

# Feeding dihydroquercetin and vitamin E to broiler chickens reared at standard and high ambient temperatures

by Pirgozliev, V., Westbrook, C., Woods, S., Mansbridge, S.C., Rose, P., Whiting, I., Yovchev, D., Atanasov, A., Kljak, K., Staykova, G. and Ivanova, S.

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**Harper Adams  
University**

1 **Feeding dihydroquercetin and vitamin E to broiler chickens reared at standard and high**  
2 **ambient temperatures**

3

4 V. Pirgozliev<sup>1a</sup>, S.C. Mansbridge<sup>1</sup>, C. Westbrook<sup>1</sup>, S. Woods<sup>1</sup>, S.P. Rose<sup>1</sup>, I.M. Whiting<sup>1</sup>, D.  
5 Yovchev<sup>2</sup>, A.G. Atanasov<sup>3,4,5,6</sup>, K. Kljak<sup>7</sup>, G.P. Staykova<sup>8</sup>, S. Ivanova<sup>9</sup>, M.R. Karagecili<sup>10</sup>, F.  
6 Karadas<sup>10</sup>, J.H. Stringhini<sup>11</sup>

7

8 <sup>1</sup>The National Institute of Poultry Husbandry, Harper Adams University, Shropshire, UK

9 <sup>2</sup>Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, Bulgaria

10 <sup>3</sup>Ludwig Boltzmann Institute for Digital Health and Patient Safety, Medical University of  
11 Vienna, 1090 Vienna, Austria

12 <sup>4</sup>Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, 05-552  
13 Magdalenka, Poland

14 <sup>5</sup>Institute of Neurobiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

15 <sup>6</sup>Department of Pharmacognosy, University of Vienna, 1090 Vienna, Austria

16 <sup>7</sup>Faculty of Agriculture, University of Zagreb, Croatia

17 <sup>8</sup>Agricultural Institute, 9700 Shumen, Bulgaria

18 <sup>9</sup>Agricultural Academy, 1373 Sofia, Bulgaria

19 <sup>10</sup>Department of Animal Science, Yuzuncu Yil University, Van, Turkey

20 <sup>11</sup>Universidade Federal de Goias, Goiania, Brazil

21 <sup>a</sup> Corresponding author: [vpirgozliev@harper-adams.ac.uk](mailto:vpirgozliev@harper-adams.ac.uk)

22

23 **Abstract**

24 The use of natural antioxidants, in particular polyphenols such as dihydroquercetin (DHQ), in  
25 animal nutrition have recently increased in popularity. This may partly be due to the risk of  
26 increased incidences of heat stress associated with raising livestock in warmer ambient  
27 temperatures, facilitated by global warming, reducing antioxidant capacity. The current  
28 research demonstrates the effect of dietary DHQ, vitamin E and standard or high ambient  
29 temperatures on growth performance, energy and nutrient metabolism, gastrointestinal tract  
30 development (GIT), jejunal villus morphometry and antioxidant status in broiler chickens. Each  
31 of the four experimental diets were fed to 16 pens of five birds, which were allocated to four  
32 rooms (four pens in each room). The temperature in two rooms was maintained at a constant  
33 35 °C (high temperature; HT), and the temperature in the other two rooms was gradually  
34 reduced from 27 °C at 7d of age to 22 °C at 20d of age (standard temperature; ST). Rearing

35 birds at HT reduced: feed intake, weight gain, weight of small intestine, total GIT, liver, spleen,  
36 heart, villus height, villus surface area and lowered blood glutathionperoxidase (GSH-Px).  
37 Dietary DHQ increased blood GSH-Px and total antioxidant status, increased heart weight and  
38 reduced caecal size. When fed separately, DHQ and vitamin E improved hepatic vitamin E  
39 concentration. Feeding vitamin E increased spleen and liver weights. When fed together, DHQ  
40 and vitamin E reduced villus height, villus height to crypt depth ratio and villus surface area.  
41 Temperature and antioxidants did not affect energy and nutrient metabolism. There were no  
42 effects of dietary antioxidants on growth performance of broiler chickens and there were no  
43 mortalities. At present it is unclear if feeding antioxidants (in particular DHQ) at different  
44 levels, using different dietary formulations, and rearing birds under a range of environmental  
45 conditions may be effective at enhancing production performance and bird health in hot  
46 ambient climates.

47

48 **Key words:** broilers, dihydroquercetin (DHQ), vitamin E, growth performance, GSH-Px,  
49 ambient temperature

50

## 51 **1. INTRODUCTION**

52 The rise in temperature due to global warming is an increasingly important consideration for  
53 poultry producers to ensure efficient production and good health and welfare of birds (Niu et  
54 al., 2009; Quinteiro-Filho et al., 2010). To reduce the impact of high temperatures, producers  
55 in hot climates typically use cooling and ventilation systems which increase production costs  
56 and are only applicable in intensive production systems (Woods et al., 2020a). However, the  
57 use of free-range rearing systems in broiler production is increasing, thus research into different  
58 approaches to alleviate the impact of heat stress on bird production performance is needed.

59

60 The use of natural antioxidants, in particular polyphenols, in food and nutrition has recently  
61 gained increased popularity (Surai, 2014). Dihydroquercetin (DHQ), also known as taxifolin,  
62 is a flavonoid, a major sub-group representing plant polyphenols, commonly found in onions,  
63 milk thistle, and various conifers (Weidmann, 2012). Dihydroquercetin has been widely  
64 applied as an antioxidant for the surface treatment of fresh meat and fish (Kamboh et al., 2019).  
65 An extensive review by Fomichev et al. (2017) reported an enhancement in growth  
66 performance of poultry and pigs when fed DHQ supplemented diets, with the responses more  
67 noticeable during summer months. Pirgozliev et al. (2019a) did not find significant differences  
68 in growth performance or physiological variables of fully-grown broilers fed DHQ, when

69 reared under industry conditions. It has been suggested, however, that where reported  
70 improvements in production variables have been noted in the literature, these may be observed  
71 when animals are exposed to heat stress (Fomichev et al. 2017). Rearing animals at  
72 temperatures outside their thermal comfort zone may deplete levels of tissue antioxidants; thus,  
73 the antioxidant status of animals may be enhanced by dietary DHQ supplementation (Surai,  
74 2014). However, there are no reported studies comparing the response to DHQ of broilers  
75 reared under standard and high ambient temperatures. In addition, there are no comparisons  
76 between the effectiveness of DHQ and other well recognised antioxidants, e.g. vitamin E, on  
77 their impact (and interactions) on growth performance and antioxidant capacity of poultry at  
78 different rearing temperatures. Dietary inclusion of supplementary antioxidants, including  
79 polyphenols and vitamin E, have been shown to reduce the adverse impact associated with high  
80 temperature (reduced antioxidant status and growth performance compared to standard rearing  
81 conditions) by improving antioxidant status and growth performance of poultry (Fomichev et  
82 al. 2017; Mazur-Kuśnerek et al., 2019).

