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Influence of Cutting Date on Phenotypic Variation in Fatty Acid Concentrations of Perennial Ryegrass Genotypes from a Breeding Population

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Abstract: Breeding forages for increased fatty acid (FA) concentrations has the potential to improve the FA profile of ruminant products (meat and milk). Twenty perennial ryegrass genotypes from an "experimental" breeding population and four genotypes from a "benchmark" mapping population were used to assess genotypic variation in FAs across a growing season. Mean total FA (TFA) concentration for cuts one through five were 29.0, 31.7, 31.1, 34.4 and 42.0 g kg^{-1} DM, respectively. Six main individual FAs, namely palmitic acid (C16:0), trans-3-hexadecenoic acid (C16:1Δ^{t3}), stearic acid (C18:0), oleic acid (C18:1Δ^{c9}), linoleic acid (C18:2$\Delta^{c9,12}$) and α-linolenic acid (C18:3$\Delta^{c9,12,15}$), accounted for between 90% to 96% of TFA. Population means differed ($p < 0.001$) for TFA and all individual FAs, expect for C18:2$\Delta^{c9,12}$ ($p = 0.106$). "Benchmark" mapping population on average had 8–44% higher FA concentrations compared to the "experimental" breeding population, except for C18:0 where the mapping population had lower concentrations. Individual genotypes from each population differed for all individual FAs and TFA ($p < 0.05$), with differences between the lowest and highest concentrations ranging from 8% to 23% amongst the mapping population genotypes and between 20% and 39% for the breeding population genotypes. Cutting date had a strong effect on population and genotype means ($p < 0.001$) with an overall trend for FA concentrations to increase through the season. However, several significant population and genotype × cutting date interactions were also found highlighting the need for further investigations to strengthen our knowledge and understanding of how genetics and environment interact for this particular trait. Nevertheless, candidate "high-lipid" genotypes were able to be identified using multivariate analysis which could be taken forward into a breeding program aimed at increasing forage FAs.

Keywords: *Lolium perenne*; temperate forage; lipids; cutting interval; seasonal pattern

1. Introduction

Grasslands support much of the world's milk and meat production owing to their basic role in feeding ruminants [1]. Permanent grasslands account for around 34% of the total utilised agricultural area (UAA) in the EU (28 countries), equivalent to just under 60 million ha [2]. In the UK, over two thirds of the UAA is classified as either permanent or temporary grassland which is predominantly used to support dairy, beef and sheep production [3]. Perennial ryegrass (*Lolium perenne* L.; PRG) is the most commonly sown species for temperate grasslands [4,5], due to its high productivity and digestibility [6,7]. This species has received a significant amount of attention and development to improve not only dry matter yield but also feed quality, rumen digestibility and livestock production efficiency [8–10]. Progress in forage breeding has been achieved through the use of various breeding

techniques that depend on geno-phenotypic variation to identify and develop new cultivars with preferential traits [11–14]. The most recent notable forage breeding success has been the development of cultivars with elevated water-soluble carbohydrate content (WSC), which have been was proposed to increase milk and meat production though improved efficiency of microbial protein synthesis, increase voluntary feed intake and potentially reduce methane emissions [15–17].

Another trait that is increasingly gaining attention is the fatty acid (FA) concentration and composition of forages, which is mainly due to the increasing consumer demand for grass-fed ruminant products (meat and milk), owing to the perceived health benefits and more "natural production" associated with these products [18,19]. Selectively breeding forages for higher FA concentrations offers a potential route to further enhance the FA profiles of meat and milk produced from forage [20–22]. Such cultivars may also provide a route for further increasing the non-fermentable energy of forages [23–25], and thus improve protein use efficiency in the rumen [15,16,26–28]. Furthermore, high lipid forages also have the potential to function as a non-seed biomass oil crop [29,30].

Several studies have highlighted the significant genetic contribution to forage FA traits, signifying the potential to selectively breed for elevated FA concentrations in forage [31–33]. Indeed, Hegarty et al. [34] recently identified regions of the PRG genome which are associated with FA concentration using marker-assisted selection carried out on a mapping population. However, integration of such genetic approaches into plant breeding programmes has been slow [13,14] meaning that many new and novel cultivars, particularly forage crop cultivars, are still being sourced from breeding populations that are predominantly based on phenotypic selection. Furthermore, a number of seasonal and environmental factors have also been shown to have a substantial influence on forage FAs [35]. It is therefore necessary to investigate how both genetic and environment factors interact with each other and how this influences FA concentrations and composition within a breeding population. In this study, we characterised the phenotypic variation and effect of cutting date on FA concentrations of genotypes from a current PRG breeding population to (1) provide insight into the variation in FA concentrations existing within the breeding population expressed under field conditions and (2) identify potential candidate genotypes that express increased FA concentrations under field conditions.

2. Materials and Methods

2.1. Experimental Design and Management

The study was conducted at the Institute of Biological, Environmental and Rural Sciences (IBERS), Gogerddan, Aberystwyth University (52°25′ N, 04°05′ W). Twenty "experimental" genotypes from an intermediate heading thirteenth generation PRG breeding population were used in the present study and compared against four "benchmark" genotypes from an Aurora × AberMagic F1 PRG mapping population which had been previously phenotyped for FA concentration [34]. All 24 genotypes had been evaluated for FA concentrations in an earlier pot experiment carried out under controlled environment conditions [33]. Four replicates of each genotype were transferred from pots to the field in August 2012, equating to a total of 96 plants, which were and planted as spaced plants in a randomised block design. The mean monthly minimum and maximum temperature and total rainfall for the experimental period (August 2012 to September 2013) are presented in Figure 1, alongside long-term mean data for the site.

