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Title: CO₂-driven changes in energy and fermentative metabolism in harvested strawberries

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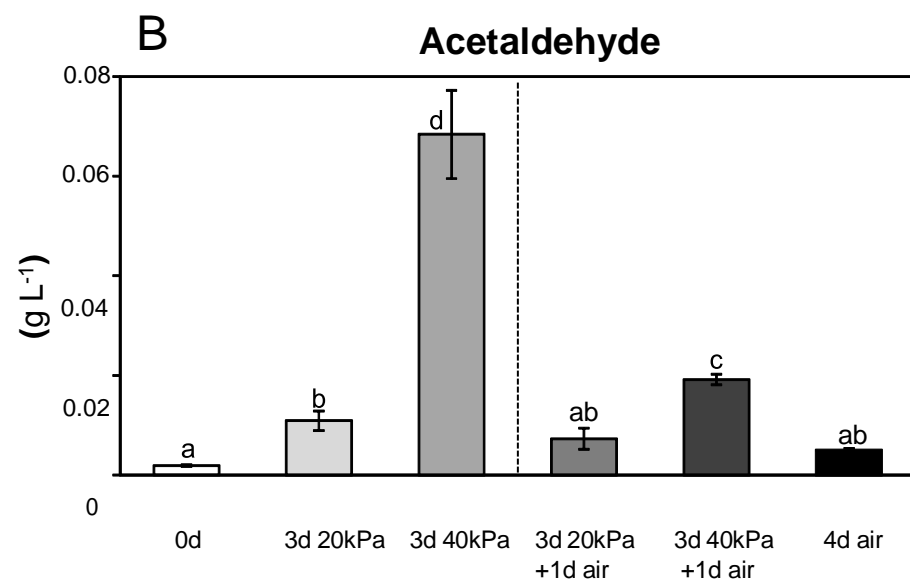
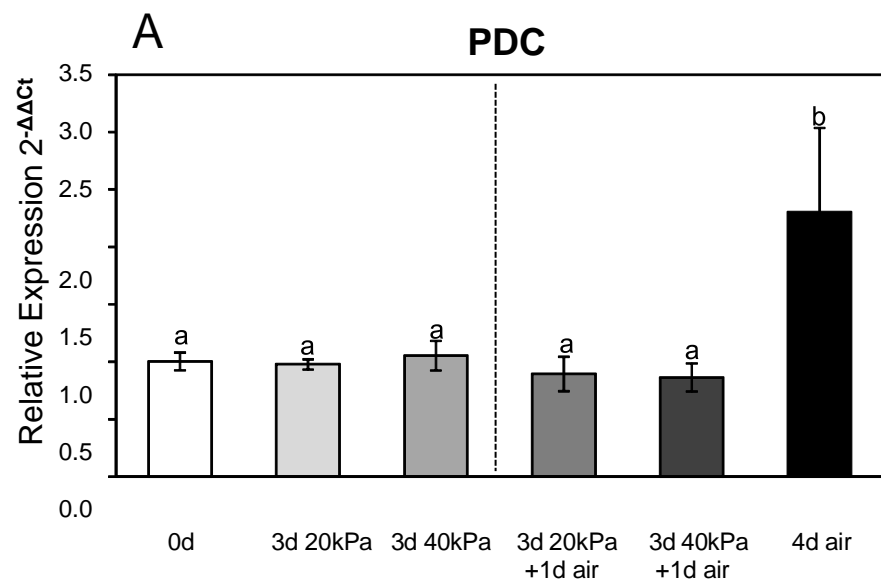
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Abstract: Short postharvest exposure of strawberries to high CO₂ levels provides significant benefits in reducing decay and controlling physiological disorders during storage at 0 °C. To define the different strategies employed by strawberries to tolerate high CO₂ concentrations, the impact of different CO₂ concentrations on energy and fermentative metabolism was studied under the same conditions of O₂ availability. Our data indicate that metabolic depression represents a strategy to effectively adapt to beneficial high CO₂ concentrations, with a decrease in ATP levels and in the energy charge, along with moderate ethanolic fermentation. Moreover, the induction of fermentative genes does not appear to be essential for the accumulation of fermentative metabolites. By contrast, when fruit is stored in air without added CO₂, the metabolism is not directed towards fermentation and is accompanied by a high ATP/ADP ratio and energy charge, favoring ATP-consuming pathways. However, when exposed to 40 kPa CO₂, the excessively low energy charge and excessive decrease in ATP could not match the ATP requirements, in a process that ultimately causes significant perturbations including a high lipid peroxidation.