83

84 The primary objectives of this experiment were to study the impact of dietary DHQ and vitamin  
85 E on growth performance variables, dietary N-corrected apparent metabolisable energy  
86 (AMEn), dry matter (DMR) and nitrogen retention (NR) coefficients, when fed to broiler  
87 chickens from 7 to 28 days of age, reared at industry recommended and high ambient  
88 temperatures. In addition, secondary objectives were to examine the impact of experimental  
89 diets and ambient temperatures on gastrointestinal tract (GIT) and relative internal organ  
90 weights, and jejunal villus morphometry. Finally, an evaluation of the influence of antioxidants  
91 and ambient temperatures on bird antioxidant status was determined.

92

## 93 **2. MATERIALS AND METHODS**

### 94 **2.1. Experimental diets**

95 A wheat-soy-based basal grower diet formulated to meet breeder's recommendations (Aviagen  
96 Ltd., Edinburgh, UK) (Table 1) was mixed for the experiment. The diet was supplied with 5  
97 g/kg of TiO<sub>2</sub> as an indigestible marker. The basal diet was then split into four batches that had  
98 1.) no additive (control diet; C); 2.) C + 0.5 g/kg extract of Siberian Larch (*Larix sibirica*) (JSC  
99 NPF Flavit, IBI RAS, Pushchino city, Moscow region, Russian Federation 142290). According  
100 to the supplier, this extract contains over 85 % pure DHQ, with the remainder including other  
101 flavonoids, saponins and water (DHQ diet); 3.) C + 0.3 g/kg vitamin E (Merck KGaA,

102 Darmstadt, Germany) (vit E diet); 4.) C + 0.5 g/kg extract of Siberian Larch (*Larix sibirica*) +  
103 0.3 g/kg vitamin E (DHQ + vit E diet).

104 **[Insert Table 1 here]**

105

## 106 **2.2. Animals, husbandry and sample collection**

107 The experiment was conducted at the National Institute of Poultry Husbandry and approved by  
108 Harper Adams University Research Ethics Committee, UK. A total of 340 day-old male Ross  
109 308 broilers were obtained from a commercial hatchery (Cyril Bason Ltd, Craven Arms, UK),  
110 allocated to a single floor pen and offered a proprietary wheat-based broiler starter feed  
111 formulated to meet Ross 308 nutrient requirements (Aviagen Ltd., Edinburgh, UK). At 7d age,  
112 320 of the birds, excluding ill and malformed, were allocated at random to the four  
113 experimental diets. Each diet was fed to 16 pens (five birds each), 64 pens in total, which were  
114 allocated to four rooms (16 pens in each room). Each of the pens had a solid floor and were  
115 equipped with an individual feeder and drinker. Feed and water were offered *ad libitum* to birds  
116 throughout the experiment. The temperature in two of the rooms was maintained at a constant  
117 35 °C (HT), and the temperature in the other two rooms was gradually reduced from 27 °C at  
118 7d age to 22 °C at 20d age (following breeder's recommendations; ST). A standard lighting  
119 programme for broilers was used, decreasing the light:dark ratio from 23h:1h from day old to  
120 18h:6h at 7d of age, which was maintained until the end of the study. The well-being of the  
121 birds was checked regularly every day.

122

123 Birds and feed were weighed on days 7 and 28 in order to determine average daily feed intake  
124 (FI), average daily weight gain (WG) and to calculate the feed conversion ratio (FCR) on a pen  
125 basis. For the last three days of the study, from day 18 to day 21, the solid floor of each pen  
126 was replaced with a wire mesh. During this period all excreta were collected each day, stored  
127 in a fridge (~5 °C), and a well-homogenised representative subsample was dried at 60 °C and  
128 then milled through a 0.75 mm screen.

129

130 At the end of the study, one bird per pen (selected at random), was electrically stunned and  
131 blood was obtained in heparin coated tubes from the jugular vein. The development of the GIT  
132 from the same birds was determined. The proventriculus and gizzard (PG), duodenum,  
133 pancreas, jejunum, ileum, caeca, liver, spleen and the heart were immediately collected and  
134 weighed. The liver (without gallbladder) was freeze dried and stored at minus 80 °C before  
135 being analysed for vitamin E content. Approximately 5 cm of the middle part of the jejunum,

136 between the point of bile duct entry and Meckel's diverticulum, of one of the birds was sampled  
137 and stored in 10 % neutral-buffered formalin.

138

### 139 **2.3. Laboratory Analysis**

140 The analysed chemical composition of the basal diet is detailed in Table 1. Dry matter (DM)  
141 in feed and excreta samples was determined by drying of samples in a forced draft oven at 105  
142 °C to a constant weight (AOAC 2000; method 934.01). Crude protein ( $6.25 \times N$ ) in samples  
143 was determined by the combustion method (AOAC 2000; method 990.03) using a LECO FP-  
144 528 N (Leco Corp., St. Joseph, MI). Oil (as ether extract) in diets was extracted with diethyl  
145 ether by the ether extraction method (AOAC 2000; method 945.16) using a Soxtec system  
146 (Foss Ltd., Warrington, UK). The gross energy (GE) value of feed and excreta samples was  
147 determined in a bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL) with benzoic  
148 acid used as the standard. Titanium in feed and excreta was determined as described by Short  
149 et al. (1996). Dietary AMEn was calculated following the method of Hill and Anderson (1958),  
150 and retention coefficients were determined as previously described (Pirgozliev et al., 2019b).

