Response to both referees' comments

We thank both referees for their detailed comments and their important criticism of the manuscript. We have included these points thoroughly in the revised version of the manuscript.

The main point of referee 1 was (1) to make clearer, which was the original source i.e. the first contribution to particular a finding or hypothesis. We have now carefully changed the wording of the manuscript and have added references to make this point clear. (2), the referee suggested to explain eqn. 1 more thoroughly. We have done that and make now clear, that "this example indicates that on a theoretical basis large isotope effects can occur through fractionation effects and metabolic branching points in the respiratory pathways, however to what extend this will be expressed *in vivo* still remains to be resolved."

Referee 2 had some formal points and offered suggestions to improve the contents of the manuscript. All formal points have been included as suggested. We are very grateful for the referee's comments and suggestions to improve the argumentation on the mechanisms potentially causing diel variation in $\delta^{13}C_{res}$. We have included the major part of the arguments and are of the opinion that the manuscript strongly benefits from these suggestions.

In the following section we give a point-by-point reply to the referees' comments.

Anonymous Referee #1

Received and published: 6 April 2011

General comments:

The authors reviewed the diel variations of the carbon isotope composition of respired CO2 at plant-organ and ecosystem levels. The manuscript is well written and summarizes the recent developments on the understanding of metabolic origin of respiratory discrimination in plants (but plant and ecosystem are missing in the title) and its diel changes. This review is thus of high relevance for publication in this journal, regarding the importance of this topic in ecosystem carbon partitioning studies. However, the authors should revise the manuscript taking into consideration the reviewer's comments with special attention to 2 major comments below:

1- Although the corresponding literature is mentioned in the manuscript, when reading the manuscript it's sometimes not clear which authors are the first contributors to the recent developments of the mechanisms of respiratory fractionations. In the abstract, where the authors state that "in this review we examine: : :: : :: : :: : :: : :.and develop mechanistic explanations: : :: : :: : :: : :: : :.", the verb "develop" should be replaced by "discuss" (see the comments below), and the term "mechanistic explanations" is linguistically awkward because explanations are not mechanistic. All the mechanisms discussed in this review are already developed and discussed in the literature, and the present manuscript is a synthetic review. So, the authors should be careful in wording.

For instance, in page 2193 (line 23), "we have a conceptual framework" is confusing because the concepts are already published. In many parts of the manuscript, the references should be replaced or added.

We have now carefully changed wording to make clear that we provide a synthesis of different mechanisms (already published in literature) and that we "[…] explore step by step whether the three main mechanisms alone or in combination can explain the observed short-term variability in $\delta^{13}C_{res}$ and $\delta^{13}C_R$."

According to the suggestion of the referee we now write in the abstract: "In this review we examine the short-term dynamics in $\delta^{13}C_{res}$ and putative substrate pools at the plant, soil

and ecosystem scales and discuss mechanisms, which might drive diel $\delta^{13}C_{res}$ dynamics at each scale."

We also state now "In conclusion, there is a conceptual framework for explaining the observed short-term variations in $\delta^{13}C$ of sugars and other fast turn-over carbon compounds" and we have added references to literature to make clear who originally contributed to a particular finding.

Further, we added a sentence it the introduction to point this out clearly:

"Thus, driven by the work of Jaleh Ghashghaie's group and others increasing knowledge on isotope fractionation during dark respiration has been acquired during the last decade (for reviews see Ghashghaie et al., 2003, Badeck et al., 2005; Bowling et al., 2008). However, marked diel variations of dark-respired $\delta^{13}C_{res}$, which occur within minutes to hours over the 24h cycle, have only lately gained scientific attention (e.g. Barbour et al., 2007; Werner et al., 2007; Wingate et al., 2010).

2- The hypotheses taken for the equation 1 (model) are not mentioned, the terms of the model are not explained, the same for equation 2. It's not clear why in the equation 1, only one part of the equation is divided by (f1+2f2) and why the f3 does not appear.

These equations and the hypotheses of the model and different terms/steps should be clearly defined and explained. The equations 1 and 2 are contradictory, mainly because of the confusion between fractionation factor and isotope effect.

The calculations follow a simple mass balance approach solved for $\delta^{13}C_{res}$. f3 did not appear as f2 equals f3 (the C-2 and C-3 carbon atoms enter the KC as one Acetyl-CoA molecule). To avoid any confusion the formula was changed showing all three fluxes and an explanation was added. Indeed all parts of the equation have to be divided by sum of the fluxes and the missing parenthesis was added. Fractionation factor (α) was substituted by the isotope effect (ε) to avoid any confusion. The calculation of the effective fractionation was done based on Hayes 2001 for fractionation in metabolic branching points. This reference was now added to the description.

