FROM AVERAGE TO INDIVIDUAL FRUIT, A PARADIGM SHIFT FOR ACCURATE ANALYSIS OF WATER ACCUMULATION AND PRIMARY METABOLISM IN DEVELOPING BERRIES

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Abstract:
Context and purpose of the study - Presentknowledge about grape development is mainly driven by the premise that a typical berry would follow the same kinetics as the population average, the principal challenge being to gather representative samples. In this frame, the elaboration of harvest quality directly reflects the impact of the GenotypexEnvironment interaction on fruit metabolism. Much energy is then being devoted to identifying the sites that regulate grape metabolism, upon screening more and more genes and metabolites, and developing virtual berry models simulating sugar and water accumulation in the future harvest. Nevertheless, successive physiological stages never coexist in a fruit and one may wonder whether the "average physiological stage" paradigm does not inevitably lead to a dead end. The strict foundations of berry developmental biology are critically revisited here.

Material and methods – Disparate literature data on the intensity and duration of the second growth period were re-interpreted, validated and clarified, upon non-destructive analysis of single berry firmness and growth, on different cultivars in the experimental vineyard of Supagro, as well as on microvines grown in greenhouses. Organic acids and sugars were measured by HPLC on thousands individual berries of Syrah, Pinot and Zinfandel.

Results - Previously unsuspected sub-periods emerged from the developmental patterns of sugar, water and malic acid flows on single berries, metabolic fluxes and kinetic data being noticeably stable among all investigated cultivars. Berries accumulated sugars at nearly constant volume during the first week following softening, indicating intense xylem back-flow at this stage. This first period of ripening was also characterized by a net malic acid/4 hexoses exchange consistent with the operation of a sucrose/H^+ exchanger at the tonoplast membrane, in usual conditions and genotypes. Aerobic fermentation and vacuolar proton pumps were induced later, while vacuolar malic acid was progressively exhausted, without compromising sugar and water accumulation. As a matter of fact, phloem unloading definitively stopped 28 days after softening. It clearly appeared that the individual fruit develops in a far more determined, reproducible and finally intelligible way than has been predicted so far, based on average samples.New phenotyping procedures were consequently designed for genetic studies, improving heritability and QTLs detection.Switching from fruit genomics and physiology to harvest quality requires a real change in scale, from the fruit to the population. The determinant role of berries asynchrony within the population can't be ignored any longer, but the impact of the GxE interaction on the population structure essentially remains *terra incognita*.

Keywords: grape, berry development, development asynchronism, metabolism, ripening

1. Introduction

The marked heterogeneity in fruit composition within a vineyard makes it a real challenge to collect samples that are truly representative of the entire harvested unit. Indeed, periodic samples of several hundred berries are necessary to limit random noise on the kinetics of evolution of the volume and composition of the future harvest. Current knowledge on berry physiology mostly relies on the assumption that this procedure provides a relevant picture of fruit development: the whole plot is conceived as an ideal berry, whose gene expression and metabolic pathways are modulated by the GxExM interaction, so variations in wine quality and terroir effects simply reflect fruit developmental

biology and vice-versa (Dai, et al.,2016 , Dal Santo et al.,2018). To set ideas, it is customary to recall that grape berry grows in a double sigmoid with a second growth period of about a month and a half (ripening), during which the sugar concentration reaches about 21 brix, and acidity decreases from 450 to ca 100 mEq. This paradigm overlooks that sugar enrichment begins with the first berry but ends with the last, so that the true duration of fruit ripening is all the more overestimated as the population is out of Sync, as evidenced by berry sorting, single fruit composition and transcriptomics (Lund et al.,2008; Rienth et al.,2016; Singleton, 1966; Bottcher et al., 2012). Indeed, veraison takes place over two to three weeks on the whole population, whether appreciated by firmness or color changes (Robin et al., 1997; Vondras et al.,2016; Herrera and Castellarin, 2016) and, albeit this incongruity has gone unnoticed, the second growth period actually turned out to last only three weeks in single berries (see fig 1&2 in Friend et al., 2009, Ollat and Gaudillere, 1998). Real time single berry diameter and firmness monitoring indicated a six days delay between softening and growth resumption (Coombe and Bishop, 1980). These disparate observations on single berries drastically change our view on berry internal clock but remain collectively consistent with whole population usual features. The time lag between fruits, as long as the second expansion period itself, resulted in physiologically chimeric samples, which dramatically distorted the berry growth pattern. In this work, the analysis of thousands of individual fruits made it possible to elucidate the kinetics of sugar accumulation and malic acid consumption in synchronized fruits, leading to a more faithful model of berry ripening.