Figure

Fig 1



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Fig 2

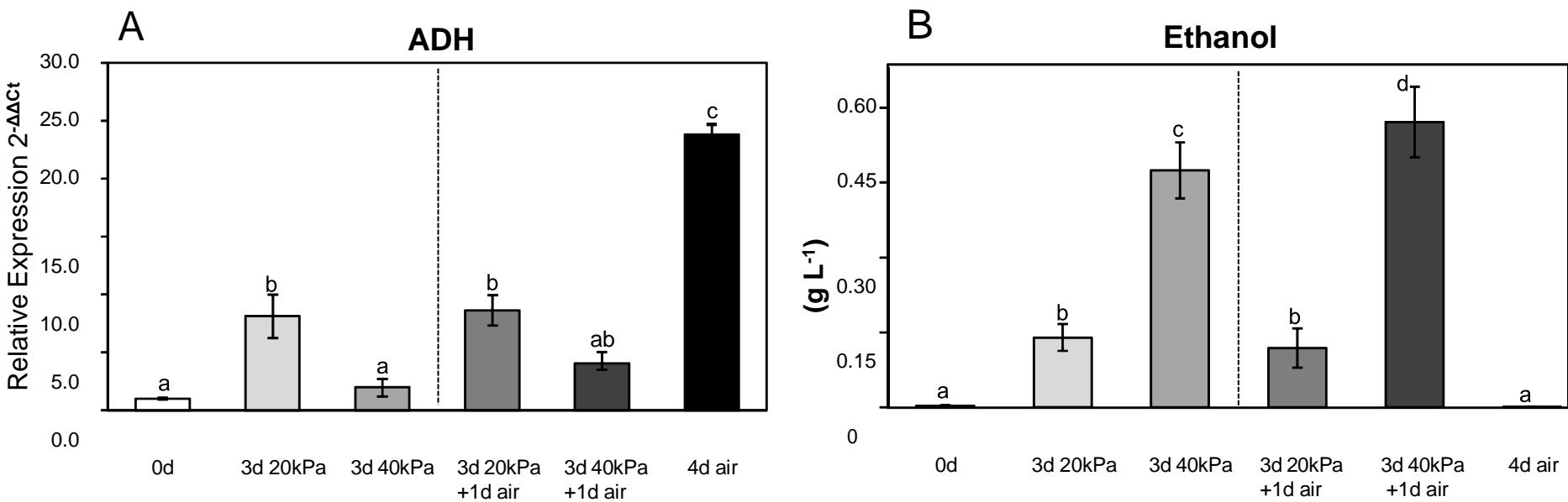
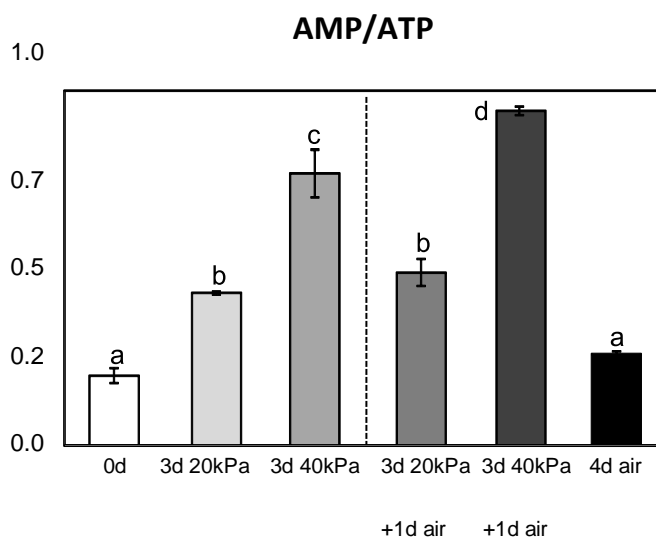
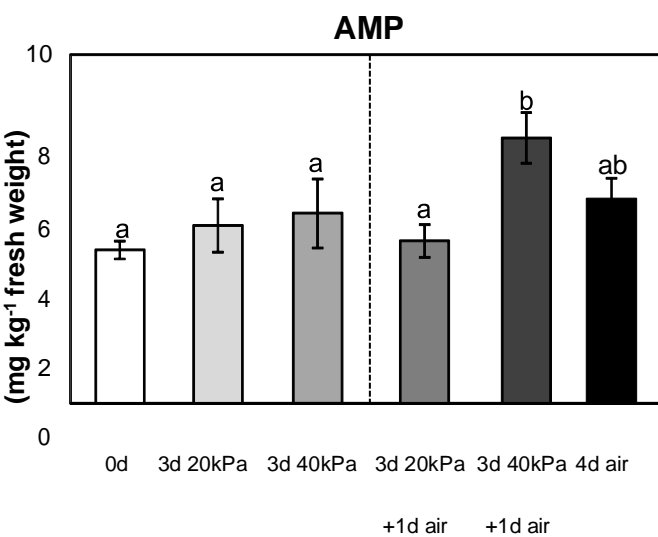
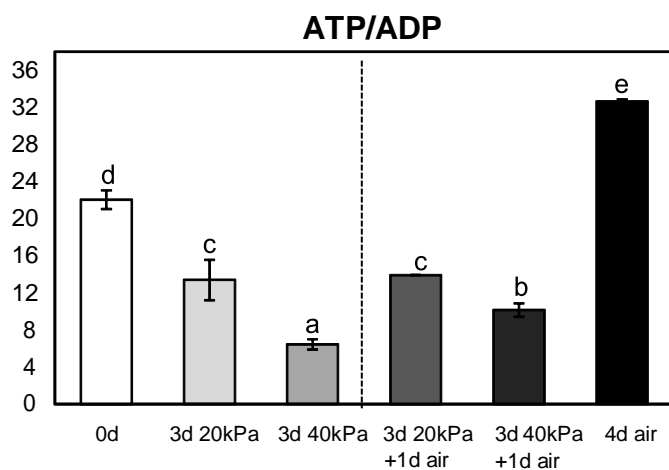
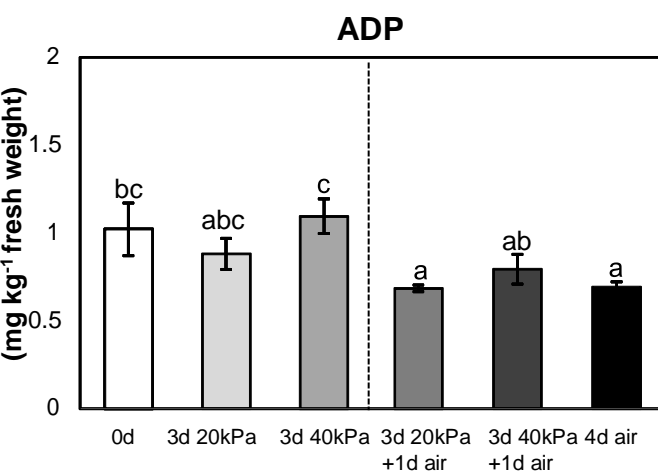
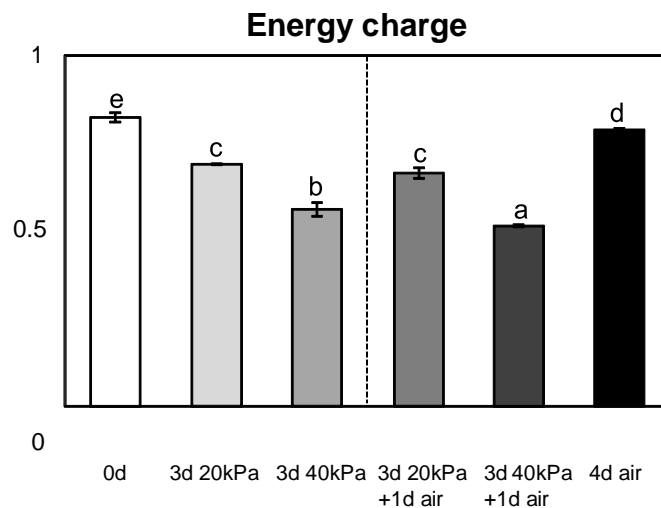
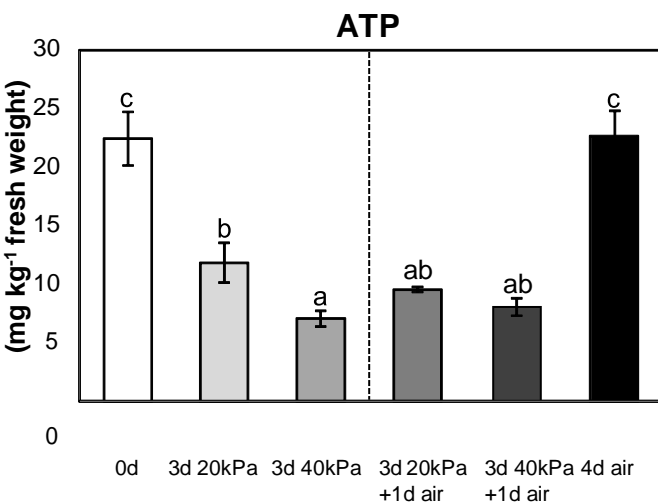
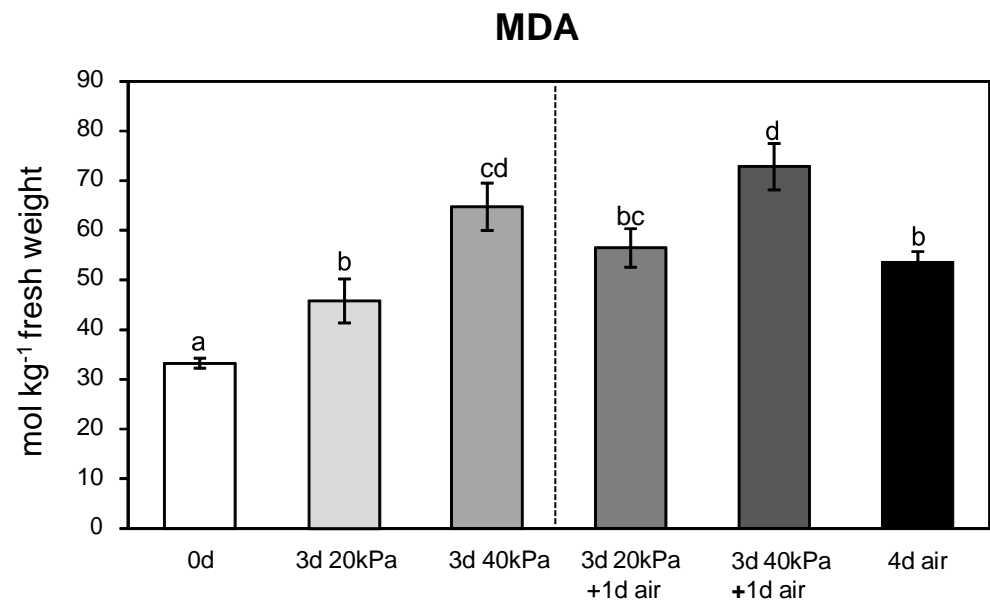


