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# PHYSIOLOGICAL BASES OF CULTIVAR DIFFERENCES IN AVERAGE GRAIN WEIGHT IN WHEAT: SCALING DOWN FROM PLOT TO INDIVIDUAL GRAIN IN ELITE MATERIAL

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**Abstract**

In recent decades, increases in wheat yield have been achieved mainly through increases in grain number per $m^2$ (GNM2) rather than through increases in average grain weight (AGW). Using AGW as a lever to increase yield would require avoidance of the negative relationship between GNM2 and AGW. It is usually supposed that this trade-off arises from an increase in the proportion of small grains as GNM2 rises. The proportional increase in small grains being the result either of (1) an increase in the proportion of secondary tillers in the spike population or (2) of an increase in the proportion of grains located in distal positions within each spike. Either or both of these two populational effects would tend to mask any true genotypic differences in AGW. The existence of these constitutive differences has already been proposed, but without considering the full extent of the populational confounding effects. Identification of a component of the constitutive genetic determinism of AGW - one that is truly independent of GNM2 - could contribute to cultivar developments that would lead to further increases in grain yield under future target environments.

To address this question, we analysed populational effects on AGW in four, modern, well-adapted bread-wheat cultivars. The four chosen cultivars show similar grain yields but contrasting AGWs. The analysis of populational effects was carried out at three hierarchical levels (the plot, the spike and the single grain) and under two contrasting environmental conditions (well-watered *vs* water-deficit conditions).

Regardless of the environment, no (or only slight) differences in individual spike size were observed between cultivars. Furthermore the weak relationship between spike size and AGWdemonstrates that AGW differences between cultivars cannot be attributed to spike-level populational effects. Meanwhile, the analysis of individual grain mass distributions, showed that the differences in AGW between cultivars, originated from shifts in the whole grain-mass distribution, rather than from shape changes in the grain-mass distribution. This clearly indicates that AGW differences between cultivars cannot be attributed to populational effects at the individual grain level.

The analysis carried out at both spike and individual grain levels indicates that the AGW differences between cultivars are largely constitutive, so that increases in grain yield through AGW may be considered independently of the GNM2 : AGW trade-off.

Taken together, these findings offer a new perspective for the genetic improvement of wheat, and one that should lead to further increases in yield.

**Keywords:** Wheat, grain number per m², average grain weight, trade-off, individual grain weight; genetic determinism.

## 1. Introduction

Yield of wheat can be decomposed into two major components: grain number per m² (GNM2) and average grain weight (AGW), often expressed as the thousand grain weight. The GNM2 is the main determinant of grain yield variation resulting from variations in environmental and/or genetic factors. Over the last fifty years grain yield increases have been achieved mainly through increases in GNM2 - rather than through increases in AGW (e.g., Fischer, 2008; Mondal et al., 2020; Slafer et al., 2014). Further increases in GNM2 are probably still possible under projected future climate conditions but the specific targeting of AGW is also a worthwhile option under climate conditions that may become less favourable and especially if these include greater levels of heat stress (Calderini and Ortiz-Monasterio, 2003; Quintero et al., 2018).

Support for the strategy of obtaining a genetic yield increase through direct manipulation of AGW is found in the observation that grain weight has a strong and heritable genetic determinism (Sadras, 2007). Indeed, some molecular-physiological studies have identified genes involved in the various processes that govern grain growth and grain filling and the effects of their allelic variations on final grain size and weight (Laudencia-Chingcuanco et al., 2007; Nadaud et al., 2010; Simmonds et al., 2016; Brinton et al., 2018; Golan et al., 2019). Moreover, numerous quantitative trait loci (QTLs) for grain weight have been identified (e.g., Breseghello and Sorrells, 2007; Gegas et al., 2010; Farré et al., 2016; Brinton et al., 2016). However, for an increase in AGW to translate into a yield increase, requires a better understanding of how the mechanisms involved in grain weight determinism (Brinton and Uauy, 2019) and, in particular, of those allowing avoidance of the critical trade-off between GNM2 and AGW under variations in both environment and genotype (Slafer et al., 2015; Slafer and Savin, 1994). This trade-off could arise from limitations imposed by the source organs and/or by the sink organs.

A source limitation (i.e., competition for assimilates between sinks) could occur either before or after anthesis. Before anthesis, reduced assimilate availability can restrict individual grain ovary size, generating a physical constraint to growth (Ugarte et al. 2007) and thus reducing final grain size (Calderini et al., 1999; 2001; 2021). After anthesis, during the grain filling phase, a grain's final size may also be constrained by its share of a limited amount of assimilate - competition between grains (Sinclair and Jamieson, 2006). Even though the exact nature on the relationship is uncertain (Calderini et al., 2021) the idea that source limitation should affect final grain size through a limitation in ovary size seems unlikely, since the relation between ovary size and final

grain size appears mainly to be correlative, not causative (Benincasa et al., 2017). On the other hand, source limitation during grain filling has usually been dismissed as a main driver of average final grain size, at least in temperate environments (Borras et al., 2004; Borrill et al., 2015; Serrago et al., 2013; Elias et al., 2016 and references therein; Reynolds et al., 2022).

The idea of a trade-off originating from sink limitation, is based on the observation that an increase in GNM2 usually leads to a higher proportion of grains of low potential weight (Acreche and Slafer, 2006; Chen et al., 2019; Feng et al., 2019). The higher proportion of small grains could be driven by processes occurring at two different levels. First, it could result from an increase in the number of secondary tillers and, consequently, an increase in the proportion of small spikes, that carry smaller grains (Xu et al., 2015) and, second, it could result from an increase in the number of grains per spike. These additional grains may be located in the basal or the apical spikelets, or in the more distal positions within each spikelet. All these locations are associated with lower potential weights and, thus, will tend to contribute to an increase in the proportion of small grains within the spike (e.g., Baillot et al., 2018; Calderini and Ortiz-Monasterio, 2003; Ferrante et al., 2017; Xie et al., 2015). These relationships seem to offer the most plausible explanation for the apparent trade-off produced by the introgression of Rht dwarfing genes (Miralles et al., 1995a). Both the processes described above, would lead to a higher proportion of small grains and so will be referred to below as 'populational' effects. The important roles of these populational effects on the trade-off between GNM2 and AGW is supported by a large number of studies (Acreche and Slafer, 2006; Chen et al., 2019; Feng et al., 2019; Ferrante et al., 2017; García et al., 2013; Li et al., 2016; Miralles and Slafer, 1995b; Quintero et al., 2018).

Given the importance of the trade-off, it appears necessary to distinguish between the part of the AGW genetic determinism that occurs *via* the populational effects described above and the part having a constitutive nature that affects each grain individually. Most quantitative genetic studies focusing on AGW do not explicitly consider the trade-off between GNM2 and AGW (e.g., Gegas et al., 2010; Brinton et al., 2018). The analytical framework of only one recent study seeks to take this trade-off into account (Quintero et al., 2018). This suggests the genetic determinism of individual grain weight is at least partly independent of the populational effects. However, their demonstration is incomplete: (i) because the study was restricted to particular spike and grain models (i.e., proximal grain (G2) and distal grain (G4) sampled on the middle spikelet of five median spikes per experimental treatment) and (ii) because neither the number of grains per spike/spikelet nor the proportions of small vs. large grains were explicitly considered.

Thus, the aim of our study was to confirm the existence of a constitutive variability in AGW due only to genetic determinism, working with different well-characterised spike and grain populations. This requires we characterise the precise distribution of individual grain weights and their association with AGW at (1) plot scale, (2) spike scale and (3) single-grain scale. With this aim, we compared four modern, well-adapted, high-yielding wheat cultivars, that express robust differences in AGW. To help ensure that any differences we might observe in AGW and its putative determinism were not specific to any particular cultivar × environment interactions, our analyses were carried out under two contrasting environmental conditions, (a) water-sufficient and (b) water-deficient.