151

152 The glutathione peroxidase (GSH-Px) assay in blood was performed using a Ransel GSH-Px  
153 kit (Randox Laboratories Ltd., UK) that employs the method based on that of Paglia and  
154 Valentine (1967). Total antioxidant status (TAS) determined in the blood serum was  
155 determined using a Randox kit, following manufacturer's recommendations (Randox  
156 Laboratories Ltd., UK). The heterophil/lymphocyte (H:L) ratio in blood was determined as  
157 described by Müller et al. (2011). The pack cell volume (PCV) test, also called the haematocrit  
158 test, was also determined (Fedde and Wideman, 1996). The vitamin E content in diets and  
159 livers was determined using an HPLC system as previously described (Karadas et al., 2010,  
160 2014).

161

162 The relative empty weights of GIT segments, including spleen and heart, of each bird were  
163 determined as previously described (Abdulla et al. 2017; Pirgozliev et al. 2019a). Jejunal  
164 samples collected in section 2.2. were embedded in paraffin wax, sectioned at approximately  
165  $5 \mu\text{m}$  and four gut segments were fixed on each slide. Morphometric measurements were  
166 determined on 20 intact well-oriented villus-crypt units for each bird as previously described  
167 (Yovchev et al., 2019).

168

### 169 **2.4. Statistical Analysis**

170 Data were analysed using Genstat (18<sup>th</sup> edition) statistical software (IACR Rothamstead,  
171 Hertfordshire, UK). Comparisons among performance, diet and temperature were performed  
172 by a split plot ANOVA procedure using a 2 X 2 X 2 factorial design. The main plots were the  
173 four rooms that were each randomly allocated to one of the two temperatures. The pens within  
174 each room were the sub-plots and these were randomly allocated to one of the four dietary  
175 treatments. The statistical analysis used the following matrix model:

176

$$177 \quad Y_{ijkl} = \mu + A_i + N_{l(i)} + B_j + C_k + (BC)_{jk} + (AB)_{ij} + (AC)_{ik} + (ABC)_{ijk} + \varepsilon_{l(ijk)}$$

178

179 Where:

180  $\mu_i$  = Grand mean

181  $A_i$  = Fixed effect of temperature

182  $N_{l(i)}$  = Whole plot (room) error

183  $B_j$  = Fixed effect of DHQ

184  $C_k$  = Fixed effect of Vit E

185  $(BC)_{jk}$  = Fixed interaction of DHQ and Vit E

186  $(AB)_{ij}$  = Fixed interaction of temperature and DHQ

187  $(AC)_{jk}$  = Fixed interaction of temperature and Vit E

188  $(ABC)_{ijk}$  = Fixed three-way interaction of temperature, DHQ and Vit E

189  $\varepsilon_{l(ijk)}$  = Split-plot error

190

191 Data were checked for normal distribution. A protected LSD test was used to separate  
192 differences in interaction means if statistical differences were evident  $p < 0.05$ . Means for  
193 interactions are only included in tables when p-values were significant.

194

### 195 **3. RESULTS**

196 All birds were healthy throughout the study period and there was no mortality.

197

#### 198 ***3.1. Growth performance and relative organ weights***

199 The overall bird weight at 28d age was 988 g, with birds reared at ST at 1196 g, and birds  
200 reared at HT at 780 g ( $p = 0.022$ ) (Table 2). Birds at HT had lower FI, (52 vs 81 grams daily;  
201  $p = 0.020$ ). Rearing birds at HT reduced their WG from 51 to 30 grams per day ( $p = 0.028$ ).

202 The FCR was not affected ( $p > 0.05$ ) by diets or temperature. There was no significant effect  
203 of vitamin E or DHQ on bird production performance characteristics.

204

205 **[Insert Table 2 here]**

206

207 The information on the GIT of the birds expressed as a relative weight of the body weight is  
208 presented in Table 3. Rearing birds at HT reduced the relative weight of jejunum, liver, total  
209 GIT, spleen and heart ( $p < 0.05$ ) and also tended ( $p = 0.091$ ) to reduce the weight of the  
210 duodenum. Feeding DHQ significantly reduced caecal weight ( $p = 0.011$ ), but increased ( $p =$   
211  $0.002$ ) relative heart weight. Feeding vitamin E increased the weight of liver ( $p = 0.011$ ) and  
212 spleen ( $p = 0.009$ ) and tended ( $p = 0.054$ ) to increase the relative weight of the PG of the birds.  
213 Birds fed vitamin E reared at ST had heavier caeca ( $p = 0.014$ ) compared to birds reared at HT  
214 (0.92% vs 0.55%), although no difference ( $p > 0.05$ ) existed in birds fed diets containing no  
215 additional vitamin E (0.77% vs 0.65%) for ST and HT respectively.

216

217 **[Insert Table 3 here]**

218

### 219 **3.2. Dietary AMEn and nutrient availability**

220 Dietary AMEn, DMR and NR were not significantly influenced by supplementary DHQ,  
221 vitamin E or rearing temperature ( $p > 0.05$ ).

222

### 223 **3.3. Jejunal villus morphometry**

224 The results of the jejunal villus morphometry of the chicks is presented in Table 4. There were  
225 many interactions between the studied treatments. In general, rearing birds at HT reduced VH  
226 and villus surface area without any mitigating effect from DHQ or vitamin E. It seems that  
227 feeding vitamin E and DHQ together changed the studied villus morphometry variables  
228 reducing VH, VH:CD and villus surface area ( $p < 0.001$ ).

229

230 **[Insert Table 4 here]**

231

### 232 **3.4. Antioxidant status of birds**

233 The hepatic vitamin E concentration was not affected by rearing temperature ( $P > 0.05$ ) (Table  
234 5). However, feeding DHQ or vitamin E, improved hepatic vitamin E concentration by 38.6 %  
235 and 23 %, respectively ( $p < 0.05$ ). The blood GSH-Px of birds reared at HT was 17 % lower ( $p$



236 = 0.039) than those of birds reared at ST, i.e. 53 vs 62 U/ml RBC. However, supplementary  
237 DQH increased GSH-Px by 13 % compared to birds fed DHQ free diets ( $p = 0.013$ ), i.e. 61 vs  
238 53 U/ml RBC. Similarly, dietary DHQ improved TAS by 33.3 % ( $p = 0.021$ ) compared to birds  
239 fed non-supplemented diets, i.e. 0.81 vs 0.54 mmol/l. The H:L ratio was not affected ( $p > 0.05$ )  
240 by experimental treatments. There was no diet by rearing temperature interactions ( $p > 0.05$ )  
241 for any of the studied variables in Table 5.