Figure 3



Figure

Fig 4



1 CO₂-driven changes in energy and fermentative metabolism in harvested
2 strawberries

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26 Abstract

27 Short postharvest exposure of strawberries to high CO₂ levels provides significant
28 benefits in reducing decay and controlling physiological disorders during storage at 0
29 °C. To define the different strategies employed by strawberries to tolerate high CO₂
30 concentrations, the impact of different CO₂ concentrations on energy and fermentative
31 metabolism was studied under the same conditions of O₂ availability. Our data indicate
32 that metabolic depression represents a strategy to effectively adapt to beneficial high
33 CO₂ concentrations, with a decrease in ATP levels and in the energy charge, along with
34 moderate ethanolic fermentation. Moreover, the induction of fermentative genes does
35 not appear to be essential for the accumulation of fermentative metabolites. By contrast,
36 when fruit is stored in air without added CO₂, the metabolism is not directed towards
37 fermentation and is accompanied by a high ATP/ADP ratio and energy charge, favoring
38 ATP-consuming pathways. However, when exposed to 40 kPa CO₂, the excessively low
39 energy charge and excessive decrease in ATP could not match the ATP requirements, in
40 a process that ultimately causes significant perturbations including a high lipid
41 peroxidation.

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43 KEYWORDS: high CO₂, ATP, energy charge, fermentative genes, MDA.

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51 Introduction

52 Strawberries have relevant economic, commercial and nutritional benefits that may vary
53 greatly depending on environmental and genetic factors (Tulipani et al., 2011).
54 Considerable effort is currently being expended on strawberry breeding programs in
55 order to develop new cultivars with enhanced flavor and health-related compounds
56 (Vandendriessche et al., 2013). Overall strawberry liking is mainly influenced by
57 sweetness and flavor intensity, which are undermined by environmental pressures that
58 reduce sucrose and the total volatile compounds content (Schwieterman et al., 2014).
59 Indeed, sucrose was proposed to be the metabolite with the single most significant
60 contribution to the overall linking.

61 Mara des Bois strawberries are highly regarded for their excellent flavor but they have a
62 very short shelf-life. Consequently, an important goal is to maintain its quality by
63 reducing detrimental effect during postharvest storage, applying different conditions of
64 refrigeration (Allais and Létang, 2009) with and without coadjuvant treatments.
65 Interestingly, a short treatment with 20 kPa CO₂ maintained higher sucrose levels than
66 when strawberries were stored in air without added CO₂ (Blanch et al., 2015a). High
67 CO₂ levels (10 to 30 kPa) also have the potential to reduce fungal decay during
68 postharvest storage of strawberries, without leaving chemical residues in the fruit (Ke et
69 al., 1991), and to increase flesh firmness (Larsen and Watkins, 1995). In addition, short-
70 term 20 kPa CO₂ treatment mitigated certain physiological and structural disorders
71 caused by low temperature storage, and effectively increasing the levels of health-
72 related compounds like proanthocyanidins and those of fructo-oligosaccharides (Blanch
73 et al., 2012a,b).

