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Scotland's Rural College

Husk to caryopsis adhesion in barley is influenced by pre- and post-anthesis temperatures through changes in a cuticular cementing layer on the caryopsis

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1 # Husk to caryopsis adhesion in barley is influenced by pre- and post-
2 anthesis temperatures through changes in a cuticular cementing layer on
3 the caryopsis

4 Running title: Barley husk adhesion is influenced by pre- and post-anthesis temperature

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16

17

Abstract

Background

At ripeness, the outer husk of “covered” barley grains firmly adheres to the underlying caryopsis. A cuticular cementing layer on the caryopsis is required for husk adhesion, however the quality of adhesion varies significantly among cultivars which produce the cementing layer, resulting in the economically important malting defect, grain skinning. The composition of the cementing layer, and grain organ development have been hypothesised to influence the quality of husk adhesion. Plants of *Hordeum vulgare* ‘Concerto’ were grown at different temperatures pre- and post-anthesis to effect changes in the development of the husk, caryopsis and cuticular cementing layer, to determine how these variables influence the quality of husk-to-caryopsis adhesion.

Results

Warm conditions pre-anthesis decreased the quality of husk adhesion, and consequently increased the incidence of grain skinning. Cool post-anthesis conditions further decreased the quality of husk adhesion. The composition of the cementing layer, rather than its structure, differed with respect husk adhesion quality. This cementing layer was produced at the late milk stage, occurring between nine and 29 days post-anthesis, conditional on the temperature-dependent growth rate. The compounds octadecanol, tritriacontane, campesterol and β-sitosterol were most abundant in caryopses with high-quality husk adhesion. The differences in adhesion quality were not due to incompatible husk and caryopsis dimensions affecting organ contact.

Conclusions

40 This study shows that husk-to-caryopsis adhesion is dependent on cementing layer
41 composition, and implies that this composition is regulated by temperature before, and
42 during grain development. Understanding this regulation will be key to improving husk-to-
43 caryopsis adhesion.

44

45 **Keywords:** Caryopsis; Cementing layer; Grain development; Grain skinning; *Hordeum*
46 *vulgare*, Husk adhesion; Malting barley

47

48

49 ## Background

50 The mature barley grain comprises the caryopsis (fruit) enclosed in an outer husk which
51 adheres to the caryopsis during grain development, and remains adherent at harvest and
52 during post-harvest handling. If the husk becomes partially or wholly detached, the grain
53 has "skinned". Grain skinning, also known as "peeling", is a quality defect for malting barley.
54 During malting, grains undergo a process of controlled germination, during which enzymes
55 "modify" the starchy endosperm by hydrolysing the cell wall and protein matrix, making
56 starch available for fermentation by yeasts during brewing and distilling. Good quality husk
57 adhesion serves to protect the embryo from mechanical damage during harvesting, and
58 processing by maltsters, brewers and distillers. As a viable embryo is essential to the malting
59 process, high levels of grain skinning leads to inefficiencies in malt production. Further, if a
60 grain has skinned but retains a viable embryo, such grains will imbibe water more quickly
61 and may over-modify with respect to intact grains, resulting in malting losses [1–3]. The
62 quality of husk adhesion is influenced by both environmental and genetic factors, and the
63 quality defect grain skinning has increased in severity in recent years with newer varieties
64 being more susceptible to the condition [4]. Ensuring the supply of high quality grain by
65 local growers is essential for the long-term sustainability of malting barley supply chains
66 worldwide, reducing the need to transport grain bulks long distances to make up any quality
67 shortfall. Reducing the risk of grain skinning by increasing the quality of husk adhesion will
68 benefit growers, barley breeders and processors within the malting supply chain.

69

70 The barley caryopsis comprises the embryo, the starchy endosperm and the outer aleurone
71 endosperm, surrounded in turn by the nucellar layer, the testa (seed coat), and the pericarp

72 (fruit coat). The outer husk is composed of two glumes, namely the lemma on the dorsal
73 side of the grain, and the palea on the ventral side of the grain [5, 6]. It has been
74 hypothesised that physical damage to malting barley grains, including grain skinning, may be
75 exacerbated by changes in grain length and width, and an incompatibility between grain size
76 and the mechanical strength of the outer husk tissues [7, 8]. Physical contact between the
77 caryopsis and the husk could be expected to differ with changes in grain or glume size.
78 Although there are limited data on differential grain development that relate directly to the
79 husk-caryopsis adhesion process, studies on the effects of temperature differences pre- and
80 post-anthesis on husk and caryopsis development are useful to understand how
81 temperature could be used to manipulate differential growth of these organs and therefore
82 contact between the caryopsis and glumes. For example, higher grain weight attained by
83 increased starch accumulation during growth at low temperatures [9] might increase grain
84 dimensions and result in better contact, and therefore adhesion, between the caryopsis and
85 the husk. Coversely, grains with reduced weights caused by high temperature stress [10, 11]
86 might be expected to have reduced husk-caryopsis contact, resulting in poor quality
87 adhesion. Equally, the size of the husk organs would contribute to the capacity for contact
88 between the husk and caryopsis. Indeed, grain size has been postulated to be determined
89 by the physical limitation of the size of the husk [12, 13], potentially due to effects of pre-
90 anthesis temperature on floret growth [14].

91

92 The husk adheres to the underlying caryopsis through a cementing layer, which is thought to
93 be composed of lipids [15–18]. The barley grain contains three internal lipid layers. The
94 thinnest is present in between the nucellar layer and the testa, whereas the thickest is

95 present between the testa and the pericarp. These tissues and the lipid layers develop as a
96 unit. The third lipid layer, the cementing layer, develops after the pericarp cuticle is formed.
97 This cementing layer is 100 to 600 nm thick [15], covering the pericarp cuticle and adhering
98 to the inner husk surfaces later in grain development. This sticky cementing substance is
99 reported to be produced from 10 days after anthesis [15, 17]. However, it has not yet been
100 determined whether this substance is always produced by 10 days after anthesis regardless
101 of the rate of grain development, or whether it is the developmental stage that governs
102 production of the cementing layer. An irregular, reticulate interface exists between the
103 pericarp cell wall and the cuticle during production of the cementing layer, indicating that it
104 is the epidermal cells of the pericarp, rather than the husk, that produces the cementing
105 material [8, 15]. Hull-less, or "naked" barley does not produce a cementing layer, and
106 naturally threshes free of the husk at harvest. Naked barley has a mutation at the *nudum*
107 (*nud*) locus on chromosome 7H, which is homologous to the *WIN1/SHN1* transcription
108 factor gene of *Arabidopsis* thought to regulate a lipid biosynthetic pathway [18]. Although
109 hull-less barley does not produce a cementing layer, it does produce a cuticle on the
110 pericarp surface [15] which is not able to be dyed by Sudan dyes, whereas the caryopses of
111 "covered" barley are dyed by Sudan Black [18]. This suggests that the cementing substance
112 is likely to be similar to, or part of, the solvent-extractable surface lipid layer of the pericarp
113 cuticle.

114

115 The location of the cementing layer, either comprising, or lying between, the surface
116 cuticles of the husk and caryopsis, suggest it is likely to be similar in nature and composition
117 to other plant cuticles. Plant surface cuticles are synthesised by epidermal cells and their

118 general structure can be described as having two domains, although it is more commonly
119 being viewed as a lipid-embedded continuation of the cell wall itself [19, 20]. The inner
120 domain is rich in the polymer cutin and is physically associated with the cell wall, often
121 referred to as the "cuticular layer" [21]. The outer domain is rich in wax compounds that are
122 soluble in organic solvents and is termed the "cuticle proper". The waxy domain has two
123 distinct layers, one with intracuticular waxes embedded in a cutin matrix, and an outer
124 epicuticular layer of waxes that coat the surface and may form crystalline structures. The
125 synthesis of cutin and cuticular wax has been well reviewed [22–26]. Cutin is a polyester of
126 hydroxy and hydroxy epoxy C_{16} and C_{18} fatty acids. Cuticular waxes comprise a complex
127 mixture of compounds including alcohols, alkanes, alkenes, aldehydes, ketones, triterpenes,
128 esters and fatty acids. Biosynthesis of plant cuticular wax is achieved by *de novo* synthesis of
129 fatty acyl chains from a malonyl-CoA precursor by the fatty acid synthase complex. Fatty
130 acids are then elongated by fatty acid elongase. Further elongation and modification to
131 major wax compounds proceeds via either of two pathways: the decarbonylation pathway
132 or the acyl reductive pathway. The major components of barley leaf surface waxes are
133 primary alcohols, with 1-hexacosanol (C_{26} alcohol) accounting for more than 75%
134 extractable cuticular wax from leaves [27, 28].

135

136 Although the composition of plant cuticles differs among organs of the same plant, and
137 even among cultivars [29, 30], the compound classes that comprise the cuticles of different
138 organs are typically the same. The pericarp cuticles of fruit undergo significant
139 compositional changes during fruit development and ripening [31–34]. Like the cuticles of
140 other plant organs, the composition of fruit cuticles is also dependent on environmental

141 factors such as temperature, light, humidity and pathogen attack [35–37]. Cuticle properties
142 such as transpiration rate and permeability, are more highly influenced by their composition
143 rather than by the thickness of the cuticle, or amount of wax. Indeed, mutations in several
144 cuticle-synthesis associated genes alter cuticle permeability, and display an organ fusion
145 phenotype [38–42], which has similarity to the process of husk adhesion, in that adjacent
146 organs adhere to one another through the cuticle [18, 43].