242

243 **[Insert Table 5 here]**

244

#### 245 **4. DISCUSSION**

246 The aim of this experiment was to evaluate the impact of dietary DHQ and vitamin E, alone  
247 and in combination, when fed to broiler chickens reared at high and standard ambient  
248 temperatures. The mean average weight of birds reared at the standard temperature at 28d of  
249 age was 1196 g; which is 27.5 % below the Ross 308 broiler target weight for commercial  
250 flocks. The birds were kept in small groups in research facilities, and fed mash diets which  
251 were further mixed before feeding, potentially compromising diet homogeneity, thus the  
252 reduced performance compared to large commercial flocks was acceptable (Pirgozliev et al.,  
253 2016; Yang et al., 2020). It is possible that lighter birds may be less susceptible to heat stress.

254

##### 255 **4.1. Growth performance and relative organ weights**

256 In agreement with previous studies (Quinteiro-Filho et al., 2010), birds reared at a constant  
257 temperature of 35 °C responded with reduced FI and WG, although FCR was not affected by  
258 rearing temperature. The results of the relative weights of the organs measured as percentage  
259 of body weight agreed with published reports (Abdulla et al. 2016; 2017). Birds in HT group  
260 with reduced WG also had a reduced relative weight of the GIT, particularly of the small  
261 intestines. Woods et al. (2020a) also found a reduction in the relative weight of the small  
262 intestine, liver, spleen and heart in birds reared at HT. A reduction in the relative heart weight  
263 of birds reared at HT has previously been observed by Yahav et al. (1999). Changes in relative  
264 organ weight may not be related to the reduced feed intake alone, since Palo et al. (1995a)  
265 found that restricted feeding only influenced absolute organ weight, not relative organ weight,  
266 and changes are transient, resulting in an improved FCR (Palo et al. 1995b). Heat stress can  
267 influence hypothalamic peptides involved in appetite regulation (Song et al., 2012) and  
268 decrease feed passage rate in the GIT, further decreasing trypsin, chymotrypsin, and amylase  
269 activity (Hai et al., 2000). Chronic heat stress can reduce blood supply of the GIT due to

270 induced peripheral vasodilation (Mckee et al., 1997), leading to a decreased size of the small  
271 intestine and absorptive capacity (Mitchell and Carlisle, 1992). High ambient temperature is  
272 therefore likely to reduce weight gain through a variety of mechanisms than the reduced feed  
273 intake alone, as noted in this study, though the effects of both factors could not be fully  
274 separated.

275

276 The enlarged hearts of the birds fed DHQ, coupled with an increase in determined GSH-Px and  
277 TAS in this study, infers that there is a potential mechanism of antioxidant protection in birds  
278 fed DHQ. However, the enlarged heart of DHQ fed birds is difficult to explain without further  
279 pathological and anatomical investigation. Korzeniowska et al. (2019) did not find differences  
280 between the relative weight of the spleen in birds fed selenium as an antioxidant. Khan et al.  
281 (2010) reported an increase in the relative weight of liver of hens with aflatoxicosis. The same  
282 authors (Khan et al., 2010) reported that a concurrent feeding of vitamin E did not ameliorate  
283 the toxic effects of aflatoxins in the hens as determined by the relative weight of the liver.  
284 Despite the liver and spleen enlargement reported in this study, no lesions and / or  
285 discolouration was observed, there was no mortality and no obvious sign of clinical disease.  
286 As previously discussed, the pathology was not determined in this study. Thus, an association  
287 cannot be made between the increase of organs size and clinical disease in this study.

288

289 The lack of response in growth performance variables to DHQ in this study is in accordance  
290 with previous research (Pirgozliev et al., 2019a), and is contradictory with the hypothesis that  
291 DHQ improves performance of birds reared under stress, i.e. during hot summer time  
292 (Fomichev et al., 2017). Published results on the effect of vitamin E on broiler growth  
293 performance are inconsistent as the use of vitamin E: improved performance of broilers (Guo  
294 et al., 2003); did not influence growth performance of broilers (Goñí et al., 2007; Niu et al.,  
295 2009) and has even reduced performance (Bölükbaşı et al., 2006). It would seem that the lack  
296 of response is prevalent in the literature and agrees with our findings in this study. However,  
297 in this current study, the determined vitamin E in the control diet was 43.86  $\mu\text{g/g}$  (65.5 IU),  
298 which is similar to the levels of dietary vitamin E recommended by the breeder (Aviagen Ltd,  
299 Edinburgh, UK) of 65 IU for this age of Ross 308. The similar levels of vitamin E in the diets  
300 compared to recommendations suggests a potential explanation for the lack of response  
301 observed in growth performance variables in this and other similar studies.

