultivar constitutively having heavier grains having less sensitivity of AGW to post-anthesis heat stress. It seemed that the plasticity of a yield component in response to heat stress was inversely related to the relevance of that component for yield determination in unheated conditions.
- However, post-AN HT effect on AGW was virtually equal to that on GN by pre-AN HT in the next season. AGW was apparently starting to explore an incipient source-limitation. In this second season, heat treatments might have affected both GN and AGW through simultaneously through direct and indirect mechanisms (i.e. reducing availability of assimilates).

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ANNEX I

Are portable polyethylene chambers reliable for imposing heat treatments in field experiments?

ANNEX I. Are portable polyethylene tents reliable for imposing heat treatments in field-grown wheat?

1. Introduction

In recent decades, there has been a growing concern about the consequences of heat effects on wheat performance (Calderini et al.,1999; Shah and Paulsen, 2003; Asseng et al., 2011, 2015; Ugarte et al.,2007), and the same problems are being projected for other crops. For instance, the number of papers published with both "wheat" and "heat" (Fig. Ai_1A) in the title and an analysis of their impact (Fig. Ai_1B) was negligible until a few decades ago and has been growing exponentially in recent times. I searched for "heat" rather than "temperature" because in some studies as the latter is not considered in terms of stress, whereas heat is implicit it its stress connotations. However, searching for both "wheat" and "temperature or heat" yielded similar exponential relationships.

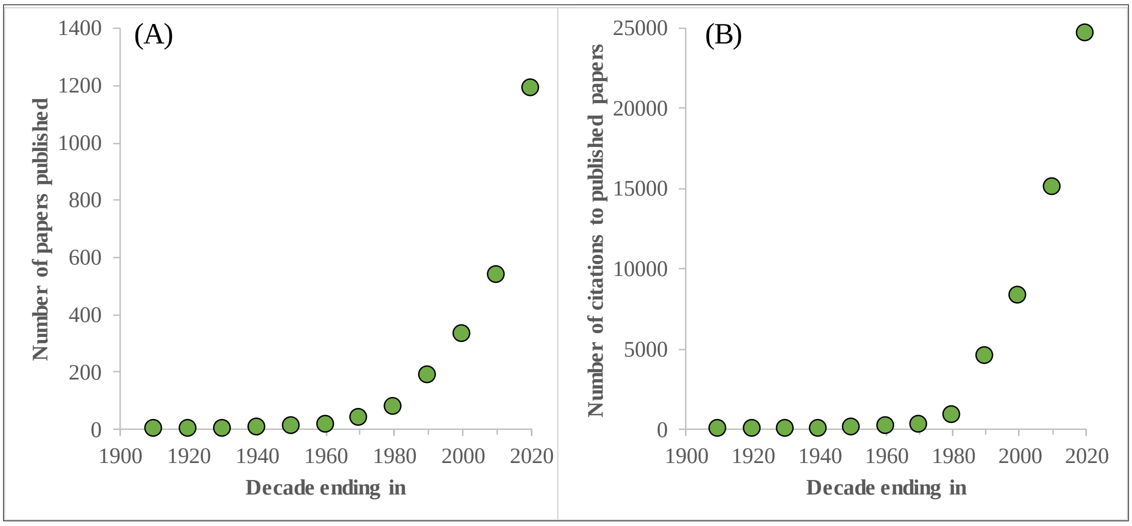

Figure Ai_1. Cumulative number of papers having in the title both "wheat" and "heat" published in JCR-journals indexed in the Core Collection of the Web of Science (A) or citations to these papers (B) during the last 12 decades (from 1901-1910 to 2011-2020). Data were obtained on 8 January 2021

This growing interest is due to an increasing certainty established over the last few decades that crops will be exposed to higher temperatures and that substantial crop yield losses are expected (Teixeira et al.,2013; Challinor et al., 2014). Indeed, the effect of high temperature on wheat yield and quality attributes has been recognised for over half a century (Chinoy, 1947; Finney and Fryer, 1958).

At the same time, environmental variability has increased more than in previous decades (Shah and Paulsen, 2003; Asseng et al., 2017), and in particular, heat waves will become more frequent and intense worldwide (Meehl and Tebaldi, 2004; Alexander et al., 2006; Orlowsky and Seneviratne, 2012; Seneviratne et al., 2014).

Despite its relevance, it is challenging to identify a simple, reliable, and cost-effective methodological approach for imposing high temperature treatments in field experiments so that the effects of heat on crop growth, yield and other complex traits can be quantified. A method converging these three qualities involves constructing portable tents of polyethylene that can be deployed over the plot. Like other methods, it has some drawbacks, the most critical one being the reduction in incoming radiation produced by the polyethylene. However, this confounding effect is debatable. These portable tents are ideally suited for experiments carried out in remote locations (e.g., in actual fields, beyond experiment stations) where there is no access to electricity and by research projects with limited funding.

In this study, I first describe the current main approaches that have been deployed to enable quantifying the effects of heat on yield and yield related traits in wheat and other crops, briefly highlighting their pros and cons; and secondly discussing whether the presumed confounding effects of reduced radiation are substantiated when temperatures are increased in field conditions by means of portable polyethylene tents and other methods.

2. Major approaches

There are indirect and direct methods to quantitatively determine the effect of heat on crop performance (for a detailed treatment of the methods, please see Lawlor, 1996; Bonada and Sadras, 2015). The indirect methods quantify the effects of heat without imposing heat treatments, while direct methods expose plants to contrasting heat treatments and measure the effects. Indirect methods are very popular because they are both simple and cost-effective. The two most common ones are: comparisons of the performance of a crop under two or more thermally contrasting conditions (i.e. different locations, different sowing dates, different seasons) and predictions offered by mathematical simulation models. Although results using these methods are usually qualitatively correct, the findings from these indirect methods are at coarse temporal and spatial scale, providing a generic quantification of heat stress impacts.

Using late sowing or a warmer season/location as a proxy for exposure to heat stress has the critical problem of unavoidable confounding effects of other variables changing at the same time (e.g. Sadras et al., 2015). Thus, differences arising in the plants grown across these contrasting conditions are confounded with the temperature effects. Furthermore, these indirect approaches do not allow the effects of slightly higher temperatures across the whole growing season (chronic effects) to be distinguished from the effects of heat waves (exposure to transient hot days) (Passioura, 2010), as warmer seasons/locations/sowing dates are likely to be associated with more frequent heat waves.

The predictions of simulation models are bound by the accuracy of the assumptions made in the models, and in turn these, are limited by the conditions under which the knowledge was gathered and the level of data fragmentation. Analyses range from speculative to semiquantitative, depending on how well the mathematical simulation model used is being validated and tested (Lawlor, 1996). Simulation models are better suited to deal with the chronic effects of high temperature than with the effects of heat waves (e.g. Gabald´on-Leal et al.,2016), simply because the latter have not yet been studied extensively (Slafer and Savin, 2018).

Direct methods manipulate the exposure to heat removing the unavoidable confounding factors mentioned above for indirect methods, although they may also pose several other problems. In order to "precisely" study the effects of high temperature (i.e. not bring about any confounding effects), the most popular approach has been growing potted plants under controlled conditions (*e.g.* Wardlaw et al., 2002; Altenbach et al., 2003, Spiertz et al., 2006; Kiss et al., 2017; Ochagavía et al., 2019; Prieto et al., 2020). While these types of experiments are useful in understanding the detailed responses of plants to a specific factor, they are relatively less reliability for drawing sound conclusions for crops when the trait considered is density-dependent like crop growth and yield. The inappropriateness of conclusions about yield and related traits has been demonstrated previously (e.g. Passioura, 2010; Sadras and Richards, 2014; Thistlethwaite et al., 2020). In part, this isdue to the yield of isolated plants not being representative of the yield attained under the normal high-density plantings present in the field (Pedró et al., 2012; Lake et al., 2016; Fischer and Rebetzke, 2018). Therefore, upscaling responses from single plants to crops is challenging and less accurate and could produce misleading conclusions (Abbaiet al., 2020). In general, yield-related responses from controlled environment studies should not be taken as representative of crop responses in the field (Sadras et al., 2020).