However we feel that currently too much emphasis was given to these theoretical calculations, which are nothing else but a numerical example for the potential isotope effect assuming all combinations of possible flux rates. Therefore, we decided to move this section to the legend of figure 6, which visualises the outcome of these calculations. Further we added an explanatory sentence to the text:

"This example indicates that on a theoretical basis large isotope effects can occur through fractionation effects and metabolic branching points in the respiratory pathways, however to what extend this will be expressed *in vivo* still remains to be resolved."

Figure legend now reads:

Theoretical isotopic fractionation effects during decarboxylation of pyruvate.
The theoretical effects were calculated utilizing a simple mass balance equation, through adding the flux-weighted isotope ratios of each carbon position (C-1-3):
$\delta^{13}C_{res} = [f_1 \times \delta^{13}C\text{-}1 + f_2 \times (\delta^{13}C\text{-}2 + \varepsilon_{eff(CS)}) + f_3 \times (\delta^{13}C\text{-}3 + \varepsilon_{eff(KG)})] / (f_1 + f_2 + f_3)$
with $f_{1\text{-}3}$ being the carbon flux (where f_2 equals f_3 as both (C-2 and C-3) carbon atoms enter the Krebs cycle as Acetyl-CoA moiety), and $\delta^{13}C$ the isotopic composition of the carbon molecules at the C-1 to C-3 positions of pyruvate and ε denotes the enzymatic isotope effect. The effective enzyme isotope fractionation (ε_{eff}) in the KC of the citrate synthase (ε_{CS}) and the α-ketoglutarate dehydrogenase (ε_{KG}) is dependent on the carbon flow in the KC (see Hayes 2001) by: $\varepsilon_{eff} = \frac{\varepsilon+1}{1+\varepsilon*f} - 1$. Potential fractionation effects were calculated by varying the carbon flow rates into KC ($f_{2,3}$) from 0-100%, assuming fractionation factor of ε_{CS} and ε_{KG} of -23‰ (see Tcherkez and Farquhar 2005 and Figure 5 for details). PDH could also potentially fractionate if the reaction is incomplete (Melzer and Schmidt

1987) which would further deplete $\delta^{13}C_{res}$, which was tested assuming f_1 of 50-100% (z-axis), but occurrence of the latter processes in vivo is unknown.

The references for different fractionation factors should be indicated in the text or reference to the figure legend where they are given added.

The reference to the fractionation factors was given at the end of the legend of figure 5, but we now explicitly added the references of each factor in the text and legends.

Other remarks:

Page2185-Line 15: Duranceau et al (1999) measured the 13CO2 res at the beginning and at the end of the night period under normal and drought conditions and showed parallel changes in 13CO2 and 13C of leaf sugars during night time (covariations)

This should be added to the manuscript in this part (also should be added to the Table 1).

Duranceau et al 1999 did not look for diel variations but focussed on day to day differences over a longer period (as affected by drought). As a consequence this citation does not fit in the context of "diel variations of dark-respired CO_2, which occur within minutes to hours over the 24 h cycle".

Page 2186 – line 2 : : : :: : :..fractionation in particular,: : :: : :: : :: : :: : :.

Has been corrected

Page 2187- line 12: one "that" should be deleted.

Has been corrected

Page 2188-line 18: Initially, Duranceau et al (1999) and Ghashghaie et al (2001) showed short term changes in respired 13_{CO2} during drought stress.

We restrict our review on diel variations of $\delta^{13}C$ in respired CO_2. Both, Duranceau et al (1999) and Ghashghaie et al (2001) explore the effect of drought on $\delta^{13}C$res on a day-to-day basis over a couple of weeks. We have now made this point (i.e. that only diel variations were taken into account) more clear in the sentence the referee refers to and now write: "Furthermore, considerable variation of diel patterns has been observed in response to changing environmental conditions (Table 1, see discussion below)."

Page 2191-line 16: Add Tcherkez et al (2010) who showed changes through the day.

Has been added

Page 2191-line 25: Add Tcherkez et al (2004)

Has been added

Page 2194- line 19: add "of":: : :: : :: : :: : :: : :: : :..in utilization of organic acid pools: : :: : :: : :: : :

Has been corrected

Page 2195-line 1-2: Add Tcherkez et al (2005) to the sentence just after " (KC) are strongly inhibited ".