Plants were subjected to a simulated grazing management regime which began in May 2013. Plant growth was poor during the beginning of 2013 due to low temperature and a lack of rainfall, especially in March and April (see Figure 1). Thus, plants were topped on the 7 May 2013 in order to encourage growth. Plants were harvested and samples collected on the mornings of 5 June, 1 July, 1 August, 28 August and 24 September 2013 within a 4-h window. Plants were topped and fertilised with GrowHow MultiCut Sulphur (N-P-K ratio of 24-4-13 plus 7 SO_3) at the rate equivalent to 55 kg N ha^{-1} within one week after each harvest.
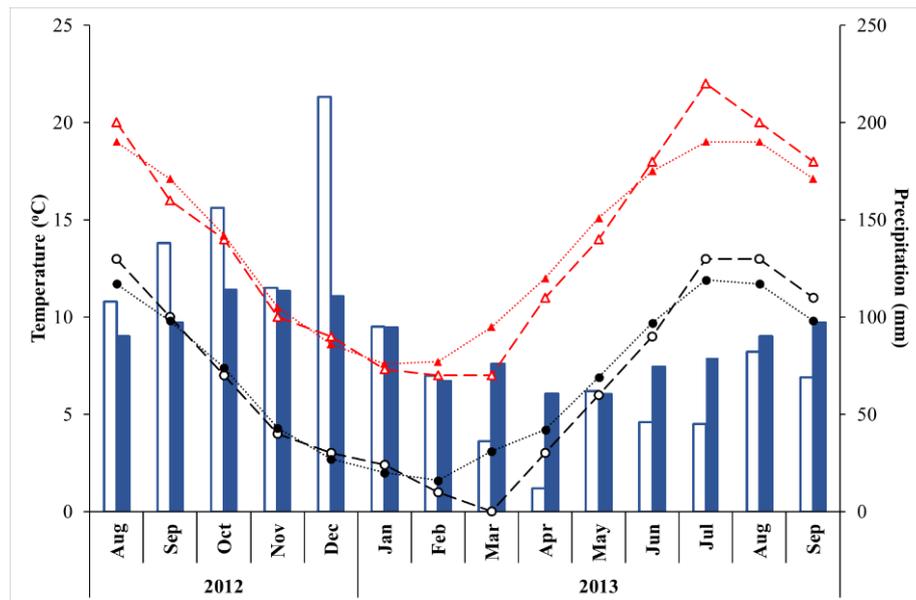
Figure 1. Observed monthly mean minimum temperature (○), maximum temperature (△) and total precipitation (□) for the experimental period; and historical averages (1945–2000) for minimum temperature (●), maximum temperature (▲) and precipitation (■).

2.2. Sample Collection and Handling

Plants were harvested using hand shears at 5 cm above the soil with all collected biomass placed in a plastic tray. Any senescent material and reproductive stems were removed from the sample before placing into a foil bag and snap frozen in liquid N. Samples were temporarily stored in a polystyrene box containing dry ice. Harvested samples were stored in the dark at −20 °C then freeze dried and ground using a Tecator Cyclotec 1093 (FOSS UK Ltd., Warrington, UK) fitted with a 1 mm screen. Ground samples were stored in the dark at −20 °C until laboratory analysis.

2.3. Fatty Acid Analysis

The one-step extraction and methylation procedure by Sukhija and Palmquist [36] was used to extract FAs and convert to fatty acid methyl esters, with tricosanoic acid (C23:0) methyl ester used as an internal standard. Fatty acid methyl esters were separated and quantified using a gas chromatograph with flame ionisation detector (CP-3800 with PAL Autosampler, Varian Inc., CA, USA) fitted with a CP-Sil 88 chemically bonded for FAME column (Agilent technologies UK Ltd., Berkshire, England, UK). Gas chromatograph conditions are described in detail by Morgan et al. [33]. Peaks were identified using a 37-component FAME standard (S37, Supelco, Poole, Dorset, UK) and quantified using the internal standard (C23:0). Varian Star v.6.41 software (Varian Inc., CA, USA) was used to capture and handle data.

2.4. Statistical Analysis

Data were analysed using Genstat 20th edition (VSN International Ltd., Hemel Hempstead, UK). Fatty acid concentrations (total and individual) were tested by ANOVA using a nested-crossed treatment design (genotype within population crossed with cut) in a randomized block with four replicates, *p*-values of ≤0.05 were deemed significant. Principal components analysis (PCA) was applied to the data to further explore the differences in FA composition amongst genotype means across harvests.

3. Results

Fatty acid results for population and cutting date are presented in Figure 2. Overall mean TFA concentration across all genotypes, populations and cuts was 35.9 g kg^{-1} DM, which ranged from 21.9 to 53.0 g kg^{-1} DM; between 90% and 96% of which was comprised of six individual FAs, namely palmitic acid (C16:0), *trans*-3-hexadecenoic acid (C16:1Δ^{t3}), stearic acid (C18:0), oleic acid (C18:1Δ^{c9}), linoleic acid (C18:2$\Delta^{c9,12}$) and α-linolenic acid (C18:3$\Delta^{c9,12,15}$). Overall mean concentration for these individual FAs was: 4.89, 0.647, 0.428, 0.595, 3.92 and 23.2 g kg^{-1} DM, respectively. Population averages differed for concentrations of C16:0, C16:1Δ^{t3}, C18:0, C18:1Δ^{c9}, C18:3$\Delta^{c9,12,15}$ and TFA ($p < 0.001$), with the "benchmark" mapping population having higher concentrations of these fatty acids except for C18:0. There was no difference between populations for C18:2$\Delta^{c9,12}$ concentration ($p = 0.106$). Cutting date had a significant effect on all individual and total FA concentrations ($p < 0.001$). Concentrations of all FAs generally increased from cut one (5 June) through to cut five (24 September), as shown in Figure 2, with average increases of 45.4%, 51.2%, 69.6%, 24.8%, 55.6%, 46.7% and 45.3% observed between the first and last cut for C16:0, C16:1Δ^{t3}, C18:0, C18:1Δ^{c9}, C18:2$\Delta^{c9,12}$, C18:3$\Delta^{c9,12,15}$ and TFA, respectively.