74 It is known that energy metabolism plays significant roles in the postharvest physiology
75 of fruit and vegetables, and that ATP, ADP and AMP levels, as well as energy status,

76 are affected by environmental factors, mainly by low oxygen levels (Saquet et al., 2001;
77 Wang et al., 2013; Huang et al., 2014). A reduction of the cellular energy charge
78 together with the accumulation of fermentative metabolites are generally considered to
79 be common responses to hypoxia in aerobic organisms, including plants, and even a
80 slight decrease in oxygen concentration provokes a drop in the cellular energy status
81 (ATP/ADP ratio) (Geigenberger, 2003). Furthermore, it was reported that ethanolic
82 fermentation also had an important function in plant species exposed to environmental
83 stresses at ambient or even at elevated oxygen concentrations (see review Tadege et al.,
84 1999), suggesting that fermentation might be an important switch in regulating
85 carbohydrate metabolism. Fermentative metabolism is essential for the production of
86 ATP, through regeneration of NAD^+ which sustains glycolysis but, due to its
87 inefficiency (Gibbs et al., 2000) a lower yield of ATP per mol of fermentative substrate
88 is produced. This means that a high ethanol production may lead to a carbohydrate
89 decline (Mustroph et al., 2006). However, in first harvest strawberries treated with high
90 CO_2 levels, the increase in the levels of fermentative metabolites was not associated
91 with a decrease in sucrose content. By contrast, lower levels of sucrose and fermentative
92 metabolites were observed in fruit stored in air without added CO_2 . Moreover, the
93 expression of genes encoding pyruvate decarboxylase (PDC) and alcohol
94 dehydrogenase (ADH) were greatly in excess of the rate of fermentation metabolites in
95 fruit stored in air (Blanch et al., 2015b). Ponce-Valadez and Watkins (2008) had also
96 previously observed that transcript levels of several genes encoding fermentative
97 enzymes were not always positively correlated with the increase in the amounts of their
98 encoded products in different strawberry cultivars.

99 It has been shown that some stressful conditions lead to changes in energy metabolism,
100 enhancing the production of reactive oxygen species (ROS: Tezara et al., 1999;

101 Blokhina et al., 2001; Dinakar et al., 2012). An imbalance in ROS can induce either
102 adaptive responses or detrimental changes in cell structure and metabolism. To prevent
103 cytotoxic damage associated with the enhanced generation of ROS oxidative stress, fruit
104 have evolved various protective mechanisms that include an enzymatic ROS scavenging
105 system and the use of non-enzymatic antioxidants. Lipid peroxidation is one of the best
106 studied consequences of the action of enhanced ROS levels on membrane structure and
107 function. The oxidative degradation of lipid membranes generates a variety of
108 aldehydes, including malondialdehyde (MDA). MDA is a secondary end product of the
109 oxidation of polyunsaturated fatty acids and it is considered a useful lipid peroxidation
110 marker. Therefore, MDA quantification by high-performed liquid chromatography
111 (HPLC) was performed to estimate lipid peroxidation in strawberries exposed to high
112 CO₂ concentrations.

113 Due to the diversity of responses to high CO₂, apart from the genetic background,
114 factors such as harvest time and ripening stage, that affect the levels of soluble
115 saccharides, might be play an important role in the response to high CO₂ levels. Our
116 hypothesis was that the ATP optimization established to preserve carbohydrate pools
117 could be crucial to maintain fruit quality during postharvest storage at low temperature.
118 Thus, the aim of this work was to analyze the energy status and the fermentative
119 metabolism in strawberries at 0 °C treated with different concentrations of CO₂ (20 or
120 40 kPa), as compared with fruit immediately after harvest. Additionally, we analyzed
121 whether the transfer to ambient CO₂ for one additional day was associated with a
122 decrease in the levels of ethanol or acetaldehyde, and in the expression of PDC, ADH or
123 the energy status In order to assess the changes in energy metabolism, the levels of
124 ATP, ADP and AMP were determined in strawberries at the end of the high CO₂
125 treatment and after transfer to air. Furthermore, the energy charge, as well as the

ATP/ADP ratio and the AMP/ATP ratio, are very sensitive indicators of compromised cellular energy status, and they were also calculated. In the case of fermentation, the levels of ethanol and acetaldehyde detected by gas chromatography, and the expression of *PDC* and *ADH* were evaluated by real-time quantitative RT-PCR. In addition, fruit deterioration was determined by HPLC, analyzing the MDA levels as a measure of lipid peroxidation.

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133 2. Materials and Methods

134 2.1. Plant material and *treatments*

Strawberries (*Fragaria vesca* L. cv. Mara des Bois) were grown at an organic orchard in San Sebastian de los Reyes (Madrid, Spain). Ripe red strawberries from the second harvest, including fruit from different inflorescences, were collected and transported to the Institute of Food Science Technology and Nutrition within two hours of harvest. The strawberries were then selected for uniform size and color, and those with 8.9% total soluble solids, 0.7% titratable acidity and an external L^*18 , a^*38 , b^*28 color were stored at 0 °C (± 0.5) and >95% RH in three sealed 1 m³ containers. Fifteen plastic boxes containing approximately 0.4 kg of strawberries per box were treated for three days at 0 °C in the presence of 20 or 40 kPa CO₂. The O₂ concentration was kept constant at 20 kPa, adjusting the N₂ concentration. CO₂ concentration was measured twice using a Check Mate 9900 O₂, O₂/CO₂ Headspace Analyser (Dansensor España, S.L.U.). After a three day exposure to the specific CO₂ concentration, the strawberries were removed from the containers, weighed and transferred to a similar container with a flux of air for a further day under the same temperature and humidity conditions (0 °C and 95% RH). Immediately after harvest, at the end of the three-day sampling period and one day after

150 exposure to air (day four), 45 strawberries were assessed for quality, while another 45
151 were randomly removed from each of the treatment groups and divided into three
152 batches of 15 berries. Each biological replicate was composed of 15 pooled strawberries
153 and each replicate was mixed, frozen in liquid nitrogen and stored at -80 °C for further
154 analysis.