## 2. Materials and methods

### 2.1 Field experiment

#### *2.1.1. Choice of cultivars*

Four cultivars expressing either low AGW (hereafter LCvs) or high AGW (hereafter HCvs) were chosen from among a range of registered, modern, awned and high-yielding winter-wheat cultivars. This choice was made by analysing a dataset compiled from a large number of post-registration field trials (J.-C. Deswartes, P. du Cheyron, Arvalis-Institut du végétal, personal communication, 2016) in which the mean yield components were calculated for each cultivar. Additional precautions were taken to minimise phenological differences between cultivars. However, it was not possible to identify cultivars that were within the exact same precocity class and still commercially available. Therefore, we selected Altigo (Nickerson, 2007) and Solehio (Momont, 2009) that exhibit higher AGWs and lower grain numbers per m² than Cellule (Florimond Desprez, 2012) and SY Moisson (Syngenta, 2012) (Table 1).

136 **Table 1.** Average yield components for selected winter bread wheat cultivars in multi-environment field trials (mean ± Standard Deviation)

| Yield component | Altigo | Solehio | Cellule | SY Moisson |
|---|---|---|---|---|
| **Number of trials** | 56 | 135 | 126 | 57 |
| **Years** | 2007 - 2014 | 2009 – 2017 | 2012 - 2018 | 2012 – 2018 |
| **Spike number per m²** | 494 ± 92 | 487 ± 72 | 555 ± 83 | 560 ± 86 |
| **Grain number per spike** | 37 ± 9 | 39 ± 5 | 43 ± 6 | 43 ± 6 |
| **Grain number per m²** | 17607 ± 2729 | 18732 ± 2688 | 23473 ± 3269 | 23740 ± 2831 |
| **Average grain weight (mg grain$^{-1}$)** | 52 ± 4 | 50 ± 5 | 41 ± 4 | 42 ± 4 |
| **Grain Yield (t ha$^{-1}$ at 15% humidity)** | 9.1 ± 1.4 | 9.3 ± 1.3 | 9.6 ± 1.4 | 9.9 ± 1.0 |

### *2.1.2. Experimental design*

The field experiment was conducted at INRAE, Clermont-Ferrand (45°78' N, 3°08' E, 401 m asl) using the Pheno3C high-throughput, field-phenotyping platform. Over the last 20 years, mean annual air temperature at this site has been 11.7°C and mean annual precipitation 570 mm. The weather conditions during the experiments are representative of the mean climate at the site (Fig. S1) and no acute environmental stress events were noticed. The tillable horizon is a clay soil with 42, 38 and 20% of clay, silt and sand, respectively and with high organic matter content (3.3%).

The Pheno3C facility consists of eight independent blocks. Four blocks are reserved for controls, maintained under the local climate, and these can be irrigated if required. The remaining four blocks can be covered by automatic rain-out shelters to generate a controlled water stress. All cultivars were sown on 10 November 2017 in plots within each of the eight blocks at a density of 250 grains $m^{-2}$. Each plot (eight rows, 100 cm long, 17 cm inter-row spacing, 2.1 $m^2$ harvestable area) was managed following local agronomic practices. Due to a high residual mineral N after winter (150 kg N $ha^{-1}$) a single N application was made to all plots at Zadock's stage Z32 (50 kg N $ha^{-1}$). Weed and pest management included one herbicide application (at Z26), two fungicide applications (at Z51 and Z69) and one insecticide application (at Z69).

In the four water-deficit blocks, the rain-out shelters of the Pheno3C facility were set in action from 1 March 2018 to divert all incoming rainfall, except for one 17 mm rain event occurring during the last week of April. From sowing to harvest, the well-watered and water-deficit plots received 396 and 175 mm of rainfall, respectively. Calculated from the start of the drought treatment, cumulative rainfall was 238 and 17 mm in the well-watered and water-deficit blocks, respectively (Fig. 1A). In the water-deficit blocks, the water stress was well established from the start of April, and deepened in intensity thereafter until harvest (Fig. 1B). The well-watered blocks were maintained at the onset of water stress (or above) by irrigation.

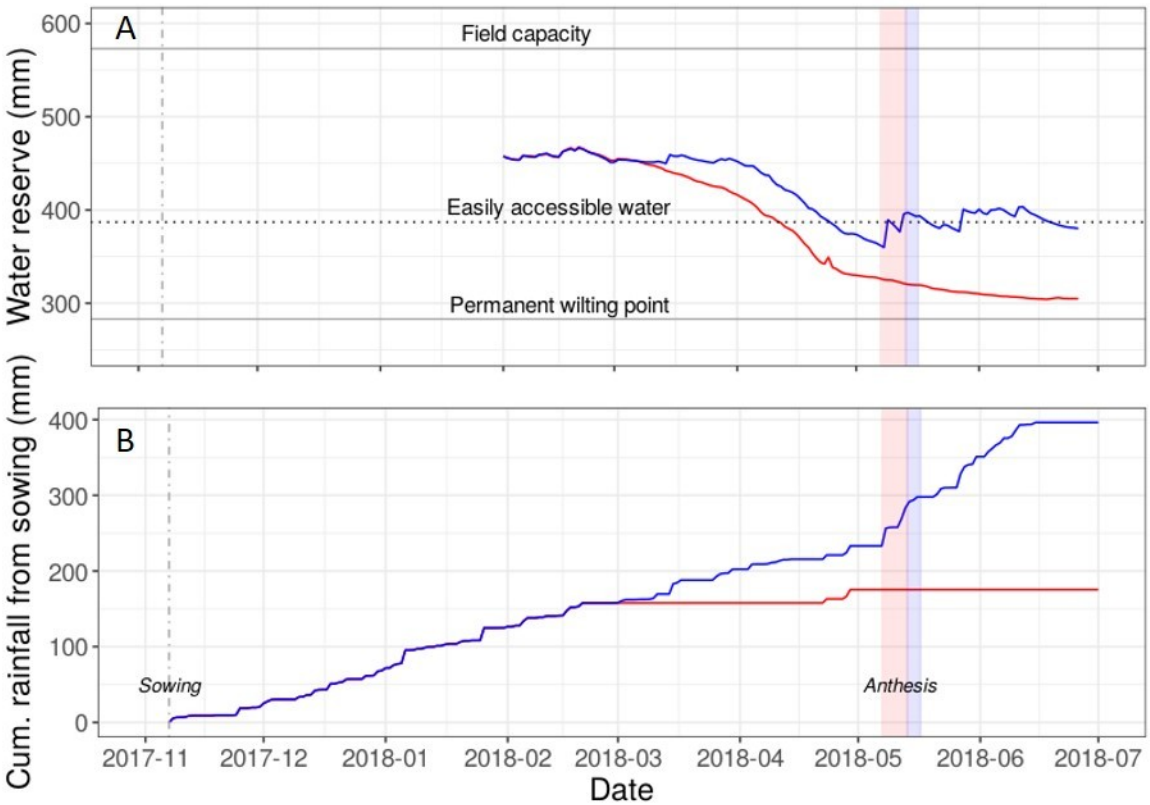


**Fig.1.** Dynamics of soil water reserve (A) and cumulative rainfall from sowing date (B) for the well-watered (blue line) and water-deficit (red line) conditions. Rectangles indicate the maximum range of observed anthesis dates for the four cultivars under well-watered (pale blue) and water-deficit (pink) conditions. Horizontal lines indicate the soil water reserves corresponding to field capacity, easily accessible water and permanent wilting point.