To produce results that can be extrapolated with confidence to field-grown crops, heat treatments must be imposed on crops grown in field experiments. Current approaches to achieve this include using infrared heaters, chambers with air conditioning, heat tents, temperature gradient tunnels, open top chambers (OTCs), and polyethylene tents placed over field plots (Table Ai_1). Each of these methods have their own advantages and disadvantages, as briefly described in Table Ai_1. For instance, some of them require relatively expensive equipment, and/or are only applicable to experiments carried out in field facilities with access to electricity, and/or impose severe restrictions in plot size and in experimental design, and/or carry a potentially confounding effect of reducing incoming photosynthetically active radiation while increasing temperature (Table Ai_1).

An additional disadvantage that is shared by all these methods for increasing the temperature in the field is that they alter the relative humidity of the air (for instance heating the air reduces the humidity while enclosing the canopy increases it) and with it the potential levels of evapotranspiration. This would imply the potential for a general confounding effect of high temperature given by the variation in humidity, making these methods more adequate for irrigated experiments (and prone to confounded effects

Table Ai_1. Main direct methods to impose high temperature in wheat crops with the aim of quantifying the effects of high temperature.

Method	Characteristics	References	
Infrared heaters placed in the field	*Pros*: likely the best system to reduce confounding effects from changes in incoming radiation. Accurate imposition of temperature treatments. Allows relatively large plots but uses many heaters. *Cons*: very high cost to build and operate. Needs access to electricity in the field. If heaters are fixed there is restriction in experimental design.	Kimball (2005); Kimball et al. (2008)	
Air-conditioned fixed chambers	*Pros*: accurate imposition of temperature treatments (and both heat and low temperature treatments). Allows relatively large plots, good for screening in breeding for tolerance to heat. *Cons*: fixed structures, thus imposing restrictions to experimental design (and experiments are always done in the same plots). High cost to build and operate. Light intensity reduced when passing through the roof and walls. Needs access to electricity in the field.	Thistlethwaite et al. (2020)	
Field-based heat tents	*Pros*: cost effective for running the experiments (once it has been built) if temperature increased passively. Spacious, good for screening in breeding for tolerance to heat *Cons*: fixed structures, thus imposing restrictions to experimental design. Relatively high cost for start-up (and to run if temperature increased with heaters). Light intensity reduced when passing through the roof and walls.	Bergkamp et al. (2018); Hein et al. (2019)	
Temperature gradient tunnels	*Pros*: intermediately costly to build. Does not need heaters (temperature increase passively). Light is equally reduced at all temperatures and is thus not be a confounding factor. Relatively good control of temperatures through dynamics of air flow. *Cons*: needs electricity in the field for fans to operate continuously. Relatively costly to operate. Limited plot sizes. Restrictions to experimental design (temperature regimes follow the flow of air from the cool end to the hot end)	Rawson et al. (1995); Aranjuelo et al. (2005)	
Open top chambers (OTCs)	*Pros*: very cost effective, cheap to build and operate. Passively increases temperature, does not need heaters or electricity. No restrictions to experimental design. They may or not have blowers. *Cons*: light intensity reduced when passing through the walls, only very slights increases in day temperature (unless using heaters, in which case there must be access to electricity), allowing treatments of slightly increased temperature but not heat waves. Limited plot size (as heating from the borders, if passive).	Kimball et al (1997); van Oijen et al. (1999); Welshofer et al. (2018); Cossani and Sadras (2021)	
Portable polyethylene chambers over the plots	*Pros*: very cost effective, cheap to build and operate. Passively increasing temperature, does not need heaters or electricity. Allows for heat wave treatments with increasing maximum temperatures of approximately 5-8ºC. Unlimited plot size. No restrictions to experimental design. *Cons*: light intensity reduced when passing through the roof and walls. Relevant increases in day temperature on sunny days.	Savin et al. (1996); Talukder et al. (2013); Elía et al. (2018)	

All pictures were reproduced, in full or in part from the original, with permission from Kimball et al. (2008), Thistlethwaite et al. (2020), Hein et al. (2019), the senior author of Aranjuelo et al. (2005), who kindly sent us an unpublished picture; Cossani and Sadras (2021), and Elía et al. (2018), from top to bottom sequentially.

under water stress). However, there are few studies in which the effects of humidity modulating the magnitude of the heat penalties have been quantified, and the existing evidence is inconclusive (Ford et al., 1976; Bhullar and Jenner, 1983;Tashiro and Wardlaw, 1990), particularly in well irrigated experiments with application of fungicides. All these methods have difficulties in producing unbiased estimate of the effect of heat when it is combined with drought. The other environmental change that is common to all methods, except for the use of infrared heaters in the field is that they interfere with wind.

3. Using portable polyethylene chambers

3.1. Producing an effective heat wave treatment

Using portable polyethylene tents is ideal when research must be done not only under field conditions, but also on plots of reasonable size (e.g. 5 m2 or more), and in remote places such as farmers' fields beyond the research organisation's experimental fields and without electricity supply. In addition, the system is very cheap to build and install and has no operational costs. However, it has the major drawback of a potential confounding effect from the reduced incoming radiation. This confounding effect could increase the yield penalty attributed to heat when at least part of the reduction in yield might be a response to reduced radiation.

These portable polyethylene tents effectively allow for a passive increase in day temperature with no effects on night temperature (Fig. Ai_2). The bottom 30 cm of the four sides of each structure were left open, in order to facilitate free gas exchange and air circulation. This prevents condensation of water on the polyethylene overnight and therefore minimised the retention of long-wave radiation (contributing to equalisation treatment`s temperature to those of the open field overnight). However, it does also effectively reduce incoming radiation by 10–12 %at noon in sunny days (Elía et al., 2018). Assuming a linear relationship of crop growth with intercepted radiation (e.g. Charles-Edwards, 1982), these tents would potentially lead to a reduction in crop growth of around 10 %.

However, the light passing through the polyethylene not only reduces the incoming radiation but also increases the proportion of diffused radiation (Cabrera et al., 2009; Soar et al., 2009). It has been speculated that the relatively small reduction in incoming radiation caused by the polyethylene in these portable tents may be compensated by a small increase in radiation use efficiency (Elía et al., 2018), as increasing the proportion of diffuse radiation enhances radiation use efficiency (Sinclair et al., 1992).

Therefore, it has been implicitly assumed that any penalty in yield and yield-related traits in the plots enclosed by these tents would have been due to the increased temperature exclusively. This assumption, to the best of our knowledge, has not been tested empirically. If validated with empiric evidence that the reduction in radiation caused by the polyethylene does not significantly alter crop yield, this factor could be disregarded as having any confounding effect on findings generated from these structures.

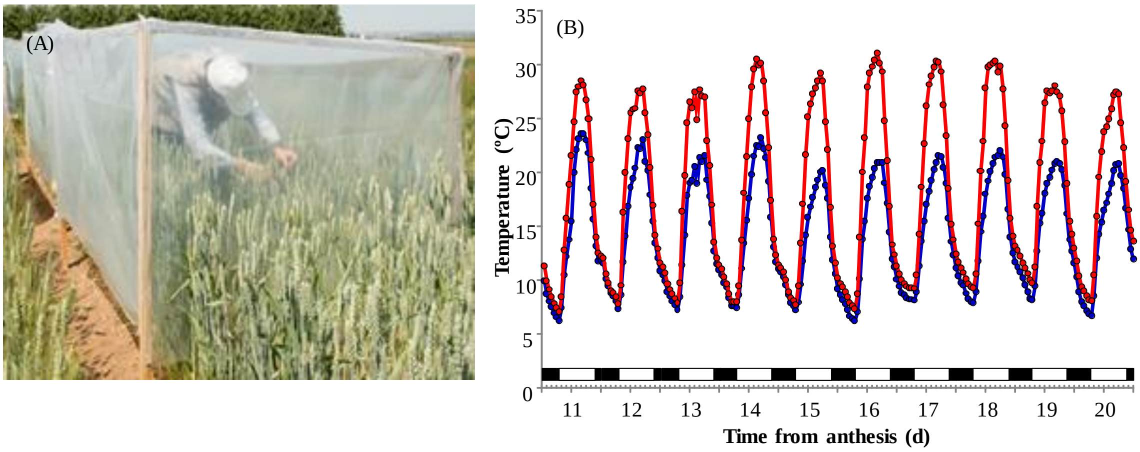

Figure Ai_2. A partial view (A) of one of the field experiments in which it can be seen a researcher inside the portable tent making it evident the transparency of the polyethylene used and the relative size of the portable tent, and (B) the changes in temperatures during the course of the day over the period of treatment in the unheated control (the open-field plots; lower blue lines) and the heated treatments (upper red lines). The alterning black and white bands on the X axis indicate the night and day hours, respectively. Taken from Elía et al. (2018), with permission (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2. Is the reduced radiation an actual confounding effect?