Has been added

Same page-Line 8: "positive delta13C" is not correct: the authors should say "high delta13C" or "less negative delta13C".

Has been corrected

Same page: The findings of Hymus et al (2005) on the relationship between the amount of photoassimilates during the day and the 13CO2resp should be underlined here.

Has been added; we now write: "Both the extent of enrichment and the subsequent 13C-depletion augment during the light period (Fig. 4, Werner et al., 2009) and have been shown to be linearly related to cumulative carbon gain during the light period (Hymus et al., 2005), even under different growth-light conditions (Priault et al., 2009)."

Same page Line 23: The verb "focussed" is too strong here!

Wording has been changed

Page 2196-second paragraph: The maximum extent of intramolecular 13C variation observed by Rossmann et al (1991) in glucose molecules (sugar beet) is around 12‰ (not 6‰.

We referred here to the maximum deviation from the average $\delta^{13}C$ value of the molecule, but the comparison was probably unclear. The differences are now shown in the new Figure 6 (see comments to referee 2) given the molecule structure and intramolecular distributions. The text has been adapted concordantly:

The maximum deviation of a particular C atom from the average $\delta^{13}C$ value of the molecule was determined as 6.3‰ for glucose in yeast (by stepwise biochemical degradation, Rossmann et al., 1991, see Fig. 6). Recently, NMR data for sucrose showed a somewhat larger enrichment at the C-3 and C-4 position (Fig. 6) and in particular a larger intramolecular deviation between the C4 to C6 positions (of 13.3‰ (Gilbert et al., 2009) compared to the data from Rossmann et al. (1991: 11.2‰).

Same page-lines 20 and 25: "New" is redundant for NMR analysis mentioned here. Change the wording.

Sentence has been reworded

Page 2197: Equation 1: d13C-1 should be d13C1 (1 as subscript).

We used the same style as Rossmann et al. 1991; Tcherkez et al. 2004 and others for defining the position of the carbon atom in a molecule i.e. C for carbon, a hyphen and then the atom number. So we prefer to leave it as it is.

Page 2200-line 15: It should be added that earlier, linear relationships between respiration rate and respiratory fractionation were observed during both leaf ageing and drought (Ghashghaie et al, 2003) and with growth (Ocheltree and Marshall 2004). Tcherkez et al (2003) should be added for linear relationship with temperature (line 16).

We have added the reference to Ghashghaie et al, 2003 and Tcherkez et al (2003) to the discussion.

Page 2201-line 26: Please replace "in our example above" by "in Rossmann et al 1991", otherwise it could be misunderstood by readers.

We have reworded the sentence to make this point clearer

Page 2205-line 2: delete "," after "new indications suggesting: : :: : :: : :: : :."

Deleted

Page 2206-line 4: delete "s" from components so it should be : component fluxes

Corrected

Figure 1: The total water soluble fraction contains metabolites other than potential substrate for respiration. If some of them are 13C depleted this could explain the dampening of signal. This is not discussed.

We have added a sentence to the figure legend to make our point clear: "Gessler et al. (2009b) and Brandes et al. (2006) showed that the δ13C of water soluble organic matter is a reasonably good proxy for δ13C of the neutral sugar fraction and thus the major respiratory substrate."

Figure 2 legend: add ";" before PAR.

Done

Brown color of the arrows is not easy to distinguish on this figure !

The arrows are blue now

Part 1 of figure 2: The environmental factors at the top of left side: it's not clear if the conductances are only affected by VPD and soil water or also light, T etc. The same for A, respiration and photorespiration. All these environmental factors affect all gas exchange parameters. This should be clarified.

Additional arrow have been added to clarify this point

Figure 5: line 6 of legend: delete "in"

Has been deleted

Same figure: In both parts of the figure, it's not clear why the enriched C (both C and delta values) is brown and not red.

Has been corrected, all symbols and values being red now

Anonymous Referee #2

Received and published: 18 April 2011

Report on the manuscript by Christiane Werner and Arthur Gessler in Biogeosciences

Discussions April 2011

The topic of the review "Diel variations in the carbon isotope composition of respired CO2 and associated carbon sources: A review of dynamics and mechanisms" is very interesting and merits publication; the more so as a good review is more than the sum of citations. The article gives a good overview on recent developments and offers a "mechanistic explanation" for the observed variations and diurnal changes of d13C of respired CO2. However, I suggest some revisions. On the one hand I would like to draw the authors' attention to more formal objections, and on the other hand I offer some proposals with regards to contents which I think could further improve the manuscript.