302

303 ***4.2. Dietary AMEn and nutrient retention***

304 Despite the reduction in feed intake and changes in GIT segment weights and villus  
305 morphometry, the results for AMEn and nutrient retention coefficients in the reported study  
306 were not significantly influenced by rearing temperature or dietary antioxidants. There was,  
307 however, a 0.9 MJ/kg difference in AMEn, between HT and ST birds. Birds reared under HT  
308 had a similar AMEn value to the expected dietary metabolisable energy (ME), though this does  
309 not consider the effect of feed intake. Published data on the impact of high ambient temperature  
310 on dietary ME and nutrient digestibility are not consistent. Bonnet et al. (1997) reported a  
311 reduction in ME and nutrient digestibility values in birds reared at 35 °C, although Woods et  
312 al. (2020a) did not observe differences when studying the same variables at the same  
313 temperature. Attia et al. (2018) reported an increase in nutrient digestibility in birds reared at  
314 high temperatures, while Koelkebeck et al. (1998) did not find an impact of rearing temperature  
315 on amino acid digestibility in laying hens. The differences may be attributed to different age,  
316 breed and type of production of the experimental birds, different dietary formulations, exposure  
317 to different temperatures for different lengths of time, ambient humidity and rearing conditions.  
318 Hai et al. (2000) reported that birds reared at a high temperature had decreased activity of  
319 trypsin, chymotrypsin and amylase, and suppressed ability to expel digesta from the crop or  
320 small intestine. Reduction in pancreatic enzyme production is usually associated with an  
321 increase in the size / weight of the pancreas in order to compensate for the reduced enzyme  
322 production (Abdulla et al., 2016). The relative weight of the pancreas in this report was not  
323 affected by rearing temperature, suggesting that the reduced release of digesta from the crop to  
324 small intestine in HT reared birds may lead to a proportional reduction in the release of  
325 pancreatic enzymes. This accounts for the AMEn and nutrient retention coefficients observed.  
326 Limited studies have reported comparisons in ME and nutrient availability in antioxidant  
327 supplemented diets. In agreement with previous reports (Goñi et al., 2007; Pirgozliev et al.,  
328 2019b), no differences were found between the broilers fed control, vitamin E and DHQ with  
329 regard to ME and nutrient retention coefficients. Studies with other antioxidants, i.e. dietary  
330 selenium, also did not detect differences in ME and nutrient retention coefficients (Choct and  
331 Naylor, 2004; Woods et al., 2020b). As ME is a measurement of the available energy in  
332 carbohydrates, fats and proteins, it is expected that dietary antioxidants would not greatly  
333 impact the ME status.

334

### 335 ***4.3. Jejunal villus morphometry***

336 The results of the villus measurements were in the expected range for birds at this age and  
337 reared under similar conditions (Santos et al., 2015; Pirgozliev et al., 2019b). Studying histo-

338 morphometric changes in the intestines of broilers during heat stress, Santos et al. (2015)  
339 indicated that the duodenum and jejunum showed more damage than the ileum. In agreement  
340 with the reported study, Santos et al. (2015) also found that when compared with  
341 morphologically normal jejunal villi, the villi of birds reared at HT had decreased height and  
342 surface area. The increase in the number and size of the intestinal villi increases the absorption  
343 surface per unit of intestinal area (Yovchev et al., 2019), thus, HT reduces the absorptive  
344 capacity of the small intestine. However, villus morphometry does not always correlate with  
345 detectable differences in bird growth (Pirgozliev et al., 2010) and retention may be considered  
346 as a more direct indicator of absorptive capacity.

347

#### 348 ***4.4. Antioxidant status of birds***

349 In many cases, the antioxidant status in birds is determined by measuring TAS and GSH-Px  
350 activity (Krawczuk-Rybak et al., 2012; Mazur-Kuśnirek et al., 2019). The antioxidant enzyme  
351 system, including GSH-Px and TAS, works in concert with free radical scavengers to quench  
352 reactive oxygen species and to protect cells from oxidative damage (Surai, 2014). The balance  
353 between the production of free radicals and the antioxidant system could be disturbed by heat  
354 stress in chickens (Lin et al., 2006). As temperature increases, oxidative stress would be  
355 expected to increase and the animal's overall GSH-Px and TAS would be expected to decrease  
356 (Sarica et al., 2017). In agreement with these reports, exposing birds to HT in this study  
357 decreased the overall GSH-Px and tended to decrease TAS.

358

359 Dietary DHQ increased GSH-Px, TAS and hepatic vitamin E which further supports the view  
360 that flavonoids can protect animal cells against oxidative stress, an action attributed to their  
361 antioxidant properties (Chen and Deuster, 2009). Supplementary vitamin E improved hepatic  
362 vitamin E, but did not affect blood antioxidant markers in this study. Feeding vitamin E with  
363 an organic source of selenium to broilers, Choct and Naylor (2004) found changes in blood  
364 GSH-Px, but not in the growth performance of the birds. Feeding dietary vitamin E at 200  
365 mg/kg, Mazur-Kuśnirek et al. (2019) observed an improved TAS which was coupled with a  
366 higher percentage content of breast muscle. It is known that inadequate vitamin E status lowers  
367 corticosteroid synthesis and thus reduces the animal's ability to cope with stress (Choct and  
368 Naylor, 2004). However, the control diet of Mazur-Kuśnirek et al. (2019) showed a very low  
369 dosage of vitamin E, 10 mg/kg vs 40 mg/kg recommendation (Aviagen Ltd, Edinburgh, 2019),  
370 which may explain the observed responses.

371

372 The intent was to apply the H/L ratio method to measure oxidative stress based on established  
373 principles (Maxwell and Robertson, 1998). Heat stress alters homeostasis by affecting the  
374 adrenal-corticoid axis and the resulting changes in hormone levels may change the numbers of  
375 lymphocyte and heterophil, thus changing the H/L. This method has however been criticised  
376 for not providing an adequate indication of stress alone (Müller et al., 2011), which agrees with  
377 our results where no significant difference in H/L was observed.

378

379 Broilers selected for an improved feed conversion ratio (e.g. Ross and Cobb strains) were  
380 shown to have more difficulty adapting to changes in their environment than in less selected  
381 birds (Scheele et al., 1991). The increased PCV in modern broiler strains is associated with an  
382 increase in blood viscosity, pulmonary arterial hypertension, ascites and death (Fedde and  
383 Wideman, 1996). The PCV values in the reported study were in the expected range (Fedde and  
384 Wideman, 1996; Hasan et al., 2015). In agreement with our results, Hasan et al. (2015) also  
385 did not find differences in PCV in a study with Cobb 500, despite high rearing temperatures  
386 (22 °C vs 35 °C). It seems that PCV can be changed when birds are exposed to extreme  
387 stressors, thus the lack of differences in PCV between birds fed DHQ or vitamin E was not a  
388 surprise.

389

390 Direct comparisons between studies using DHQ are difficult because there is no consistency in  
391 dietary concentrations (Pirgozliev et al., 2019a). In the reported study, DHQ was added at 0.5  
392 g per kg feed. On average birds were consuming approximately, 67 g feed per day, and their  
393 average daily weight gain was approximately 41 g. Thus, the average daily consumption of  
394 DHQ was 0.03 g per bird, or 0.73 g per kilogram daily growth. The lack of adverse effects on  
395 animals fed relatively high dietary DHQ concentrations in the reported and in previous studies  
396 (Pirgozliev et al., 2019a) gives an opportunity for further research, including various dietary  
397 DHQ concentrations. Studying the potential interactions between DHQ and exogenous  
398 enzymes, or comparing DHQ of different purities may also be of interest.