2. Material and methods

Single berries of Syrah were harvested at the indicated dates in the experimental vineyard of Montpellier-SupAgro (France) , individually weighed and crushed in water, before HPLC analysis of sugar and organic acids (Rienth et al., 2016). Individual softening dates were manually determined before tagging berries. Fruit volumes were evaluated from pixel counts on periodic pictures, using the ImageJ software (http://rsb.info.nih.gov/ij). Kinetics of individual berry diameter of Pinot Noir and Cabernet Sauvignon were retrieved from Friend et al. (2009) before volume calculation.

3. Results and discussion

Image analysis, that avoids the deformation of flaccid ripe berries as a possible artefact of caliper measurement, confirmed that the second growth period started 6-8 days after softening and lasts three weeks only, in unlimited watering conditions (Coombe and Bishop,1980 ; Friend et al., 2009 ; Ollat et al.,2002). Comparable growth patterns were obtained following normalization of the maximal berry volume, irrespective of the considerable variations in fruit size on the same or on different cultivars (Syrah, Meunier, Pinot noir and Cabernet Sauvignon) (Fig 1). These fluctuations depending on early fecundation events affecting cell divisions and seed number (Ojeda et al.,1999; Vondras et al., 2016) exceeded two fold variations at a similar sugar concentration (fig 2a). Sugar did not accumulate above 0.12 M on hard and green berries, with a glucose/fructose ratio higher than 1.6 (not shown), at which point malic acid was maximum (450 and 350 ± 50 mEq in Meunier and Syrah, respectively). On soft berries, malic acid displayed a limited and erratic decline till 0.2 M hexose, then it linearly dropped below 100 mEq at 0.8 M hexose, showing the same slope on Meunier and Syrah (fig 2 & 3), above which point sugar concentration continued while malate decay was very slow. Heterogeneity in sugar and malate concentration was considerable inside individual clusters : at mid-veraison, green hard berries that didn't reached the peak in malic acid co-existed with soft and colored ones, some of them as concentrated as 0.8 M sugars. This experiment was repeated on Syrah berries which softening dates have been individually identified, showing that the heterogeneity in sugar and malate concentrations was essentially a matter of a-synchronicity in the trigger of sugar loading, but not the result of different velocities in the ripening process. All berries on lines passing through the origin just differ by dilution. Berry weight was then plotted with respect to solute concentration as a proxy for osmotic pressure, in order to resynchronize berries with imprecise or unknown individual softening dates, including the green and hard ones, before calculating the amount of sugar and malic acid per berry (conc.xvol.) (fig 4 a,b,c). One can notice that the upper part of the growth curve is well resolved and that smaller berries are more concentrated, at each sampling date. Whatever, growth proved very limited during the first week following the onset of sugar accumulation and malate breakdown, and one should wait the accumulation of 25 % final sugar and the loss of 50 % malate till growth resumption for a three weeks period. Growth and sugar accumulation stopped simultaneously, at a rather low sugar concentration (0.9 M), what can be interpreted as the definitive arrest of phloem unloading, above which sugar