155 2.2. Relative gene expression assessed by quantitative RT-PCR (RT-qPCR)

156 Total RNA was extracted three times from 0.4 g of each sample with CTBA based
157 extraction buffer, according to the protocol of Yu et al. (2012). The quality and purity of
158 the total RNA was evaluated by agarose gel electrophoresis and spectrometry
159 (NanoDrop 2000, Thermo Scientific). The RNA was then treated with DNase (DNase I,
160 RNase-free, Thermo) to remove any genomic DNA and cDNAs were synthesized from
161 1 µg of each sample using the iScript™ Reverse Transcription Supermix for RT-
162 qPCR (Bio-Rad). RT-PCR amplification was carried out in a 96 well-plate iCycler iQ
163 thermal cycler (Bio-Rad) and quantified using the iCycler iQ™-associated software
164 (Real Time Detection System Software, version 2.0), evaluating each gene in at least
165 two independent runs. The parameters for PCR were: one cycle at 50 °C for 2 min; one
166 cycle at 95 °C for 10 min; 40 cycles at 95 °C for 20 s and 60 °C for 1 min. Sequences
167 from the NCBI database and from the available literature were used to design the
168 following gene specific primers using Primer3 software.

169 The primer pairs used in the RT-qPCR for pyruvate decarboxylase (XM_004302484)
170 were FvPDC_F: GTTGCTTGAGTGGGGGTCTA and FvPDC_R:
171 ATCTGTGAATGCGAATGAAGG; and for alcohol dehydrogenase (XM_004290520),
172 were FvADH_QFw2: GCCCTTCTATACTGTGTCCTC and FvADH_QRv2:
173 ACTGTTCTGGCTGACTGGTT.

174 The relative expression of the genes studied was assayed by RT-qPCR. To calculate the
175 efficiency of the reaction (optimal range 90–110%) and to establish the most suitable
176 template concentration, the cDNAs synthesized from serial dilutions between 40 ng and
177 2.5 ng of total RNA were amplified. Standard curves and linear equations were
178 determined by plotting cycle threshold (Ct) values (y-axis) against the logs of the total
179 RNA (x-axis). The specificity of the products was validated by analyzing the
180 dissociation curves (evaluated in agarose gels) and by sequencing the products
181 (Genomic Department of the CIB-CSIC). The *actin-97-like* housekeeping gene from *F.*
182 *vesca* (XM_004307470) was not regulated by low temperature or high CO₂ levels (data
183 not shown) and therefore, it was used as the internal reference gene to normalize the
184 transcript profiles according to the $2^{-\Delta\Delta C_t}$ method and relative to the calibrator sample
185 (fruit at harvest). The *actin-97-like* mRNA was amplified with the primers FvActin_Fw
186 GGGTTTGCTGGAGATGATG and FvActin_Rv CACGATTGGCCTTGGGATTC.
187 Similarly, the specificity of the products was validated by analyzing the dissociation
188 curve in agarose gels and by sequencing.

189 2.3. Ethanol and acetaldehyde content

190 Ethanol and acetaldehyde were analyzed from the headspace of the juice of three
191 replicates of 15 strawberries without calyx, immediately after harvest and after each
192 period in storage. An aliquot (5 mL) of juice was transferred to 10 mL vials, closed
193 tightly with crimp-top caps and TFE/silicone septum seals, and frozen at -80 °C. Gas
194 Chromatography (Thermo Trace, Thermo Fisher Scientific) was used to measure the
195 ethanol and acetaldehyde according to the procedure of Valencia-Chamorro et al.,
196 (2009), expressing the results as **g per L of juice**.

197 2.4. Chromatographic determination of MDA

198 Frozen fruit samples (ca. 1 g) were homogenized in 10 mL of ultra-pure water,
199 centrifuged for 20 min at 30,000×g and after filtering through a 0.45 µm pore size
200 membrane, the supernatants were collected to quantify the malondialdehyde (MDA)
201 content. MDA was measured by HPLC as its hydrazone using 2,4-
202 dinitrophenylhydrazine (DNPH) for derivatization and following the method previously
203 described by Mateos et al., (2005), with slight modifications in the chromatographic
204 conditions (Blanch et al., 2015b). The concentrations were expressed as **mol MDA per**
205 **kg fresh weight**.

206 2.5. Determination of ATP, ADP and AMP

207 Frozen fruit samples (ca. 1 g) were homogenized with 5% (v/v) cold perchloric acid
208 (1:2.4; w/v), and the homogenate was centrifuged for 10 min at 6000×g and 4 °C. The
209 supernatant was neutralized to pH 6.5–6.8 with KOH and incubated for 15 min at 4 °C
210 before it was centrifuged for 10 min at 6000×g and 4 °C. After centrifugation, the
211 supernatant was filtered through a 0.22 µm nylon filter, and ATP, ADP and AMP were
212 analyzed by HPLC according to Palma et al., (2015). A relative calibration procedure
213 (0–20 µg mL⁻¹) was used to determine the ATP, ADP and AMP in the samples, and the
214 results were expressed as **mg of ATP, ADP or AMP kg⁻¹ of fresh weight**. The adenylate
215 energy charge was calculated according to Pradet and Raymond (1983): $([ATP] + 0.5 \times$
216 $[ADP])/([ATP] + [ADP] + [AMP])$.

217 2.6. Statistical analysis

218 **An analysis of variance (one-way ANOVA)** was performed using SPSS v. 19.0 and a
219 multi-comparison of the means was performed using Tukey's test, with the level of
220 significance set at $P < 0.05$.