To empirically test the assumption that the increased radiation use efficiency would have compensated the reduced incoming radiation, I conducted a small field experiment in the 2018/2019 growing season (with irrigation, fertiliser and control of weeds, pests, and diseases). I compared the yield and yield components of two cultivars with contrasting grain number and average grain weight (Pistolo characterised by heavier grains, and Sublim having larger number of grains per m^2), under a “roof-only” treatment, imposed either at booting (DC 47, Zadoks et al., 1974) or at the onset of grain filling (DC 73) and lasting 7 days, and untreated control conditions in which plots were not covered (Fig. 3A). These treatments were randomised in three complete blocks. Temperature sensors with cover shields (connected to dataloggers Decagon Devices) were distributed at regular positions to monitor air temperatures inside and outside the structures at the height of the spikes (Fig. Ai_3A).

The roof-only treatment did effectively reduce incoming photosynthetically active radiation by 10.7 ± 0.19 % at noon of sunny days (from1481 ± 3.38–1322 ± 0.82 μmol m^{-2} s^{-1} in the uncovered plot and in the roof-only treatment, respectively), which is in close agreement with measurements made independently few years ago (Elía et al., 2018). This is a common value reported from other types of structures used to increase temperature in the field. For instance, Cossani and Sadras(2021) reported that the walls of their open-top chambers (made of polycarbonate) had a 90 % transmittance of photosynthetically active radiation, and the rather sophisticated fixed chambers used by Thistlethwaite et al. (2020), also made of clear polycarbonate, reduced incoming radiation at noon by around 10–20 % (Richard Trethowan, personal communication, 2020). On the other hand, the polyethylene roofs used in our experiments did not alter the temperature of the air at the top of the canopy at any time of the day (Fig. Ai_3B).

As expected, Sublim had more grains than Pistolo but the average weight of these grains was lower: the uncovered controls had 16,567 ±745 and 13,731 ± 283 grains m^{-2}; and 39.2 ± 0.6 and 45.6 ± 1.0 mg $grain^{-1}$, respectively). In both cultivars, it was clear that the approximate11 % reduction in incoming radiation did not result in any statistically significant yield penalty (Fig. Ai_3C).

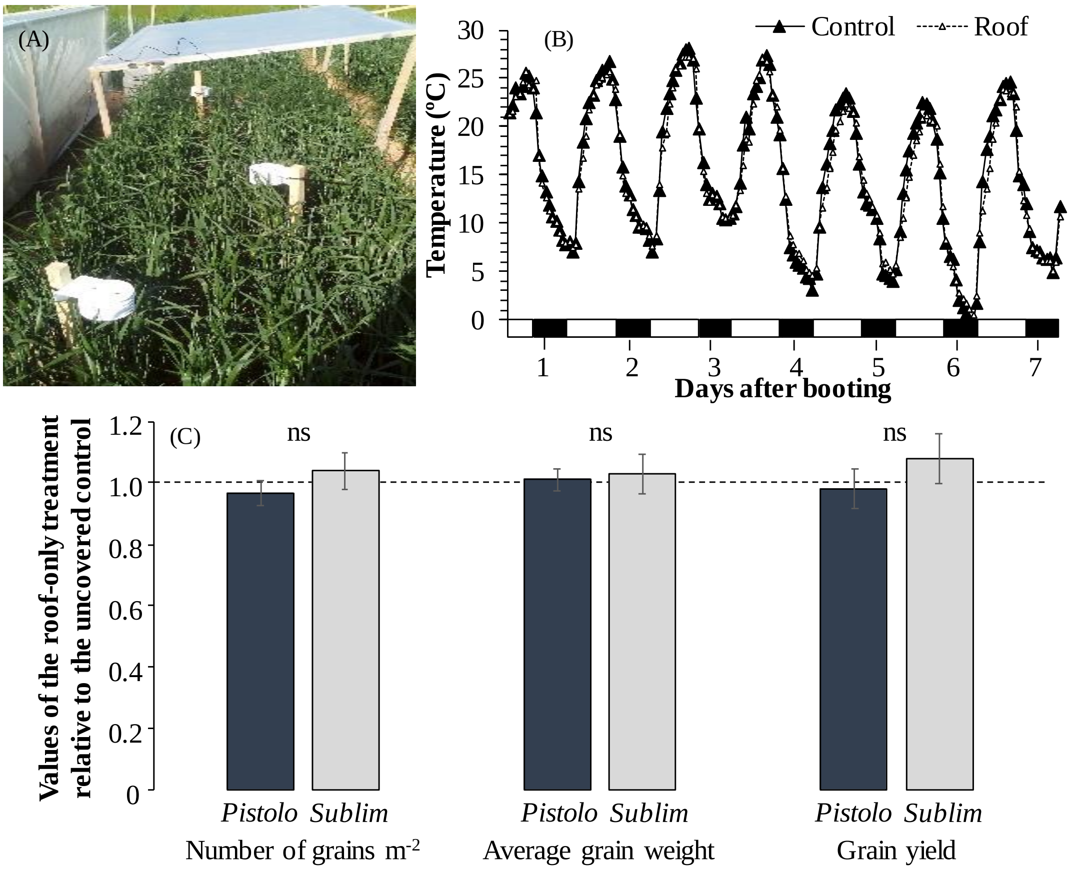

Figure Ai_3. A partial view of the field experiments showing a roof-only treatment (a polyethylene roof covered the plot) and an uncovered control plot (A) and the temperatures during the course of the day during the period of the treatment for the roof only (open triangles joined by a dashed line) and control plots (closed triangles joined by a plain line) illustrating the example of the treatment imposed at booting (B). The bottom panel (C) shows the lack of any significant effect on yield of the roof-only treatments and its major components, in any of the two contrasting cultivars.

This lack of effect of reducing around 10 % of the incoming radiation on crop performance is not exceptional. In the study carried out by Thistlethwaite et al. (2020), with chambers in the field that also reduced the incoming radiation, there was no difference in yield or yield components between crops grown inside the chambers or outside in an uncovered control, if the temperature regime inside the chamber was equal to that outside (as mentioned in the paper and further confirmed by a personal communication by Richard Trethowan, 2020).

Therefore, the high temperatures generated under portable tents with transparent polyethylene film should not produce any confounding effect in field-grown wheat due to the small loss of incoming radiation. This potentially negative side effect of these tents is likely balanced by a concomitant increase in radiation use efficiency due to the increased proportion of diffuse radiation underneath these tents. This means that when day temperatures are increased with the portable tents, the direct effect of reduced radiation would be negligible and therefore it is possible to assume that all effects recorded were due to the increased temperature. However, I acknowledge that this assumes no interactive effect between temperature and radiation, which cannot be easily deconvoluted in the field experiments.

Another interesting aspect that can be considered for research using transparent tents in the field is that polyethylene films with specific optical properties (selectively blocking ultraviolet, visible, near infrared, or middle infrared radiation; Espi et al., 2006) are now available.

Portable polyethylene tents are therefore a reliable method to impose heat treatments in field experiments under irrigated conditions; and would be less suitable under non-irrigated conditions as water stress would produce a confounded effect, as recognised for field based heat tents as well (Bergkamp et al., 2018; Hein et al., 2019).Although other methods are more sophisticated and may be perceived as having fewer confounding factors, it seems that the use of these portable polyethylene tents is appropriate when there is no access to electricity at the field site and the budget to run the experiment is limited. Further, plots can be of sufficient size to produce reliable yield results.

