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1 **Comparison of growth performance and immune parameters of three commercial**
2 **chicken lines used in organic production**

3

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15

16 **Abstract**

17 Owing to the higher demands for avoiding medication and antibiotics, health status of the
18 production animals plays an important role in the poultry industry, especially in organic
19 poultry systems. Immunity plays a major role in keeping the host free from disease, and it is
20 evident that the host's genetic make-up influences immunity and disease
21 resistance/susceptibility in chickens.

22 Previously, breeding strategies aimed at selection for resistance against specific diseases
23 with the risk of creating less disease resistance against other pathogens. Changing breeding
24 strategies towards selection of chickens with a more general and broad disease resistance
25 or robustness may therefore improve the overall health status, animal welfare, and food
26 security in the poultry production. The aim of this study was therefore to compare the
27 immunocompetence of the presumed "robust" Hellevad chickens with two chicken lines
28 widely used in organic production, Bovans Brown (Bovans) and Hisex White (Hisex). The
29 chickens were subjected to a routine vaccination program comprising one parasite and four
30 viral vaccines.

31 The current study indicates that considerable differences in immunocompetence may exist
32 between commercial layer lines used in organic production. The Hellevad chickens were
33 found to have higher body weight at the end of the experiment (17 weeks of age) than the
34 other two lines. Furthermore, Hellevad and Hisex chickens were found to have higher levels
35 of humoral innate immunity with regard to sample to positive ratio of natural antibodies in
36 serum and concentration of mannose-binding lectin in serum as compared to Bovans.
37 Moreover, indications of an inflammatory response were observed in the Bovans at week 5,
38 corresponding to 1 week after vaccination with live infectious bursal disease virus. With
39 regard to adaptive immune parameters such as IgY concentration in blood and infectious

40 bursal disease virus (IBDV)-specific antibody titres, the Hellevad and Hisex chickens had
41 lower levels than the Bovans. How the differences observed in growth and immune
42 parameters in the three chicken lines influence the immune protection against infection
43 needs to be studied further.

44 **Keywords**

45 Chicken, immunocompetence, Bovans brown, Hellevad chicken, Hisex white

46 **Abbreviations**

47	DP	Double positive
48	IBDV	Infectious bursal disease virus
49	IBV	Infectious bronchitis virus
50	KLH	Keyhole Limpet Hemocyanin
51	MBL	Mannose-binding lectin
52	NDV	Newcastle disease virus
53	NAb	Natural antibody
54	RT	Room temperature
55	SP	Sample to positive control

56 **Introduction**

57 During the last two decades, rearing laying hens under organic conditions has advanced
58 increasingly, partly due to the consumer's desire for food from animals free from antibiotics
59 and at the same time an increased interest in animal welfare. EU regulations define specific

60 and precise standards for the organic egg production (EU, 2007) which mainly affect the
61 three areas: housing, feeding and medication. Organically raised hens must have access to
62 free-range areas or grazing which lower rearing intensity (Kucukyilmaz et al., 2012). The
63 feed has to be organically grown and regarding medication, the use of antibiotics is limited.
64 This requires high management quality and chickens well adapted to the organic rearing
65 system.

66 Chicken genotypes performing well in conventional production systems may not perform
67 well in organic production systems (Sørensen et al., 2004). The genotypes used for
68 conventional production systems depend on feeding and disease control strategies, which
69 are unfeasible in organic poultry systems. Also, factors like behaviour, egg laying and
70 immunocompetence must be compatible with the production systems in which the hens are
71 kept. Immunocompetence plays an important role in the poultry industry, especially in
72 organic poultry systems because of higher demands for using less medication.