From a formal point of view, I think more attention to exact wording is necessary and would facilitate the readers' understanding in the following instances:

1) Page 2184 line 13: I know that the term "post-photosynthetic fractionation" is used quite often. Regardless the fact that a very wide range of anabolic and catabolic reactions of the secondary metabolism are "post-photosynthetic" or downstream to carbon assimilation, please replace the term by "post-photosynthetic isotope fractionation".

The term has been replaced

Please add "isotope" to "fractionation" also at other places in the manuscript.

The term "isotope" has been added to "fractionation" where applicable.

2) Page 2184 line 25-27: Please use either ": : : alter the carbon isotope ratio : : :" or ": : : alter the 13C/12C ratio : : :" not both descriptions in one sentence. The d13C value is the relative deviation of the 13C/12C ratio of a sample from the 13C/12C ratio of an international standard.

Has been corrected. The sentence now reads as follows: "As carbon travels from the atmosphere through plants and is respired back to the atmosphere by leaf, stems, roots and soil there are many processes, which alter the carbon isotope ratio (generally expressed in the δ-notation ($\delta^{13}C$) in ‰ as the relative deviation of the $^{13}C/^{12}C$ ratio of a sample from the $^{13}C/^{12}C$ ratio of an international standard)."

3) Page 2187 line 20-21: Please replace "carbohydrates, starch" by something more appropriate as starch is

also a member of the “carbohydrate family”. Perhaps “soluble and storage carbohydrates” or “mono-, oligo- and polysaccharide” etc.

Has been replaced; we now refer to “soluble and storage carbohydrates” as suggested by the referee.

4) Page 2200 line 14: Please rephrase “higher enrichment of d13Cres” with e.g. “more positive d13Cres values”. Pls check at other places in the manuscript.

Has been changed here and throughout the manuscript

Page 2197 line 11: “leading to a depletion of d13Cres” –> more negative d13C ?

Has been corrected

5) Page 2192 title: Please replace “M1.3: Isotope effects during : : :” by something like “M1.3: Isotope fractionation during : : :”. Not every isotope effect will be expressed in vivo because of its dependence on turnover rates and metabolic branching points : : :.

Has been corrected

See also 14).

6) Page 2196 line 18: Please rephrase. The fragmentation fractionation per se cannot lead neither to an “isotope effect” (pls see definition of isotope effects: http://goldbook.iupac.org/I03327.html) nor to an “isotope fractionation”. Perhaps to a difference in d13C-values because of different d13C-values of the educts ?

We have changed the wording of the sentence. It now reads as follows: “The potential 13C enrichment of PDH-derived CO2 above the whole glucose molecule as a result of the “fragmentation fractionation” depends on the extent of intramolecular ^{13}C variation.”

7) Page 2198 line 17/18: Please rephrase “Inversely, in the dark the reaction can be assumed to go to completion resulting in little net isotope effects”. Isotope effects do not depend on completion of reaction. They can depend on reaction conditions like temperature, pH etc. Isotope fractionation is depending on branching points and turnover rates.

Has been corrected: "In the dark when the KC is reorganized again, the impact of fragmentation fractionation together with potential kinetic isotope discrimination *in vivo* will depend on how much of the respiratory substrate is oxidized to CO_2 and which portion is used for biosyntheses."

The following points might improve the content of the review and therefore support the

authors’ reasoning and argumentation:

8) Page 2188 lines 11-16: Perhaps the article by Villar & Merino on leaf construction costs (New Phytol 151, 213-226, 2001) or related work would be helpful for the discussion on why there are “largest diel variations in d13Cres” in not fast-growing plant species. Additionally helpful could also be the article by Pinelli and Loreto (J exp Bot 54, 1761-1769, 2003) regarding the ratio of CO2 emission in the light/ CO2 emission in the dark being smaller for Quercus relative to two herbaceous species. The working group of F. Loreto has also published articles on the production of volatile organic emissions by plants.

We think that the relation between leaf construction costs and short term variation in $\delta^{13}C$ would be really worth to be explored. However, we believe that a discussion about the relations and potential mechanism would be to speculative – especially because Villar and Merino (2001) conclude that there is no clear and straightforward relation between functional group and leaf construction costs (“However, functional groups (evergreens vs. deciduous) and ecosystems showed small differences in [leaf construction costs]) – which is in contrast to our postulate. As a consequence we did not include

this point to the discussion. We, however, acknowledge that there is further research needed to explore functional group specific patterns in $\delta^{13}C_{res}$.