However, significant interactions between population and cutting date were also observed for all individual FAs and TFA ($p < 0.05$) owing to deviations between populations at particular cuts from the overall trend for FA concentrations to increase from cut one though to cut five. Population × cutting date interactions were particularly dominant for C16:0, C16:1Δ^{t3} and C18:1Δ^{c9} ($p < 0.001$). In the case of C16:0 this interaction occurred at cut three and cut four, where the "experimental" breeding population had similar mean values for both these cuts (4.65 g kg^{-1} DM) whereas the "benchmark" mapping population had a higher mean concentration at cut three relative to cut four (5.64 and 5.49 g kg^{-1} DM, respectively). Mean concentrations for C16:1Δ^{t3} for the "experimental" breeding population increased between cut one and cut two, decreased to the lowest value at cut three, then increased through to the highest value at cut five. In contrast, the mapping population had similar C16:1Δ^{t3} concentrations at cuts one and two (0.72 g kg^{-1} DM), which decreased to almost identical values for cuts three and four (0.66 g kg^{-1} DM), then sharply increased to 1.05 g kg^{-1} DM at cut five. For C18:1Δ^{c9}, concentrations decreased slightly between cuts one and two, then increased through to cut five for the "benchmark" mapping population, whereas for the "experimental" breeding population, lower values were observed at cuts one and four and higher values at cuts three and five.