73 Genetics influences both innate and adaptive immune responses. Innate humoral immune
74 components such as level of so-called natural antibodies (NAb) have previously been shown
75 to differ between lines and may be indicative for “robustness” in laying hens (Star et al.,
76 2007). Other innate humoral immune components such as mannose-binding lectin (MBL)
77 are also influenced by the genetic background of the chickens. MBL is a plasma protein
78 important for opsonophagocytosis and complement activation, and we have shown that the
79 MBL serum concentrations in chickens vary largely between individuals (Juul-Madsen et al.,
80 2003; Juul-Madsen et al., 2011; Kjaerup et al., 2013; Norup et al., 2009). This variation is
81 genetically determined and associated with disease susceptibility (Kjaerup et al., 2014b;
82 Kjaerup et al., 2013; Norup et al., 2009; Ulrich-Lynge et al., 2015). We have also shown that
83 levels of pathogen-specific antibodies after infection or vaccination are influenced by the

84 genetic background (Juul-Madsen et al., 2006; Norup et al., 2013). Finally, genetics,
85 especially MHC polymorphisms, influences the T cell-mediated immune responses
86 (Dalgaard et al., 2010a; Dalgaard et al., 2010b; Norup et al., 2011).

87 Several layer chickens with different genetic background have been tested and used in
88 organic production systems. The Hisex chickens were first introduced in the 1970s and
89 developed by the Dutch breeding company Euribrid (Hendrix-genetics, 2011). Hisex
90 chickens originate from White leghorn strains (Hendrix-genetics, 2011). The Bovans was
91 first introduced by the Dutch breeding company Bovans (now part of Institut de Sélection
92 Animale (ISA), Hendrix Genetics). The Bovans chickens are a sex-link hybrid line with the
93 parents being selectively bred from a Rhode Island Red rooster line and a Rhode Island
94 White hen line (Hendrix-genetics, 2009). The Hellevad chickens were first introduced in the
95 1970s and developed by the Danish Hellevad hatchery (Rugeriet Hellevad, 2014). They are
96 hybrids of the dual purpose breed New Hampshire Red (hen line) and the layer breed White
97 Leghorn of the Skalborg line (rooster line) (Rugeriet Hellevad, 2014). The Hellevad chickens
98 have been bred for several generations without the use of therapeutics or vaccines and have
99 been described as a robust chicken (Norup et al., 2009; Seges P/S, 2005; Sørensen et al.,
100 2004). Currently, the parental lines are vaccinated against Newcastle disease virus
101 according to the Danish legislation introduced in 2004. Hellevad chickens are also known to
102 lay eggs for a longer period than other chickens and they are gaining popularity among
103 smallholder egg producers. The aim of this study was therefore to compare parameters
104 related to immunocompetence of the Hellevad chickens with Bovans and Hisex during the
105 first 17 weeks of age under influence of a Danish standard vaccination program used for
106 commercial layers.

107 **Material and methods**

108 *Animal material*

109 The three lines with different genetic background Bovans, Hellevad and Hisex were used.
110 The parents of Bovans and Hisex were 55 weeks of age and the parents of Hellevad were
111 54 weeks of age at the time of collecting eggs for the experiment. The parents of the
112 chickens received vaccinations according to Table S1. Fertilised eggs were bought from Top
113 Æg ApS, Denmark (Bovans Brown and Hisex White) and from the Hellevad Hatchery,
114 Denmark (Hellevad) and incubated under the same conditions at the Dept. of Animal
115 Science, Aarhus University. The number of fertilised and hatched eggs was recorded. Thirty-
116 five offspring from each line (all hens) were reared together in a biosecure environment. The
117 chickens were fed diets that met or exceeded National Research Council requirements
118 (Subcommittee on Poultry Nutrition et al., 1994). Feed and water were provided *ad libitum*.
119 The chickens were kept in a cage containing perches and with 6.8 m² of floorage (15
120 chickens per m² which is the maximal number of chickens per m² according to Danish
121 legislation for organically raised chickens). The experimental chickens received routine
122 vaccinations according to the recommendations for layers from the Knowledge Centre for
123 Agriculture, Denmark (Table 1).