We emphasise that the aim of the study was not to analyse responses to water deficit but merely to create two contrasting background environments to explore the consistency of any differences that might appear in AGW and its determinants, so bringing a greater degree of confidence and robustness to our conclusions.

## 2.2 Plant sampling and measurements

Within each plot, the number of spikes per m² (SNM2) was determined after anthesis, at about anthesis + 500°C days (base 0°C) by counting all spikes within two, 100 cm long, transects in the two central rows.

At physiological maturity, in July 2018, 100 spikes per plot were sampled from the central 1.5 m of the two central rows to avoid possible edge effects. To avoid bias in the selection of spikes, the 100

178 spikes were sampled as four separate samples of 25 adjacent spikes. All spikes were oven-dried to
179 constant weight (60°C, 48 h) and for each individual spike, average grain weight ($AGW_{spike}$) and the
180 number of grains per spike ($NGS_{spike}$) were determined with an automatic seed-weighing/-counting
181 device (Opto Agri2, Optomachine, France). The weight of each individual grain within each spike
182 was then measured with an automatic seed-weighing device developed in our laboratory. This
183 device comprises a six-axis robotic arm (Yaskawa MH3, Yaskawa, Japan) integrated with a
184 precision weight module (WMC15-SH, Mettler-Toledo, Switzerland).

185 Additional variables were calculated at the plot level. Mean number of grains per spike per plot
186 ($NGS_{plot}$) and AGW per plot ($AGW_{plot}$) were calculated as the means of the 100 spikes of the sample.
187 GNM2 was estimated as the product of SNM2 and $NGS_{plot}$ and grain yield (GY) was calculated as
188 the product of GNM2 and $AGW_{plot}$.

189

190 **2.3 Statistical analyses**

191 Results were analysed at different scales depending on the variable considered. Cultivar effects on
192 mean yield components (SNM2, $NGS_{plot}$, GNM2, $AGW_{plot}$ and GY) were analysed using mean
193 variable values at the plot scale. Average grain weight per spike ($AGW_{spike}$) and the individual grain
194 weight per spike were analysed using individual spike values considering the nesting effect of plots.
195 All analyses were carried out separately for the two environmental conditions.

196

197 *2.3.1. Cultivar effects on mean yield components at plot scale*

198 Within each environmental condition, comparison of the mean yield components (SNM2, $NGS_{plot}$,
199 GNM2, $AGW_{plot}$, GY) between cultivars was carried out using Tukey's *post-hoc* test following an
200 ANOVA. For each environmental condition, the ANOVA model corresponded to a randomised
201 complete block design with cultivar and block as fixed effects (n=4):

202 $$Y = \mu + X_1Cv + X_2B + \epsilon \qquad \text{(Eq.1)}$$

203 where Y is the vector of each mean yield component value, μ is the mean effect, Cv the fixed effect
204 of each cultivar, B the fixed effect of the block, $X_i$ the incidence matrix and ϵ is the residual
205 following a normal distribution $(0, \sigma^2_e)$.

206

207 *2.3.2. Cultivar effects on average grain weight ($AGW_{spike}$) at spike scale*

208 To compare the differences in average grain weight per spike ($AGW_{spike}$) between cultivars at spike
209 scale, a linear model was developed including the effect of the covariable $NGS_{spike}$. The
210 experimental design was also considered. To test the effect of the AGW genotypic level on
211 $AGW_{spike}$, the four cultivars were pooled into two groups: HCvs (i.e., Altigo and Solehio) and LCvs
212 (i.e., Cellule and SY Moisson).

213 The following linear model was then used with the package ASREML (Gilmour et al., 1995):

214 $$Y = \mu + X_1Cov + X_2G + X_3Cv + X_4B + X_5Cov\times G \times B + \epsilon \qquad \text{(Eq.2)}$$

215 where Y is the vector of average grain weight per spike ($AGW_{spike}$) values, μ is the intercept, Cov
216 the fixed effect of the covariable ($NGS_{spike}$), G the fixed effect of the group of cultivars, Cv the fixed
217 effect of each cultivar nested within each group of cultivars, B the fixed effect of the block, $X_i$ the
218 incidence matrix and ϵ is the residual following a normal distribution (0, $\sigma^2_e$). A Wald test was
219 computed on this linear model to estimate the sizes of the different effects. Based on coefficients
220 calculated with the linear model, slopes and intercepts were calculated for each cultivar and under
221 each environmental condition.

222

223 *2.3.3. Cultivar effects on individual grain weight*

224 To analyse the putative populational effects associated with AGW differences requires going
225 beyond just the differences in means, to try to understand how the distributions actually differ.
226 Comparing quantiles is well suited to this purpose as they provide informative, robust and intuitive
227 information (Rousselet et al., 2017). Rousselet et al. (2017) proposed plotting a 'shift function' that
228 represents the differences between the quantiles of the distributions of two groups as a function of
229 the quantiles of one of the groups. Quantiles are estimated with the Harrell-Davis estimator (Harrell
230 and Davis, 1982 *in* Wilcox, 1995) and then quantiles differences between groups and their
231 associated confidence interval are estimated using a bootstrap method (as described in Rousselet et
232 al., 2017). In our study we extended this approach to provide a graphical representation of each
233 cultivar distribution to compare with the distribution of a pooled population comprising all of the
234 four cultivars. This offered a visual representation of the cultivar differences in spike size ($NGS_{spike}$)

and individual grain weight. The same approach was used to compare individual grain weight distribution of the two groups of cultivars (HCv and LCv).

To complete the graphical comparison, we carried out an analysis using the deciles as a categorical variable with the following linear model using the package ASREML (Gilmour et al., 1995):

$$Y = \mu + X_1GrD + X_2G + X_3Cv + X_4B + X_5GrD\times G\times Cv\times B + \epsilon \qquad \text{(Eq. 4)}$$

where Y is the vector of individual grain weight values, $\mu$ is the intercept, GrD the fixed effect of the grain decile, G the fixed effect of the group of cultivars, Cv the fixed effect of each cultivar nested within each group of cultivars, B the fixed effect of the block, $X_i$ the incidence matrix and $\epsilon$ is the residual following a normal distribution $(0, \sigma^2_e)$. A Wald test was computed on this linear model to estimate the sizes of the different effects.

All statistical analyses were carried out with R statistical software (R version 3.5.0, R Development Core Team (2005)).

## 3. Results

### 3.1. The four cultivars achieved similar grain yields through different combinations of AGW per plot and GNM2

Within each environmental condition (well-watered *vs* water-deficit), there were no differences in grain yield among the four cultivars (Table 2). These results agree with those of the multi-environment trials used to select the cultivars for the experiment (Table 1). As expected, water-deficit led to a decrease in grain yield (~29% averaged among the four cultivars; Table 2). The expected small differences in cultivar precocity (Table 1) were also observed. Under well-watered conditions, the anthesis date difference between the earliest and the latest cultivars was only four days (Table 2). Under water-deficit conditions, anthesis dates of the four cultivars occurred over a seven-day period (Table 2).