147

148 This study used differential temperatures during husk and grain development to separately
149 effect changes in husk and grain size, the structure and composition of the surface cuticles
150 and therefore the cementing layer. It was hypothesised that the quality of husk adhesion,
151 and therefore the severity of grain skinning, would be influenced by one or all of the above,
152 and that by measuring these we would gain insight into critical grain and cuticle
153 developmental stages that influence husk-to-caryopsis adhesion

154

155 Methods

156 Plant growth and sampling

157 A two-row spring barley (*Hordeum vulgare* cv. Concerto) known to be susceptible to
158 skinning [4] was sown directly in Levingtons No. 2 compost, at a density of seven plants per
159 pot in a total of 36 four-litre pots, and established in a glasshouse until the first leaf came
160 through the coleoptile (GS 10). Developmental growth stages (GS) referred to throughout
161 the study are those of the decimal code described by Tottman and Broad [44]. Thereafter,
162 pots were moved into two Sanyo Fitotron (SGC097 CSX.F) growth cabinets set at day/night

163 temperatures of 18°C/13°C, 79% relative humidity, 200 µmol $m^{-2}s^{-1}$ photosynthetically
164 active radiation and grown for four weeks under a 10 h photoperiod to reduce tillering [10,
165 45]. The cabinet conditions were then changed to "cool" or "warm" temperatures set to
166 day/night 13°C/7°C or 28°C/22°C respectively, with relative humidity 79% and a 16 h
167 photoperiod. The position of the pots within the cabinets was rearranged weekly to reduce
168 positional effects by moving every pot one position to the left each week in the manner of
169 MacNicol et al.[10]. The date of anthesis (GS 65, flowering half-way complete) was
170 determined by visual inspection of the florets. Half of the pots (18) from each cabinet were
171 then switched between the warm and cool cabinets at anthesis, giving a total of nine pots
172 for each of the following four treatments depending on temperatures pre- and post-
173 anthesis, respectively: cool, cool = "CC"; cool, warm = "CW"; warm, warm = "WW" and
174 warm, cool = "WC". Growth stages were monitored from booting (GS 45) through to
175 harvest-ripeness (GS 92). Thermal time (°C days) for development was calculated using the
176 mean hourly temperature for the cool (16.3°C) and warm (26.0°C) cabinets, multiplied by
177 the number of days until that development stage was reached. Three ears from the main
178 shoots were harvested at growth stages 45, 51 and 65 from the two warm and cool pre-
179 anthesis treatments, and at post-anthesis growth stages 75, 77, 85 and 92 from the four
180 treatments listed above. The three ears at each growth stage were harvested from separate
181 pots to ensure spatial replication. The three central florets or grains from one side of each
182 ear were processed for electron microscopy. The five central florets or grains from the
183 opposing side of the ear were used to measure dimensions and fresh weights of the organs
184 (palea, lemma and caryopsis) with a micrometer (accuracy ± 0.05 mm) and Mettler Toledo
185 XP6 microbalance (accuracy ± 1 µg), and then processed for surface lipid analysis.

186

187 A separate experiment was done to examine the caryopsis surface after development of the
188 cementing layer using scanning electron microscopy. Plants of Concerto, and the hull-less
189 variety Nudinka, were grown in pots in a glasshouse as described above. Grains from both
190 cultivars were harvested at GS 77, when in covered barley, the caryopsis is sticky to the
191 touch. These grains were then fixed and processed for scanning electron microscopy as
192 described below.

193

194 **Skinning quantification**

195 Grain skinning was assessed using an in-house procedure, where a threshold of 20% or
196 greater husk loss by area was used to distinguished skinned grains from intact grains (less
197 than 20% husk loss) [4]. Remaining ears from the main shoots and tillers were harvested at
198 ripeness and the ear length, floret number, grain number and grain weights per ear were
199 measured. Hand-threshed ears were then further threshed in a Wintersteiger LD 180
200 laboratory thresher (Wintersteiger AG, Ried, Austria) for five s, and grains scored for
201 skinning.

202

203 **Transmission and scanning electron microscopy**

204 For transmission electron microscopy of husk material, segments (3 mm long × 1 mm wide ×
205 1 mm thick) were cut from the centre of the lemma, and spanning one vascular bundle from
206 the palea. Segments of the same size were cut from the centre of the dorsal side of the

207 caryopsis, taking care to ensure that the segments excised included all cell layers down to
208 the starchy endosperm, and avoiding the area over the embryo which does not produce a
209 cementing layer. Segments were fixed in 4% (w/v) paraformaldehyde and 2% (w/v)
210 glutaraldehyde in 100 mM sodium 1,4-piperazinediethanesulfonic acid (PIPES) buffer (pH
211 7.2) for four hours at room temperature, then overnight (18 h) at 4°C. Fixed tissue was
212 washed three times in 0.1 M sodium cacodylate buffer (pH 7.3) for 10 minutes each time.
213 Tissue was then post-fixed in 1% osmium tetroxide in sodium cacodylate for 45 minutes at
214 room temperature, then washed in three 10 minute changes of sodium cacodylate buffer.
215 Washed tissue was dehydrated in an aqueous ethanol series (50, 70, 90, and three × 100%)
216 for 15 min each step, and then twice in propylene oxide for 10 min each time. Samples were
217 then embedded in TAAB 812 resin (TAAB laboratories, Berks, England). Sections, 1 µm thick,
218 were cut on a Leica Ultracut ultramicrotome (Leica Microsystems, Milton Keynes, UK),
219 stained with 1% aqueous toluidine blue in 1% borax and viewed on a light microscope to
220 select suitable areas for investigation. Ultrathin sections, 60 nm thick, were cut from
221 selected areas, stained in 1% aqueous uranyl acetate and Reynolds lead citrate then viewed
222 in a Philips CM120 BioTwin transmission electron microscope (Philips Electron Optics,
223 Eindhoven, The Netherlands). Images were taken on a Gatan Orius CCD camera (Gatan,
224 Oxon, UK). The thickness of the inner and outer cuticles (palea and lemma), outer cuticle
225 (caryopsis) and cementing layer (whole grain) was measured using the open-source
226 software Image J [46]. The mean thickness of the cuticular layers for each of the three
227 replicate ears was calculated by taking the mean of five measurements from each of five
228 micrographs per replicate.
229

230 For scanning electron microscopy, tissue was cut into 4 mm x 4 mm segments from the
231 dorsal side of the caryopsis and fixed and dehydrated as above. Samples were dried in a
232 Polaron Critical Point Drier (Quorum Technologies Ltd, Lewes, UK), mounted on aluminium
233 stubs, and sputter coated with 20 nm gold palladium in an Emscope SC500A sputtercoater
234 (Emscope, Kent, UK) before examining with a Hitachi S-4700 scanning electron microscope
235 (Hitachi, Japan).
236

237 **Surface lipid analysis**

238 Surface lipid extracts were prepared from the husks (pooled paleas and lemmas) and
239 caryopses of five central grains from replicate ears as described above. Organs were dipped
240 in dichloromethane (puriss. p.a. grade for GC ≥99.9%, Sigma-Aldrich, UK) for 20 s at room
241 temperature and the extract evaporated to dryness under N_2 (British Oxygen Company,
242 99.995%). Extracts were re-solubilised in isohexane (HPLC Plus grade for GC ≥98.5%, Sigma-
243 Aldrich, UK) containing 50 ppm BHT (2,6-di-tert-butyl-4-methylphenol) (Sigma-Aldrich, UK)
244 and evaporated to dryness as above. Methyl nonadecanoate (0.5 µg) (Sigma-Aldrich, UK)
245 was added to each sample as an internal standard. Compounds in the extracts with free
246 hydroxyl and carboxyl groups were derivatised to TMSi ethers and esters esters by addition
247 of 25 µl *N*-*O*-bis-trimethylsilyltrifluoroacetamide (BSTFA, ThermoScientific, UK) and 25 µl
248 anhydrous pyridine (Sigma-Adrich, UK) at 50°C for 90 min with agitation every 30 min. Wax
249 constituents were analysed by gas chromatography-mass spectrometry (GC-MS) using a
250 Trace DSQ™ II Series Quadrupole system (Thermo Electron Corporation, Hemel Hempstead,
251 UK), fitted with a CTC CombiPAL autosampler (CTC Analytics, Switzerland). Samples (1 µl)
252 were injected into a programmable temperature vaporising (PTV) injector operating in