399

400 The mode of action of flavonoids is usually associated with their antioxidant properties (Surai,  
401 2014), but flavonoids do not behave the same way in vitro and in vivo (Veskoukis et al. 2012).  
402 In the present study, birds fed DHQ or vitamin E had no interaction with rearing temperatures.  
403 Thus, the antioxidant properties of DHQ and vitamin E did not benefit the overall growth  
404 performance variables of birds reared at high ambient temperature. It should be noted, however,

405 that in the reported study, the determined level of dietary vitamin E in the control diet was close  
406 to the daily recommendations of the breeder.

407

## 408 **5. CONCLUSIONS**

409 Rearing birds at a high ambient temperature reduced daily feed intake and weight gain but did  
410 not affect the efficiency of feed utilisation. Feeding DHQ or vitamin E improved various  
411 aspects of antioxidant status of the birds, although it did not affect growth performance, energy  
412 or nutrient availability. There were no observed interactions between dietary antioxidants and  
413 rearing temperature in the variables studied. At present it is unclear if feeding antioxidants (in  
414 particular DHQ) at different levels may be effective at enhancing production performance and  
415 bird health in hot ambient climates.

416

## 417 **ACKNOWLEDGEMENTS**

418 Special thanks to Richard James and Rose Crocker of the National Institute of Poultry  
419 Husbandry (Harper Adams University) for their technical support in conducting the study.

420

## 421 **DISCLOSURE STATEMENT**

422 The authors report no potential conflicts of interest. This work was not sponsored by any  
423 funding agency or commercial company.

424

## 425 **DATA AVAILABILITY STATEMENT**

426 The data that support the findings of this study are available from the corresponding author,  
427 upon reasonable request, subject to restrictions and conditions.

428

## 429 **ETHICS STATEMENT**

430 The authors confirm that they have followed all appropriate EU and UK standards and  
431 regulations for the protection of animals used for scientific purposes. All mandatory laboratory  
432 health and safety procedures have been complied with in the course of conducting this  
433 experimental work. This manuscript complies with the ARRIVE guidelines (Kilkenny et al.,  
434 2010).

435

## 436 **ORCID**

437 VR Pirgozliev - <https://orcid.org/0000-0002-4213-7609>

438 SC Mansbridge - <https://orcid.org/0000-0003-4246-9782>

439 SP Rose - <https://orcid.org/0000-0001-6459-597X>  
440 S Ivanova - <https://orcid.org/0000-0002-6226-1287>  
441 AG Atanasov - <https://orcid.org/0000-0003-2545-0967>  
442 F Karadas - <https://orcid.org/0000-0002-8187-349X>  
443 Jose Henrique Stringhini - <https://orcid.org/0000-0002-3710-6963>

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661 Table 1. Ingredient composition [g/kg 'as fed'] and nutritional analysis of the basal diet for  
 662 broiler chickens

Ingredients	g/kg
Wheat	550
Soya bean meal [crude protein = 48 %]	230
Barley	79
Full fat soya meal	50
Soya oil	45
Limestone	12.5
Monocalcium phosphate	12.5
Salt	2.5
Sodium bicarbonate	1.5
L Lysine HCL	3
DL Methionine	3.5
L Threonine	1.5
Vitamin/Mineral premix*	4
Titanium dioxide	5
Total	1000
Calculated values [as fed]	
Crude protein [Nx6.25]	201
Crude fat [g/kg]	68
Metabolisable energy [MJ/kg]	12.99
Calcium [g/kg]	9.3
Available Phosphorus [g/kg]	4.2
Available Lysine [g/kg]	11.8
Methionine + Cysteine [g/kg]	9.4
Determined values	
Dry matter	894
Gross energy [MJ/kg]	17.43
Crude protein [N x 6.25]	194
Crude fat [g/kg]	69
Vitamin E [ $\mu\text{g/g}$ ] <sup>†</sup>	43.86

663 \*The vitamin and mineral premix contained vitamins and trace elements to meet the  
 664 requirements specified by NRC [1994] except vitamin E. There was no vitamin E  
 665 supplemented to the control diet. The premix provided [units/kg feed]: retinol 3600  $\mu\text{g}$ ,  
 666 cholecalciferol 125  $\mu\text{g}$ , menadione 3 mg, thiamine 2 mg, riboflavin 7 mg, pyridoxine 5 mg,  
 667 cobalamin 15  $\mu\text{g}$ , nicotinic acid 50 mg, pantothenic acid 15 mg, folic acid 1 mg, biotin 200  $\mu\text{g}$ ,  
 668 iron 80 mg, copper 10 mg, manganese 100 mg, cobalt 0.5 mg, zinc 80 mg, iodine 1 mg,  
 669 selenium 0.2 mg and molybdenum 0.5 mg.

670  
 671 <sup>†</sup>The determined values of vitamin E [ $\mu\text{g/g}$ ] for diets 2, 3 and 4 are 54.31, 87.51 and 83.49,  
 672 respectively.

673

674 Table 2. Effect of bird rearing temperature [T°C], dietary dihydroquercetin [DHQ] and vitamin  
675 E [Vit E] on bird final body weight [BW], bird daily feed intake [FI], bird daily weight gain  
676 [WG], feed conversion ratio [FCR], N-corrected apparent metabolisable energy [AMEn], dry  
677 matter [DMR] and nitrogen [NR] retention coefficients, when fed to broiler chickens from 7 to  
678 28d age \*