Results for genotypes and cutting date from the "benchmark" mapping population are presented in Figure 3. Genotypes from this population differed for concentrations of C16:0, C18:0, C18:1Δ^{c9}, C18:2$\Delta^{c9,12}$, C18:3$\Delta^{c9,12,15}$ and TFA ($p < 0.001$), with respective differences of 13.5%, 26.4%, 26.1%, 22.0%, 18.2% and 15.1% observed between the means for the lowest and highest genotypes. Mapping population genotypes also differed to a lesser extent for concentrations of C16:1Δ^{t3} ($p = 0.026$), with a difference of 8.7% observed between the genotypes with the lowest and highest mean concentrations of this FA. Cutting date had a significant effect on all individual and total FA concentrations for these "benchmark" mapping population genotypes ($p < 0.001$), with an overall trend for FA concentrations to increase from cut one through to cut five. However, significant genotype × cutting date interactions were found for the "benchmark" mapping population genotypes for C16:1Δ^{t3} ($p = 0.015$) and C18:0 ($p = 0.01$), driven by differences in the relative rankings of genotypes at each cutting date, as shown in Figure 3. In the case of C16:1Δ^{t3}, the interaction can be seen between cuts one and two where some genotypes decrease in C16:1Δ^{t3} concentration while other genotypes increase. For C18:0, a "pinch-point" is noticeable at cut four where the genotypes have very similar C18:0 concentrations, whereas in the preceding cuts, the genotypes are differentiated. No genotype × cutting date interactions were found for C16:0, C18:1Δ^{c9}, C18:2$\Delta^{c9,12}$, C18:3$\Delta^{c9,12,15}$ and TFA ($p > 0.05$).
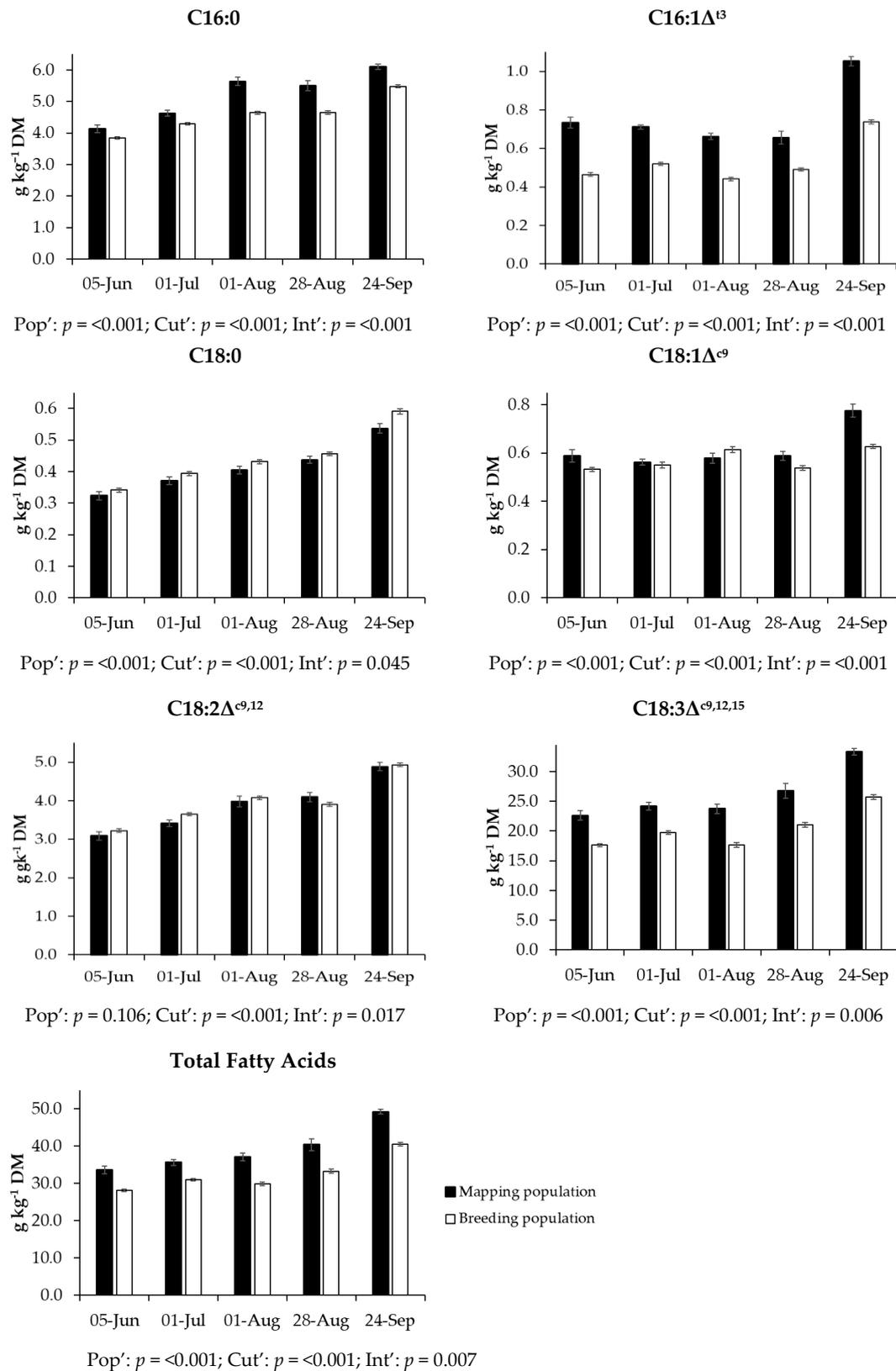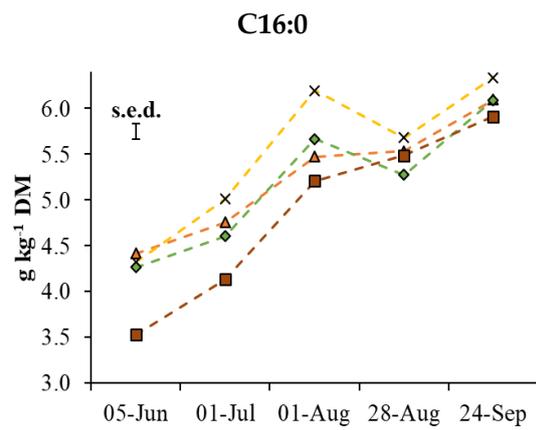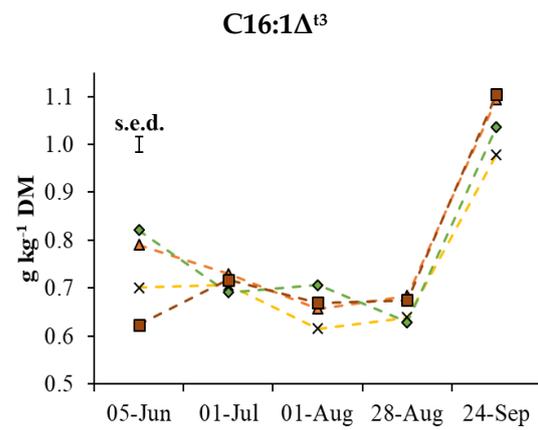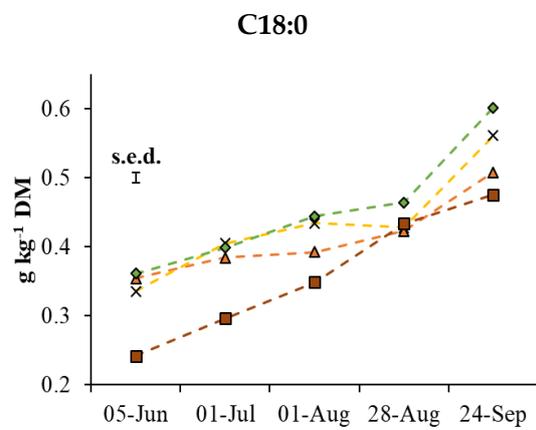
Figure 2. Mean fatty acid concentrations (g kg^{-1} DM) in perennial ryegrass from a "benchmark" mapping population (*n* = 16) and an "experimental" breeding population (*n* = 80) across five harvests collected in 2013. Bars indicated ± 1 standard error of the mean. Pop', population; Cut', cutting date; Int', interaction; *p*, probability.
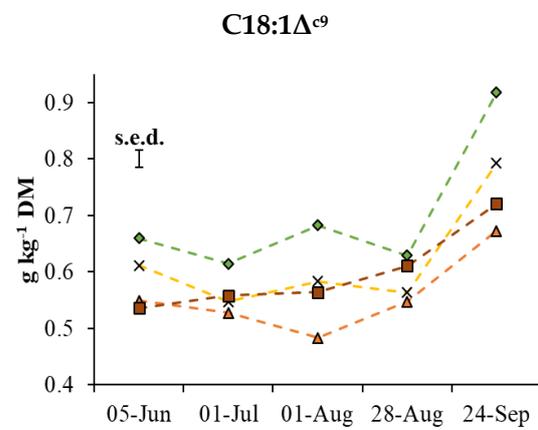
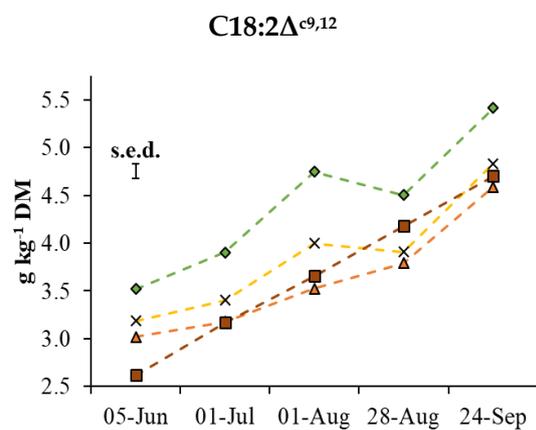**C16:0**

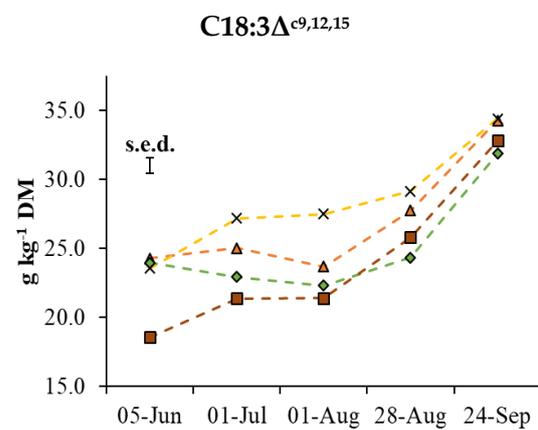Geno': *p* = <.001; Cut': *p* = <0.001; Int': *p* = 0.173