253 splitless mode and fitted with a Merlin Microseal™ High Pressure Septum and a Siltek™
254 deactivated metal PTV liner (120 mm × 2 mm internal diameter × 2.75 mm external
255 diameter, Thermo Scientific, UK). The PTV conditions were injection temperature 132°C for
256 1 min, transfer rate 14.5°C s^{-1}, transfer temperature 320°C for 1 min, clean rate 14.5°C s^{-1}
257 and clean temperature 400°C for 2 min. Chromatography was effected on a DB5-MS™
258 column (15 m × 0.25 mm × 0.25 µm; Agilent Technologies, UK) using helium at 1.5 ml min^{-1}
259 (constant flow). The GC temperatures were 100°C for 2.1 min, 25°C min^{-1} to 320°C, then
260 isothermal for 3.5 min. The GC-MS interface temperature was 325°C. Mass spectrum
261 acquisition conditions were electron impact (EI) ionisation at 70 eV, solvent delay 1.3 min,
262 source temperature 230°C, mass range 35 to 900 a.m.u. at 6 scans s^{-1}. Acquisition rates
263 were set to give approximately 10 data points across each chromatographic peak. Data were
264 acquired and analysed using Xcalibur™ 2.0.7 (Thermo Electron Corporation, Hemel
265 Hempstead, UK). Specific ions characteristic of each compound in the husk and caryopsis
266 samples, including the internal standard (IS), were selected following examination of total
267 ion chromatograms (TIC) for several raw data files of both types of sample. Ion selection was
268 on the basis that they should have as high a relative abundance as possible and should be
269 unique to the compound and/or be well resolved from other ions with the same m/z [47].
270 These ions were used for compound detection and quantification in a processing method
271 created in Xcalibur™. For each compound a time window was defined, centred on the
272 chromatographic peak apex and a summed selected ion chromatogram (SIC) for all of the
273 chosen ions was generated within the time window. Response ratios for each analyte were
274 calculated relative to the IS using the calculated SIC areas for both components. Processed
275 data were checked for correct peak assignment and adjusted where necessary. Compounds
276 were identified by comparison of their mass spectra and retention times with reference

277 standards, MS libraries (Palisade 600k, Palisade Corporation, USA; NIST05, National Institute
278 of Standards, USA), by comparing with retention and MS data for known compounds and by
279 reference to published data. A total of 121 compounds were identified, the masses used for
280 compound identification and quantification are given in Additional file 1, Table S1. The
281 "abundance" of each compound was calculated from the summed relative response of the
282 selected ions for that compound, divided by the number of organs extracted for that
283 sample; the abundance of each compound was then used for statistical analysis as below,
284 abundance data are given in Additional file 1, Table S2.

285

286 For each class of compound present in the samples, specific ion groups were selected as
287 follows:

288 Fatty acids: Two characteristic ion groups were used for identification and quantification of
289 fatty acids as their TMS ester derivatives. The intense ion group *m/z* 117, 129, 132 and 145
290 are common to all fatty acids and for most acids were used for quantification. The
291 prominant molecular ion $[M]^+$ and $[M\text{-}15]^+$ ions were used to confirm identification of
292 individual acids. However, 14-methylhexadecanoic acid co-eluted with 8-Heptadecenoic
293 acid, and both contribute to *m/z* 117, 129, 132 and 145. The ratio of the *m/z* 117, 129, 132,
294 145 ion group to $[M]^+$ plus $[M\text{-}15]^+$ for the earlier eluting 10-methylhexadecanoic acid was
295 calculated. Assuming the same ratio for 14-methylhexadecanoic acid, the abundance of $[M]^+$
296 and $[M\text{-}15]^+$ ions for this compound were used to estimate its abundance of *m/z* 117, 129,
297 132 and 145, and hence the abundance of *m/z* 117, 129, 132, 145 due to 8-Heptadecenoic
298 acid. Octacosanoic acid co-elutes with the TMS derivative of campesterol, both of which
299 share *m/z* 129. Consequently, only ions *m/z* 117 and 132 were used for measurement of the

300 abundance of octacosanoic acid, and the measured abundance was multiplied by a
301 correction factor of 1.76 to account for the absence of *m/z* 129 and 145. The correction
302 factor was deduced from data for campesterol-free octacosanoic acid.

303

304 Fatty alcohols: The characteristic homologue-specific $[M-15]^+$ ions were used for
305 measurement of the abundance of the TMS ether derivatives of long chain alcohols.

306

307 Wax esters: The long chain alky esters were identified using two characteristic ions, the
308 molecular ion $[M]^+$ from which the overall carbon number could be deduced, and the
309 prominent McLafferty rearrangement ion $[RCO_2H_2]^+$ arising from the acid portion of the
310 intact ester from which the carbon numbers of the individual acid:alcohol combination
311 could be deduced [48]. Abundance measurements were based on the McLafferty ion.

312

313 Alkanes and alkenes: The identity of the most prominent alkane and alkene homologues
314 were determined from their molecular ions $[M]^+$ from which the identities of the minor
315 homologs could be deduced by interpolation. Abundance measurements were based on the
316 characteristic series of fragment ions of mass $[C_nH_{2n+1}]^+$ for alkanes and $[C_nH_{2n-1}]^+$ for alkenes
317 which were common to all homologues.

318

319 Ketones, ß-diketones, enols and hydroxy-ß-diketones: Mass spectral fragmentation patterns
320 for these compounds are shown in Additional file 2, in which diagnostic Ions seen in the

321 mass spectra are marked with an asterisk and those used for quantification are underlined.
322 The mass spectra of nonacosan-14-one and hentriacontan-14,16-dione are dominated by
323 ions arising from fragmentation α or ß to the carbonyl group, the latter also involving
324 hydrogen transfer, typical of ketones and ß-diketones [49, 50]. In the MS of ß-diketones a
325 prominant ion of *m/z* 100 forms from sequential fragmentations on opposite sides of the
326 molecule ß to the diketo group. Other diagnostic fragmentations include loss of 18 from the
327 molecular ion or other fragments. In Additional file 2 diagnostic ions seen in the mass
328 spectra are marked with an asterisk and those used for quantification are underlined. Mass
329 spectral fragmentation schemes for the different enol and $(enol)_2$ tautomers of
330 hentriacontan-14,16-dione are dominated by ions arising from fragmentation α or ß to the
331 carbonyl or OTMS groups, with fragments incorporating OTMS being favoured [51]. The
332 mass spectra of the four enols appear to be very similar and it was not possible to
333 distinguish between them on the basis of their mass spectra since the same ion groups were
334 used for identification and quantification. The same was the case for the three $(enol)_2$
335 tautomers. Rather than attempting to separate the selected ion chromatogram trace for the
336 chosen ions into separate portions for each tautomer, the whole trace was integrated to
337 provide an abundance measurement for all tautomers in combination. Finally, the
338 abundance measurements for the enol and $(enol)_2$ components were combined with that
339 for hentriacontan-14,16-dione to provide an overall abundance measurement for the ß-
340 diketone. Fragmentation α to the OTMS group gives rise to the major diagnostic ions in the
341 mass spectra of 8- and 9-hydroxyhentriacontan-14,16-diones, although some ions arising
342 from fragmentation to the carbonyl groups are also evident.

343

344 5-Alkyl resorcinols: Members of the two homologous series of 5-alky resorcinols are
345 distinguished by the intense fragment ions at *m/z* 268 for unsubstituted homologues and
346 *m/z* 282 for methyl substituted homologues in the mass spectra of the diTMS derivatives,
347 arising from fragmentation between C1 and C2 of the 5-alkyl chain [52, 53]. Individual
348 homologues are identified from their relatively intense molecular ions. The exact position of
349 the methyl group in the methyl substituted compounds cannot be distinguished from the
350 mass spectra, but must be either within the aromatic ring or at C1 of the alkyl side chain.

351

352 Terpenes: Ions characteristic of each of the terpenes including free squalene and cholesta-3,
353 5-diene and sterols and γ-tocopherol as TMS ethers were used for compound identification
354 and characterisation. For most sterols, *m/z* 129 was used as one of the ions, but was
355 excluded for campesterol due to co-elution with octacosanoic acid.

356

357 **Statistical analysis**

358 The data were analysed using the open-source software R [54]. The fit of models described
359 below were checked by plotting residuals against fitted values, and also by plotting fitted
360 values against observed values. The effect of treatment on skinning severity was analysed
361 by fitting a generalized linear model [55] to the binomial counts of skinned and total grains
362 for each ear, using "treatment" as the predictor variable. Calculation of 95% profile
363 likelihood confidence intervals was used to determine significant differences among
364 treatments. The effect of the four treatments on ear measurements at harvest ripeness was
365 determined by analysis of variance (ANOVA) ($\alpha = 0.05$) followed by post-hoc Tukey's HSD

366 tests (α = 0.05) where a significant effect was found. During plant development, the effect
367 of treatment and growth stage on organ weights and dimensions were analysed separately
368 for three phases of development: husk development (pre-anthesis), grain development and
369 husk adhesion. For each phase a generalized linear mixed effects model was built with the
370 measurement as the response variable, treatment, growth stage and their interaction as
371 fixed effects, with the random effect being ear nested within pot. The final minimally-
372 adequate models were selected by dropping non-significant variables (α = 0.05) on
373 comparison of hierarchical models using ANOVA, and significant differences among samples
374 determined by least squares means comparisons [56]. The effect of treatment and growth
375 stage on the composition of surface lipid extracts was tested for each organ type (husk and
376 caryopsis) separately using a linear mixed effects model. Compound abundance was the
377 response variable, with treatment, growth stage and their interaction as fixed effects, and
378 pot number as the random effect. Hierarchical models were compared by ANOVA and non-
379 significant terms (α = 0.05) were sequentially dropped from each model to find the
380 minimally adequate model for each compound. Significant differences in compound
381 abundance among samples were determined from least-squares means contrasts (α = 0.05).