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ANNEX II

Weight of individual wheat grains estimated from high-throughput digital images of grain area

ANNEX II. Weight of individual wheat grains estimated from high-throughput digital images of grain area

1. Introduction

Wheat yield comprises two major components: the number of grains per m^2 (GN) and their average grain weight (AGW) (Frederick and Bauer, 1999). Most studies analyzing the effects of genetic and/or environmental factors determine these components. The most frequent procedures are (i) to estimate yield through weighing the threshed grains from the sample, then counting them and finally estimating AGW as the yield-to-GN ratio, or (ii) to determine yield directly from the combine-harvested plot and measuring AGW (from sub-samples of a certain number of grains) and then estimating grain number as the yield-to-AGW ratio. In many cases, these two components are enough to reach sound interpretations for the particular aim of the studies. However, in other cases, AGW may be insufficient and the effects of treatments on the final size of individual grains (that collectively determine the AGW) may be needed.

For researchers attempting to offer a mechanistic explanation for differences in AGW, the level of detail of having the weight of each individual grain, with which to analyse the grain weight distribution in particular treatments, may be more relevant than having only their average weight. For instance, a reduction in average size due to a particular treatment may well reflect that all grains are smaller in that treatment, but also that a particular subset of the population of grains was reduced, and inferences on the causes of AGW reductions would be different. Among a longer list of examples, it was necessary to determine not only AGW but also the individual weight of particular grains for a mechanistic explanation of (i) a negative relationship between AGW and GN in response to yielding condition (e.g. Acreche and Slafer, 2006), (ii) the effects of awns on potential grain size (Sanchez-Bragado et al., 2020), (iii) the relevance of pericarp characteristics in determining grain size (Hasan et al., 2011; Brinton et al., 2017; Herrera and Calderini, 2020), and (iv) the genetic and molecular basis of trade-offs between AGW and spike fertility (Zhai et al., 2018). Indeed, it seems more frequent than not that treatments modifying GN bring about concomitant changes in AGW (Frederick and Bauer, 1999), regardless of whether the effects are due to genetic or environmental factors (Slafer et al., 2015b, and references quoted therein). Changes in AGW may reflect an increase in competition during grain filling (Sakai and Sakai, 2005; Cartelle et al., 2006; Labra et al., 2017), or a reduction in the potential size of the grains (Slafer et al., 2015; Elía et al., 2016), or simply a change in the relative contribution of small grains to the final AGW (Slafer and Savin, 1994; Acreche and Slafer, 2006; Ferrante et al., 2017). Discerning the actual cause of the changes in AGW, for which analyzing the weight of individual grains would contribute, is clearly relevant for the appropriate interpretation of results and suggestions to be considered for more efficient breeding or management.

A major inconvenience is that to determine the individual weight of thousands (or tens of thousands) of grains, that are normally considered in AGW determination, would require an amount of time and other resources that are simply impossible to handle. Therefore, when scientists are interested in having such detail of the weight of individual grains, either they (i) do consider only a minor proportion of particular grains of the canopy (Miralles and Slafer, 1995; Acreche and Slafer, 2006; Philipp et al., 2018), or (ii) make individual determinations of grain linear dimensions (length, width, area) (Gegas et al., 2010; Philipp et al., 2018) that can be easily achieved with image digitalization procedures (for hundreds of grains in few minutes, including the preparation of grains for the image). The problem with the former approach is that the individual grains sampled for the determination may not cover all sizes of the populations of grains in a complete sample, causing more differences between the sample and population variance, and even considering a very small sub-sample it is time-consuming and painfully laborious. The inconvenience of the second approach is that the explanation for the differences in AGW comes from measurements of dimensions, but not from the actual weight of the individual grains in that complete sample.

As the density of grains (weight per unit volume) is virtually constant across genotypes and environments, at least considering grains above a minimum threshold of c. 20 mg grain^{-1}[1], the ratio of volume and weight is very conservative (Millet and Pinthus, 1984; Hasan et al., 2011; Walker and Panozzo, 2011), implying that the weight of each grain in a sample may be confidently estimated from the individual volumes. However, determining the volume of each individual grain is likely more laborious and more time-consuming (Millet and Pinthus, 1984; Hasan et al., 2011; Herrera and Calderini, 2020) than actually weighing grain by grain with a precision balance, or requires of highly sophisticated equipment (e.g. can be obtained from computed tomography scanning image segmentation and reconstruction; Strange et al., 2015). However, as the volume is related to the grain dimensions of length, width and height (Miralles et al., 1998; Hasan et al., 2011; Walker and Panozzo, 2012; Xie et al., 2015; Benincasa et al., 2017; assuming the shape is an ellipsoid), it is highly likely that considering the dimensions of easily, quickly and affordably obtained 2-D images of grains the weight of individual grains could also be trustworthily estimated. These dimensions are length and width from which the area may be estimated. Indeed, although length and width have been laboriously measured by hand (e.g. Ramya et al., 2010; Hasan et al., 2011), the use of a simple and affordable equipment to quickly obtain a 2-D images of individual grains in a relatively large sample estimating (among other things) the length, width and area of each individual grain are available (e.g. Tanabata et al., 2012; Whan et al., 2014; Komyshev et al., 2017). The most frequently used equipment of this type for the determination of wheat grain dimensions is likely the Marvin seed analyser (e.g. Gegas et al., 2010; Neuweiler et al., 2020; Sanchez-Bragado et al., 2020). Taking advantage that grains of modern cultivars do not vary appreciably in shape (Gegas et al., 2010), a single equation might be generated to transform the dimensions taken from 2-D images of individual grains into their dry weights that could be reasonably valid across genotypes and growing conditions.

[1] This threshold implies that the ratio of volume and weight may not be that conservative when severe water or heat stress very strongly affect grain growth

In this study, I aimed to generate a single model equation to trustworthily convert grain linear dimensions to weight for wheat grains. For this purpose, I (i) used a set of data to establish linear relationships between dry weight and either length, width, or area of individual grains of different wheat cultivars grown in different experiments to select a calibrated model to be used to predict grain weight from 2-D images, and then (ii) validated the calibrated model with independent data (including using cultivars and environmental factors not considered in model calibration).

2. Materials and methods

2.1. Experimental setup

Field experiments were carried out at Bell-lloc d'Urgell (41.64°N, 0.79°E), Catalonia, North-East Spain. Firstly, I carried out three experiments, each one combining different wheat genotypes and two doses of nitrogen (N) fertilisation. From each of these experiments, some cultivars were used to develop a model whose parameters could be used to predict individual grain weights from the individual grain dimensions (so that the model would be based on a wide range of G x E conditions). For developing a model to estimate grain weight, grains were harvested from 48 combinations of G x E conditions: six treatments in Experiment 1 (3 cultivars x 2 N fertilisation levels), two treatments in Experiment 2 (1 cultivar x 2 N) and four treatments in Experiment 3 (2 cultivars x 2 N) (Table Aii_1). Data of the other cultivars (under the two contrasting N fertilisation regimes) within each of these three experiments were used to validate the model generated with independent data representing 96 combinations of G x E conditions: eight treatments in Experiment 1 (4 cultivars x 2 N), two treatments in Experiment 2 (1 cultivar x 2 N) and six treatments in Experiment 3 (3 cultivars x 2 N) (Table Aii_1). Then I decided to use data from other completely independent experiments to complement the validation exercise expanding the dataset to cultivars never used before (experiment 4) and cultivars that were grown in previous experiments but subjected to a completely different environmental treatment, heat stress (Experiment 5) (Table 1). In all experiments, the plot size was 1.2 m wide (6 rows, 0.20 m apart) and 4 m or 6 m long. Nitrogen fertilisation treatments consisted in an unfertilised control and a N fertilised treatment with a dose of 200 KgN ha^{-1}. Heat treatments consisted of an unheated and two heated treatments (one starting at early booting, the other one 15 days after anthesis) increasing maximum daily temperatures (but not the minimum ones) over 10 days. The heat treatment was imposed by installing over the designated plots wood structures of 1.5 m height above the whole plot covered with transparent polyethylene film (structures and type of heat like those described in detail by Elía et al., 2018). The mean difference of temperature between inside and outside of chamber in daytime was 5.20 ± 0.03 and 5.39 ± 0.03 ºC at pre- and post-anthesis respectively, while during the night, the average temperature difference was negligible (0.30 ± 0.01 and 0.40 ± 0.01 ºC at pre- and post-anthesis respectively).