124 *Experimental design*

125 All 105 chicks were weighed at 1, 3, 5, 7, 11 and 17 weeks of age. Blood samples were
126 taken from the jugular vein of 12 chickens from each line (36 in total) at 1, 3, 5, 7, 11, 13
127 and 16 weeks of age. The same 36 chickens were used for continuous blood sampling
128 throughout the experiment. At the termination of the experiment (at 17 weeks of age) the
129 heart, spleen and liver from the 36 chickens used for blood sampling were weighed.
130 Unstabilised blood for serum and stabilised blood (sodium heparin) for immune-phenotyping

131 were collected at all the blood sampling time points and EDTA-stabilised blood for MHC
132 genotyping was collected at 7 weeks of age. Furthermore, heparin-stabilised blood was used
133 for haematocrit level determination at 16 weeks of age. The experiment was conducted
134 following the protocols approved by the Danish Animal Experiments Inspectorate in
135 compliance with the Danish Ministry of Justice Law no. 382 (10th June 1987) and Acts 739
136 (6th December 1988) and 333 (19th May 1990) concerning animal experimentation and care
137 of experimental animals. The license to conduct the animal experiment was obtained by
138 Helle R. Juul-Madsen. The experiment was conducted according to the ethical guidelines.

139 *MHC genotype determination*

140 Putative MHC genotypes of the chickens were determined using the LEI025 microsatellite
141 locus (McConnell et al., 1999) by a PCR-based fragment analysis (Fulton et al., 2006) as
142 previously described (Dalgaard et al., 2005).

143 *ELISA measurements*

144 So-called natural antibodies (Nabs) in serum were detected using Keyhole limpet
145 hemocyanin (KLH) as antigen. as previously described (Sun et al., 2011). Briefly, Nunc 96-
146 well polypropylen MicroWell plates (Thermo Fisher Scientific, Slangerup, Denmark cat. no.
147 442587) were coated with 100 µL of coating buffer (carbonate buffer, pH 9.6) containing
148 KLH (2 µg/mL; Merck Milipore, Copenhagen, Denmark, cat. no. 374825), and incubated at
149 4°C overnight. After washing three times in washing buffer (H₂O with 0.05% Tween 20)
150 serum samples stepwise-diluted (1:30, 1:90, 1:270, and 1:810) in buffer (PBS containing
151 0.05% Tween 20 and 0.5% horse serum) were applied to the wells. Standard positive serum
152 samples stepwise-diluted in buffer (PBS containing 0.05% Tween 20 and 0.5% horse serum)
153 were also applied to the plate. The plates were incubated for 1 h at room temperature (RT).
154 After washing three times, the plates were incubated with 1:20,000-diluted rabbit-anti-

155 chicken IgG_{H+L} (RACH/IgG/Fc/PO) (Nordic, Tilburg, the Netherlands), and incubated for 1.5
156 h at RT. After washing three times, binding of the antibody in the serum sample to KLH was
157 visualized by adding 100 µL of substrate (< 0.05 % (wt/wt) 3,3',5,5'' tetramethylbenzidin).
158 After 10 min, the reaction was stopped with 100 µL of 1 M H₂SO₄ solution. The colour
159 development was determined by reading the absorbance at 405 nm with absorbance at 650
160 nm as reference. Results were calculated as sample to positive standard control (SP) ratio
161 based on the OD-values obtained for the 1:90 dilutions.

162 The MBL serum concentration was measured by ELISA as previously described (Juul-
163 Madsen et al., 2003; Norup and Juul-Madsen, 2007) using the monoclonal chicken anti-MBL
164 antibody HYB 182-01 (Bioporto®, Copenhagen, Denmark, cat. no. HYB 182-01).

165 Total IgY and IgM antibody concentrations in serum were measured as previously described
166 (Juul-Madsen et al., 2007) using the chicken IgY ELISA kit and chicken IgM ELISA kit from
167 Bethyl Laboratories, Inc. (Montgomery, TX, USA, cat. no. E30-104 (IgY) and cat. no. E30-
168 102 (IgM)) according to the manufacturer's instructions.

169 The specific antibody titres against Newcastle disease virus (NDV) in serum were measured
170 as previously described (Dalgaard et al., 2010a) using the ProFLOK® NDV Plus NDV
171 Antibody Test Kit from Synbiotics Corporation (San Diego, CA, USA, cat. no. 96-6547)
172 according to the manufacturer's instructions.

173 The specific antibody titres against infectious bursal disease virus (IBDV) in serum were
174 measured as previously described (Juul-Madsen et al., 2006) using the ProFLOK® IBD
175 Antibody Test Kit from Synbiotics Corporation (San Diego, CA, USA, cat. no. 54-81-01)
176 according to the manufacturer's instructions.