382

383 # Results

384 **Plant growth**

385 Development of main shoots and tillers were recorded every few days from booting until
386 ripening and are shown in relation to date of anthesis, which is indicated by a horizontal
387 dotted line in Fig. 1. Ears from plants grown under warm conditions pre-anthesis took an

388 average of 84 days (2184 °C days) from sowing until anthesis, whereas ears from plants
389 grown under cold conditions took an average of 105 (1715 °C days) days to reach anthesis.
390 Development rates also differed among the four post-anthesis treatments, with plants
391 grown in cool post-anthesis conditions taking longer to reach ripening than those grown in
392 warm post-anthesis conditions. The CC plants took 71 days from anthesis to ripening (1160
393 °C days), the WC plants 87 days (1421 °C days), the WW plants 24 days (624 °C days) and the
394 CW plants 27 days (702 °C days). Across all treatments, the caryopsis became sticky to the
395 touch at GS 77, when the caryopsis is nearing maximum volume. The CC plants took 29 days
396 from anthesis to GS 77 (474 °C days), the WC plants 24 days (392 °C days), the WW plants 19
397 days (494 °C days) and the CW plants only nine days (234 °C days). These data suggest that
398 the growing conditions pre-anthesis have an influence on the subsequent rate of
399 development, and length of grain-filling period of the caryopsis. The metabolic processes
400 responsible for production of the sticky cementing material are likely to come into play
401 shortly before the caryopsis becomes noticeably sticky, therefore the period between GS 75
402 and GS 77 is marked by vertical dashed lines in Fig. 1 to indicate the developmental period,
403 and range in days after anthesis, that the critical period for husk adhesion is likely to span.

404

405 **Effect of temperature on grain skinning**

406 Both pre-anthesis and post-anthesis temperatures had a significant impact on skinning, with
407 all treatments having significantly different proportions of skinned grains per ear ($P < 0.001$).
408 Differences in skinning among treatments as determined by comparing confidence intervals
409 from the generalised model are shown in Fig. 2. Ears of plants grown in warm pre-anthesis

410 conditions had a higher proportion of skinned grains than those grown in cool conditions
411 pre-anthesis, with the WC plants having the highest proportion of skinned grains (> 0.75).

412

413 **Grain development**

414 Measured weights and dimensions of the grain components in the five central florets,
415 specifically the caryopsis and the two glumes of the husk (palea and lemma), are
416 summarised in Table 1. For comparisons of significant differences among measurements,
417 the data were separated into three developmental phases: husk development, grain
418 development, and post-adhesion. Linear mixed effects models were compared to determine
419 the significance of treatment, growth stage and their interaction on each measurement
420 within these three phases. The final, minimally adequate models were determined by
421 comparing hierarchical models using ANOVA, dropping one variable each time (Additional
422 file 1, Table S3). Predicted means and standard error of the difference between means from
423 the minimally adequate models are given in Additional file 1, Table S4.

424

425 During husk development (ie pre-anthesis, GS 45, 51 and 65), only two temperature
426 treatments could be compared, warm (W) and cool (C). Growth stage had a significant effect
427 on palea length ($P < 0.001$), width ($P = 0.021$), and weight ($P = 0.013$). At GS 41, paleas were
428 shorter and less wide than at GS 51 or 65. Treatment had no effect on palea weights or
429 dimensions. For lemma length, GS was the only significant variable ($P = 0.017$), but post-hoc
430 tests indicated no significant differences among samples. Lemma width on the other hand
431 was significantly affected by treatment only ($P < 0.001$), with C plants having wider lemmas

432 than W plants. Although the interaction between treatment and GS was significant for
433 lemma weight (*P* = 0.049), post-hoc tests showed no significant differences among samples.

434

435 During grain development (GS 75 and 77), treatment significantly affected palea length (*P* =
436 0.011) width (*P* < 0.001), and weight (*P* = 0.003), with GS also being a signficant factor of
437 palea width (*P* = 0.022). Palea length was highest in the WW plants compared with the other
438 three treatments. Within treatments, there was no difference between palea widths
439 between GS 75 and 77, but both growth stages in WC plants had wider paleas than those in
440 CW plants. Palea weight was greater in the CW plants than in WC or WW. Treatment did
441 have a significant effect on lemma weights (*P* < 0.001), with CW lemmas weighing
442 significantly less than those grown under WC, WW or CC conditions. The interaction
443 between GS and treatment was significant for both caryopsis length (*P* < 0.001) and weight
444 (*P* = 0.047), with caryopsis width being affected by treatment (*P* < 0.001) and GS (*P* = 0.014).
445 WW caryopses were significantly shorter, wider and heavier at GS 77 than GS 75. Caryopsis
446 width also increased significantly between GS 75 and 77 for the WC, CC and CW treatments.
447 Only CC and WW plants both had a significant increase in caryopsis weight between GS 75
448 and 77.

449

450 During the post-adhesion phase, the husk could not be removed from the caryopsis without
451 causing damage to the husk, or the underlying tissues of the caryopsis; “caryopsis”
452 measurements therefore include the husk organs, and are more accurately “grain”
453 measurements. Growth stage had a significant effect on grain width (*P* < 0.001) and weight

454 (P < 0.001). Treatment also had a significant effect on grain width (P < 0.001) and weight (P
455 < 0.001), with the interaction between treatment and growth stage significantly affecting
456 grain length (P = 0.004). For all treatments, grain length and width decreased between GS
457 85 and ripeness (GS 92), with CC plants having wider grains at harvest ripeness than the
458 other treatments. Grain weight also significantly decreased between GS 85 and 92 for all
459 four treatments, with CC plants having significantly heavier grains than the other three
460 treatments. Treatments with the highest or lowest grain weight, length or width at harvest
461 did not correspond to the treatments that induced the highest or lowest skinning.

462

463 **Ear measurements at harvest**

464 Measurements of harvest ripe ears are summarised in Table 2. Temperature had a
465 significant effect (P < 0.001) on all measured ear traits at harvest. The conditions pre-
466 anthesis determined ear length and grain number, with plants grown in warm conditions
467 pre-anthesis having significantly shorter ears, with a lower floret number and high infertility
468 resulting in a significantly lower number of grains compared with plants grown in cool
469 conditions pre-anthesis. Within each pre-anthesis temperature, there were no significant
470 differences in ear length, floret number or grain number between the different post-
471 anthesis treatments. Grain weight on the other hand, was significantly affected by the post-
472 anthesis temperatures. Grain weight was highest in ears from CC plants, with the other
473 three treatments not having significantly different grain weights from each other.

474

475 **Structure of the husk and pericarp cuticles, and the cementing layer**

476 The structure of the cuticular layers and cementing layer was similar across all treatments,
477 but differed among growth stages. A light micrograph of a barley grain section post-
478 adhesion is provided in Additional file 3, showing a low-powered orientation of the husk and
479 caryopsis tissue organisation. At GS 75, there was typically an irregular interface between
480 the electron-dense cuticle proper and the pericarp cell wall, signifying ongoing production
481 of cuticular material (Fig. 3a, arrow). At GS 77, the interface between the cuticle and the cell
482 wall was often smoother in appearance, although the cuticle proper was still typically
483 electron-dense. By GS 85, after husk adhesion is complete, production of further cuticular
484 material had ceased, and the cuticular layer comprised the pericarp cuticle, the husk cuticle
485 and a layer of material in between that was either amorphous and electron dense, or a
486 lamellated structure with alternating layers of electron dense and electron translucent
487 material (Fig. 3b). The inner and outer surfaces of the husk cuticles do not undergo
488 increased production of cuticular material during critical periods of husk adhesion, and do
489 not have a lamellated structure at any developmental stage when the husk can still be
490 removed from the pericarp. An example of the inner lemma surface cuticle at GS 45 is
491 shown in (Fig. 3c). The pericarp surface cuticle of the hull-less variety “Nudinka” at GS 77
492 was examined for comparative purposes, and was found to have a thin cuticular layer of ~30
493 nm which is shown in Fig. 3d. The interface between the cuticle and the pericarp cell wall of
494 Nudinka is smooth, with no indication of continuing production of cuticular material.

495

496 The surface cuticles often became separated from the underlying cell wall in both the husk
497 and pericarp samples, typically at the corners where adjacent epidermal cells join (Fig. 4a,
498 arrow), although this separation sometimes extended across the whole sample. From GS 77

499 onwards, the pericarp and husk cuticles began to adhere together, and were sometimes
500 seen to be detached from both husk and pericarp cell walls, but remaining firmly attached
501 to each other. From GS 85, large spaces between the cuticles often had an electron dense
502 layer between them which did not always extend to fill the space between the husk and
503 pericarp when the separation distance was particularly large (Fig. 4b). This electron dense
504 material later became lamellated with either parallel striations, or lamellations with random
505 orientations at regions of husk-caryopsis separation (Fig. 4c). These lamellations measured
506 between four to 10 nm thick. One region where separation of the cuticular layers was
507 common was regions adjacent to "tubelike" cells, which are most likely trichomes, and
508 potentially cause some mechanical stress between the husk and caryopsis (Fig. 4d).
509 Lamellae were not observed in cuticles of either the inner or outer surfaces of the palea or
510 lemma.

511

512 The frequent separation of the cementing layer from the pericarp and husk cell walls during
513 the adhesion phase made it difficult to measure (shown in Additional file 3), with only a
514 small number of samples retaining sufficiently intact cementing layers to calculate the
515 standard error of the mean (Table 3). Pre-husk adhesion, the inner and outer cuticles of the
516 husk organs were thinner than the pericarp surface cuticle. From GS 85, after husk adhesion
517 is complete, the inner husk cuticle was measured together with the pericarp cuticle as part
518 of the entire cementing layer, with the entire layer measuring approximately 100 nm in
519 thickness. In regions of evident husk-caryopsis separation however, the cementing layer
520 could be up to several hundred nm in thickness.