Table Aii_1. Summary of the experiments performed under field conditions. Treatments were always different commercial wheat cultivars, in experiments 1, 2 and 3 cultivars were combined with two contrasting N fertilisation regimes, and in experiment 5 they were subjected to unheated conditions and heated either in pre- or in post-anthesis.

Experiment	Sowing date and rate	Treatments: Cultivars	Treatments: Environment	No. grains sampled	Ranges included in the samples (Min, [*Average*], Max): Length (mm)	Width (mm)	Area (mm^2)	Dry weight (mg)
Development of model to estimate grain weight								
EXP1	20 Nov 2015 350 seeds m^{-2}	Marcopolo Ingenio Kilopondio	0 KgN ha^{-1} 200 KgN ha^{-1}	30[a] 90[b]	4.58 [6.44] 7.65	2.40 [3.65] 4.32	8.39 [17.89] 23.32	15.40 [50.31] 69.30
EXP2	16 Nov 2016 350 seeds m^{-2}	Avelino	0 KgN ha^{-1} 200 KgN ha^{-1}	130[a] 130[b]	5.15 [7.10] 8.01	2.15 [3.53] 3.98	9.50 [18.08] 22.91	17.80 [45.46] 62.5
EXP3	17 Nov 2017 380 seeds m^{-2}	Pistolo Sublim	0 KgN ha^{-1} 200 KgN ha^{-1}	60[a] 120[b]	5.30 [6.59] 7.62	2.43 [3.55] 4.63	10.70 [17.78] 24.17	18.05 [43.17] 66.02
Validation of model with independent data								
EXP1	20 Nov 2015 350 seeds m^{-2}	Alabanza ArthurNick Bologna Sar32	0 KgN ha^{-1} 200 KgN ha^{-1}	30[a] 120[b]	4.42 [6.16] 7.43	2.10 [3.46] 4.32	7.36 [16.21] 22.57	9.4 [43.28] 71.30
EXP2	16 Nov 2016 350 seeds m^{-2}	Ingenio	0 KgN ha^{-1} 200 KgN ha^{-1}	130[a] 130[b]	5.60 [7.50] 8.34	2.80 [3.70] 4.21	11.6 [20.05] 24.46	22.9 [54.6] 69.17
EXP3	17 Nov 2017 380 seeds m^{-2}	Avelino Ingenio MarcoPolo	0 KgN ha^{-1} 200 KgN ha^{-1}	60[a] 180[b]	5.07 [6.56] 7.74	2.43 [3.57] 4.51	9.56 [17.83] 24.34	16.44 [43.61] 61.15
EXP4	17 Nov 2017 125 Kg_{seeds} ha^{-1}	Garcia Paledor	-	50[a] 100[b]	4.60 [6.32] 7.60	2.90 [3.63] 4.50	10.80 [17.21] 24.40	24.79 [40.32] 62.07
EXP5	20 Dec 2018 400 seeds m^{-2}	Avelino Pistolo Sublim	Unheated control Heated at pre-AN Heated at post-AN	45[a] 135[b]	5.50 [6.48] 7.21	2.84 [3.73] 4.71	12.0 [18.59] 24.00	26.20 [51.13] 67.30

[a] *grains sampled from each cultivar*
[b] *total number of grains used for generating (top part of the Table) or validating (bottom part) the model*

All treatments in each experiment were arranged in a randomised complete block design (RCBD) with three replications. All experiments were irrigated to avoid any water stress and biotic interferences were avoided through controlling weeds, insects and diseases following usual practices.

2.2. Sampling, measurements, and model generation/validation

From each particular case (each particular treatment in each particular experiment), I had a large sample at physiological maturity from the agronomic study (a whole 1 m from a central row) involving thousands of grains. In these samples, I analysed the dimensions (length, width, and area) of each of the grains using the MARVIN 5.0 optical seed analyser (GTA Sensorik GmbH, Germany), after evenly distributing grains over the tray of the seed analyser. I did not accommodate all grains to a single position (either with the ventral furrow downwards or by lying on one side, individual grains adopted one of these positions at random). Accommodating the grains one-by-one to a single position would have produced an unrealistic situation when compared to what researchers do when measuring thousands of grains. Furthermore, as grains are mostly elliptic accommodating the grains to a single of the two positions would have only slightly affected the measured dimensions (Mabille and Abecassis, 2003). Knowing the distribution of grain morphometric measurements, I took from each sample a sub-sample at random and from it, I selected grains for analysis representing all classes of sizes in the large sample from the agronomic study. In these sub-samples (Table Aii_1), I measured, grain by grain, not only the morphometric measurements with the seed analyser but also each individual grain weight with a precision balance with an accuracy of 0.01 mg (VWR International, SMG 2285Di-ION, Italy). Indeed, the dimensions and weights of grains used for the development of the model and for its validation ranged widely in all cases (Table Aii_1).

Then using the data of each individual grain dry weight, length, width and area from the dataset used for the development of the model (Table Aii_1), I established the relationships between grain dry weight and each of the three dimensions. For that purpose, I fitted regressions between grain weight and either grain length, grain width, or grain area using RStudio (RStudio Team 2020). I firstly fitted linear regressions ($\hat{y}=a+b\,x$) and then tested a power relationship ($\hat{y}=a\,x^b$); for computing the latter, I actually fitted the linear regression to the log-transformed data of both the independent and the dependent variables ($\log \hat{y}=a+b \log x$) (Kvålseth, 1983). Then, with the best predictor, I estimated grain weight for each grain considered in the validation exercise from the morphometric determinations based on the digital images and using the model developed. Finally, I plotted the predicted *vs* the actually measured weight of each grain with RStudio.

I analysed the frequency of the distribution of both actual and predicted grain weights. Once the average and the standard deviation of each case were estimated, I adjusted a normal distribution curve converting within each distribution the value of each individual grain to a probability density function (i.e. the likelihood of each particular grain weight within the population of grains considered with that particular average and standard deviation) (Bellido et al., 2006).

3. Results

3.1. Predictive model

I regressed measured grain weight against the three size dimensions measured: grain length (Fig. Aii_1A), grain width (Fig. Aii_1B), and grain area (Fig. Aii_1C), considering a universe of 340 data-points of individual values measured in 6 different cultivars (three of them in experiment 1, one in experiment 2 and two in experiment 3), grown under two contrasting N fertilisation levels in all these experiments (Table Aii_1).

Naturally, all relationships were positive and, at first sight, they all appeared to have a strong linear component (Fig. Aii_1). Indeed, all three linear regressions between actual grain weight and grain size dimensions considered here were highly significant (Table Aii_2), with the relationship with grain area (Fig. Aii_1C) explaining a higher proportion of the variation in grain weight than those with grain length (Fig. Aii_1A) or width (Fig. Aii_1B; Table Aii_2).

Not only the percentage of variation in grain size was better explained by the area than by either the length or the width of grains, but also the magnitude of variation in grain area (from c. 8 to c. 24 mm^2) was more proportional to that in grain weight (from c. 15 to c. 69 mg $grain^{-1}$) than variation in grain length (from c. 4.5 to c. 8 mm) or width (from c. 2 to c. 4.6 mm) (Fig. 1; Table. 1).

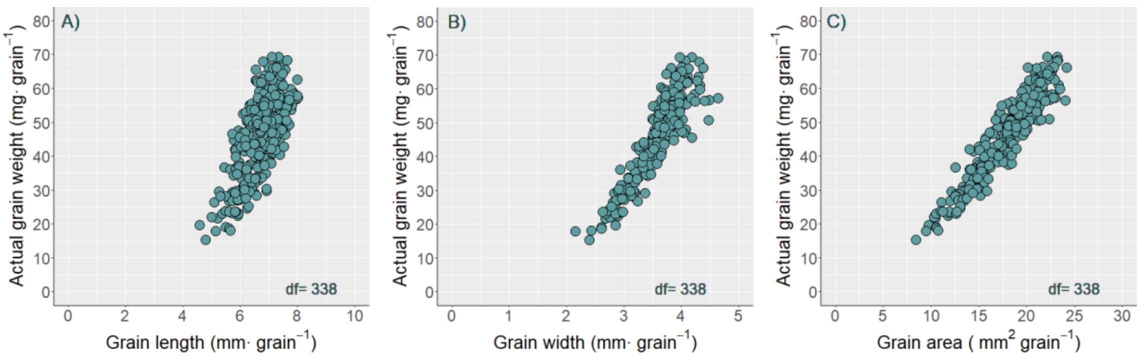

Figure Aii_1. Relationships between dry weight and A) length, B) width and C) area of individual grains. Data-points of individual values measured in 6 cultivars (three of them in experiment 1, one in experiment 2 and two in experiment 3), grown under two contrasting nitrogen fertilisation levels in all these experiments (see details in Table Aii_1).