**Table 2 –** Average yield and yield components for the four selected winter bread wheat cultivars under well-watered or water-deficit conditions.

| Condition | Group of cultivars[1] | Cultivar | Flowering date (days from sowing) | SNM2[2] (spike per $m^2$) | $NGS_{plot}$[2] (grains $spike^{-1}$) | GNM2[2] (grains $m^{-2}$) | $AGW_{plot}$[2] (mg $grain^{-1}$) | GY[2] (t $ha^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| **Well-watered** | HCv | Altigo | 137 | 368 ± 24 a [3] | 35.9 ± 0.6 b | 13182 ± 837a | 51.96 ± 0.77 d | 7.0 ± 4 a |
| | | Solehio | 137 | 430 ± 84 ab | 34.5 ± 1.3 a | 14821 ± 3007 ab | 49.83 ± 0.79 c | 7.5 ± 14a |
| | LCv | Cellule | 133 | 378 ± 32 ab | 40.5 ± 0.8 c | 15336 ± 1209 ab | 40.43 ± 0.86 a | 6.3 ± 4a |
| | | SY Moisson | 136 | 485 ± 78 b | 35.5 ± 1.0 ab | 17146 ± 2478 b | 42.54 ± 0.82 b | 7.5 ± 10a |
| **Water-deficit** | HCv | Altigo | 134 | 362 ± 18 a | 28.7 ± 1.2 a | 10397 ± 648 a | 48.77 ± 2.44 b | 5.1 ± 5 a |
| | | Solehio | 134 | 344 ± 45 a | 30.2 ± 1.8 a | 10440 ± 1892 a | 48.62 ± 1.03 b | 5.1 ± 9 a |
| | LCv | Cellule | 127 | 394 ± 50 a | 33.1 ± 1.0 a | 13042 ± 1645 b | 38.43 ± 1.52 a | 5.0 ± 7a |
| | | SY Moisson | 130 | 380± 21 a | 31.2 ± 4.8 a | 11793 ± 1141 ab | 40.20 ± 1.34 a | 4.8 ± 5a |

[1]: HCv: high-AGW cultivar; LCv low-AGW cultivar

[2]: **SNM2**: Spike number per $m^2$; **$NGS_{plot}$**: number of grains per spike determined on a plot basis; **GNM2**: grain number per $m^2$; **$AGW_{plot}$** : Average Grain Weight determined on a plot basis; **GY**: Grain Yield.

[3]: Per condition, different letters indicate statistical significance at level P=0.05 within the same column (according to a Tukey post-hoc test following ANOVA).

265 The homogeneity among cultivars for yield, masked strong differences in the individual
266 yield components between cultivars. Again, as expected from the cultivar choices, strong
267 differences in AGW between cultivars were observed irrespective of the environmental conditions.
268 Based on $AGW_{plot}$, under well-watered conditions, two classes of cultivars could be distinguished:
269 on the one hand Altigo and Solehio (HCvs) had an average $AGW_{plot}$ of ~51 mg $grain^{-1}$ and on the
270 other hand Cellule and SY Moisson (LCvs) had an average $AGW_{plot}$ of ~41 mg $grain^{-1}$ (Table 2).
271 This distinction was emphasised under the water-deficit conditions with $AGW_{plot}$ averages of ~48.7
272 mg $grain^{-1}$ for HCvs and ~39.0 mg $grain^{-1}$ for LCvs (Table 2).

273 Given the trade-off between AGW and GNM2, strong differences in GNM2 were also
274 expected between genotypes. Even though exhibiting a tendency, these differences in GNM2 were
275 not always significant within the groups created on a $AGW_{plot}$ basis. For example, within the well-
276 watered experimental conditions, Solehio (HCv) and Cellule (LCv) showed similar values of
277 GNM2 but different ones of $AGW_{plot}$. Similarly, under water-deficit condition, SY Moisson (LCv)
278 showed GNM2 values not significantly different from those of Altigo or Solehio (HCvs) (Table 2).

279 No clear trend was observed for the source of cultivar variation in GNM2. The differences
280 could be attributed either to genotypic variation of NGS at plot scale ($NGS_{plot}$) that varied between
281 34.5 and 40.5 grains per spike under well-watered conditions or to SNM2 depending on the
282 genotype and the environmental conditions (Table 2).

283 Therefore, with similar grain yields but contrasting $AGW_{plot}$, $NGS_{plot}$ and SNM2 under both
284 environmental conditions, the four cultivars allow exploration of the main sources of variability in
285 cultivar $AGW_{plot}$ and, in particular, the putative populational effects on variability of cultivar AGW.

286

287 **3.2. $AGW_{spike}$ cultivar differences were not explained by differences in the distributions of**
288 **spike size**

289 To explore spike population effects on AGW our methodology requires information at the
290 individual spike level. To this end, 100 individual spikes were sampled for each experimental unit,
291 and $NGS_{spike}$ and $AGW_{spike}$ were determined for each spike sampled. This information obtained at the
292 single spike scale allows us to study the contributions of spike population to cultivar $AGW_{plot}$
293 following a two-step evaluation. First, the distributions of spike size between cultivars were

compared using $NGS_{spike}$. Second, we examined whether between-spike variations in $NGS_{spike}$ significantly affected $AGW_{spike}$.