C16:1Δ^{t3}

Geno': *p* = 0.026; Cut': *p* = <0.001; Int': *p* = 0.015

C18:0

Geno': *p* = <0.001; Cut': *p* = <0.001; Int': *p* = 0.01

C18:1Δ^{c9}

Geno': *p* = <0.001; Cut': *p* = <0.001; Int': *p* = 0.210

C18:2Δ^{c9,12}

Geno': *p* = <0.001; Cut': *p* = <0.001; Int': *p* = 0.334

C18:3Δ^{c9,12,15}

Geno': *p* = <0.001; Cut': *p* = <0.001; Int': *p* = 0.151

Figure 3. *Cont.*

Total Fatty Acids

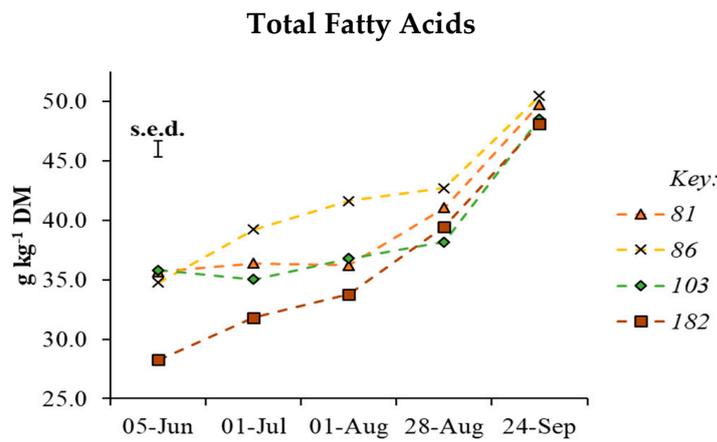Geno': *p* = <0.001; Cut': *p* = <0.001; Int': *p* = 0.190

Figure 3. Mean fatty acid concentrations (g kg^{-1} DM) for individual perennial ryegrass genotypes (*n* = 4) from a "benchmark" mapping population across five harvests collected in 2013. s.e.d, standard error of the difference; Geno', genotype; Cut', cutting date; Int', interaction; *p*, probability.

Figure 4 presents the results for each genotype and cutting date for the "experimental" breeding population. Genotype, cutting date and genotype × cutting date interaction effects were found for all individual and total FAs (*p* < 0.001). Concentrations of C16:0, C16:1Δ^{t3}, C18:0, C18:1Δ^{c9}, C18:2$\Delta^{c9,12}$, C18:3$\Delta^{c9,12,15}$ and TFA differed by 21.9%, 46.7%, 48.6%, 40.0%, 26.4%, 43.2% and 32.2%, respectively, for the lowest and highest genotypes from the "experimental" breeding population. Fatty acid concentrations followed the same overall trend across cuts, with all individual and total FAs increasing from cut one through to cut five. The strong genotype × cutting date interactions are again driven by sizable shifts in the relative rankings of genotypes at each cutting date, as presented in Figure 4, which is mostly evident at cut three.
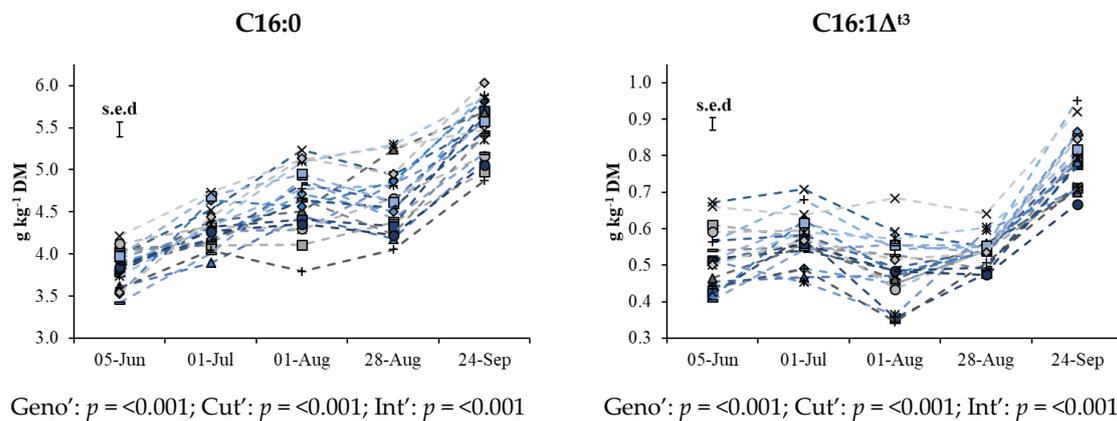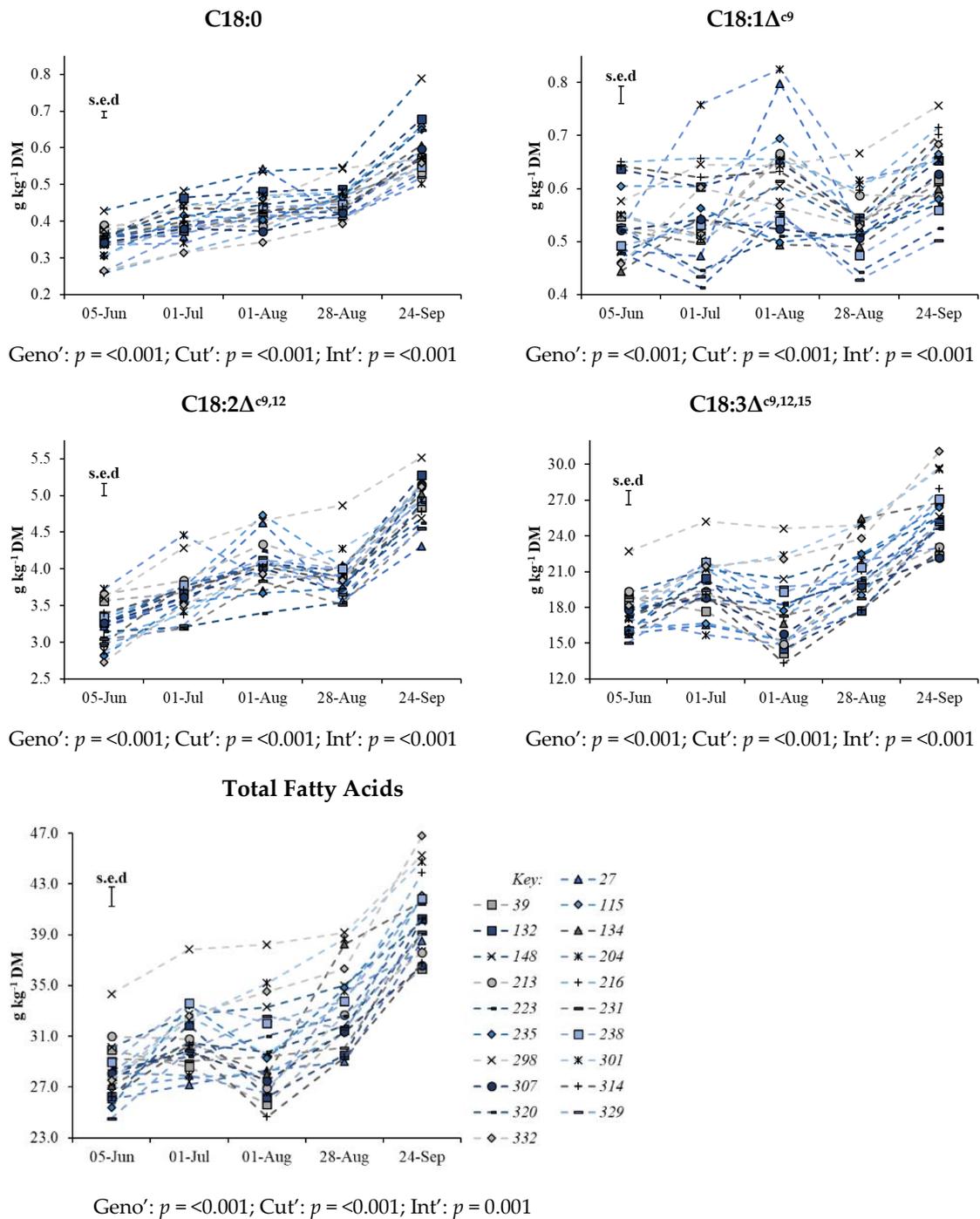
C16:0　　　　　　　　　　　　　　　　　　　　　**C16:1Δ^{t3}**