	Initial BW [g]	BW 28d [g]	FI [g/d]	WG [g/d]	FCR	AMEn [MJ/kg] †	DMR †	NR †
T°C								
ST‡	119	1196	81	51	1.589	11.74	0.703	0.659
HT#	123	780	52	30	1.683	12.64	0.743	0.650
SEM§	2.2	44.0	2.9	2.6	0.0295	0.324	0.0152	0.0078
DHQ								
No	122	987	66	41	1.631	12.15	0.719	0.651
Yes	121	990	67	40	1.640	12.24	0.728	0.658
SEM§	0.6	17.5	0.9	1.0	0.0183	0.083	0.0050	0.0059
Vit E								
No	121	1001	67	41	1.629	12.16	0.721	0.652
Yes	121	975	66	40	1.642	12.22	0.726	0.657
SEM§	0.6	17.5	0.9	1.0	0.0183	0.083	0.0050	0.0059
<i>p</i> -Values								
T°C	0.112	0.022	0.020	0.028	0.152	0.189	0.201	0.481
DHQ	0.242	0.903	0.881	0.723	0.727	0.427	0.209	0.384
Vit E	0.677	0.300	0.259	0.479	0.612	0.604	0.471	0.574
T°C x DHQ	0.469	0.723	0.499	0.448	0.854	0.903	0.965	0.979
SEM§	2.2	47.3	3.1	2.8	0.0347	0.335	0.0160	0.0098
T°C x Vit E	0.609	0.068	0.086	0.095	0.429	0.224	0.379	0.313
SEM§	2.2	47.3	3.1	2.8	0.0347	0.335	0.0160	0.0098
DHQ x Vit E	0.544	0.974	0.274	0.899	0.096	0.261	0.257	0.587
SEM§	0.8	24.7	1.3	1.4	0.0259	0.118	0.0070	0.0084
T°C x DHQ x Vit E	0.222	0.996	0.843	0.890	0.350	0.330	0.361	0.899
SEM§	2.4	53.4	3.3	3.1	0.0433	0.355	0.0175	0.0129

679 \* Each mean average represents values from thirty two replicate pens for main effects; †AMEn,  
680 DMR and NR were determined between 25 and 28 days of age; ‡ST = the ambient temperature  
681 was gradually reduced from 27 °C at 7d age to 22 °C at 20d age; #HT = high rearing temperature  
682 of constant 35 °C; §SEM = pooled standard errors of mean.

683  
684

685 Table 3. Effect of bird rearing temperature [T°C], dietary dihydroquercetin [DHQ] and vitamin E [Vit E] on the relative organ weight expressed  
 686 as the percent of body weight [BW] of gastrointestinal tract, liver, spleen and heart of 28d old broiler chickens\*.

	BW	PG	Duodenum	Pancreas	Jejunum	Ileum	Caeca	GIT	Liver	Spleen	Heart
T°C											
ST <sup>‡</sup>	1231	2.42	1.06	0.30	1.91	1.52	0.85	8.05	2.31	0.08	0.71
HT <sup>#</sup>	779	2.48	0.90	0.31	1.45	1.27	0.60	7.01	1.77	0.05	0.47
SEM <sup>§</sup>	45.2	0.057	0.035	0.012	0.043	0.105	0.049	0.110	0.030	0.001	0.035
DHQ											
No	1011	2.41	0.96	0.31	1.67	1.43	0.78	7.57	2.03	0.07	0.56
Yes	1000	2.49	1.0	0.30	1.69	1.34	0.66	7.49	2.05	0.08	0.61
SEM <sup>§</sup>	21.5	0.051	0.027	0.010	0.055	0.050	0.034	0.144	0.036	0.003	0.010
Vit E											
No	996	2.38	0.95	0.30	1.70	1.38	0.71	7.43	1.97	0.06	0.60
Yes	1015	2.52	1.01	0.31	1.66	1.41	0.73	7.63	2.11	0.07	0.58
SEM <sup>§</sup>	21.5	0.051	0.027	0.010	0.055	0.050	0.034	0.144	0.036	0.003	0.010
<i>p</i> -Values											
T°C	-	0.552	0.091	0.654	0.018	0.231	0.071	0.022	0.006	0.001	0.040
DHQ	-	0.214	0.354	0.887	0.845	0.188	0.011	0.689	0.695	0.873	0.002
Vit E	-	0.054	0.165	0.580	0.570	0.717	0.678	0.322	0.011	0.009	0.257
T°C x DHQ	-	0.147	0.625	0.300	0.604	0.528	0.387	0.777	0.262	0.791	0.187
SEM <sup>§</sup>		0.076	0.044	0.016	0.070	0.012	0.060	0.181	0.047	0.003	0.037
T°C x Vit E	-	0.627	0.741	0.376	0.636	0.900	0.014 <sup>†</sup>	0.750	0.428	0.556	0.787
SEM <sup>§</sup>		0.076	0.044	0.016	0.070	0.012	0.060	0.181	0.047	0.003	0.037
DHQ x Vit E	-	0.136	0.683	0.182	0.923	0.465	0.823	0.350	0.797	0.319	0.264
SEM <sup>§</sup>		0.072	0.038	0.014	0.077	0.071	0.048	0.204	0.050	0.004	0.014
T°C x DHQ x Vit E		0.969	0.898	0.228	0.832	0.311	0.814	0.734	0.609	0.744	0.654
SEM <sup>§</sup>		0.104	0.058	0.021	0.104	0.136	0.077	0.273	0.069	0.006	0.039

687 \*Each mean average represents values from thirty two replicate pens for main effects; <sup>‡</sup>ST = the ambient temperature was gradually reduced from  
 688 27 °C at 7d age to 22 °C at 20d age; <sup>#</sup>HT = high rearing temperature of constant 35 °C; <sup>§</sup>SEM = pooled standard errors of mean; <sup>†</sup>Birds fed vitamin  
 689 E reared at ST had heavier caeca [*p* = 0.014] compared to birds reared at HT [0.92 vs 0.55], although no difference [*p* > 0.05] existed in birds fed  
 690 diets containing no additional vitamin E [0.77 vs 0.65] for ST and HT respectively.  
 691



692 Table 4. Effect of bird rearing temperature [T°C], dietary dihydroquercetin [DHQ] and vitamin  
 693 E [Vit E] on the villus height [VH], villus width [VW], crypt depth [CD] and villus surface  
 694 area [Area] of 28d old broiler chickens\*.  
 695