9) Page 2192 lines 8-9: Gleixner et al. (Planta 207, 241-245, 1998) suggested that transitory starch is 13C enriched relative to soluble sugars because of the isotope effects on the "aldolase-reaction" measured originally by Gleixner and Schmidt (1997). Interesting for the M1.2 discussion is certainly also the fact that transitory starch "does not provide substrates for respiratory and photorespiratory decarboxylations in irradiated photosynthesizing leaves." (Ivanova et al., Photosynthetica 46, 84-90, 2008).

The referee's suggestions have been fully included. The section now reads as follows: "Gleixner et al. (1998) suggested that transitory starch is ^{13}C enriched relative to soluble sugars because of the isotope effects on the "aldolase-reaction" determined originally by Gleixner and Schmidt (1997). As a consequence, ^{13}C-depleted triose phosphates are exported from the chloroplast, which are used for sucrose production during the light period and thus influence $\delta^{13}C_{res}$. It has also to be mentioned that the ^{13}C enriched transitory starch does not provide substrates for respiratory and photorespiratory decarboxylation in irradiated photosynthesizing leaves (Ivanova et al., 2008)."

10) Page 2195 line 1ff: Please give citations for the light-inhibition of glycolysis (!) and Krebs-Cycle. Citations for the light-reduction of the TCA cycle activity could be e.g. Techerkez et al. (Plant Physiol. 138, 1596-1606, 205) and Nunes-Nesi et al. (Physiol. Plant. 129, 45-56, 2007) etc. According to Tcherkez et al. (Plant Physiol 151, 620- 630, 2009) and Sweetlove et al. (Trends in Plant Science 15, 462-470, 2010) and cited literature therein, the Krebs-Cycle is not a real cycle during illumination.

We have added citations (Tcherkez et al. 2007; Nunes-Nesi 2007) referring to the light-inhibition of glycolysis and Krebs-Cycle. In addition, we have added information that the TCA cycles is not really a cycle anymore in the light. The section now reads as follows: "In the light, both glycolysis and particularly the Krebs cycle (KC) are strongly inhibited (Tcherkez et al., 2005; Nunes-Nesi et al, 2007). Moreover, during illumination probably only a non-cyclic Krebs "cycle" operates in autotrophic tissues (Tcherkez et al., 2009, Sweetlove et al., 2010) because three key enzymes i.e. the mitochondrial isocitrate dehydrogenase (Igamberdiev & Gardeström, 2003), the succinate dehydrogenase (Popov et al., 2009) and the 2-oxoglutarate dehydrogenase (Gessler et al., 2009b) are inhibited."

11) Page 2196 1st paragraph: Please be aware that pyruvate is also the starting point of corresponding biosyntheses e.g. biosynthesis of the amino acids alanine (and valine). The PDH reaction has been recognized to imply kinetic isotope effects on all 3 carbon atoms of pyruvate (Melzer and Schmidt 1987). In case of a non-quantitative conversion of pyruvate to acetyl-CoA and CO2 + a branching point at pyruvate, the KIE on C1 of pyruvate will be expressed in vivo and the released CO2 will be depleted in 13C relative to C3/C4 of glucose.

This information has been included as suggested by the referee; the section now reads as follows:

"Any change in the relative contribution of CO_2 decarboxylated in the KC versus by PDH to total CO_2 production may thus cause variations in $\delta^{13}C_{res}$. Furthermore, kinetic and equilibrium isotope fractionation in glycolysis and KC may also occur. Pyruvate is also the substrate for amino acid synthesis and the PDH reaction has been recognized to imply kinetic isotope effects on all 3 C atoms of pyruvate (Melzer and Schmidt 1987). In case of a non-quantitative conversion of pyruvate to acetyl-CoA and CO_2 and a metabolic branching point at pyruvate, the kinetic isotope effect on the C-1 of pyruvate will be expressed in vivo and, as a consequence, the released CO_2 will be depleted in ^{13}C relative to C-3 and C-4 of glucose. In conclusion, it is most likely a mixed influence of fragmentation fractionation and enzymatic isotope effects related to metabolic flux rates (e.g. see Fig. 5) which together drive $\delta^{13}C_{res}$ variations."

Please check also on Page 2198 line 9ff: The membrane of the mitochondria is permeable to pyruvate. This

has as consequence the possibility for pyruvate to be distributed to other pathways besides the Krebs cycle.

We now refer to the detailed information added as detailed above.

This metabolic branching (upstream to the PDH reaction) is certainly not only regulated by the substrate availability regulation of the PDH reaction.