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222 3. Results

223 3.1. Effect of different CO₂ concentrations on the PDC and ADH gene transcript 224 profiles

225 A cDNA fragment was obtained by RT-PCR from Mara des Bois strawberries using
226 specific primers designed from very conservative PDC sequences present in the
227 Rosaceae Genome Database (GDR) and the EST database, and it was then confirmed as
228 a PDC sequence. Specifically, we analyzed the expression of the gene encoding the
229 Fvpdc isoform 2-like (XP_004302532), which is the PDC isoform in *Fragaria vesca*
230 with the highest homology to Fapdc1 (AF333772) from *Fragaria x ananassa*, a gene
231 known to be induced by stress conditions (Moyano et al., 2004). The relative expression
232 of *PDC*, as well as changes in acetaldehyde levels were assessed in strawberries stored
233 at 0 °C in the presence of either 20 or 40 kPa CO₂ for three days, maintaining an O₂
234 concentration of 20 kPa, as well as after transfer to air for one further day (Fig. 1).
235 These results were compared with those from fruit of the same chronological age stored
236 in air for 4 days, and any changes were established relative to the fruit at harvest (day
237 0).

238 After three days in the presence of CO₂, the strawberries maintained the same levels of
239 *PDC* transcripts as those seen in the fruit at harvest (day 0), with only a slight decrease
240 in these transcripts after transfer to air. By contrast, a sharp increase in *PDC* expression
241 was observed in fruit stored in air for four days, whereby the fruit stored in air had 2.6
242 times more *PDC* transcripts than that of the same chronological age previously treated
243 with CO₂. Clearly, both CO₂ treatments (20 kPa and 40 kPa) reduced the accumulation
244 of *PDC* transcripts during storage at 0 °C with respect to the fruit stored in air. While
245 acetaldehyde accumulated in strawberries as the CO₂ concentration increased, the most

246 marked change in acetaldehyde levels was observed in strawberries exposed to 40 kPa
247 CO₂ at the end of treatment (Fig. 1B). These high levels of acetaldehyde diminished on
248 transfer to air, while low acetaldehyde content was quantified in the fruit stored in air
249 for four days. When these data are compared (Fig. 1A and B), there is no clear
250 correspondence between the low acetaldehyde content and the high accumulation of
251 *PDC* transcripts in strawberries stored in air.

252 Fig. 2 shows the relative quantification of *ADH* expression levels (A) and the changes in
253 ethanol levels (B) in Mara des Bois strawberries. We studied the expression of the gene
254 coding for the predicted *Fragaria vesca* ADH isoform XP_004290568, the sequence
255 with the highest homology to the ADH from *Fragaria x ananassa* (P17648) involved in
256 cold storage (Koehler et al., 2012). *ADH* expression increased irrespective of the CO₂
257 concentration to which the fruit was exposed, with more transcripts quantified in fruit
258 maintained in 20 kPa CO₂ than in 40 kPa CO₂. Like *PDC* and its corresponding
259 metabolite, the strongest expression of *ADH* was detected in fruit stored in air, in
260 conjunction with the lowest levels of ethanol (Fig. 2B). In addition, an inverse
261 relationship between ethanol and *ADH* expression was observed in fruit exposed to 40
262 kPa CO₂. The marked ethanol content in fruit maintained in 40 kPa CO₂ was therefore
263 associated with weaker *ADH* expression, with the ethanol levels even increasing after
264 transfer to air. Again, there was apparently no correspondence between the ethanol
265 content and *ADH* expression in strawberries during storage at 0 °C in air.

266 3.2. Effect of different CO₂ concentrations on ATP, ADP and AMP levels

267 The levels of ATP, ADP and AMP were assessed in strawberries at the end of the 3-day
268 exposure to either 20 or 40 kPa CO₂ (maintaining an O₂ concentration of 20 kPa), and
269 after transfer to air for one additional day (Fig. 3). The results after transfer were

270 compared with those from fruit stored in air of the same chronological age (4 days) and
271 any changes were considered relative to the fruit at harvest (day 0). There was a
272 progressive and significant depletion in ATP as the concentration of CO₂ increased.
273 Consequently, a stronger decrease in ATP was seen in fruit exposed to 40 kPa CO₂ than
274 that treated with 20 kPa CO₂. Conversely, high ATP levels were detected in strawberries
275 stored at 0 °C in air for four days. It is interesting to note that the high levels of ATP in
276 fruit stored in air were associated with low levels of ADP. By contrast, ADP levels were
277 higher in CO₂-treated fruit, mainly in those maintained at 40 kPa CO₂. With respect to
278 AMP, the highest levels were quantified in 40 kPa CO₂-treated fruit after transfer to air
279 for one day. The energy charge, and the ratio of both ATP/ADP and AMP/ATP, was
280 also evaluated (Fig. 3), and the energy charge was lower in CO₂-treated fruit than in
281 fruit at harvest. Interestingly, the depletion in the energy **charge** was as significant as the
282 increase in the levels of CO₂. Thus, the energy charge was significantly higher in fruit
283 exposed to 20 kPa CO₂ than to 40 kPa CO₂, with the lowest energy charge quantified at
284 the end of the 40 kPa CO₂ treatment. A high energy charge was detected in fruit stored
285 in air, with values above those of the fruit previously treated with CO₂. Moreover, these
286 fruit stored in air presented the highest ATP/ADP ratio. When the AMP/ATP ratio was
287 calculated it was highest in the fruit maintained in 40 kPa CO₂, with this value
288 increasing after transfer to air.

289 3.3. *Effect of different CO₂ concentrations on lipid peroxidation*

290 The impact of high CO₂ levels on lipid peroxidation was measured in terms of the MDA
291 content, detecting the DNPH derivative by HPLC-UV. MDA levels were assessed in
292 strawberries at the end of the 3-day treatment with either 20 or 40 kPa CO₂ and after
293 transfer to air for one additional day (Fig. 3). The level of MDA was significantly
294 higher in fruit stored at 0 °C without added CO₂ than that detected in strawberries at

295 harvest (33.2 g/kg fresh weight), reaching values of 53.4 g/kg fresh weight (Fig. 4). The
296 increase in MDA was 23% higher in fruit maintained in 20 kPa CO₂ at the end of the 3-
297 day treatment, reaching values of 45.8 g/kg fresh weight. Moreover, the MDA in the
298 strawberries increased after transfer to air. When maintained in 40 kPa CO₂ the MDA
299 levels in the fruit were as high as 64.7 g/kg fresh weight, increasing even further after
300300 transfer to air.