521

522 **Pericarp surface morphology**

523 As the surface cuticles were often observed to have pulled away from the cell walls, grains
524 of Concerto at GS 77 were examined by scanning electron microscopy to determine whether
525 this separation was likely to be happening during physical removal of the husk from the
526 caryopsis, or during sample processing. The hull-less variety "Nudinka" was examined at GS
527 77, the growth stage at which the pericarp surface becomes sticky in covered varieties, to
528 compare pericarp surfaces between grains that produce a cementing layer with a grain that
529 does not. At GS 77, the surface of the Nudinka pericarp is smooth (Fig. 5a, Additional file 3),
530 whereas the surface of the Concerto pericarp is damaged from the action of pulling the husk
531 away from the already adhesive pericarp surface (Fig. 5b). The surface of Concerto at GS 77
532 is covered by a further layer of globular material Fig. 5 c), most likely the cementing
533 material, which does not smoothly coat the entire pericarp surface, possibly due to
534 disruption by removal of the husk at this stage. The tubelike cells on the pericarp surface are
535 not fully covered over by the cementing material (Fig. 5d) in agreement with transmission
536 electron microscopy results which show the cementing layer does not entirely fill the spaces
537 between the pericarp and husk around these cells (Fig. 4d). The extent of surface damage in
538 grains of Concerto at GS 77, combined with the absence of damage to Nudinka at GS 77,
539 indicate that separation of the cementing layer from the cell walls is likely to be due to the
540 strength of the adhesion through the cementing layer being higher than that between the
541 cuticle and the cell wall, rather than an artefact of sample processing.

542

543 **Surface lipid composition**

544 In the surface lipid extracts from the husk and caryopses, a total of 121 different compounds
545 were identified, belonging to the following structural classes: fatty acids; fatty alcohols; wax
546 esters; alkanes and alkenes; ketones, diketones, hydroxydiketones and related compounds;
547 5-alkylresorcinols; and triterpenes. For each structural class except the esters, abundances
548 of compounds in each sample are shown by carbon-number distribution in Additional file 4.
549 A total of 20 fatty acids were present, mostly saturated straight chain homologues (even
550 carbon numbers of C_{14} to C_{32}, and odd carbon numbers of C_{15}, C_{17} and C_{25}). Two branched
551 chain C_{17} acids were identified as 14- and probably 10-methylhexadecanoic acids on the
552 basis of their equivalent chain lengths of 16.72 and 16.40 [48, 57]. Of the 14 long-chain
553 alcohols detected, the most abundant were even carbon homologues (C_{16} to C_{28}), with odd
554 carbon compounds (C_{21} to C_{31}) as minor constituents. A total of 37 long chain esters in the
555 range C_{32} to C_{48} were detected as minor components which consisted of fatty acids (C_{14} to
556 C_{24}) esterified to alcohols (C_{16} to C_{30} and C_{21} to C_{25}). Among the total of 22 aliphatic
557 hydrocarbons detected, odd carbon alkanes (C_{21} to C_{37}) were most abundant with even
558 carbon homologues (C_{22} to C_{38}) and odd (C_{27} to C_{31}) and even (C_{30}) carbon alkenes as minor
559 constituents. Nonacosan-14-one, the β-diketone hentriacontan-14,16-dione, and both 8-
560 and 9-hydroxy-hentriacontan-14,16-dione were detected. In addition, several components
561 were detected which appear to be consistent with 4 individual enol tautomers and 3
562 individual $(enol)_2$ tautomers of hentriacontan-14,16-dione. The interrelationship between
563 these is shown in Additional file 5. Such compounds are not usually reported as being
564 constituents of plant waxes along with ß-diketones and it is unclear whether these
565 components were genuine constituents within the wax, or were artefacts of wax extraction
566 or derivatisation. Keto-enol tautomerism can be both acid and base catalysed, and it is
567 possible that the presence of pyridine in the derivatisation medium facilitated the reaction.

568 However, we have evidence to suggest that the enol and (enol)$_2$ forms were also present in
569 the absence of pyridine (data not shown). Since these tautomers are all considered to be
570 derived from hentriacontan-14,16-dione, their abundances were combined and included
571 within that of the diketone. Two homologous series of 5-alkyl resorcinols were detected. In
572 the first the alkyl substituents consist of odd carbon straight chains (C_{15} to C_{27}), whereas in
573 the second the molecule carries an additional methyl substituent, either within the benzene
574 ring or on C1 of the predominantly odd carbon 5-alkyl side chain (C_{15} to C_{27}, and C_{20}). Six
575 terpenes were detected including the sterol precursor squalene, cholesterol, campesterol
576 and ß-sitosterol, a component tentatively identified as cholesta-3,5-diene and γ-tocopherol.
577 In addition, 6 compounds of unknown identity were present in the samples.

578

579 No compounds were identified that were unique to either the husk or caryopsis samples,
580 treatments or growth stages, although alkylresorcinols and ketones were typically most
581 abundant in the husk extracts. There were significant differences in the abundance of
582 several compounds among treatments and growth stages as described below. Table S5 in
583 Additional file 1 gives the probabilities of treatment, growth stage or their interaction
584 having a significant effect on the abundance of each compound, and minimally adequate
585 models chosen for determining significant differences in abundance among samples. The
586 additional tables S6, S7 and S8 in Additional file 1 give the fitted means, standard error of
587 the difference and whether compounds differed significantly in abundance among variables
588 of the minimally adequate models for the husk and caryopsis samples.

589

590 **Differences in compound abundance between growth stages**

591 A number of compounds differed significantly in abundance between GS 75 and 77,
592 independent of treatment (Additional file 1, Table S6). In the caryopsis samples, the
593 abundance of tetracosanol, pentacosane, heptacosane and nonacosane was lower in GS 77
594 than 75. The fatty acids 9, 12-octadecadienoic acid and eicosanoic acid were present in
595 increased amounts in GS 77 caryopses, as were the alkylresorcinols 5-heneicosylresorcinol,
596 5-pentacosyresorcinol and methyl-5-pentacosylresorcinol. The abundance of compound
597 "Unknown-3" also increased between GS 75 and GS 77. In husk samples, the abundance of
598 hexadecanol and hentriacontane was higher in GS 77 than GS 75. The abundance of
599 tetradecanoic acid and branched 10-methylhexadecanoic acid also increased by GS 77.
600 Esters and alkylresorcinols also increased in abundance in husk samples between GS75 and
601 77, specifically these were the following esters: docosyl tetradecanoate, eicosyl
602 octadecanoate, docosyl tetracosanoate and the alkyresorcinols: 5-pentacosylresorcinol,
603 methyl-5-pentacosylresorcinol and methyl-5-heptacosylresorcinol.

604

605 **Differences in compound abundance among treatments**

606 The abundance of only a small number of compounds from caryopsis surface lipids changed
607 significantly among treatments, independently of growth stage (Additional file 1, Table S7).
608 Nonacosanol was present in greater abundance in caryopses from the WC treatment than
609 those from the CC treatment. The abundance of hexacosyl octadecanoate was higher in CW
610 caryopses compared with CC caryopses. Methyl-5-pentadecylresorcinol was more abundant
611 in CC caryopses than in any other treatment, whereas methyl-5-heptadecylresorcinol was in

612 greater abundance in CC caryopses than CW or WW. Nonacosan-14-one was more
613 abundant in CC caryopses than WW.

614

615 A greater number of compounds from husk surface lipids had significant changes in
616 abundance among treatments independent of growth stages compared with the caryopses.
617 Docosanol was less abundant in CW husks than WW husks, whereas hexacosanol was less
618 abundant in CW husks than WC husks. The fatty acids pentacosanoic acid and octacosanoic
619 acid were more abundant in CC husks than CW husks, docosanoic acid was more abundant
620 in the WW husks than either CC or CW husks, and the longer-chain hentriacontanoic acid
621 was more abundant in WC husks that WW or CW. The abundances of nonacosan-14-one, 5-
622 heptadecylresorcinol and methyl-5-heptadecylresorcinol were greatest in CC husks over all
623 other treatments, with 5-pentadecylresorcinol being greatest in CC husks only compared
624 with WC and CW husks. A large number of esters differed significantly in abundance among
625 treatments, for all compound difference see Table S3 in Additional file 1. All identified esters
626 derived from hexacosanol, known to be the most abundant barley surface lipid, had
627 differences in abundance among treatments, with CC husks always having lower abundance
628 of these esters than WW and WC husks, and WC having the highest abundance of these
629 esters among all treatments although differences were not always significant. The
630 compound “Unknown-4” had higher abundance in CC husks than all other treatments.
631 “Unknown 6” had higher abundance in WW and WC husks than CC or CW husks.

632

633 **Differences in compound abundance where treatment and growth stage are significant**
634 **factors**

635 Those compounds where treatment and growth stage both have a significant effect on
636 compound abundance, either individually or where the interaction are significant, are given
637 in Additional file 1, Table S8. Within each of the four treatments, the abundance of
638 hexadecanol increased significantly between GS 75 and 77 caryopses, but there were no
639 differences in the abundance of hexadecanol among treatments within each growth stage.
640 The WW caryopses had significantly more tricosanol than CC caryopses at GS 75, but at GS
641 77 WW caryopses had more tricosanol than both CC and CW caryopses. The abundance of
642 both hexacosanol and octacosanol significantly decreased between GS 75 and GS 77 for all
643 treatments, but there were no differences in abundance among the treatments within each
644 growth stage. The same pattern was observed for octacosane, which decreased in
645 abundance between GS 75 and 77, but was not different among treatments within each
646 growth stage. At both GS 75 and 77, WW caryopses had a greater abundance of triacontane
647 than CC caryopses. Both Unknown compounds 1 and 4 significantly increased in abundance
648 from GS 75 to GS 77.