concentration continued to increase through berry shriveling. The average growth pattern of these individual berries noticeably fits the growth kinetics previously observed through continuous monitoring of single fruits (see above). One malic acid was exchanged with four hexoses during the first part of ripening (not shown), suggesting a net 1 H^+/sucrose exchange at the tonoplast membrane. The acidity gradient formed in green stage may then suddenly energize sugar loading as long as malic acid is available, before being relayed by the vacuolar H^+ pumps that are actually activated later during ripening (Terrier et al., 2001). Moreover, only one eight of accumulated sugar can be formed from malic acid by the neoglucogenesis pathway during the first week of ripening, assuming that no malic acid would be oxidized during this period, what would be surprising (Famiani et al., 2014). By the way, as summarized in figure 4 d, the unloading of phloem sucrose must proceed at full rate since the onset of ripening, but berries refuse to grow before one week, indicating major xylem back flow at this stage. Such an adaptation may help late neighbor berries that would be otherwise disadvantaged by higher osmotic pressure to complete their first growth period. Following growth resumption, sugars discharge at a constant rate in an expanding volume so that the concentration increase less and less rapidly, without indicating that the late fruits would ripen faster than the first. Finally, although a slight accumulation of sugar can't be formally rejected when growth arrested, present data do not indicate intense reverse xylem backflow at ripe stage (Keller et al., 2015). Further work is needed to elucidate this point, since melting late berries still growing and accumulating sugars with older, shriveling ones may have been interpreted as the continuation of hexose loading at constant volume.

4. Conclusions

The quantitative analysis of the respective flows of water, sugar and malic acid in the single berry paves the way for testing the possible regulatory role of membrane bioenergetics on sugar import and malate breakdown during berry ripening. Past physiological interpretations of results obtained on non synchronized samples should be regarded with extreme circumspection.

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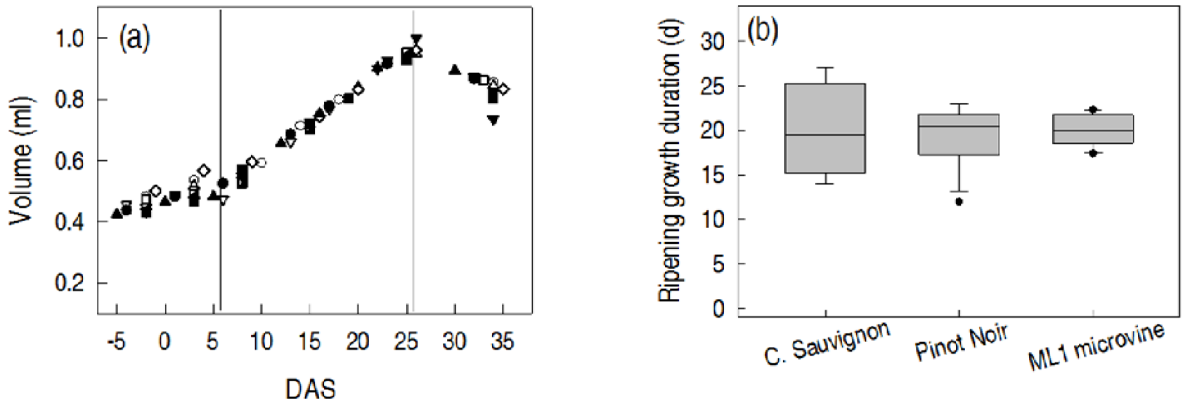

Figure 1. (a) Normalized growth of ten individual berries of ML1 microvines as a function of days after softening (DAS). Each symbol corresponds to one berry which growth was calculated on successive pictures (b) Duration of the second growth period in ML1 microvine (n= 10, this work), Pinot Noir and Cabernet Sauvignon berries (n= 12, recalculated from Friend et al.,). Differences between varieties are not significant (ANOVA, P>0.05). Normalized berry volume = V/(V_{max}) where V_{max} is maximum berry volume.