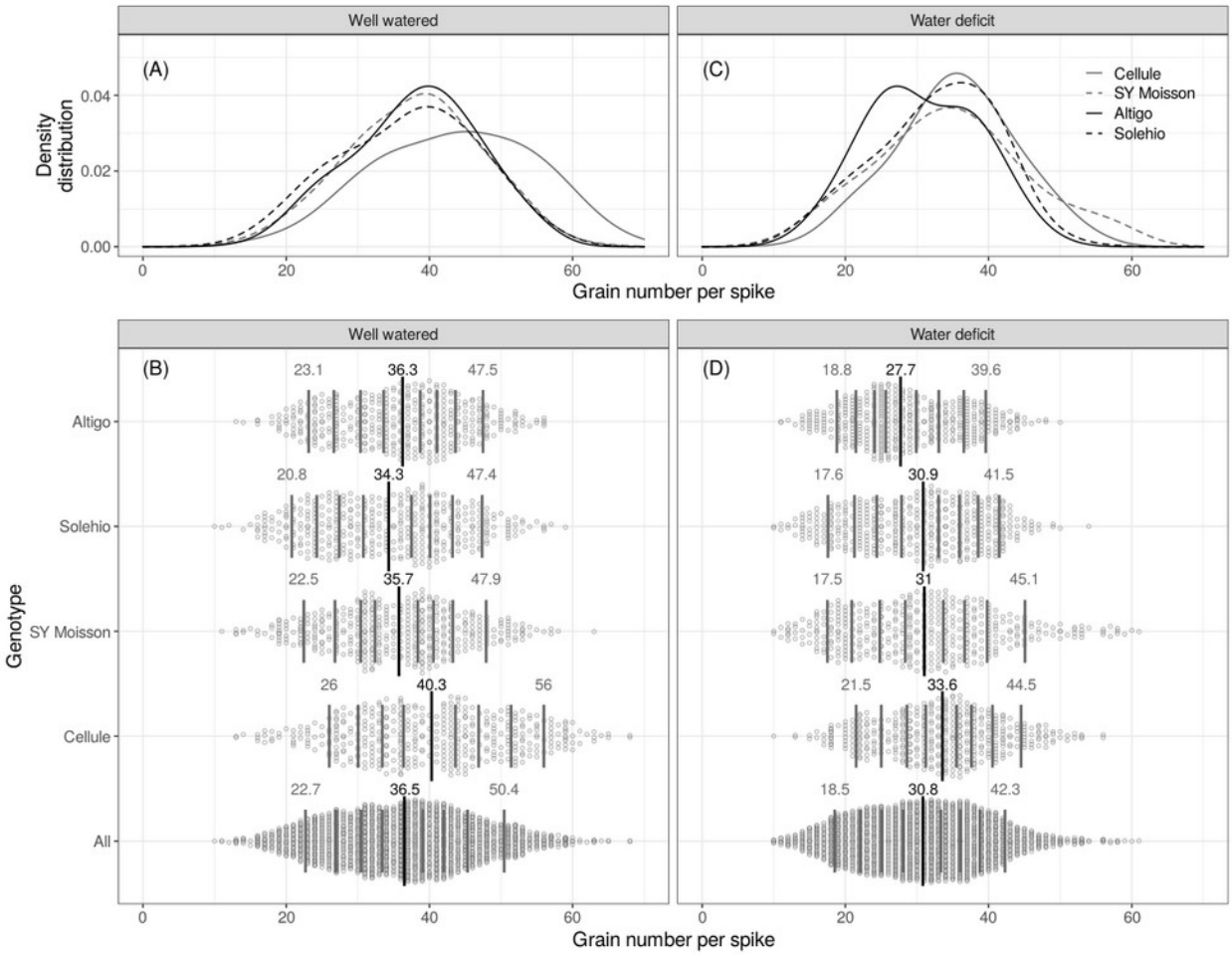


**Fig. 2**: Distributions of the grain number per spike ($NGS_{spike}$) of the four cultivars under well-watered (A, B) and water-deficit (C, D) conditions. Top panels (A, C) represent the density distribution of $NGS_{spike}$ per cultivar. Distribution parameters (mean, variance, skewness) for each condition and each cultivar: (A): Altigo (mean= 35.85, variance= 85.00, skewness= -0.02); Solehio (34.50, 105.36, 0.01); Cellule (40.53, 134.67, 0.06); SY Moisson (35.47, 92.11, -0.04); (D): Altigo (mean= 28.71, variance= 64.95, skewness= 0.24); Solehio (30.17, 81.29, -0.11); Cellule (33.11, 76.79, 0.02); SY Moisson (31.28, 115.18, 0.33). Bottom panels (B, D) show the decile values of $NGS_{spike}$ distributions for the four cultivars and the whole spike population pooling the four cultivars (All). Figures in the bottom panels stand for the values of the first decile, median and last decile.

For the well-watered condition, all distributions of $NGS_{spike}$ were close to symmetrical (i.e., exempt from strong negative skewness) and were relatively similar except Cellule, for which $NGS_{spike}$ values were shifted towards higher values (Fig. 2A) for all deciles (Fig. 2B). Regardless of Cellule specificity, no link between SNM2 differences and $NGS_{spike}$ distributions could be inferred. For example, SNM2 was 32% higher in Altigo (HCv) than in SY Moisson (LCv) (Table 2) whereas the $NGS_{spike}$ distributions of these two cultivars were nearly identical (Fig. 2A).

Similar conclusions can be drawn under the water-deficit condition (Fig. 2C and 2D). The distributions of $NGS_{spike}$ were very similar for the four cultivars with only a small shift of Altigo towards smaller spikes. This small difference does not appear to be correlated with NSM2, as differences among the genotypes were marginal and not significant (Table 2).

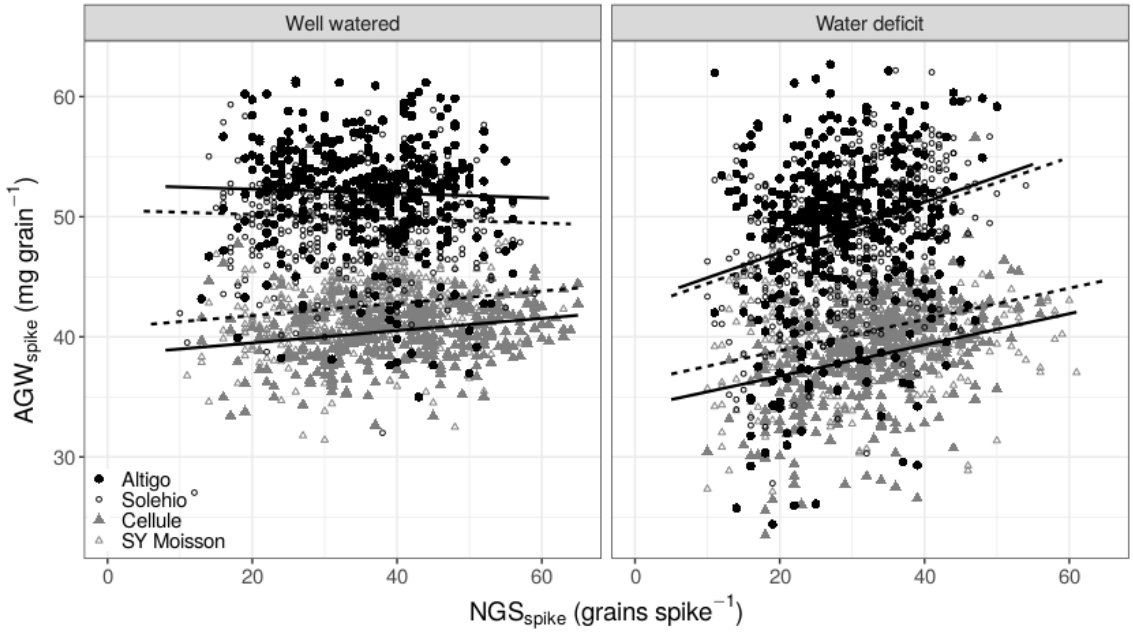


**Fig.3.** Relationship between $NGS_{spike}$ (grains spike$^{-1}$) and $AGW_{spike}$ (mg grain$^{-1}$) for each cultivar under well-watered (A) and water-deficit conditions (B). For each condition, slope and intercept are calculated following the model described in the Material and Methods section: (A) Altigo (slope= - 0.018; intercept= 52.65); Solehio (-0.018, 50.55); Cellule (0.051, 38.48); SY Moisson (0.051, 40.75); (B) Altigo (slope= 0.21, intercept= 42.82); Solehio (0.21, 42.34); Cellule (0.13, 34.13); SY Moisson (0.13, 36.25). The $R^2$ of the models are 0.67 (A) and 0.56 (B). Altigo and Solehio are High-AGW cultivars (HCv); Cellule and SY Moisson are Low-AGW cultivars (LCv).

There were no relationships (Fig. 3A) between $AGW_{spike}$ and $NGS_{spike}$ for the well-watered conditions and only weak positive (Fig. 3B) ones for the water-deficit conditions. For both conditions, an analysis of variance (Table 3) reveals that the differences in $AGW_{spike}$ are explained mainly by the *a priori* classification of the cultivars into HCv or LCv with 61.3% of total variance explained for the well-watered and 47.9% for the water-deficit conditions. Although highly significant, the other effects never explained more than 4.2% of the variance in $AGW_{spike}$ (Table 3). In particular, the interaction term between $NGS_{spike}$ and the group of cultivars (NGS×G) explained only 0.4 and 0.2% under well-watered and water-deficit conditions, respectively. Taken together, these results indicate that the cultivar differences in $AGW_{spike}$ were not due to populational effects at spike level.