649

650 In the husk samples, the abundance of hexadecanoic acid was greater at GS 77 than GS 75
651 for all treatments. Similarly, the abundance of cholesterol was greatest at GS 77 than GS 75
652 for all four treatments. At GS 75, octacosyl hexadecanoate was more abundant in the CC
653 and CW treatments than in WW husks, but at GS 77 the trend was reversed, with WW husks
654 having significantly more of this ester than CC and CW husks. Hexacosanoic acid was more

655 abundant in CW husks at both GS 75 and 77, than CC husks. Octadecananoic acid was less
656 abundant in CC husks at GS 75 and 77 compared with WC husks.
657

658 **Differences in compound abundance where treatment and growth stage interact**

659 The abundances of octadecanol, tritriacontane, campesterol and ß-sitosterol were
660 significantly greater in the CW caryopses at GS 77 than in all other samples. Similarly,
661 heptacosanol was most abundant in WW caryopses at GS 77 than all other samples.
662 Pentacosanol was more abundant in WW caryopses at GS75 than in all other samples except
663 for WC caryopses at GS 75. The alkenes hetacosene, nonacosene and hentriacontene were
664 typically most abundant in CC caryopses at GS 75, than in all other samples. In the husk
665 samples, octadecanol was significantly more abundant in CW organs at GS 77,
666 corresponding to the same trend in the caryopses.
667

668 **Changes in surface lipid composition with respect to skinning**

669 As the WC plants had the highest proportion of skinned grains, and CW plants had the least
670 proportion of skinned grains, compounds with significantly different levels of abundance
671 between these treatments in the caryopsis extracts are of particular interest. Those
672 compounds that change significantly at GS 77 in particular, when the caryopsis becomes
673 sticky to the touch, might indicate which compounds are correlated with good quality husk
674 adhesion. The CW caryopsis extracts at GS 77 have four compounds that increase
675 significantly in abundance to all other samples. The estimated model coefficients (relative

676 abundance to WC) and partial residuals for these compounds are shown in Fig. 6;
677 octadecanol (Fig. 6a), tritriacontane (Fig. 6b), campesterol (Fig. 6c) and ß-sitosterol (Fig. 6d).

678

679 Discussion

680 Grain skinning with different severities can be induced by imposing different temperatures
681 during development of the husk and the caryopsis. Previous studies have reported that the
682 critical production of sticky material on the caryopsis occurs from 10 days after anthesis [15,
683 17]. However, by relating developmental stages [44] and production of sticky material to the
684 date of anthesis, we have shown that it is the developmental stage that is critical for husk
685 adhesion, and that this can be reached over a wide range of days post-anthesis depending
686 on the rate of development. Even the thermal time from anthesis until GS 77 varied widely
687 among treatments, suggesting that pre-anthesis conditions also influence the development
688 of the cementing layer by altering rate of grain development. As seen in previous studies
689 using temperature to manipulate barley growth, similarly elevated temperatures shortened
690 the period of the developmental stages to which they were applied and reduced the final
691 grain weight [45, 58]. Ugarte et al. [14] found that short-term heat treatment pre-anthesis
692 also decreased subsequent rates of grain development, although the present study shows
693 that temperature during the grain filling period has a greater effect on the rate of grain
694 filling than pre-anthesis temperatures, as the time from GS 65 to ripeness for the warm (CW
695 and WW) or cool (CC and WC) post-anthesis conditions were more similar to each other,
696 than treatments with the same pre-anthesis temperature. The temperature treatments that
697 resulted in the most severe skinning levels (WC and WW), although not having a significant
698 effect on the size of grain components, did have a marked effect on ear physiology. These

699 treatments resulted in shorter ears with fewer fertilised florets developing to grains, and is
700 likely due to heat stress at the time of anthesis in a similar manner to that reported in rice
701 by Shi et al. [59]. A reduction in the number of grains produced per ear has previously been
702 reported for barley and other grains grown at elevated temperatures [60]. It is likely that
703 the low grain weights seen in the WW and CW treatments in the current study was due to a
704 reduction in starch synthesis, as observed in other studies of elevated temperature during
705 grain filling [45, 61].

706

707 Warm conditions during husk growth and carpel development resulted in higher skinning
708 levels, with cool conditions during caryopsis development and grain filling further
709 exacerbating skinning. It is unlikely that the different skinning levels were caused by an
710 incompatibility between grain size and strength of the husk in this study as proposed by
711 Rajasekaran et al. [7], as the weights and dimensions of husk components did not differ
712 significantly among treatments once they had reached GS 77, and the treatment with the
713 largest, heaviest grains at harvest (CC) did not correspond to the treatment with the most
714 severe grain skinning. Only one variety was assessed in the current study however,
715 examination of a number of diverse genotypes may find that skinning severity is correlated
716 with varying husk and caryopsis dimensions in some varieties. Interestingly, the cool post-
717 anthesis temperature of the WC treatment did not result in grains with a similar weight to
718 those of CC plants, despite the similar grain filling duration of these two treatments. This
719 result supports the finding of Calderini et al. [11] that higher temperature from booting to
720 anthesis significantly reduces final grain weight. The effect of pre-anthesis temperature on
721 grain weight has been assumed to be due to an effect on floret development [11, 14]; the

722 results of the current study provide supporting data that this effect is independent of the
723 size or weight of the husk, and therefore is probably due to development of the carpel, as
724 reported in wheat by Xie et al. [62]. As the variation in skinning among the different
725 treatments was not correlated with the size or weights of the grain components, it is likely
726 that skinning was mediated through the effects that the different temperature treatments
727 had on the cementing layer.

728

729 Until now, the most detailed study of the cementing layer has been completed by Gaines et
730 al. [15]. Although others have since examined the cementing layer using light microscopy
731 [16, 18] and electron microscopy [8]. The cementing layer is very thin, between 100 and 600
732 nm [15] and not reaching more than 120 nm in the present study. As the limit for resolution
733 of light microscopy is reached at 200 nm, it is essential to use electron microscopy to gain
734 the best understanding of differences in cementing layer structure. The structure of the
735 pericarp and husk cuticles changed throughout grain filling in a manner consistent with the
736 general description of cuticle development put forward by Jeffree [21, 63]. The pericarp
737 cuticle at GS 75 had a reticulate boundary with the pericarp cell wall (Fig. 3a), consistent
738 with continuing development of surface cuticles during cell wall expansion. Gaines et al. [15]
739 described the cementing layer as a thin, electron-lucent layer with cell-wall material
740 embedded in it, and suggested that it formed as early as two days after anthesis. However
741 this description, and accompanying micrographs, correspond to what we currently know is a
742 plant surface cuticle mid-development [21, 63], and may not necessarily correspond with
743 production of cementing material as distinct from the pericarp cuticle. Indeed, a thin,
744 electron-dense layer was visible on the outer surface of both the Nudinka and Concerto

745 cuticles at GS 77 (Fig. 3d, Concerto not sown), similar to that described by Gaines et al. [15]
746 as the cementing material. It was difficult to precisely distinguish boundaries between the
747 pericarp surface cuticle and any cementing material until GS 85, when the husk and
748 caryopsis adhere to each other and a layer with contrasting electron-lucency could be
749 observed between the two cuticles as reported by Hoad et al. [64].

750

751 It is not clear from the results of this study, whether the lamellations seen in the cementing
752 layer, when present, formed as part of the pericarp or husk cuticle, or whether they were
753 only formed after contact of the glume with the caryopsis. These lamellations were often
754 parallel, as observed by Gaines et al. [15], but in the regions of the greatest separation
755 where the cementing material did not entirely fill the space between the husk and pericarp
756 cuticle they became disordered. Typically, lamellations remained on the side of the pericarp
757 at regions of separation, indicating that the cementing material is likely to be present on
758 their surface, and that they are not part of the cementing layer itself. Extraction of
759 eucalyptus spp. leaf cuticles with dichloromethane and ethanol resulted in lamellae
760 becoming amorphous [20], indicating that such lamellae could be chemically distinct,
761 although similar treatment of *Eucalyptus* spp. leaf cuticles left the lamellae intact [20].
762 Regions next to tubelike cells, most likely trichomes, always had some degree of separation
763 between the husk and caryopsis. Gaines et al. [15] reported that tubelike cells did not
764 produce a distinct cementing layer, and the cuticle of these cells in the present study was
765 distinctly thinner than that of the surrounding pericarp epidermal cuticle. Although cuticle
766 lamellations remained on the pericarp side, Gaines et al. [15] reported the cementing
767 material always remained on the pericarp, the present study found that sometimes both the

768 husk and the pericarp cuticle separated from the underlying cell wall. This demonstrates
769 that the strength of adhesion between the two cuticles is greater than that between the
770 surface cuticle and the cell wall, and suggests that the interface between the cuticle and the
771 cell itself might be a point of weakness that influences skinning severity. Olkku et al. [16]
772 reported that skinning was likely to be due to breakage of the large thin-walled parenchyma
773 of the husk rather than separation along the cementing layer. Cell breakage was not
774 observed in the present study, and may have been avoided by differences in sample
775 preparation, however as husk thickness and the number of husk parenchyma cell layers
776 were not measured in this study, we can not determine whether the different treatments
777 had any effect on skinning in this way.