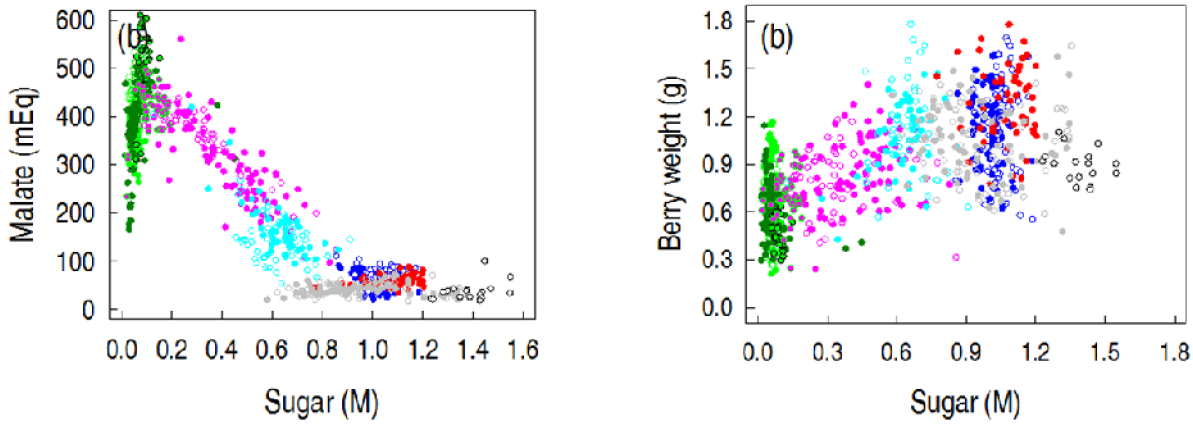

Figure 2. Development of Meunier berries (1 point/ berry). Each color corresponds to one cluster harvested at the indicated days after 50% veraison (DAV) . -10 DAV (3 clusters: green, dark-green and open-green circles), 0 DAV (2 clusters: pink and open pink circles), 10 DAV (2 clusters: cyan and open-cyan circles), 20 DAV (2 clusters: blue and open blue circles), 30 DAV (1 cluster: red circles) and 40 DAV (2 clusters: gray and open gray circles).

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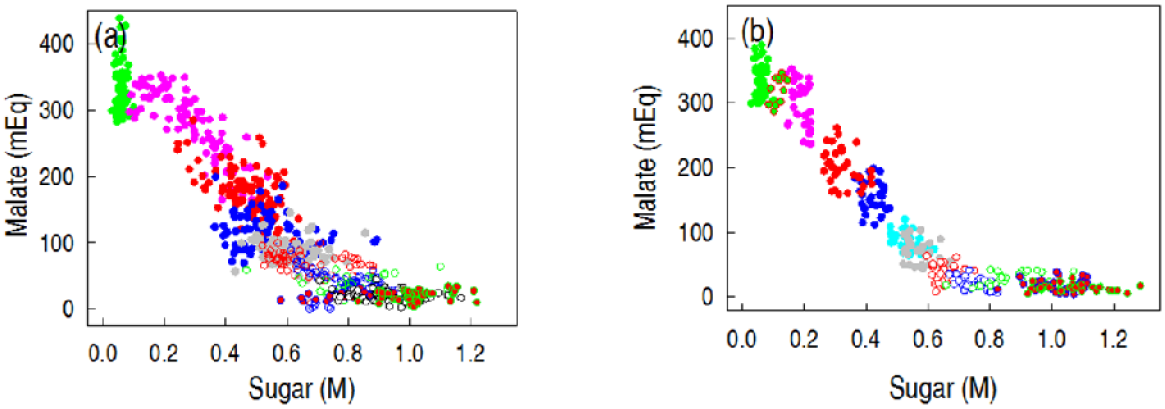

Figure 3. Development of Syrah berries (a) Each point corresponds to one berry sampled: -10 days after 50% veraison (DAV) (green), 0 DAV (pink), 5 DAV (red), 8 DAV (blue), 13 DAV (gray), 18 DAV (open red), 25 DAV (open blue), 32 DAV (open green), 39 DAV (blue red), 46 DAV (open black) and 55 DAV geen red). (b) Each point corresponds to one berry sampled : before softening (green), during softening (pink green), 1 Day After it Softened (DAS) (pink), 3-5 DAS (red), 5-6 DAS (blue), 7-8 DAS (cyan), 8-11 DAS (gray), 16-18 DAS (open red), 19-22 DAS (open blue), 29-30 DAS (open green), 37-38 DAS (cyan pink) and 52-54 DAS (green red)