**Table 3**. Analysis of variance (ANOVA) followed by the statistics of Wald test for $AGW_{spike}$ (mg $grain^{-1}$) under well-watered and water-deficit conditions.

| Condition | Source of variation [1] | Degrees of freedom | Percentage of variance explained | Wald statistic | P-value[2] |
|---|---|---|---|---|---|
| **Well-watered** | **Number of grains per spike (NGS)** | 1 | 0.7 | 35.74 | *** |
| | **Block (B)** | 3 | 0.8 | 40.31 | *** |
| | **Group (G) of cultivars (Cv)** | 1 | 61.3 | 2992.91 | *** |
| | **Cv nested in G** | 2 | 3.0 | 147.32 | *** |
| | **NGS × G** | 1 | 0.4 | 17.44 | *** |
| | **NGS × B** | 3 | 0.2 | 7.14 | ns |
| | **G × B** | 3 | 0.5 | 22.59 | *** |
| | **G × B × NGS** | 3 | 0.1 | 2.79 | ns |
| | **Residual** | 1618 | 33.1 | | |
| **Water-deficit** | **NGS** | 1 | 0.9 | 34.49 | *** |
| | **B** | 3 | 4.2 | 155.77 | *** |
| | **G** | 1 | 47.9 | 1758.29 | *** |
| | **Cv nested in G** | 2 | 1.1 | 41.77 | *** |
| | **NGS × G** | 1 | 0.2 | 6.89 | ** |
| | **NGS × B** | 3 | 0.3 | 9.32 | * |
| | **G × B** | 3 | 0.8 | 28.07 | *** |
| | **G × B × NGS** | 3 | 0.2 | 7.03 | ns |
| | **Residual** | 1631 | 44.4 | | |

[1]: **G**: HCv = High AGW cvs (Altigo and Solehio) ; LCv = Low AGW cvs (Cellule and SY Moisson)

[2]: ns : P> 0.05; *: P<0.05; **: P<0.01 ; *** : P<0.001

335 **3.3. Cultivar differences in individual grain weight were observed, irrespective of grain decile**

336 The second populational effect proposed as a possible driver of cultivar differences in
337 $AGW_{plot}$, occurs within the spike, at the individual grain level. Under this assumption, cultivars with
338 larger spikes (higher NGS) will present higher proportions of small grains within spikes and this
339 will negatively affect $AGW_{plot}$. Assessing individual grain weight distributions per spike is made
340 difficult due to (i) the limited number of grains within a spike and (ii) the large variation in the
341 numbers of grains between spikes. Given the absence of clear a relationship between $NGS_{spike}$ and
342 $AGW_{spike}$, the individual grain weight distribution was assessed only at the whole population level.

343 As expected, given that the choice of the four cultivars was based on their *a priori* differences in
344 AGW, clear differences in individual grain weight could be observed between Altigo and Solehio
345 (HCv cultivars) on the one hand and SY Moisson and Cellule (LCv cultivars) on the other hand
346 under both environmental conditions (Fig. 4A and 4D). All distributions were negatively skewed,
347 with an excess of small grains. The shift function allowed comparison of the individual grain weight
348 decile values, on the one hand of the whole-grain population made by pooling the four cultivars
349 and, on the other hand, with each cultivar independently. This representation allows to visualisation
350 of both the strong differences between the HCv and the LCv cultivars and of the relative
351 homogeneity of the cultivars within each of these groupings, in spite of the divergent behaviours of
352 the first four deciles (Fig. 4C and 4F) that relate to small skewness differences among cultivars.

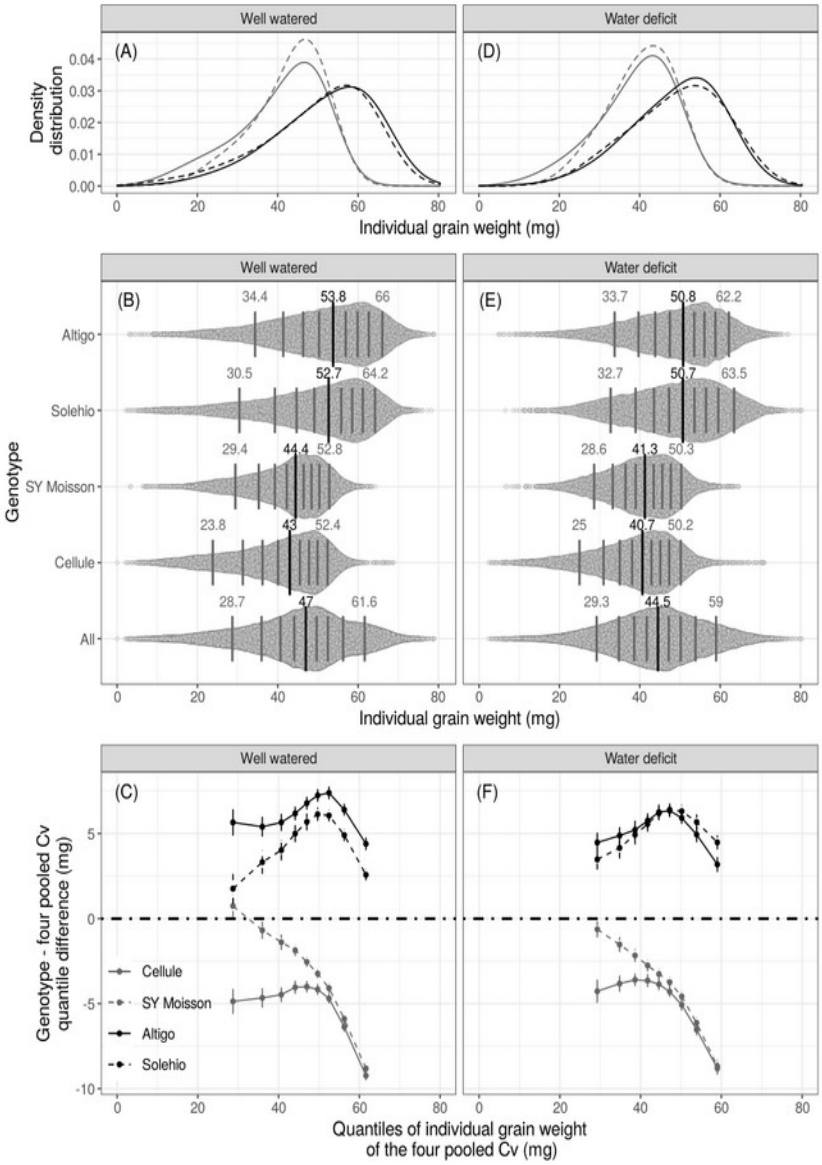


**Fig. 4**: Distributions of the individual grain weight (mg) of the four cultivars under well-watered (A, B, C) and water-deficit (D, E, F) conditions. Top panels (A, D) represent the density distribution of individual grain weight per cultivar. Distribution parameters (variance, skewness) for each condition and each cultivar: (A) Altigo (mean= 51.79, variance= 156.91, skewness= -0.69); Solehio (49.91, 178.58, -0.85); Cellule (40.60, 122.71, -0.73); SY Moisson (42.78, 83.04, -0.68); (D) Altigo (mean= 49.16, variance= 125.47, skewness= -0.56); Solehio (49.34, 136.68, -0.40); Cellule (38.96, 98.21, -0.65); SY Moisson (40.38, 70.07, -0.44). Middle panels (B, E) show the deciles values of individual grain weight distributions for the four cultivars and the whole grain population mixing the four cultivars (All). Numbers are given for the first decile, median and last decile. Bottom panels represent the shift function i.e., the differences between the deciles of each cultivar from the deciles of whole population.

Individual grain weight differences between the HCv and LCv cultivars are even more striking when averaged per group of cultivars. Under both conditions, individual grain weight from all deciles is higher for HCv than for LCv, regardless of the decile considered (Fig. 5A and 5C). Remarkably, these differences between cultivar groups tended to increase with grain weight (Fig. 5B and 5D) and these relationships were very similar under both environmental conditions.