Therefore, at first, a single linear model using grain area as a predictor of grain weight ($\hat{y} = -15.99 + 3.46\ x$; Table 2, top left; Fig. 1C) seemed appropriate with grain area explaining more than 88% of the variation in grain weight.

Table Aii_2. The output of the regression analyses (parameters a and b, with their standard errors estimated, and coefficients of determination) for the relationships between grain weight and either grain length, grain width, or grain area, fitting a linear or a power curve model (top and bottom part of the Table, respectively) with either a free intercept or forcing the regressions through the origin (left and right parts of the Table, respectively).

	Independent variable	Free intercept estimated			Forced through the origin	
		a	***b***	R^2	***b***	R^2
Linear model	Length	-40.27±4.80***	12.78±0.71	0.492***	6.86±0.07	0.385***
	Width	-50.48±2.42***	27.02±0.68	0.827***	13.04±0.11	0.603***
$\hat{y}=a+b\,x$	Area	-15.99±1.24***	3.46±0.07	0.885***	2.59±0.01	0.828***
Power model	Length	0.73±0.09^{ns}	2.15±0.11	0.541***	1.99±0.01	0.538***
	Width	2.34±0.03***	2.32±0.05	0.865***	2.99±0.01	0.794***
$\hat{y}=a\,x^b$	Area	0.76±0.03***	1.42±0.03	0.906***	**1.32±0.00**	**0.901*****

*** or ^{ns} for the intercepts indicate that they were highly significantly ($p<0.001$) or not significantly different from zero; while *** for the R^2 values indicates that all coefficients of determination were highly significant ($p<0.001$); $n = 340$. In bold it is the regression model selected to predict grain weight ($\hat{y}$) from grain area (x); $\hat{y}=x^{1.32}$ (see text).

Regardless of the highly significant coefficients of determination, all these linear regressions had a major drawback in that the estimated intercept was highly significantly negative. Forcing the linear regressions through the origin removed this incongruity, but not only reduced the proportion of the variation in grain weight explained (though only moderately when using grain area as the independent variable; Table Aii_2) but also would have produced a consistent overestimation of grain weight in the range of small grains and a consistent underestimation for the large grains, making it unsuitable for producing a generalised model to be trustworthily employed.

The negative intercept of the linear regression reflected that the true relationship was not linear. Indeed, when fitting the data with a power curve, all coefficients of determination increased (Table Aii_2) and, more importantly, residuals were more randomly distributed. As with the linear relationships, again the grain area was a better predictor of grain weight than both grain length (having a substantially greater R^2) and grain width (the R^2 was only slightly higher but the intercept was much closer to zero) (Table Aii_2). Even though 0.76 mg grain^{-1} was close to zero, it was significantly higher, and it must be zero in reality (if there is a dimension there must be a weight and *vice-versa*). Then, I forced the intercept to zero with the log-transformed data and, as in the previous fitted models, grain area was the dimension best predicted grain weight (Table Aii_2). In this particular case, where the free intercept was very low (less than 1 mg grain^{-1}), the coefficient of determination was only negligibly lower than when fitting the relationships with a free intercept (Table Aii_2). Therefore, the model equation best predicting grain weight in this calibration exercise was $\hat{y} = x^{1.32}$ where $\hat{y}$ was the predicted grain weight and x was the measured grain area (Fig. Aii_1C; Table Aii_2).

As this relationship was obtained using data from different growing seasons, contrasting N fertilisation regimes and a range of different cultivars, it was proposed that the weight of any individual grain could be estimated from high-throughput measurements of grain area (as its area raised to 1.32), at least for a very wide universe of cases including different cultivars and growing conditions.

3.2 Validation across different cultivars and growing conditions

To test the validity of the model proposed to appropriately estimate the weight of grains from measurements of their area beyond the particular G x E used to generate it, I validated the model $\hat{y} = x^{1.32}$ against a range of independent data. This range included (i) independent data from the same studies used to build the model (experiments 1, 2 and 3); (ii) data from other completely independent experiments (experiments 4 and 5) that included some cultivars never used in the calibration of the predicting model as well as not only different seasons as variation in environmental conditions but also in one experiment the imposition of heat stresses (Table Aii_1); and furthermore (iii) I discussed the more universal validity considering data reported in the literature (in the Discussion section).

The relationship between predicted and actual weight of the grains for the cultivars and N regimes not used to develop the predicting model in the three first experiments produced a positive validation supporting the trustable use of the grain measured area raised to 1.32 to estimate the weight of the grains (Fig. Aii_2).

Naturally, the coefficients of determination were highly significant and actually very high in the validations carried out in each of the three experiments (Fig. Aii_2A, B, C). Although not always the regressions of the data resulted in the ideal expected output (slope = 1 and intercept = 0), the distribution of the data was actually close to the 1:1 ratio in all cases (Fig. Aii_2A, B, C). As a consequence, when I compared the frequency distribution of the actual and predicted grain weights for the 430 independent data-points validating the predicting model proposed from the same experiments used to build the model (i.e. same experiments but independent data; Table Aii_1), both were rather overlapped (Fig. Aii_2D); resulting in a predicted average grain weight that differed from the actual average grain weight by less than their standard errors; and the difference between these distributions were not significant (*p-value* = *0.37*).