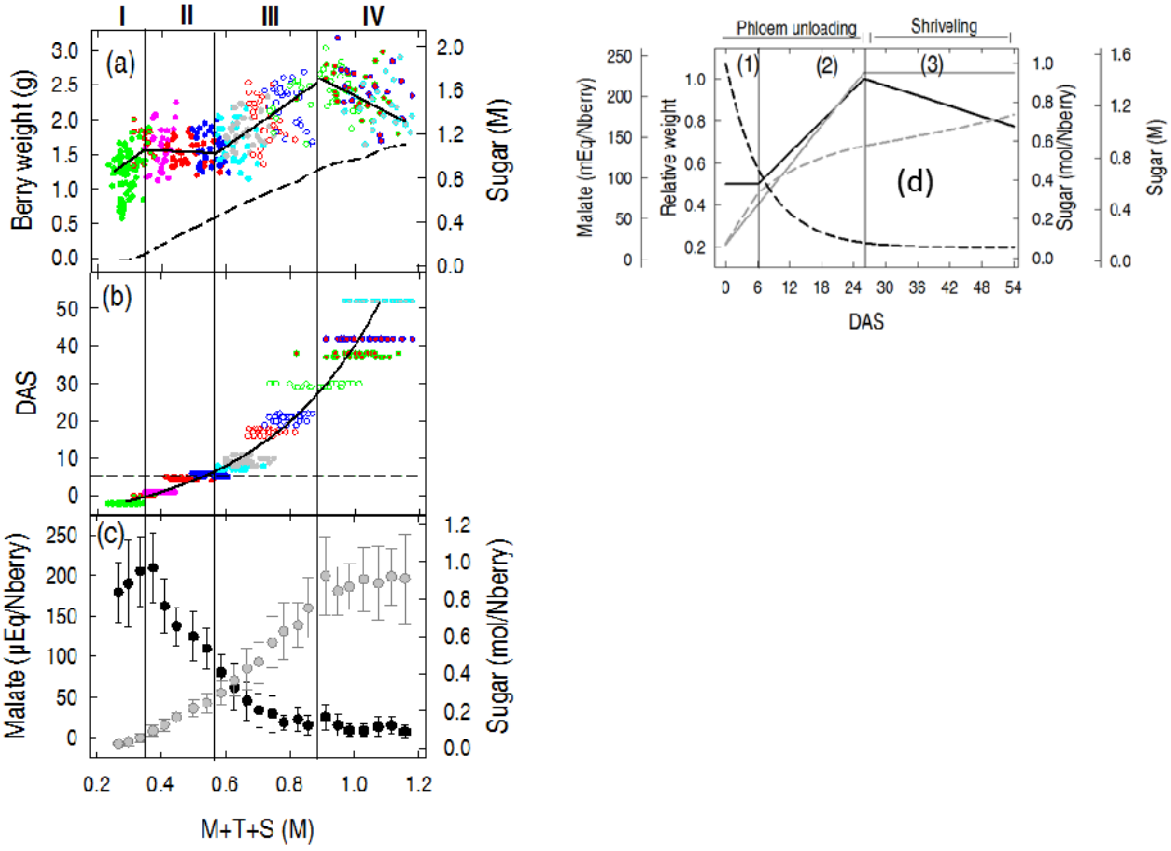

Figure. 4. Development of synchronized Syrah berries with respect to malate + tartrate + glucose + fructose (M+T+S) as a proxy for osmotic pressure. (a) : Individual berry weight, each point refers to one berry which date after softening (DAS) is specified in (b), black line : mean berry weight, dotted line : sugar concentration; (b) day after softening (DAS), black line: exponential fit; (c) average malate (black) and sugar (gray) contents of 385 fruits, reaching 1 Kg at the completion of phloem unloading. Mean (±SD) were calculated on successive 0.05M (M+T+S) intervals including 9 to 26 berries (16 on average). Phases : (I) Green stage (unsynchronized hard berries), (II) Synchronized soft berries starting to ripen at constant volume, (III) Growth of synchronized ripening berries, (IV) Arrest of sugar loading and berry shriveling. (d) generalized kinetic model : black : net water balance, gray net sugar storage , dotted black : malic acid ,dotted gray : sugar concentration.