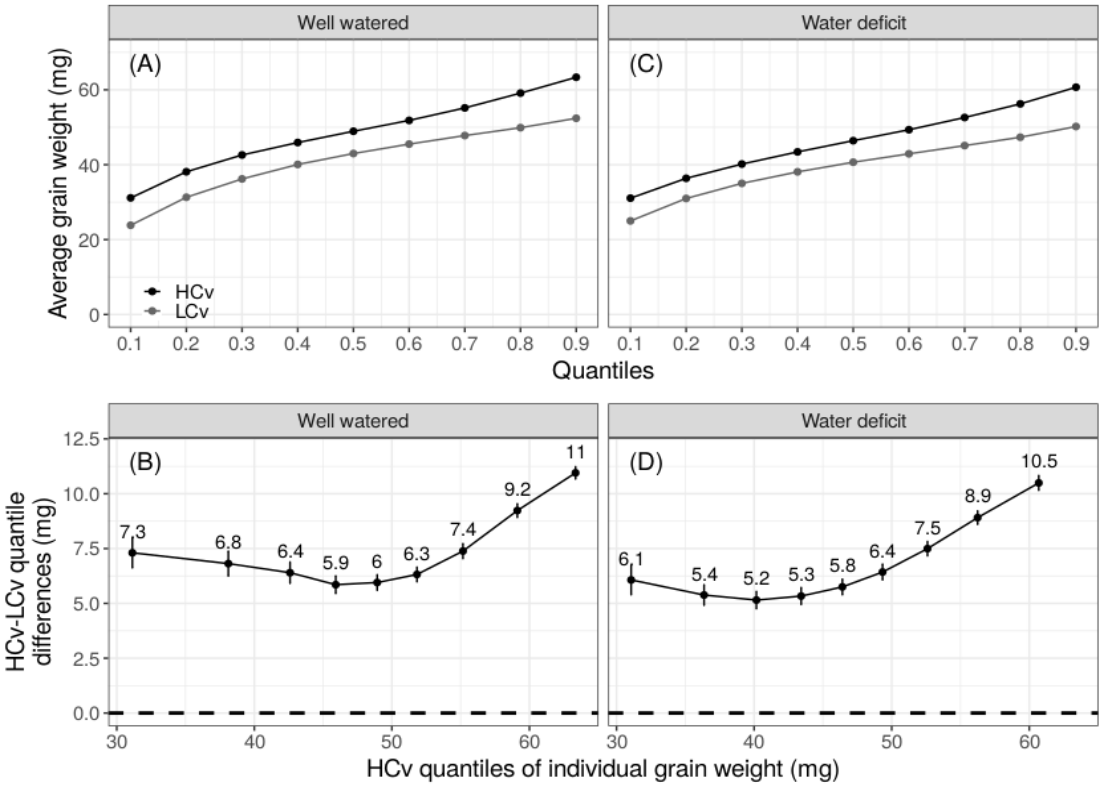


**Figure 5**: Mean individual grain weight per decile for two groups of cultivars HCv and LCv under well-watered (A, B) and water-deficit (C, D) conditions. HCv are Altigo and Solehio; LCv are Cellule and SY Moisson. Top panels (A, C) represent the mean individual grain weight per decile per group. Bottom panels represent the shift function i.e., the differences between the deciles of the HCv cultivars and the deciles of the LCv cultivars as a function of the HCv deciles. The numbers indicate the differences (mg) for each decile.

The conclusions drawn from the graphical analysis of the shift function are supported by an analysis of variance (Table 4). Indeed, apart from the large effect of grain decile, the main factor affecting individual grain weight was the group of cultivars (14.2 for the well-watered conditions and 16.9% for the water-deficit conditions). This result is independent of grain weight decile, as indicated by the small interaction term (<1% of the variance explained in both environmental conditions). In other words, HCvs had higher AGW than LCvs due to their having constitutively heavier grains.

**Table 4.** Analysis of variance (ANOVA) followed by the statistics of Wald test for individual grain weight (mg) under well-watered and water-deficit conditions.

| Condition | Source of variation [1] | Degrees of freedom | Percentage of variance explained | Wald statistic | P-value [2] |
|---|---|---|---|---|---|
| **Well-watered** | **Block (B)** | 3 | 0.16 | 704.47 | *** |
| | **Grain Decile (GrD)** | 9 | 70.69 | 316262.95 | *** |
| | **Group (G) of Cultivars (Cv)** | 1 | 14.17 | 63398.26 | *** |
| | **Cv nested in G** | 2 | 0.69 | 3101.77 | *** |
| | **G × B** | 3 | 0.09 | 410.19 | *** |
| | **G × GrD** | 9 | 0.86 | 3832.66 | *** |
| | **B × GrD** | 27 | 0.01 | 56.03 | *** |
| | **G × B × GrD** | 37 | 0.02 | 69.84 | *** |
| | **Residual** | 59582 | 13.32 | | |
| **Water-deficit** | **B** | 3 | 1.68 | 3903.87 | *** |
| | **GrD** | 9 | 54.70 | 127044.86 | *** |
| | **G** | 1 | 16.94 | 39343.56 | *** |
| | **Cv nested in G** | 2 | 0.21 | 482.45 | *** |
| | **G × B** | 3 | 0.35 | 819.85 | *** |
| | **G × GrD** | 9 | 0.38 | 891.54 | *** |
| | **B × GrD** | 27 | 0.06 | 133.76 | *** |
| | **G × B × GrD** | 37 | 0.02 | 40.47 | ** |
| | **Residual** | 59582 | 25.66 | | |

[1]: Grain deciles (GrD) ; G: HCv = High AGW cvs (Altigo and Solehio) ; LCv = Low AGW cvs (Cellule and SY Moisson))

[2]: **: P<0.01 ; *** : P<0.001.

384 **4. Discussion**

385 **4.1. Relevance of the selected cultivars to exploring the grain number/AGW relationship**

386 Most studies comparing wheat cultivars and showing differences in grain number and AGW
387 have focused on the dichotomy between old cultivars and new ones, or between tall cultivars and
388 semi-dwarf ones (Chen et al., 2019; Ferrante et al., 2017; Miralles and Slafer, 1995b), or they have
389 explored cultivars showing wide-ranging yield potentials (e.g., Peltonen-Sainio et al., 2007;
390 Quintero et al., 2018). All these studies, together with an abundance of studies that have focused on
391 yield responses to different management practices (e.g., Acreche and Slafer, 2006; Feng et al.,
392 2019; Fischer, 1993; Li et al., 2016) have concluded that yield variations are mainly related to
393 variations in GNM2 (e.g., Slafer et al., 2014 and references therein). Given the negative relationship
394 that commonly exists between GNM2 and AGW (Slafer et al., 2014; Slafer and Savin, 1994),
395 studies that rely on a high variability in GNM2, only highlight the dominant effect of GNM2 on
396 AGW. Therefore, this limits their ability to determine the component of AGW genetic variation,
397 that can be attributed to constitutive genetic differences.

398 One way to resolve this problem is to drastically restrict the range of variation in GNM2, so
399 as to allow a focus on AGW. In the studies cited above (Ferrante et al., 2017; Peltonen-Sainio et al.,
400 2007; Quintero et al., 2018), the range of values of GNM2 varied by more than ten-fold across the
401 cultivars and/or conditions they tested. For example, Ferrante et al. (2017) observed GNM2 values
402 that varied between 2 500 and 25 000, Peltonen-Sainio et al. (2007) between 2 300 and 19 000 and
403 Quintero et al. (2018) between 11 000 and 25 000 grains $m^{-2}$. In our study, the range in GNM2 was
404 much narrower i.e., between 13 000 and 17 000 grains $m^{-2}$ under well-watered conditions and
405 between 10 000 and 13 000 grains $m^{-2}$ under water-deficit conditions (Table 2). Nevertheless,
406 significant differences in AGW were still evident (Table 2). This suggests the observed differences
407 in AGW were not driven solely by GNM2 differences. The four modern, high-yielding winter bread
408 wheat cultivars we used, expressed consistently high (Altigo and Solehio) and low (Cellule and SY
409 Moisson) values of AGW. However, these strong differences in yield components are also
410 accompanied by earliness differences between LCv and HCv cultivars (Table 2). Earliness
411 differences between cultivars are suspected to have a strong confounding effect on the
412 establishment of yield components, in particular when developmental differences generate
413 differences in exposure to climatic stresses. In our experiment, no acute uncontrolled abiotic stress
414 event occurred during the crop cycle (Fig. 1, Fig. S1). Therefore, we do not believe that the limited

415 precocity differences observed will have caused significant differences in yield component
416 establishment.