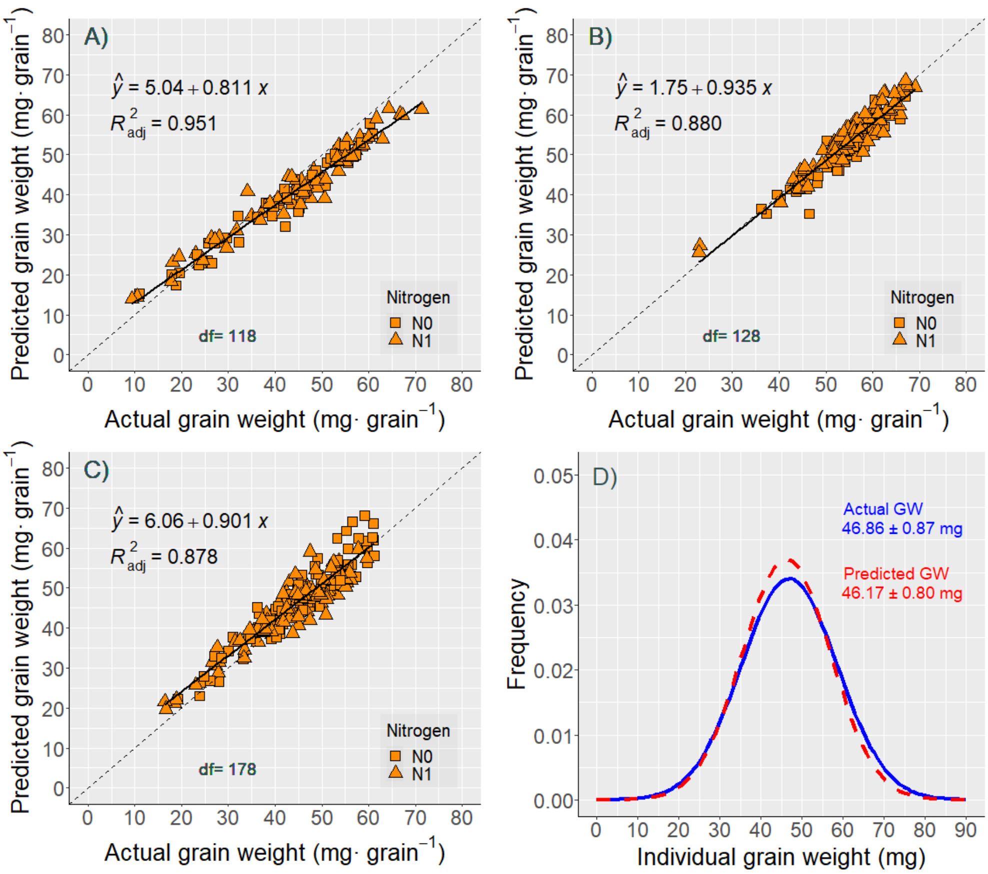

Figure Aii_2. Relationships between predicted and actual grain weight for independent data (cultivars x N conditions not used to develop the predicting model) of experiments 1 (A), 2 (B), and 3 (C); and the frequency distribution of both predicted (dotted line) and actual (plain line) grain weights across overall cultivars x N conditions of these experiments (n=430) that were not used to build the predicting model (D). Data from Experiment 1 comprises four cultivars and two N regimes, in Experiment 2, a single cultivar under two contrasting N regimes, and in Experiment 3, three cultivars and two N regimes (Table Aii_1). Dashed lines in panels A, B and C are the 1:1 ratio where predicted and actual grain weights are equal; and solid lines stand for the linear regression between them (and equations representing these lines are included inset). Lines in panel D stand for the normal distribution adjusted to the frequencies observed; and the averages and their standard errors are given inset for both distributions.

Even though the cultivars in experiment 4 were never used in the calibration of the predicting model (and they were grown in a field close, but independent, to the field where Experiment 3 was carried out), the model $\hat{y}=x^{1.32}$ predicted grain weights rather accurately (Fig. Aii_3A). Indeed the regression line was quite close to the 1:1 ratio line.

In addition, the predicted weights for a completely independent experiment carried out one growing season later than Experiment 3 and subjecting three cultivars to a completely different environmental treatment (heat waves) also were quite close to the actual weights (Fig. Aii_3B). Consequently, when analyzing the frequency distribution of the actual and predicted grain weights for the 235 data-points from completely independent experiments they were rather overlapped (Fig. Aii_3C), and the difference between these distributions was not significant (*p-value= 0.44*).

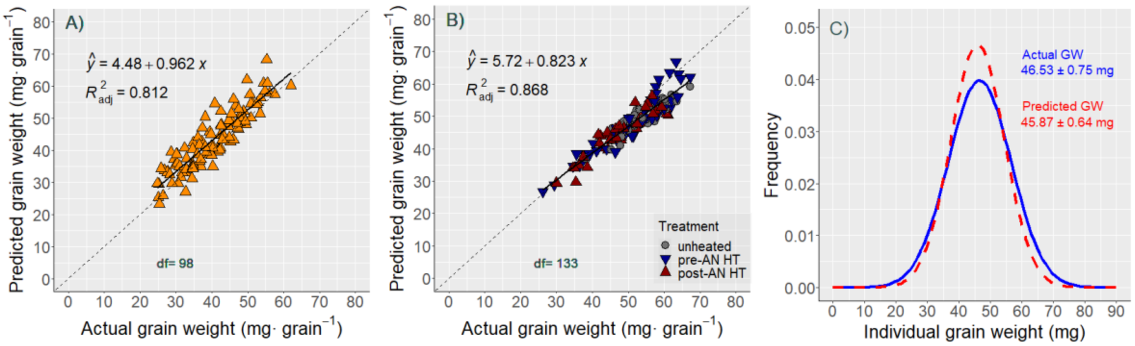

Figure Aii_3. Relationships between predicted and actual grain weight for data taken from two completely independent studies: Experiments 4 (A) and 5 (B), and the frequency distribution of both predicted (dotted line) and actual (plain line) grain weights (C) across overall treatments of these completely independent studies (n=235). Experiment 4 comprised two modern cultivars never considered in Exps 1-3, and Experiment 5 consisted of three cultivars subjected to heat waves during pre-and post-anthesis (Table Aii_1). Dashed and solid lines in panels A, B and lines in panel C are as in Figure. Aii_2.

When pooling all independent datasets (Experiments 1-5) the overall robustness of the model proposed to predict grain weight is clearly illustrated (Fig. Aii_4A). Considering a rather large set of independent data-points (n =665) the data were very close to the 1:1 ratio (Fig. Aii_4A) and the frequency distribution of the actual and predicted grain weights were rather overlapped (Fig. Aii_4B). The difference in average grain weight between actual and predicted grain weight was only 0.68 mg grain^{-1}, well within the 95% confidence interval for the difference [-0.46 and 1.82 mg grain^{-1}] and representing a difference of *c.*1.5% of the average grain weights. The two distributions were obviously not significantly different (*p-value = 0.24*; Fig. Aii_4B).

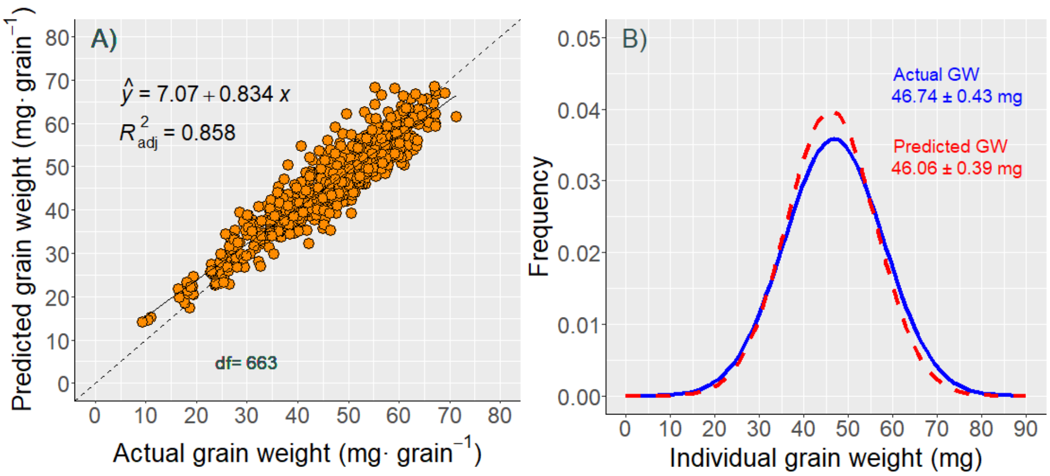

Figure Aii_4. Relationships between predicted and actual grain weight for independent data across all experiments (1-5) (A) and the frequency distribution of both predicted (red dotted line) and actual (blue plain line) grain weights across all experiments (n=665) (B). Dashed lines in panels A, B and lines in panel C are explained in Figure Aii_2.

4. Discussion

Previous studies have mostly developed high-throughput image scanning for directly determining grain dimensions (Whan et al., 2014; Strange et al., 2015) and elucidated phenotypic variance in them (Gegas et al., 2010; Neuweiler et al., 2020). However, to discuss the effects of treatments on grain yield it is more relevant to consider grain weight, which is a direct yield component and therefore a more appropriate agronomic trait than grain dimensions. Therefore, it would be valuable to count with a reliable estimate of grain weight from grain dimensions (that can be easily and affordably obtained in detail from image scanning methods). I found that grain area was a better predictor of grain weight than length and width of grains. This seems appropriate as when considering only length or width of the grains studies disagree in which of the two is the most relevant in explaining differences in grain weight: while it has been grain length in some cases (e.g. Lizana et al., 2010; Hasan et al., 2011), it was grain width in others (e.g. Gegas et al., 2010). Then using grain area, that integrates both linear dimensions, would likely always improve the prediction, and grain area is a direct output of image scanning tools.

Then using data from different cultivars under contrasting N fertilisation conditions and grown over three growing seasons I calibrated a prediction model ($y=x^{1.32}$) that not only produced a high coefficient of determination (implying that more than 90% of the variation in grain weight was explained by differences in grain area) but also it had a zero intercept (that is mandatory as it would not be possible to have grain area without weight or *vice-versa*). This power regression naturally implies that the prediction in grain weight from measured grain area was non-linear, i.e. when grain area increases, grain weight does so more than proportionally. And this is effectively expected if the specific weight (dry weight per unit volume) of the grains is very conservative (i.e. grain weight increases strictly linearly with grain volume; (Millet and Pinthus, 1984; Hasan et al., 2011; Walker and Panozzo, 2011), due to the fact that the geometric ratio of volume to area increases with size (as illustrated by Marshall et al., 1984).