778

779 The composition of the surface lipids of the barley caryopsis has not yet been reported.
780 However, Kakeda et al. [65] compared thin-layer chromatographs of surface lipids extracted
781 from the caryopses of covered and naked barley, and found little evidence of solvent-
782 extractable compounds being present on the naked barley caryopses, despite there being a
783 thin cuticle present on naked barley caryopses. With the findings of Taketa et al. [18] that
784 the cementing material could be dyed by Sudan Black dye, this indicates that the cementing
785 material itself is likely to be extractable using organic solvents. The relative abundance of
786 some compounds differed among treatments in relation to skinning severity in this study, in
787 particular for the treatment with the lowest skinning (CW). The thickness of the cuticular
788 layers and cementing layer is unlikely to have influenced the quality of husk-caryopsis
789 adhesion among treatments in this study, suggesting that the ability of these cuticles to

790 adhere to each other is significantly influenced by an altered composition rather than the
791 thickness or amount of cuticular material.

792

793 Lack of correlation between cuticle thickness and physical properties is not unusual. Several
794 studies have reported that plant cuticle composition is more correlated with physical or
795 mechanical properties such as permeability and transpiration than cuticle size [66–69]. In
796 this study, the triterpenoids and their derivative sterol compounds, particularly ß-sitosterol
797 and campesterol had the most strikingly altered increase in abundance at GS 77 among
798 treatments that resulted in different skinning severities. Some plants with higher
799 proportions of triterpenoids in their cuticles have higher cuticle permeability [66], and
800 mutants with altered surface lipid compositions that result in higher cuticle permeability
801 often display an organ fusion phenotype, particularly among floral organs. The long-chain
802 alkane tritriacontane was also associated with good quality husk adhesion; *n*-alkanes have
803 also been linked to cuticular permeability [66], however in tomato fruits, higher-chain
804 alkanes with C_{31} to C_{34} were associated with lower cuticle permeability [67]. Organ fusion
805 has been previously noted to be a similar phenotype to husk adhesion [43]. In the maize *ad1*
806 mutant, where the cell walls of floral parts adhere to each other, the cuticle appears to
807 remain intact between the cell walls [41], similar in appearance to the cementing layer
808 comprising the husk and pericarp surface cuticles. In the case of the *Arabidopsis* mutants
809 *atwbc11* and *bdg* (*bodyguard*) however, the cuticle is disrupted and the cell walls merge [40,
810 70]. Neither disruption of cuticular layers or direct adhesion of husk and pericarp cell walls
811 was observed in the present study. Organ fusion has been induced by expression of a fungal
812 cutinase in *Arabidopsis*, where cuticular layers either remaned intact between cell walls, or

813 walls of epidermal cells came in direct contact with each other [71]; organ fusion has also
814 been induced by repression of *WIN1/SHN1*, a gene with homology to *Nud*, in *Arabidopsis*
815 and *Tourenia fournieri* [72]. The increase in abundance of compounds known to increase
816 cuticle permeability in samples with the highest quality husk adhesion, supports the
817 observations of Duan et al. [43] that the process of husk adhesion is similar to organ fusion,
818 and may be due to an increase in cuticle permeability. Although the present study suggests
819 that cuticular wax composition has a role in the quality of husk adhesion, it is possible that
820 the entire matrix of cell-wall, cutin and cuticular waxes contribute to husk adhesion.

821

822 **Conclusions**

823 In this study, we were able to determine that adhesion of the barley husk to the underlying
824 caryopsis is directly dependent on the developmental stage of the grains regardless of the
825 rate of grain development. It is not until the caryopsis nears maximum volume (GS 77) that
826 the adhesive cementing layer comes into contact with the husk and adhesion is initiated.
827 Therefore it is vital to account for developmental variation in future studies of husk
828 adhesion, and not just the number of days post-anthesis. We now have evidence that the
829 composition of the cuticular waxes on the caryopsis influences the quality of husk adhesion,
830 and therefore has a tangible effect on the severity of the malting quality defect grain
831 skinning. The quality of the husk adhesion is influenced by environmental conditions, but it
832 is likely that there is a genetic influence on husk adhesion, as we know that different
833 cultivars of malting barley have differential susceptibility to grain skinning [4]. As the current
834 work has indicated that differences in skinning risk are likely due to cuticle composition,
835 rather than differences in grain dimensions among cultivars, research efforts to minimise

836 skinning risk in future cultivars should focus on determining the genetic controls of
837 cementing layer composition.

838

839 Abbreviations

840 **ANOVA:** Analysis of variance **CC:** cool, cool treatment **CW:** cool, warm treatment **GS:** growth
841 stage **TMS:** trimethylsilyl **WC:** warm, cool treatment **WW:** warm, warm treatment

842

843 Declarations

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853 *Availability of data and material*

854 All data generated or analysed during this study are included in this published article [and its
855 supplementary information files].

856 ***Author’s conributions***

857 MB designed and performed the experiment, analysed the data and wrote the manuscript.
858 TS derivatised the lipid extracts, and identified and quantified the compounds using GC-MS.
859 SM and MB prepared the samples for electron microscopy. CFET contributed to analysis of
860 the data, and CFET and SH participated in revisions of the manuscript. All authors have read
861 the manuscript, given comments and approved the final version of the manuscript.

862 ***Competing interests***

863 The authors declare that they have no conflict of interest.

864 ***Consent for publication***

865 Not applicable.

866 ***Ethics approval and consent to participate***

867 The seeds used in this study were grown from material kindly proved by Dr. WTB Thomas,
868 and are part of the Association Genetics of UK Elite Barley (AGOUEB) collection maintained
869 by the James Hutton Institute. As the AGOUEB collection is currently maintained as above, a
870 voucher of the material has not been deposited in a herbarium.

871

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1054

1055 **Tables**

Table 1 Dimensions[a] and fresh weights[b] of organs at selected growth stages across four treatments

Phase			Measurement								
Husk development	Treatment	GS	Palea length	Palea width	Palea weight	Lemma length	Lemma width	Lemma weight	Caryopsis length	Caryopsis width	Caryopsis weight[c]
	W	45	8.93 ± 0.39	4.28 ± 0.25	2.58 ± 0.24	10.13 ± 0.33	5.45 ± 0.33	5.55 ± 0.29	-	-	-
	W	51	9.56 ± 0.17	4.73 ± 0.15	3.12 ± 0.02	10.57 ± 0.18	5.69 ± 0.41	6.20 ± 0.65	-	-	-
	W	65	9.44 ± 0.30	4.81 ± 0.37	2.48 ± 0.11	10.45 ± 0.24	5.82 ± 0.19	4.96 ± 0.30	-	-	-
	C	45	8.68 ± 0.47	4.46 ± 0.15	2.46 ± 0.43	9.48 ± 0.42	6.30 ± 0.04	5.29 ± 0.65	-	-	-
	C	51	9.42 ± 0.11	4.90 ± 0.04	2.67 ± 0.22	10.14 ± 0.11	6.64 ± 0.13	5.43 ± 0.10	-	-	-
	C	65	9.83 ± 0.20	5.07 ± 0.08	2.62 ± 0.08	10.74 ± 0.23	6.69 ± 0.15	5.84 ± 0.28	-	-	-
Grain development											
	WC	75	9.86 ± 0.16	5.33 ± 0.13	2.81 ± 0.24	10.60 ± 0.14	6.55 ± 0.14	5.88 ± 0.41	8.45 ± 0.17	3.00 ± 0.10	32.56 ± 1.32
	WC	77	10.13 ± 0.15	5.03 ± 0.20	2.98 ± 0.22	10.74 ± 0.22	6.52 ± 0.09	6.03 ± 0.13	9.07 ± 0.13	3.56 ± 0.12	48.87 ± 5.07
	WW	75	10.49 ± 0.34	5.08 ± 0.12	2.97 ± 0.27	10.71 ± 0.34	6.34 ± 0.23	5.70 ± 0.54	9.65 ± 0.38	3.37 ± 0.39	35.21 ± 2.73
	WW	77	10.01 ± 0.20	5.21 ± 0.16	2.98 ± 0.13	10.26 ± 0.76	6.38 ± 0.37	6.04 ± 0.36	8.53 ± 0.39	3.69 ± 0.05	50.23 ± 1.28
	CC	75	10.12 ± 0.07	4.88 ± 0.07	2.69 ± 0.02	10.78 ± 0.31	6.72 ± 0.04	5.91 ± 0.07	9.94 ± 0.12	3.34 ± 0.21	45.69 ± 5.59
	CC	77	9.87 ± 0.31	4.56 ± 0.06	2.68 ± 0.21	10.32 ± 0.31	6.74 ± 0.13	5.87 ± 0.56	9.59 ± 0.28	4.13 ± 0.09	64.96 ± 2.14
	CW	75	9.52 ± 0.18	4.60 ± 0.05	2.33 ± 0.08	10.04 ± 0.10	6.40 ± 0.10	4.58 ± 0.23	8.88 ± 0.32	3.10 ± 0.13	35.54 ± 3.87
	CW	77	9.62 ± 0.19	4.24 ± 0.20	2.44 ± 0.14	9.83 ± 0.18	6.44 ± 0.29	4.58 ± 0.40	8.63 ± 0.01	3.36 ± 0.07	39.34 ± 1.26
Post-adhesion											
	WC	85	-	-	-	-	-	-	9.83 ± 0.22	4.24 ± 0.04	76.42 ± 4.75
	WC	92	-	-	-	-	-	-	7.41 ± 0.21	3.40 ± 0.08	46.24 ± 3.63
	WW	85	-	-	-	-	-	-	9.80 ± 0.17	4.18 ± 0.00	75.29 ± 0.92
	WW	92	-	-	-	-	-	-	8.05 ± 0.15	3.43 ± 0.09	41.07 ± 1.52
	CC	85	-	-	-	-	-	-	9.49 ± 0.09	4.62 ± 0.13	94.63 ± 6.02
	CC	92	-	-	-	-	-	-	8.43 ± 0.18	4.03 ± 0.02	68.73 ± 2.84
	CW	85	-	-	-	-	-	-	9.48 ± 0.08	4.30 ± 0.02	77.96 ± 3.11