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302 4. Discussion

303 Cultivar variation has been reported in reference to the tolerance of strawberries to high
304 CO₂ concentrations and in the accumulation of fermentation products (Watkins et al.,
305 1999; Pelayo et al., 2003). Furthermore, differences in ATP and ADP content have been
306 attributed to different physiological states of avocados (also a fruit tolerant to high CO₂)
307 and to the length of CO₂ exposure (Lange and Kader, 1997). Here, the impact of
308 maintaining Mara des Bois strawberries from the second harvest in different high
309 concentrations of CO₂ were evaluated in terms of fermentation and energy metabolism.

310 The data indicate that the most significant changes in *PDC* expression were evident in
311 fruit stored at 0 °C in air without added CO₂, although low acetaldehyde content was
312 quantified. By contrast, there was no increase in *PDC* transcripts detected in
313 strawberries stored in 20 and 40 kPa CO₂, either at the end of the treatment or after
314 transfer to air for one day, although a rise in acetaldehyde levels was evident. CO₂ was
315 reported to inhibit *PDC* expression in Jewel strawberries during the first days of storage
316 at 2 °C (Ponze-Valadez and Watkins, 2008). Our results also indicate that there is no
317 correlation between *ADH* transcript accumulation and ethanol levels, showing that fruit
318 stored in air underwent the strongest changes in the expression of *ADH* and that it
319 accumulated the lowest levels of ethanol, even lower than at harvest. *ADH* expression in

Jewel strawberries also increased after storage at low temperature, although it was not correlated with the accumulation of ethanol (Ponze-Valadez and Watkins, 2008). Elevated acetaldehyde and ethanol concentrations have been reported in fruit held for extended periods in high CO₂ conditions (Wszelaki and Mitcham, 2000). Changes in fermentative gene expression have been detected in strawberries and in cultured cells subjected to anoxia and stress conditions (Moyano et al., 2004). Moreover, the induction of *ADH* and *PDC* expression appears to be low-temperature specific, as reported for *ADH* mRNA accumulation in several plant species (Christie et al., 1991; Jarillo et al., 1993). In terms of acetaldehyde and ethanol accumulation, it seems that fermentative metabolism is activated by high CO₂ concentrations, while fruit stored in air at low temperature do not support such rates of ethanol and acetaldehyde production. The induction of chilling tolerance by endogenous and applied ethanol has been reported (Frenkel and Erez, 1996), and our data show that a higher degree of fermentation occurs in 40 kPa CO₂ than in 20 kPa. Indeed, fermentative metabolism in fruit exposed to 40 kPa CO₂ was not depressed even after transfer to air for one further day.

In the absence of oxygen, the ability to maintain an active fermentative metabolism, by fuelling the glycolytic pathway with readily fermentable carbohydrates, is certainly crucial (Magneschi and Perata, 2009). Furthermore, it has been reported by Tadege et al., (1999) that under different stress conditions, which damages the mitochondrial ATP-generating machinery, the cells resort to ethanolic fermentation to regenerate NAD⁺ for the support of glycolytic ATP production. These authors suggest that fermentation might be an important switch in regulating carbohydrate metabolism. In Mara des Bois strawberries maintained in a high CO₂ environment, the activation of fermentative metabolism was associated with a marked decrease in the ATP/ADP ratio

345 and a low energy status, which favors ATP-generating catabolic pathways. A lower
346 ATP/ADP ratio in low O₂-treated pear discs was also reported (Nanos and Kader,
347 1993), indicating a lower energy charge. By contrast, in strawberries stored in air
348 without added CO₂ there was a sharp increase in the ATP/ADP ratio, coupled to a
349 significant decrease in soluble sugars (Blanch et al., 2015a). Similarly, a decrease in
350 sucrose content was reported in different varieties of strawberries during cold storage at
351 6 °C (Cordenunsi et al., 2003). Considering that respiration in heterotrophic plant tissues
352 is mostly regulated by intracellular ADP levels (or the ATP/ADP ratio) and the supply
353 of substrates (Shugaev and Bukhov, 1997), the decrease in sucrose content coupled to
354 the high ATP levels and a high ATP/ADP ratio could suggest higher respiration and
355 metabolic activity in fruit stored at 0 °C in air than in that stored in high CO₂ conditions.
356 In this sense, the ATP content of avocado fruit was reported to be closely connected to
357 the increase in the respiration rate (Bennett et al., 1987), and a positive correlation
358 between energy status and the respiration rate was shown (Huang et al., 2014). An
359 increase in the respiration rate immediately after removal from extended cold storage,
360 possibly indicating chilling injury, has also been reported in other fruit (Galli et al.,
361 2009). Moreover, chilled fruit exhibit higher ATP levels than ripe fruit, as quantified by
362 phosphorus NMR (Muñoz et al., 2001). Here, a decrease in the ATP/ADP ratio and the
363 adenylate energy charge was observed in strawberries as the CO₂ concentration
364 increases. Thus, while there are numerous examples where low O₂ conditions
365 effectively suppress the intensity of respiration, there are quite varied responses in terms
366 of the effect of elevated CO₂ (Kubo et al., 1990). Blanke (1991) indicated that fruit
367 subjected to CO₂ shock had a progressive reduction in respiration.

368 The decrease in energy charge in CO₂-treated strawberries, determined in our results,
369 may reflect the inhibition of a wide range of ATP-consuming processes. It is clear that

370 by reducing the demand for ATP to a threshold level, 20 kPa CO₂-treated fruit not only
371 diminish the depletion rate of fermentative substrates but also, they reduce the rate of
372 excessive anaerobic end product formation. Accordingly, the reported perturbation due
373 to the direct or indirect effects of enhanced ethanol and/or acetaldehyde could be
374 minimized, including those on the membrane (Slater et al., 1993; Pesis, 2005).
375 However, significant differences in the adenylate pools were observed in strawberries
376 exposed to 20 and 40 kPa CO₂. Consequently, exposure to 40 kPa in the absence of any
377 additional adjustments provoked an excessively low energy charge, and the fruit was
378 not able to avoid harmful fermentation