We have omitted this statement

12) Page 2196 2nd paragraph and corresponding comment of Referee 1 (“around 12‰ ”): Perhaps it would be good to include a chemical drawing of the monosaccharide and state there which C-atom has which relative 13C-enrichment or 13C-depletion according to Rossmann et al. (1991) and recent articles by the working group of R. Robins. This would be helpful also for the discussion in 1st paragraph on page 2197 and for the corresponding discussion of the PPP cycle on page 2199.

It would also facilitate the fragmentation fraction issue (Page 2196 line 18). Please see attached draft (Fig. 1) as a non-binding suggestion, and in case pls check the correctness of the numbers.

We agree that this will facilitate the reading and have added a new figure as suggested:

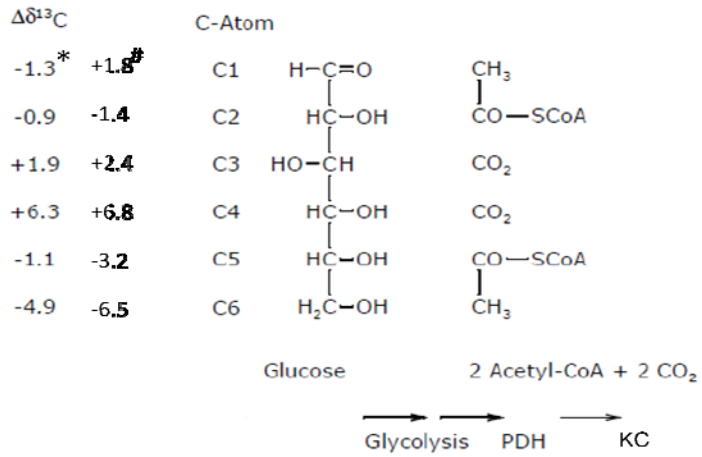

Figure 6. Relative intramolecular ^{13}C distribution for different carbon positions calculated as deviation from the mean $\delta^{13}C$ of the whole molecule. Examples are given for glucose (*), determined by stepwise fermentation by Rossmann et al. (1991) and for sucrose (#) determined by NMR by Gillbert et al. (2009). Glucose enters glycolysis where two CO_2 molecules from the C-3 and C-4 positions are decarboxylated by pyruvatedehydrogenase (PDH) and the remaining ^{13}C-depleted Acetyl-CoA molecules enter the Krebs cycle (KC).

13) Page 2197 line 10, literature citation Hayes 2001: Savidge and Blair (Oecologia 139, 178-189, 2004) discuss the possibility to use the 13C-enrichment of the glutamate carboxyl groups and the C4 carboxyl group of aspartate as proxy to estimate the amount of anaplerotically fixed carbon in C3-plants. Melzer and O‘Leary show similar results (Plant Physiol 84, 58-60, 1987; Planta 185, 368-371, 1991).

We agree with the referee that this is an interesting point. However, we are of the opinion that this side note would distract the readers from that main focus of this section as additional information would have to be included to make the point clear. As a consequence we have decided to leave this point out.

14) Page 2197 line 10-11: Regarding “: : :equilibrium and kinetic isotope effects: : :” in the Krebs cycle. Several isotope effects on reactions catalyzed by enzymes of the Krebs cycle have been measured (see

Tcherkez & Farquhar (2005) and cited literature therein). But not in every case an isotope effect will lead to an observable isotope fractionation. E.g. the citrate synthase reaction in the mitochondrium will most probably not lead to an isotope fractionation in the acetyl part of the produced citrate molecule as the inner mitochondrial membrane is impermeable for acetyl-CoA (see e.g. cited Voet & Voet or other (plant) biochemistry text book). Acetyl-CoA can only be transported to the cytosol in form of citrate. This has as consequence that acetyl-CoA will react quantitatively with oxaloacetate to citrate! Quantitative (enzymically catalyzed) reactions can "have" isotope effects (as a sort of chemical property) but will not show a corresponding isotope fractionation. See e.g. JM Hayes' chapter 2.4 "isotopic fractionations" in http://www.nosams.whoi.edu/docs/IsoNotesAug02.pdf.

A discussed carbon isotope fractionation by supposed isotope effects on other reactions catalyzed by enzymes from the Krebs cycle is questionable because of the (discussed) channeling principle of the Krebs cycle (e.g. Srere et al. 1996, Channeling in the Krebs tricarboxylic acid cycle. In: L Agius, HSA Sherratt (eds.): Channeling in intermediary metabolism. Portland Press, London, pp. 201-217). Intermediates of the Krebs cycle are transferred from one enzyme to the other in a manner that allows only restricted mixing of the enzyme-bound intermediates with free intermediates. Please check equation (1) and related text passages and also Figures 5 and 6 under this premise.