Geno': *p* = <0.001; Cut': *p* = <0.001; Int': *p* = <0.001　　　　Geno': *p* = <0.001; Cut': *p* = <0.001; Int': *p* = <0.001

Figure 4. *Cont.*

Figure 4. Mean concentrations of fatty acids (g kg^{-1} DM) for individual perennial ryegrass genotypes (*n* = 4) from an "experimental" breeding population across five harvests collected in 2013. s.e.d, standard error of the difference; Geno', genotype; Cut', cutting date; Int', interaction; *p*, probability.

The first two principal components (PC) combined explained 79.4% of the variation between genotype means across all harvests, as shown in Figure 5. Over half of the variability within the data (55.7%) was explained by PC1 which was comprised of C16:0, C16:1Δ^{t3}, C18:3Δ^{c9,12,15} and TFA, all of which had approximately equal positive loadings on PC1. The remaining individual FAs (C18:0, C18:1Δ^{c9} and C18:2Δ^{c9,12}) characterised PC2 which explained a further 23.7% of the variation, all of which had negative loadings. There was a clear divide between the genotypes from the "experimental" breeding population and the "benchmark" mapping population along PC1, with the former located to

the left-hand side of PC1 and the latter to the right. It is also worth noting that the breeding population genotypes were evenly clustered around the central point where the PCs intersect (except for genotype 298 from the breeding population). Genotypes from both populations were evenly dispersed across PC2. Two genotypes stood out on the PCA biplot, namely 298 from the breeding population and 103 from the mapping population, both of which are located in the bottom-right quadrant, reflecting their expression of much higher levels of FAs compared to their counterpart genotypes. Conversely, genotypes 27, 307, 314 and 329 from the breeding population appear to express lower levels of FAs.
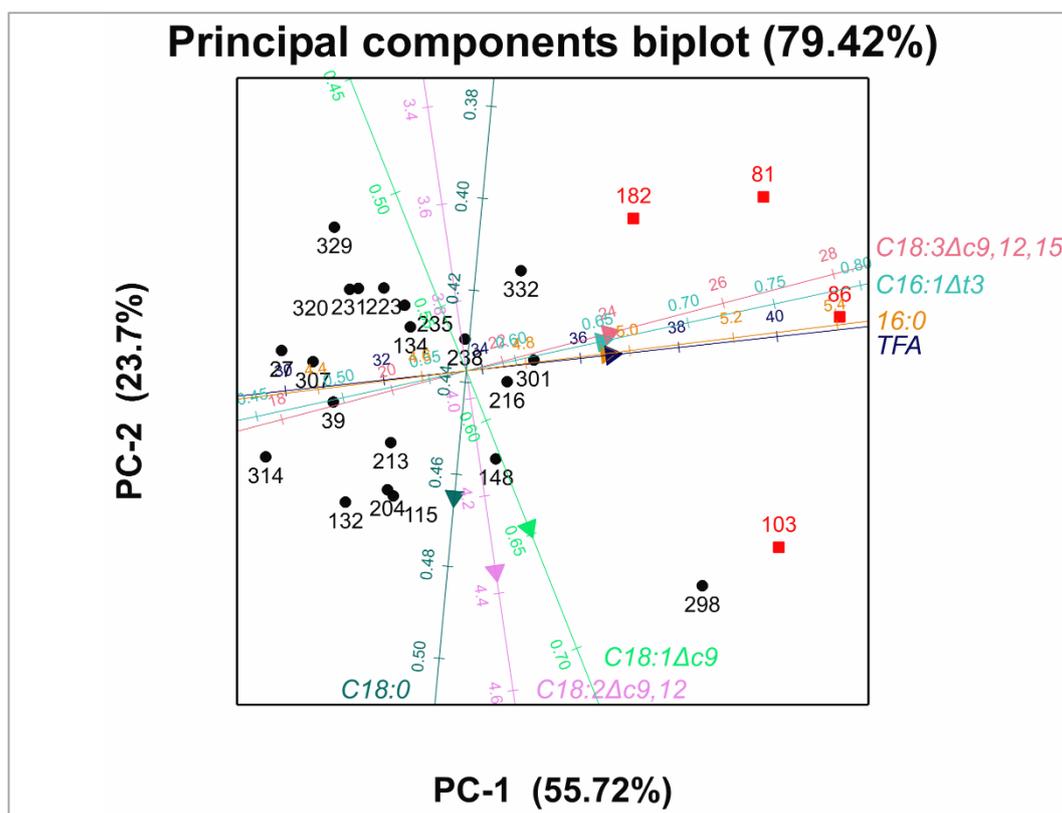

Figure 5. Biplot of the first and second principal components. Genotypes from the "experimental" breeding population are indicated by a black circle [●] and genotypes from the "benchmark" mapping population are indicated by a red square [■]. Vector labels: C16:0, palmitic acid; C16:1Δ^{t3}, *trans*-3-hexadecenoic acid; C18:0, stearic acid; C18:1Δ^{c9}, oleic acid; C18:2$\Delta^{c9,12}$, linoleic acid; C18:3$\Delta^{c9,12,15}$, α-linolenic acid; TFA, total fatty acids.

4. Discussion

Differences in FA concentration and composition have previously been reported between plant families, species and cultivars [31,37–40], and more recently at a genotypic level for PRG [33,34]. In the present study, we investigated differences between genotypes from a current "experimental" breeding population relative to "benchmark" genotypes from a mapping population across multiple harvests under field conditions. Differences between PRG populations and amongst genotypes were found for most individual FAs and for TFA. However, FA concentrations were notably higher in the present study compared to previously reported values for harvests of PRG from field experiments. Dewhurst et al. [31] reported TFA values of between 9 to 15 g kg^{-1} DM for harvests of PRG collected between June and September, which is approximately 65% lower than the TFA concentrations observed in the present study. Results presented by Elgersma et al. [40] and Van Ranst et al. [41] for PRG were intermediate between those reported by Dewhurst et al. [31] and the present study, with TFA concentrations being approximately 35% lower than the present study (ranging from 22 to 33 g kg^{-1} DM). Differences of a similar magnitude were also observed between concentrations of the main

individual FAs. The limited number of studies and inconsistencies in experimental design make it difficult to openly compare forage FA results [35]. Several factors which are known to affect FA concentrations of forage have likely contributed to the disparity between results from the present and previous studies, including differences in climatic conditions [42], length of regrowth interval [31,43], plant maturity (leaf:stem ratio) [37,39,44] and fertiliser regime [39,45,46], along with sample preparation and method of analysis [35]. Although there are many environmental factors that can substantially influence FA concentrations, most of these studies have reported differences between species, cultivars and genotypes, signifying the strong genetic contribution to this trait.

Several studies have also previously investigated seasonal variation in FA concentrations and composition of forages with the majority reporting strong cutting date effects [31,40,41]. Broadly speaking, they have found FA concentrations to be higher early in the growing season while plants are in a vegetative state. Fatty acid concentrations then gradually decrease as the plants enter the reproductive stage (usually during late spring/early summer), followed by an increase towards the end of the season when plants return to a vegetative state. Strong effects of cutting date on individual and total FA concentrations were also found in the present study; however, no distinct decrease was observed during late spring/early summer, as has been reported in other studies. This may be due to the first harvest in the present study happening too late in the growing season thus missing the time-period where plants were in an entirely vegetative state; or it may be related to differences in sample composition. The present study determined FA concentrations of purely leaf material from single plants, whereas earlier studies have sampled whole plants or plots which, in some instances, would have included stemmy material in addition to leaf material. As previously mentioned, leaf:stem ratio is known to affect FA concentrations of forages due to the majority of the lipids and FAs being localised within the chloroplasts [42,47]. Consequently, leaves are richer in FAs, particularly unsaturated FAs, compared to stems [48,49]. Management practices that promote a higher leaf:stem ratio, such as frequent cutting and shortened regrowth intervals, may be a strategy to elevate FA concentrations in forages. Other factors that may have contributed to the disparity between results of the present study and those reported by previous studies could also include differences in light intensity, temperature, precipitation and N fertilisation [39,42,45,46].