379 The excessively low energy charge and the excessive decrease in ATP in fruit exposed
380 to 40 kPa CO₂ could not match the ATP requirements for anabolic synthesis of critical
381 antioxidant compounds, a process that ultimately increases ROS formation and the
382 successive peroxidation of lipids. In this sense, a threshold in ATP synthesis has been
383 found, below which potato cells under anoxia become committed to hydrolysis of their
384 membrane lipids (Rawlyer et al., 1999). Since the detrimental changes in the cell
385 structure and metabolism as a consequence of oxidative stress are manifested through
386 enhanced lipid peroxidation, the significant formation of MDA in 40 kPa CO₂-treated
387 fruit indicates that oxidative damage is part of the stress induced by high CO₂
388 concentrations. Moreover, the increase in MDA levels was higher after transfer to air,
389 further indicating that the anabolism of the radical-scavenging system is impaired and
390 membrane lipids are attacked more easily by oxidative stress than in the fruit at the end
391 of treatment. The increase in AMP/ATP ratio in stressed high CO₂-treated fruit might
392 act as a signal of the activation of specific metabolic pathways that should be further
393 studied.

394 It has been reported that re-aeration of highly reduced anoxic tissues leads to the
395 formation of harmful oxygen radicals and toxic oxidative products, resulting in rapid
396 peroxidative damage (Biemelt et al., 1998). The effects of controlled or modified
397 atmosphere on oxidative stress appear to be commodity specific, yet if applied correctly
398 they could greatly reduce or suppress oxidative stress (Hodges et al., 2004). At the
399 cellular level, the increase in lipid peroxidation is responsible for the alterations to the
400 physical properties of the membrane. Moreover, these results confirm our previous data
401 showing that when the concentration and/or length of exposure to high CO₂ was above
402 the tolerance threshold, excess of ethanolic fermentation potentially caused a loss of the
403 bound water fraction, which resembles cellular water stress (Blanch et al., 2015a). Our
404 results also indicate that excessively high CO₂ (40 kPa) accelerates the loss of
405 membrane integrity, provoking oxidative damage, concomitant with the appearance of
406 higher levels of fermentative volatiles, as mentioned above.

407 In conclusion, the changes to fruit energy metabolism can be interpreted as adaptations
408 in order to tolerate high CO₂ concentrations levels regardless of the O₂ present. These
409 adaptations include lowering the adenylate status to a desirable level, the activation of
410 fermentative metabolism and the depression of metabolism giving priority to ATP-
411 generating catabolic pathways. In terms of the activation of fermentative metabolism,
412 the induction of fermentative genes does not seem to be essential. By contrast, there is a
413 marked increase in the ATP/ADP ratio and a lack of fermentation metabolism in fruit
414 stored in air, associated with a high energy charge, which favors ATP-consuming
415 anabolic pathways. However, in the presence of excessively high concentrations of CO₂
416 (40 kPa), the accumulation of fermentative products above a threshold and the overly
417 low energy status that is associated with a strong depression of the anabolic processes

418 requiring ATP (such as an excessive reduction of defense strategies), could explain why
419 the oxidative damage and formation of MDA increases markedly in such fruit.

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576 Figure Legends

577 Figure 1. Relative expression of the PDC gene XM-004302484 (A) and the
578 acetaldehyde content (B) in Mara des Bois strawberries stored at 0 °C with 20 or 40 kPa
579 CO₂ for 3 days, and after transfer to air for one further day. The O₂ concentration was
580 kept at 20 kPa throughout. The results of transfer to air were compared with the fruit of
581 the same chronological age stored in air throughout (four days) and any changes were
582 relative to the fruit at harvest (day 0). The transcripts were measured by quantitative
583 RT-PCR and normalized against those of *actin-97-like* used as a reference gene. The
584 results were calculated relative to a calibrator sample (fruit at harvest, day 0) using the
585 formula $2^{-\Delta\Delta C_t}$ and the values represent three biological replicates per three repeated
586 measures. (B) Changes in acetaldehyde (g L⁻¹) content under the same conditions
587 described above. Each letter indicates significant differences between the means, as
588 determined with Tukey's test ($P < 0.05$).

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590 Figure 2. Relative expression of the ADH gene XM-004290520 (A) and the ethanol
591 content (B) in Mara des Bois strawberries stored at 0 °C for 3 days with 20 or 40 kPa
592 CO₂ and after exposure to air for 1 further day. The O₂ concentration was kept at 20 kPa
593 throughout. The results of the transfer to air were compared with the fruit of the same
594 chronological age stored in air throughout (four days) and any changes were relative to
595 the fruit at harvest (day 0). The transcripts were measured by quantitative RT-PCR and
596 normalized against those of *actin-97-like* used as a reference gene. The results were
597 calculated relative to a calibrator sample (fruit at harvest, day 0) using the formula $2^{-\Delta\Delta C_t}$
598 and the values represent three biological replicates per three repeated measures. (B)
599 Changes in ethanol (g L⁻¹) content under the same conditions described above. Each

600 letter indicates significant differences between the means, as determined with Tukey's
601 test ($P < 0.05$).

602

603 Figure 3. The ATP, ADP and AMP levels in Mara des Bois strawberries stored at 0 °C
604 for 3 days in the presence of 20 or 40 kPa CO₂ and after exposure to air for 1 further
605 day. The O₂ concentration was kept at 20 kPa throughout. The results of transfer were
606 compared with the fruit of the same chronological age stored in air throughout (four
607 days) and any changes were relative to the fruit at harvest (day 0). The data represent
608 two repeated measures from three biological replicates and the letters indicate
609 significant differences between the means, as determined with Tukey's test ($P < 0.05$).

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611 Figure 4. Change in MDA determined by HPLC in Mara des Bois strawberries stored at
612 0 °C for 3 days in the presence of 20 or 40 kPa CO₂ and after exposure to air for 1
613 further day. The O₂ concentration was kept at 20 kPa throughout. The results of transfer
614 were compared with the fruit of the same chronological age stored in air throughout
615 (four days) and any changes were relative to the fruit at harvest (day 0). The data
616 represent two repeated measures from three biological replicates and the letters indicate
617 significant differences between the means, as determined with Tukey's test ($P < 0.05$).

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