			VH [μm]	VW [μm]	CD [μm]	VH:CD	Area (mm <sup>2</sup> )
T°C							
ST <sup>‡</sup>			1300	172	336	4.0	0.706
HT <sup>#</sup>			856	155	167	5.2	0.413
SEM <sup>§</sup>			2.8	0.4	0.2	0.01	0.0015
DHQ							
No			1136	169	227	5.2	0.601
Yes			1020	158	276	4.0	0.517
SEM <sup>§</sup>			2.4	1.6	1.3	0.04	0.0067
Vit E							
No			1081	166	229	4.9	0.565
Yes			1076	161	274	4.3	0.553
SEM <sup>§</sup>			2.4	1.6	1.3	0.04	0.0067
T°C	DHQ						
ST	No		1408	171	300	4.7 <sup>a</sup>	0.758
ST	Yes		1193	173	371	3.3 <sup>b</sup>	0.653
HT	No		865	167	153	5.6 <sup>c</sup>	0.445
HT	Yes		847	143	182	4.7 <sup>a</sup>	0.381
SEM <sup>§</sup>			3.7	1.7	1.3	0.04	0.0069
T°C	Vit E						
ST	No		1363	169	305	4.6 <sup>b</sup>	0.725
ST	Yes		1237	174	367	3.4 <sup>c</sup>	0.687
HT	No		799	164	153	5.3 <sup>a</sup>	0.406
HT	Yes		914	147	182	5.1 <sup>a</sup>	0.420
SEM <sup>§</sup>			3.7	1.7	1.3	0.04	0.0069
DHQ	Vit E						
No	No		1035	173	198	5.3 <sup>a</sup>	0.545
No	Yes		1238	165	256	5.1 <sup>a</sup>	0.658
Yes	No		1127	160	260	4.5 <sup>b</sup>	0.585
Yes	Yes		913	156	293	3.4 <sup>c</sup>	0.449
SEM <sup>§</sup>			3.5	2.3	1.9	0.05	0.0095
T°C	DHQ	Vit E					
ST	No	No	1326 <sup>d</sup>	155 <sup>a</sup>	260 <sup>a</sup>	5.1	0.647 <sup>b</sup>
ST	No	Yes	1489 <sup>g</sup>	186 <sup>c</sup>	341 <sup>c</sup>	4.4	0.869 <sup>f</sup>
ST	Yes	No	1400 <sup>e</sup>	183 <sup>c</sup>	350 <sup>d</sup>	4.0	0.803 <sup>e</sup>
ST	Yes	Yes	985 <sup>a</sup>	163 <sup>ab</sup>	393 <sup>f</sup>	2.5	0.504 <sup>a</sup>
HT	No	No	744 <sup>b</sup>	190 <sup>d</sup>	135 <sup>b</sup>	5.5	0.443 <sup>d</sup>
HT	No	Yes	987 <sup>f</sup>	144 <sup>ab</sup>	171 <sup>c</sup>	5.8	0.446 <sup>d</sup>
HT	Yes	No	854 <sup>c</sup>	138 <sup>a</sup>	171 <sup>c</sup>	5.0	0.368 <sup>bc</sup>
HT	Yes	Yes	841 <sup>c</sup>	149 <sup>b</sup>	192 <sup>e</sup>	4.4	0.394 <sup>c</sup>
SEM <sup>§</sup>			5.1	2.8	2.3	0.06	0.0117
<i>p</i> -Values							
T°C			<0.001	0.001	<0.001	<0.001	<0.001
DHQ			<0.001	<0.001	<0.001	<0.001	<0.001
Vit E			0.133	0.015	<0.001	<0.001	0.219
T°C x DHQ			<0.001	<0.001	<0.001	<0.001	0.034
T°C x Vit E			<0.001	<0.001	<0.001	<0.001	0.008
DHQ x Vit E			<0.001	0.443	<0.001	<0.001	<0.001
T°C x DHQ x Vit E			<0.001	<0.001	<0.001	0.305	<0.001

696 \*Each mean average represents values from thirty two replicate pens for main effects; <sup>‡</sup>ST =  
 697 the ambient temperature was gradually reduced from 27 °C at 7d age to 22 °C at 20d age; <sup>#</sup>HT  
 698 = high rearing temperature of constant 35 °C; <sup>§</sup>SEM = pooled standard errors of mean.  
 699 <sup>a-c</sup> Values within a column not sharing the same superscripts differ significantly at  $p \leq 0.05$ .

700 Table 5. Effect of bird rearing temperature [T°C], dietary dihydroquercetin [DHQ] and vitamin  
 701 E [Vit E] on hepatic vitamin E, blood plasma glutathione peroxidase [GSH-Px], total  
 702 antioxidant status [TAS], blood heterophil to lymphocyte [H:L] ratio and packed cell volume  
 703 [PCV] in 28d old broiler chickens\*.

	Hepatic vitamin E [µg/g]	GSH-Px [U/ml RBC]	TAS [mmol/l]	H:L	PCV
T°C					
ST‡	52	62	0.72	1.09	31.8
HT#	84	53	0.63	1.22	26.0
SEM§	11.5	1.3	0.103	0.111	1.45
DHQ					
No	57	53	0.54	1.11	29.3
Yes	79	61	0.81	1.20	28.5
SEM§	4.8	2.3	0.078	0.063	0.57
Vit E					
No	61	57	0.74	1.13	29.1
Yes	75	57	0.61	1.17	28.7
SEM§	4.8	2.3	0.078	0.063	0.57
<i>p</i> -Values					
T°C	0.185	0.039	0.606	0.485	0.107
DHQ	0.002	0.013	0.021	0.298	0.315
Vit E	0.043	0.964	0.219	0.655	0.643
T°C x DHQ	0.858	0.094	0.819	0.470	0.388
SEM§	12.4	2.6	0.129	0.128	1.56
T°C x Vit E	0.061	0.248	0.223	0.778	0.869
SEM§	12.4	2.6	0.129	0.128	1.56
DHQ x Vit E	0.575	0.603	0.084	0.829	0.716
SEM§	6.8	3.3	0.110	0.089	0.80
T°C x DHQ x Vit E	0.634	0.102	0.746	0.190	0.914
SEM§	14.2	4.2	0.169	0.156	1.75

704 \*Each mean average represents values from thirty two replicate pens for main effects; ‡ST =  
 705 the ambient temperature was gradually reduced from 27 °C at 7d age to 22 °C at 20d age; #HT  
 706 = high rearing temperature of constant 35 °C; §SEM = pooled standard errors of mean.  
 707