177 The specific antibody titres against infectious bronchitis virus (IBV) in serum were measured
178 as previously described (Juul-Madsen et al., 2011) using the ProFLOK® IBV Antibody Test
179 Kit from Synbiotics Corporation (San Diego, CA, USA, cat. no. 96-6506) according to the
180 manufacturer's instructions.

181 *Flow cytometry*

182 The absolute numbers of different T cell subsets in peripheral blood were determined at 1,
183 3, 5, 7 and 15 weeks of age using a no-lyse no-wash flow cytometric method. Fifty microliters
184 of 25 times diluted heparin-stabilised blood was mixed with 50 µL of antibody solution
185 containing anti-CD3-SPRD (clone CT-3), anti-CD4-RPE (clone CT-4), anti-TCRγδ-FITC
186 (clone TCR-1) and anti-CD8α-Cy5 (clone 3-298) in FACS buffer (0.2 % BSA, 0.2 % sodium
187 azide and 0.05 % normal horse serum in PBS). All monoclonal antibodies were obtained
188 from Southern Biotech (Birmingham, AL, USA). The samples were incubated at room
189 temperature for 20 min in a 4 mL tube in darkness, and immediately before acquisition 400
190 µL FACS-buffer and 100 µL PERFECT-COUNT Microspheres™ (Cytognos, S.L.,
191 Salamanca, Spain, cat. no. CYT-PCM-50) diluted 1:2 in FACS buffer were added.

192 The absolute numbers of B cells, monocytes and heterophils were determined at 1, 3, 5, 7
193 and 15 weeks of age. The protocol was as described above but using anti-Bu-1-FITC (clone
194 AV20), anti-Monocyte/Macrophage-R-PE (clone KUL01) and anti-CD45-PerCP-Cy5.5
195 (clone UM16-6) in FACS buffer. The monoclonal CD45 antibody was obtained from AbD
196 Serotec® Bio-Rad Laboratories, Inc (Kidlington, UK) and the other monoclonal antibodies
197 were obtained from Southern Biotech (Birmingham, AL, USA). Conjugation of anti-CD45
198 with PerCP-Cy5.5 was performed using Lightning-Link® from Innova Bioscience
199 (Cambridge, United Kingdom, cat. no. 763-0030) according to the manufacturer's
200 instructions.

201 All flow cytometric analyses were performed on a BD FACSCanto™ (BD Biosciences, San
202 Jose, CA, USA) equipped with a 488 nm blue laser and a 633 nm red laser. Using the
203 FACSDiva software each sample was acquired and recorded for 1 min at medium flow rate.
204 Titration of all antibodies was performed prior to the experiment in order to determine the
205 optimal staining concentrations. Single-stained compensation controls as well as negative
206 fluorescence minus one (FMO) controls were included. Leucocytes in whole blood were
207 identified through singlet gating (see Supplementary Figure S1 for gating strategy). The
208 PERFECT-COUNT Microspheres™ were identified by FITC/PE gating (R1) and
209 FITC/PerCP-Cy gating (R2). The absolute number of cells in the samples was calculated
210 according to the manufacturer's instructions as: cells/ μ L = (total number of cells counted x
211 dilution factor x μ L Microspheres added x assayed concentration of Microspheres) / (total
212 number of Microspheres counted x total volume).

213 *Statistics*

214 All dependent data were analysed for being normally distributed, and they were all except
215 the KLH data found to have normal distribution. A normal distribution was obtained for the
216 KLH data after transposing the dependent variable to logarithm A. Interaction effects are
217 included in the model if they have a significant influence on the dependent variable.

218 The model used for statistical analyses of the chicken lines and time effect on IBDV titre,
219 IBV titre and MBL concentration was: $W_{ijk} = \mu + H_i + T_j + HT_{ij} + e_{ijk}$, where μ = overall means,
220 H_i = fixed effect of chicken line, T_j = fixed effect of chicken age. The same model was used
221 for the flow cytometric data, except that the number of chicken ages was two less.

222 The model used for statistical analyses of the effect of the chicken lines on haematocrits,
223 chicken weight, and relative weight of heart, liver or spleen was: $W_{ijk} = \mu + H_i + e_{ijk}$, where
224 μ = overall means, H_i = fixed effect of chicken line.