417 We thus conclude that the four cultivars we study here will allow us to determine if a
418 proportion of the determination of the AGW differences could be constitutive. In addition, the two
419 contrasting growing conditions (well-watered and water-deficit) will allow us to determine if our
420 conclusions are reasonably independent of the growing environment. In this case it will allow a
421 degree of generalisation for our results.

422

423 **4.2. AGW cultivar differences are poorly explained by populational effects reflecting their**
424 **constitutive nature**

425 The results of numerous studies published in the last few years support the idea that a large
426 portion of the trade-off between GNM2 and AGW originates from populational effects. In other
427 words, an increase in GNM2 will be associated with an increase in the relative contributions of
428 grains having a lower weight potential than the rest of the grain population (Acreche and Slafer,
429 2006; Miralles and Slafer, 1995a) and this will directly and negatively affect AGW.

430 From the putative populational effects suggested above, the one acting at the spike
431 population level has often been mentioned but rarely studied directly. Xu et al. (2015) showed that
432 tillers of higher order, produce spikes with lower grain numbers per spike and lower AGW. In
433 particular, spikes from the first primary tillers have (depending on genotype) between 4 and 12%
434 less grains per spike than spikes from the main stem. This effect is even stronger for tillers of higher
435 order. The same observation was highlighted by Thorne and Wood (1987) with a much higher
436 (28%) reduction in NGS in tillers compared to main stems. Such results imply that any increase in
437 SNM2 made through an increased proportion of tillers (of any order) would induce a higher
438 proportion of smaller spikes in the population. In the present study, the number of grains per spike
439 ($NGS_{spike}$) of individual spikes was recorded but information on the tiller order that produced these
440 spikes was not. Nevertheless, given that the plant density at sowing under our conditions (250
441 plants $m^{-2}$) and the observed SNM2, are within the range 344-485 spikes $m^{-2}$ (Table 2), our spike
442 populations would have been composed mainly of spikes originating from the main stem with the
443 calculated number of spikes per plant within the narrow range of 1.4 to 1.9 spikes per plant,
444 depending on genotype and condition. The low SNM2 observed in our experiment probably
445 explains the absence of clear genotypic differences in the NGS distribution (Fig. 2). This absence of
446 populational effect observed at the spike level is likely to explain the absence of a relationship

447 between NGS and $AGW_{spike}$ in spite of the large range of $NGS_{spike}$ observed under both conditions
448 and in all genotypes (Fig. 3). This implies that the hypothesis that spike populational effects on
449 AGW probably relies on the effects of tiller order on AGW, rather than on a direct effect of $NGS_{spike}$
450 on AGW. To fully validate this proposition, the characterisation of both the individual NGS (such
451 as in the present experiment) and the order of the tillers carrying each spike (as made by Xu et al.,
452 2015 and Thorne and Wood, 1987) would require additional experimental work.

453 The second populational effect suggested in the literature having a putative effect on AGW,
454 operates at the within-spike level. From this point of view, increasing grain number per $m^2$ through
455 an increase in NGS is accompanied by a higher proportion of small grains positioned in basal or
456 apical spikelets within the spike (e.g., Feng et al., 2019; Ferrante et al., 2017; Miralles and Slafer,
457 1995a). It is well established that, within spikelets, the grains in distal florets are smaller than in
458 basal florets (e.g., Baillot et al., 2018; Miralles and Slafer, 1995a; Rawson and Evans, 1970). Taken
459 together both relationships lead to a decrease in $AGW_{spike}$ (Acreche and Slafer, 2006).

460 A problem is the estimation of the proportion of small grains within a population of grains is
461 not straightforward, as the weight threshold criterion for 'small' is necessarily arbitrary. An
462 alternative used by Acreche and Slafer (2006) is to define the proportion of small grains as the ratio
463 between the number of grains located in distal positions as a proportion of the total number of
464 grains in the spike. However, this method does not account for actual individual grain weights and
465 can be misleading as an additional grain in a distal position on a central spikelet is not necessarily
466 smaller than a proximal grain in a more basal or apical spikelet, as suggested by the results of Feng
467 et al. (2018; Fig. 4).

468 Therefore, we used a method based on an analysis of individual grain weight deciles to
469 compare cultivars. Within this analytical framework, the hypothesis of cultivar AGW differences
470 driven by populational effects should result in higher weight differences between cultivars for the
471 first deciles than for the last deciles. Our results clearly don't support this hypothesis as cultivar
472 differences in individual grain weight tend to increase with deciles in a similar fashion under the
473 two environmental conditions (Fig. 5, Table 4). In other words, the LCv cultivars do not have lower
474 AGWs than the HCv cultivars due to an increased proportion of small grains but because all grains
475 are smaller, in particular the larger ones.

476 In conclusion, we note that our data did not expose either of the two populational effects
477 previously suggested as the main determinants of AGW difference between cultivars. This does not
478 mean that such populational effects never occur (they would likely dominate the trade-off between

479 GNM2 and AGW when large changes in GNM2 are produced) but that a component of the AGW
480 differences observed between cultivars has a constitutive nature, as already suggested by Quintero
481 et al. (2018). However, these authors' conclusions were based exclusively on the observation that
482 both the proximal and distal grains (G2 and G4) of the two central spikelets were larger in the high
483 AGW cultivars than in the low AGW cultivars. Thus, their analyses did not account for the diversity
484 observed either in the spike population (variation in spike grain number distribution) or in the grain
485 population caused by variation in spikelet number per spike. Our study explicitly evaluated the
486 populational effects and showed that these were extremely weak and thus not sufficient to explain
487 the cultivar differences in AGW. The constitutive nature of grain size difference has thus been
488 highlighted for all grains (Fig. 5), independently of their weights and independently of the spike
489 from which they originated. In agreement with Calderini et al. (2021), our conclusion implies that
490 AGW can to some extent be manipulated independently of grain number.

491

492 **4.3. Towards new targets for grain-weight breeding**

493 It is widely accepted that the grain yield increases achieved in recent decades have come
494 about mainly through increases in grain number per m². Meanwhile, AGW has been increased only
495 marginally over the same period (e.g., Brancourt-Hulmel et al., 2003; Philipp et al., 2018; Shearman
496 et al., 2005) and has been selected principally for its effects on grain quality, rather than on grain
497 yield (Philipp et al., 2018; Xie et al., 2018). The existence of a significant part of AGW genetic
498 variation that can be attributed to constitutive cues (i.e., independent of variations in grain number)
499 offers new avenues for manipulating yield. First, gene manipulation (in particular, expansin gene
500 manipulation) has been proposed as a way to increase GW (Lizana et al., 2010), without having a
501 negative effect on GN, and so increase GY (Calderini et al., 2021). Second, this approach could rely
502 on the QTLs already identified for grain weight (e.g., Yan et al., 2017), and especially on those that
503 have been reported to be independent of the trade-off between AGW and grain number (Griffiths et
504 al., 2015). Nevertheless, such QTLs should be used with care since the AGW quantification on
505 which they rely were potentially compromised by populational effects that negatively influences the
506 mean AGW. Using a methodology that provides access to single grain weights (as does the present
507 study) should allow application of GWAS approaches, focusing specifically on the mean weights of
508 the larger grains (defined, for example, as the 25% of larger grains). Our results suggest that this
509 particular grain sub-population is unaffected by putative populational effects. This approach should
510 be validated, independently of the target environment.

## Declaration of Competing Interest

The authors declare they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.


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## Supplementary material

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