We have now included both points (impermeability of the inner mitochondrial membrane and chanelling) to the discussion. We make clear that theoretically possible kinetic isotope effects may not lead to in vivo isotope fractionation. We also discuss this in relation to eqn 1 and Figure 6. We now introduce our equation 1 more carefully: "f pyruvate is not fully respired, both equilibrium and kinetic isotope effects that occur in the KC (Tcherkez and Farquhar, 2005) could lead to more negative δ13Cres."

The section in which we discuss the points raised by the referee reads as follows:

"Only recently, however, Werner et al. (2011) postulated that the isotope effects associated with the KC enzyme reactions do most probably not lead to in vivo isotope discrimination. On the one hand, the inner mitochondrial membrane is impermeable for acetyl-CoA (Voet and Voet, 1995) and thus acetyl-CoA will react quantitatively with oxaloacetate to citrate. As a consequence, the citrate synthase reaction should not lead to any isotope fractionation in the acetyl-part of citrate (Werner et al. 2011). On the other hand, the whole KC is assumed to work as an organised enzyme complex (Srere et al., 1996). The proposed channelling of the KC substrates at reduced concentrations would avoid metabolic branching to other enzymatic reactions (Srere et al., 1996). As a consequence, theoretically possible kinetic isotope effects on KC enzyme reactions would not be expressed in vivo (Werner et al. 2011). We need at that time clear experimental evidence if kinetic isotope fractionation occurs in the KC under physiological conditions as postulated by Tcherkez (2010) or not as concluded by Werner et al. (2011)."

15) Page 2198 line 17/18: A more "philosophical" objection towards that sentence could be: At what time during a normal 24 h rhythm (illumination – darkening) the plants will grow? According to A. Walter's group (e.g. Walter et al. Annu Rev Plant Biol 60, 279ff, 2009) there are plants which mainly grow during dawn and others during dusk. According to Michael et al. (PLOS 6, 1887-1898, 2008) the gene expression for phytohormone growth pathway increases during the night with a maximum at dawn where the stem elongation is fast. There is obviously a not inconsiderable percentage of growth during night. This would most probably shift the Krebs cycle towards the anaplerotic side. But also alanine will be produced for proteins and other functions.

We have now carefully reworded this section and no longer postulate that the respiratory substrate is fully oxidised in the night. The section now reads as follows: "In the dark when the KC is reorganized again, the impact of fragmentation fractionation together with potential kinetic isotope discrimination in vivo will depend on how much of the respiratory substrate is oxidized to CO2 and which portion is used for biosyntheses."

16) Page 2198 line 27: “fatty acids”. Please be aware that most fatty acids will be biosynthesized in the chloroplast and not in the mitochondria. According to Tovar-Méndez et al. (2003) “the de novo synthesis of fatty acids” in green plant parts “is lightdriven and occurs exclusively” in plastids. Acetyl-CoA for this synthesis is supplied by plastidial PDH and the released CO2 is most probably re-fixed by photosynthesis??

We have removed fatty acids from this passage

17) Page 2198 line 24ff and page 2200 line 1ff: Interesting approach. During isoprenoid biosynthesis through the DOXP/MEP pathway in chloroplast the 13C-enriched C-1 of pyruvate will be released and under illumination possibly re-fixed by photosynthesis.

During biosynthesis of isorenoids through the mevalonate pathway starting from acetyl-CoA in the cytosol as for the biosynthesis of flavonoids, polyketids, plant sterols etc. 13C-enriched CO2 from pyruvate will be released. A corresponding release of the 12C-enriched acetyl-moiety of the pyruvate in form of e.g. terpenes would partly compensate the 13C-enrichment. Ghirardo et al. (PLOS ONE 6(2), e17393, 2011) give some numbers on BVOC production (under illumination) and show that the C loss in form of isoprene is mainly originating from recently assimilated CO2.