That accepted model to predict grain weight from grain area was evaluated with independent data in a two-step process. The robustness of the model to predict the weight of grains was successfully validated from either treatments not considered for building the model though from the same experiments, or from completely independent experiments involving different genotypes and environmental treatments (in addition to different background environmental conditions) to those considered in the generation of the model. Considering the diversity of genotypes and environments in the model generation and validation would allow assuming that it could be trustworthily used to estimate grain weights from measured areas more or less widely, at least considering modern bread wheat genotypes.

To further test the robustness of the model proposed to be used with other G x E conditions it would be ideal to count with data from independent studies for validating it more widely. Regretfully, as far as I am aware, there are no data in the literature on area and weight measured on individual grains. However, there are a few studies in which the authors attempted to explain the effects of particular treatments on average grain weight considering the area of the grains (Table Aii_3).

Then, I took the reported values for average grain area from these experiments and predicted the corresponding grain weight using our model (area raised to 1.32) and then further validated the model proposed with data from the literature. The model actually predicted rather well the reported weights from the values of grain area in all these studies (Fig. Aii_5) and the difference between the distributions of actual and predicted grain weights was not significant (*p-value= 0.322*) offering more guarantees that the proposed model can be used trustworthily when it is relevant to analyses not only the average weight of all grains from a treatment but also the individual grains, based on high throughput determinations of grain area.

Table Aii_3. Datasets from the literature reporting average grain weight and grain area of wheat in response to particular treatments used to validate the model proposed to predict grain weight ($\hat{y}$) from grain area (x); $\hat{y}=x^{1.32}$

Reference	Treatments, location and seasons	Range in average grain weight[b] (mg $grain^{-1}$)	Range in average grain area[b] (mm^2 $grain^{-1}$)
(Brinton et al., 2017)	Near isogenic lines for QTL on wheat chromosome 5A associated with grain weight, grown in UK in 2015 and 2016[a]	42.73-51.27	18.04-20.61
(Philipp et al., 2018)	Different genetic resources and elite varieties, grown in Gatersleben, Germany in 2015[c]	30.35-60.54	13.70-22.40
(Wang et al., 2018)	Genome editing on TaGW2-A1 allele which is pleiotropic for thousand-grain weight and grain number, grown in Kansas in 2017	28.79-46.33	12.88-17.24
(Calderini et al., 2020)	Transgenic lines expressing different levels of expansins, grown in Chile in 2012-2015	32.45- 59.06	13.57- 20.40
(Sanchez-Bragado et al., 2020)	Near isogenic lines for the presence of awns, grown in Lleida, Spain in 2019	32.20-53.10	13.54-18.46

[a] the study also comprised some other previous seasons, but we used only two seasons as the measuring scale before 2015 was different (personal communication with Dr. Jemima Brinton).
[b] sample size was 400 grains (Brinton et al., 2017), 10 spikes per genotype (Philipp et al., 2018), 5 ~ 53 grains (Wang et al., 2018), 20 data points between different grain positions and genotypes (Calderini et al., 2020), 15 plants (Sanchez-Bragado et al., 2020). [c] year of the experiment was not reported in the paper, but we obtained the information from the author.

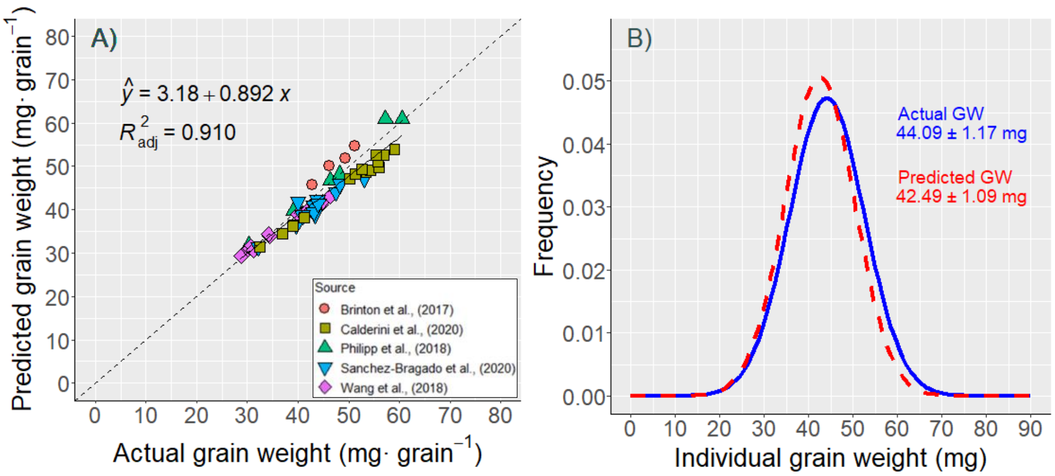

Figure Aii_5. Relationships between predicted and actual grain weight for data taken from five completely independent studies (Table Aii_3; A) and the frequency distribution of both predicted (dotted line) and actual (plain line) grain weights (B) across datasets reported in these papers (n=52). Dashed and solid lines in panel A, and lines in panel B are as in Figure Aii_2.

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Abstract

Generally, wheat yield can be divided into two main components; grain number per m^2 (GN) and average grain weight (AGW). Yield is commonly strongly associated with GN, but there is also a common negative relationship between AGW and GN. As this may represent a penalty in achievable yield gains should grain growth be limited by the source strength, it will be important to elucidate whether the reduction in AGW in response to increases in GN represents a case of source-limitation, and to recognize whether AGW in elite germplasm is limited by resource availability to fill the grains. GN and AGW showed a noticeable difference in phenotypic plasticity in response to the contrasting nitrogen condition. Across the three field studies, GN and yield showed to be very plastic, while AGW was extremely conservative. The proportion of distal grains, that are constitutively smaller than those that are proximal was increased when GN increased, indicating that the negative relationship was at least in part due to a non-competitive cause. However, proximal grains were also affected as the consequence of increased GN, indicating the size of all grain classes was affected when GN increased. To explore this more specific negative relationship, source-sink manipulations were imposed. Overall, AGW did not respond to de-graining, while defoliation resulted in small but consistent decreases in AGW. Further supporting these results, GN was dramatically increased in response to halving the competition during stem elongation, while AGW was only slightly reduced. This result indicates that the trade-off between AGW and GN across elite material must have been mainly due to differences in the intrinsic potential weight of the grains. Also, even though grain growth was dominantly sink-limited, current cultivars are approaching to experience some degree of source-limitation to grain growth.

Exposing wheat cultivars to pre- and post-anthesis heat stress resulted in yield penalties associated with the components mainly being determined at those stages, GN and AGW, respectively, but yield was more sensitive to pre- than to post-anthesis heat stress, consistently across both locations and genotypes. The penalty of AGW by post-anthesis heat stress did not result from competition for resources as (i) heavier grains were reduced similarly to, or more than small grains by the heat stress, and (ii) grain weight did not clearly respond to the increase in assimilate availability, indicating that the reduction of AGW caused by heat stress was not related to source strength changes. Therefore, the reduction of AGW caused by heat stress at post-anthesis might have been simply due to a direct effect reducing PGW *per se*. When transient heatwaves were imposed twice at both pre- and post-anthesis, the reduction of AGW caused by that post-anthesis heat stress was diminished compared with the effect of the same post-anthesis heat treatment imposed solely, indicating that the effect of transient heatwaves was not additive during the whole growing period of wheat, but to some extent, there was sort of a priming effect of an earlier heat stress on the magnitude of the effect of a succeeding heat stress.

All in all, considering all experiments carried out it is suggested that breeders may need to consider not only further improvements in sink-strength (through either higher GN or higher PGW) but also simultaneously in source-strength during grain filling. This is because it was found in the different approaches that modern cultivars may still be sink-limited during grain filling but close to experience some degree of source limitation (in general AGW not responding to de-graining but being reduced in response to defoliations).

Supervisor

Dr. Roxana Savin

Dr. Gustavo A. Slafer

CROP PHYSIOLOGY LABORATORY

School of Agriculture and Forestry Engineering

Department of Crop and Forest Sciences

University of Lleida, Spain