CW	92	-	-	-	-	-	-	8.08 ± 0.38	3.46 ± 0.05	47.39 ± 3.54

1056 Values are expressed as mean ± standard error of the mean

1057 [a]Measurement in mm

1058 [b]Measurement in mg

1059 [c]Note that at GS 85 and GS 92, caryopsis measurements include the palea and lemma and are therefore more accurately "grain" measurements

1060 "-" Indicates that measurements could not be made due to the growth stage

1061

1062

Table 2 Differences[a] in harvest-ripe ears among treatments

Treatment	N	Ear length (mm)	Floret numberb	Grain number[b]	Grain weight (mg)	% Infertility
WC	37	6.1 ± 0.2 a	19.1 ± 0.6 a	4.7 ± 0.6 a	43.5 ± 2.0 a	75.4
WW	92	5.8 ± 0.1 a	17.3 ± 0.4 a	7.3 ± 0.5 a	43.6 ± 1.9 a	57.8
CC	37	7.5 ± 0.2 b	28.8 ± 0.5 b	15.5 ± 1.2 b	66.9 ± 1.0 b	46.2
CW	96	7.6 ± 0.1 b	29.2 ± 0.4 b	22.7 ± 0.9 b	37.4 ± 1.2 a	22.3

1063 [a]Values are expressed as mean values for each ear ± standard error of the mean. Significant differences among treatments (post-hoc Tukey's HSD, $\alpha = 0.05$)
1064 are designated by lowercase letters

1065 [b]These are mean values and therefore not integers

1066

1067

1068

Table 3 Measurement of surface cuticle thickness of grain components

Treatment	GS[a]	Palea inner	Palea outer	Lemma inner	Lemma outer	Caryopsis/pericarp
W	51	42.90 ± 13.01	25.88 ± 7.68	24.87 ± 10.42	52.97 ± 6.03	-
C	51	28.54 ± 14.58	51.64	27.80	62.58 ± 5.74	-
W	65	25.38 ± 4.37	31.87 ± 4.39	36.67 ± 7.12	35.63 ± 4.84	-
C	65	37.83 ± 6.85	62.84 ± 7.66	26.30 ± 1.22	67.43 ± 4.40	-
WC	75	34.15 ± 5.00	35.27 ± 1.14	48.16	22.87 ± 9.32	76.29 ± 20.67
WW	75	30.89 ± 2.69	51.42 ± 22.18	34.41 ± 6.77	79.68 ± 11.08	83.27 ± 6.56
CC	75	39.85 ± 3.04	52.40	32.78 ± 8.23	m.v.	90.12
CW	75	17.30 ± 5.36	m.v.	43.87	m.v.	53.44
WC	77	m.v.	25.34 ± 1.26	36.55 ± 13.25	86.83 ± 44.67	93.70 ± 45.86
WW	77	32.59 ± 3.01	27.22 ± 5.02	30.93 ± 1.96	83.80 ± 33.69	82.62 ± 12.48
CC	77	38.45 ± 6.43	27.00	31.07 ± 1.34	58.80	91.40
CW	77	59.04	24.96	30.99 ± 19.85	m.v.	50.33
WC	85	-	-	-	-	118.98
WW	85	-	-	-	-	84.06
CC	85	-	-	-	-	113.12 ± 20.00
CW	85	-	-	-	-	131.22 ± 16.20

1069 Where three samples could be measured, values are expressed as mean ± standard error of the mean; where fewer than three samples could be measured,
1070 only the mean is given.

1071 "-" Indicates that measurements were not made due to the growth stage

1072 "m.v." Indicates missing data due to cuticle separation from cell walls

1073 [a]Note that at GS 85, caryopsis measurements comprise the husk and pericarp cuticles, whereas only the pericarp is measured on the caryopsis surface at

1074 earlier growth stages

1075

1076 **Figures:**

1077 **Fig. 1** Developmental stage of *Hordeum vulgare* cv. Concerto, in relation to anthesis date
1078 (dotted line), grown under four different treatments depending on pre- and post-anthesis
1079 temperatures. Number of days from sowing until harvest is displayed on the right hand side.
1080 The critical developmental stages for husk adhesion fall within the dashed lines.

1081

1082 **Fig. 2** Mean proportion skinned grains per ear for each of the four treatment types. A high
1083 proportion of skinned grains indicates poor quality adhesion of the husk to the caryopsis.
1084 Significant differences among treatments were determined by comparison of 95% profile
1085 likelihood confidence intervals (error bars), and are denoted by different lowercase letters.

1086

1087 **Fig. 3** Transmission electron micrographs of husk and caryopsis surface cuticles. **a** Caryopsis
1088 at GS 75 from WC treatment. There is an electron-dense cuticle proper and underlying
1089 flocculate cuticular layer (black arrow). **b** Well-adhered cementing layer at GS 85 from CC
1090 treatment. The cementing layer comprises the caryopsis cuticle (black arrow), and the inner
1091 cuticle of the husk (black and white arrow). Alternating electron-dense and electron-lucent
1092 lamellae are present between the cuticles (white arrow). **c** Inner surface cuticle of a lemma
1093 at GS 45 grown in warm conditions. The interface between the electron-lucent cuticle and
1094 cell-wall of the lemma is smooth (black arrow). **d** Nudinka caryopsis at GS 77. A thin,
1095 electron-lucent surface cuticle is present (black arrow). Le = lemma cell wall, Pe = pericarp
1096 cell wall.

1097

1098 **Fig. 4** Transmission electron micrographs of husk and caryopsis cuticles at regions of poor
1099 adhesion. **a** Inner surface cuticle of a palea at GS 51 grown in cool conditions. The cuticular
1100 layer (solid black arrow) often began to detach from the underlying cell wall (detachment
1101 shown by black and white arrow) at the corners between adjacent epidermal cells. **b** Region
1102 of separation between the husk (lemma) and pericarp at GS 85. The cementing layer does
1103 not fill the entire space between the pericarp and the husk, evidenced by gaps in the
1104 cementing material (black arrow). The interface between the pericarp cell wall and the
1105 pericarp cuticle is not smooth, but does not show globular deposits of cutin (black and white
1106 arrow). A thin, electron-lucent outer cuticle separates the pericarp cuticle from the
1107 electron-dense cementing material (white arrow). **c** Caryopsis at GS 85 from CC treatment.
1108 The surface cuticles of the husk and caryopsis are separated (black arrow). The underlying
1109 cuticular layer of the pericarp has a slightly flocculate appearance (black and white arrow)
1110 with electron-dense lamellae in the cuticle proper (white arrow). At the point of separation,
1111 these lamellae are not all in parallell, but occur at several orientations. Lamellae do not
1112 occur on the inner cuticle of the lemma. **d** Tubelike cell at a region of separation between
1113 the husk and caryopsis (black arrow) at GS 85 in a CC grain. CC = cell corner, Le = lemma cell
1114 wall, Pe = pericarp cell wall, T = tubelike cell.

1115

1116 **Fig. 5** Scanning electron micrographs of caryopsis surfaces at GS 77. **a** The pericarp surface
1117 of Nudinka at GS 77 is smooth and un-damaged. **b** The pericarp surface of Concerto has
1118 been damaged by removal of the husk, evidenced by broken cells (black arrow). **c** At higher
1119 magnification the surface of the cementing material can be seen. It is a layer of globular
1120 material that has been damaged by removal of the husk. Where the cementing material has

1121 been pulled away, the underlying surface is smooth (black arrow). **d** The junction between a
1122 tubelike cell and the pericarp surface is shown (black arrow), demonstrating that the
1123 tubelike cell is not covered by the globular cementing material.

1124

1125 **Fig. 6** Contrast plots of model coefficients showing relative changes in abundance among
1126 treatments of the following compounds between GS 75 and 77 among the four treatments.
1127 **a** Octadecanol. **b** Tritriacontane. **c** Campesterol. **d** β-Sitosterol.

1128

1129 **Additional files:**

1130 **Additional file 1** "Additional file 1.xlsx" is an excel workbook containing 8 sheets, with
1131 supplementary tables of data or analysis results. These are numbered Table S1 through
1132 Table S8, and are each invididually referred to in the tex as, for example "...shown in
1133 Additional file 1, Table S2".

1134

1135 **Additional file 2** "Additional file 2.pdf" Is a schematic of mass spectrometric fragmentation
1136 patterns to enable readers to understand how certain lipid compounds where identified
1137 using their characteristic fragmentations.

1138

1139 **Additional file 4** "Additional file 4.pdf" Is a large series of graphs showing the mean
1140 abundance of compounds for each structural class, ordered by carbon-number, for each
1141 treatment and growth stage sampled. Some readers may be interested in compound

1142 distributions as displayed this way, although we do not feel it was essential to the main

1143 message of the text.

1144

1145 **Additional file 4** “Additional file 4.pdf” Is a schematic showing the different tautomers of

1146 enol compounds used to quantify the enols as described in the text.