Indeed at the organ level the emission of the ^{13}C-enriched CO_2 will be compensated by the emission of the volatile ^{13}C-depleted compounds, which has been traced by a pyruvate positional labelling experiment (Jardine et al. 2010a). However, it will still have an effect on the emitted CO_2 (if not fully refixed), which could have a noticeable effect for large-scale flux networks. For example, it has been shown that isoprene and monoterpene emissions alone account for 2% of net primary production (NPP) (Guenther et al. 1995), and relative emissions can increase by at least an order of magnitude during stress events that decrease photosynthetic rates (Kesselmeier et al. 2002). However, we feel that by going to deep into this issue we may lose the focus of this review. Therefore we have just added a few sentences:

While the ^{13}C-enriched C-1 from pyruvate will be released as CO_2, pyruvate positional labelling showed that the ^{13}C depleted C-2 and C-3 carbon atoms of the acetyl-moiety are emitted as a variety of volatile isoprenoids and oxygenated VOCs (such as isoprene, acetaldehyde, ethanol, or acetic acid, Jardine et al., 2010a). VOC emissions generally range about 2-5% (Guenther et al., 1995) of mostly recently fixed carbon (Ghirardo et al., 2011), but can increase at least by an order of magnitude during stress conditions (e.g. Kesselmeier et al., 2002), which may potentially cause a large effect on the emitted $\delta^{13}CO_2$.

18) Page 2199 line 19ff and literature citation Priault et al. (2009): Please be aware that in case of a non-cyclic Krebs cycle under illumination the C atom(s) released as CO2 connected to the Krebs cycle will not originate from the carbon skeleton of acetyl- CoA (see the “way of carbon atoms” through the Krebs cycle on p. 539 in Voet & Voet 1995 cited by the authors). The (diurnal) CO2 released under illumination by the isocitrate dehydrogenase (and the 2-oxoglutarate dehydrogenase, if applicable) will originate from C-1 and C4 of oxaloacetate and not from acetyl-CoA. In any case there should be no possibility for an observable 13C-enrichment of this CO2 during illumination in case of addition of pyruvate labelled with 13C in C-2 or C3 position.

Instead of this the “acetyl moiety” of pyruvate can be used for biosynthesis purposes (also for biosynthesis of BVOC).

The measurements in Priault et al. (2009) were conducted in leaves which were shortly darkened (for 5 min) before measurements, thus during the period where the PDH was activated again. We added a note to point this out more clearly.

19) Page 2232 Figure 5: Please be aware that PEPc will bind HCO3- to PEP. The resulting product is

oxaloacetate and not malate as it is shown in Figure 5.

Corrected

20) Interesting for me personally would be: why is the d13C value of the CO2 respired by plants during darkness getting more and more negative in the course of the night?

We agree that this is an interesting point but is far from being understood. There are observations, that some time after darkening leaf, $\delta^{13}C$ of leaf respired CO2 gets constant (e.g. Barbour et al. 2007; Plant Cell and Environm. doi: 10.1111/j.1365-3040.2007.01634.x) but also a longer-term decrease in $\delta^{13}C_{res}$ has been observed. On the other hand for tree trunks for example also an increase in $\delta^{13}C_{res}$ has been observed during the night (see figure 1A).

For leaves we may suggest to refer the referee to the discussion of Barbour et al (2011; New Phytologist doi: 10.1111/j.1469-8137.2010.03635.x). They write:

"We recognize that the LEDR peak in respiration rate, and its associated decarboxylation of ^{13}C-enriched malate, is expected to last for 15–20 min only after darkening (Atkin et al., 1998b; Barbour et al., 2007), although the timing and magnitude of the LEDR peak are known to be sensitive to temperature (Atkin et al., 1998a). However, we observed here that the decline in $\delta^{13}C_{Rl}$ continued for at least 60 min after darkening. The reasons for such slow isotopic kinetics under our conditions remain unclear. It has been demonstrated in bean leaves (*Phaseolus vulgaris*) that in the steady state (say, after 1 h in darkness) leaf-evolved CO_2 is ^{13}C enriched by nearly 6‰ compared with sucrose (Duranceau et al., 1999) and then slowly decreases by c. 2‰ within 8 h (Tcherkez et al., 2003). Similarly, Nogues et al. (2004) observed a slow decline of 2.5‰ within 3 h in darkness in the same species. Plausibly then, in the species used here, transitions between the three phases (LEDR, steady-respiration rate and then slow decline) were slow and could not be distinguished. Further, under our conditions, CO_2 evolved by leaves reached a minimal value after, c. 3 h (Fig. 2a–c). Future studies of $\delta^{13}C_{Rl}$ and LEDR should include measurements of concentrations and isotope compositions of key leaf metabolites, such as malate and sucrose, to allow mechanistic interpretation."

We also think that more intensive studies combining metabolic flux analysis, compound specific isotope analyses and online measurements of $\delta^{13}C_{res}$ are necessary to address this question.