It is important, however, to also highlight the significant interactions of population and genotype with cutting date found in the present study, which have also been noted by some previous studies. For example, Dewhurst et al. [31] found significant species × cutting date interactions for all main individual FAs and TFA, whereas significant interactions were found only for C16:0, C16:1, C18:3 and TFA in the study by Van Ranst et al. [41] and for C16:0, C16:1, C18:0 and C18:3 in the study by Garcia et al. [50]. In the present study, the main cause of the significant population and genotype × cutting date interactions appear to relate to changes in the relative ranking of genotypes within each cutting date. This is especially evident for the "experimental" breeding population genotypes where shifts in genotype rankings can be seen particularly at cut three with some "low" genotypes jumping up the rankings and vice versa for "high" genotypes. The months of June and July were dryer and July hotter than the long-term averages (see Figure 1), which may have resulted in some plants experiencing borderline drought-stress at the time of cut three on 01-Aug. The effect of drought-stress on lipid and FA content and composition of leaves and chloroplasts has been previously studied in a wide range of plant species, including wheat (*Triticum durum* Desf.) [51], coconut (*Cocos nucifera* L.) [52], white lupin (*Lupinus albus* L.) [53], cotton (*Gossypium hirsutum*) [54] and rape (*Brassica napus*) [55], with many studies reporting a decrease in total lipid/FA content and a reduction in the degree of lipid/FA unsaturation in response to drought stress. Xu et al. [56] investigated the association between drought tolerance and FA composition in Kentucky Bluegrass (*Poa pratensis* L.) and found that FA unsaturation decreased in both the drought-tolerant and -susceptible cultivars during drought stress; however, this decrease was less pronounced in the drought-tolerant cultivar which also recovered quicker after re-watering to pre-drought levels of FAs compared to the drought-susceptible cultivar. Thus, the notable changes in relative ranking of genotypes observed at cut three in the present study

may likely be due to the dry weather and differences in drought tolerance between the genotypes combined. In contrast, Hegarty et al. [34] found little evidence of genotype × cut interactions for any of the FAs reported in their study; however, this may be due to only analysing material from two cuts (June and September) across three years.

Mapping population genotypes were stacked toward the right-hand end of PC1, signifying that all four genotypes had higher concentrations of C16:0, C16:1Δ^{t3}, C18:3$\Delta^{c9,12,15}$ and TFA compared to the breeding population. Interestingly, these genotypes were dispersed across PC2, suggesting that the differences between these genotypes were mainly due to variation in concentrations of C18:0, C18:1Δ^{c9} and C18:2$\Delta^{c9,12}$. In contrast, the breeding population genotypes were evenly distributed around the central point where the variable axes (PCs) of the biplot intersect, demonstrating the heterogeneity in FA concentrations amongst these genotypes around the overall mean. The exception to this was genotype 298, which particularly stood out from the rest of the breeding population genotypes. This specific genotype expressed much higher levels of all individual FAs and TFA compared to the other genotypes, making it a potential candidate for the development of a high-lipid cultivar. Genotypes that expressed lower levels of FAs can also be identified in the PCA biplot—for example, genotypes 27, 307, 314 and 329 from the breeding population.

Hypothetically, the total FA intake for a 400 kg beef animal with a DM intake of 10 kg/day would be 295 g/day if the grazing pasture consisted of the mean lowest breeding population genotype (314), whereas total FA intake if consuming pasture of the highest genotype (298) would be 390 g/day—a difference of 95 g/day. Although a significant proportion of the dietary unsaturated FAs are lost through lipolysis and biohydrogenation in the rumen [57,58], there is a large body of evidence demonstrating that increasing the unsaturated FA content of ruminant diets, and thus intake, does still positively impact the FA composition of meat and milk [22,59]. The same divergent genotypes were also classified as such in an earlier study investigating the FA concentrations of the same sample of genotypes under controlled environment conditions [33]. For the "experimental" breeding population, genotype 314 ranked lowest for mean TFA concentration in both studies while genotype 298 ranked highest with some variation in ranking for the "mid-range" genotypes. The four genotypes from the "benchmark" mapping population also ranked the same in both studies relative to each other, with genotype 182 expressing lower mean TFA concentration and genotype 86 having higher TFA concentration.

Additionally, there is a clear segregation of FAs into two groupings within the PCA biplot, aligning with either PC1 or PC2. The biological explanation for this may relate to the types and roles of the lipids with which these FAs are predominantly associated. Palmitic acid (C16:0), C16:1Δ^{t3} and C18:3$\Delta^{c9,12,15}$ are present in higher proportions in the galactolipids of plants, which are key components of the thylakoid and chloroplast membranes, whereas C18:0, C18:1Δ^{c9} and C18:2$\Delta^{c9,12}$ are proportionally higher in the phospholipids which form the cell membranes [60,61]. Total FAs are grouped with C16:0, C16:1Δ^{t3} and C18:3$\Delta^{c9,12,15}$ due to C18:3$\Delta^{c9,12,15}$ contributing such a large proportion (>50%) to the TFA. However, further investigation into the lipid composition of PRG is required to confirm this hypothesis.

The present work has primarily focused on investigating the variation in FA concentrations of genotypes from the most current breeding population under field conditions. The results presented here and elsewhere provide increasing assurance that alterations in FA concentrations and composition can be selectively bred for in PRG [33,34]. However, despite the strong genetic basis for this trait, the present study also demonstrates the sizeable effect of environment and G × E interactions on FA concentrations of PRG. Strengthening our knowledge and understanding of how genetics and environment interact with each other is a vital next step in the pathway to creating distinctive "high-lipid" grass varieties with increased FA concentrations and/or altered FA composition.

5. Conclusions

Concentrations of FAs generally increased during the season. However, the significant G × E interactions imply that responses were not consistent between populations and genotypes across the season. Nonetheless, notable differences between genotypes were still detectable and the multivariate analysis showed which genotypes tended to express higher versus lower FA concentrations across the season as whole. Furthermore, the multivariate analysis revealed that the higher TFA concentrations in the mapping population genotypes were mostly driven by higher concentrations of individual FAs that are primarily present in the galactolipids of thylakoid and chloroplast membranes. Further investigations are required to gain a deeper understanding of how genetics and environment interact and influence FA content and composition in forages, and to identify the underlying biological mechanisms that are driving these differences. This study provides further insight into the typical levels and variation across genotypes and cutting dates in FA concentrations present in a current PRG breeding population and proposes some candidate genotypes which could be taken forward into a breeding program aimed at increasing forage FAs.

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