225 **Results**

226 The fertilisation and hatchability were recorded and are shown in Table 2. Hellevad had a
227 higher percentage of fertilisation (100% versus 90% and 80%) than the other two chicken
228 lines with Bovans having the lowest percentage of fertilization. Bovans also had the lowest
229 percentage of hatched eggs (88% versus 91% and 94%) and Hisex had the highest
230 percentage of hatched eggs.

231 *Weight, haematocrits and relative weight of organs*

232 The weight of the chickens during the experimental period is shown in Table 3. At 5 and 7
233 weeks of age Hellevad had a significantly higher weight than Bovans and Hisex ($P < 0.01$)
234 whereas all three chicken lines differed significantly in weight at 11 and 17 weeks of age (P
235 < 0.01) with Hellevad being the heaviest and Hisex the lightest chickens.

236 The haematocrit values were measured to 30.6 ± 0.4 (Bovans), 30.9 ± 0.3 (Hellevad) and
237 30.1 ± 0.5 (Hisex) when the chickens were 16 weeks of age. No statistically significant
238 difference was observed between the different lines ($P > 0.05$).

239 At the termination of the experiment the relative weight of heart, liver and spleen was
240 compared between the three chicken lines (Table 3). For heart and spleen the relative
241 weight was similar in all three lines, but the relative weight of the livers from the Hisex
242 chickens was significantly higher ($P < 0.05$) than the relative weight of the livers from the
243 Bovans and Hellevad chickens.

244 *Determination of MHC genotypes*

245 A total of 12 different genotypes (based on eight different alleles) were found in the chickens
246 with two of the genotypes (with the PCR product lengths 254/332 and 332/332) recurring in
247 the Hisex and Hellevad chickens. In the Bovans and Hisex four different genotypes were
248 found and six different genotypes were found in the Hellevad (data is shown in
249 supplementary Table 2).

250 *ELISA measurements*

251 *Natural antibody titres.* For all three line strains a decrease in Nabs was observed from 1 to
252 7 weeks of age followed by an increase (Figure 1). A significant difference for the SP ratio
253 between Hellevad and Bovans chickens was observed at 1, 5, 11 and 16 weeks of age (P
254 < 0.05). A significant difference in the SP ratio between Hellevad and Hisex chickens was
255 observed at 1 and 3 weeks of age ($P < 0.05$).

256 *MBL concentrations.* No significant difference was observed between MBL serum
257 concentrations (Figure 1) in the chicken lines at 1 week of age. However, the serum
258 concentration for the Hisex chickens increased by 76.4% after 1 week of age whereas the
259 serum concentrations of the other two chicken lines did not increase until after 3 weeks of
260 age by 148.9% (Bovans) and 8.69% (Hellevad). The increase in MBL serum concentration
261 in the Bovans chickens resembled that of an acute phase response. After 5 weeks of age
262 the increases in serum concentrations in Hellevad and Hisex chickens were fairly stable
263 throughout the experiments ending with MBL serum concentrations of 16.14 (Hisex) and
264 17.61 (Hellevad) $\mu\text{g/mL}$ at 16 weeks of age. After the peak in MBL serum concentration in
265 the Bovans chickens the same MBL level was observed throughout the experiment with the
266 Bovans having a significantly different MBL serum concentration (8.24 $\mu\text{g/mL}$) compared to
267 the other two chicken lines at 16 weeks of age ($p < 0.01$). The MBL serum concentrations of

the parents at the time of collecting the eggs for the experiment were also measured and found to be 12.2 ± 1.25 µg/ml for Bovans (n = 30), 26.7 ± 1.97 for Hisex (n = 30), 14 ± 1.45 for New Hampshire (n = 20) and 38.5 ± 1.34 for Skalborg (n = 20).

Total IgY and IgM concentrations. From 3 to 11 weeks of age Bovans had a significantly higher amount of total IgY (Figure 2) in the serum than Hellevad and Hisex ($P < 0.01$) and at 13 and 16 weeks of age all three chicken lines differed significantly from each other ($P < 0.05$) with Bovans having the highest amount of IgY and Hellevad the lowest amount of IgY. Except at the age of 5 weeks all three chicken lines had the same amount of IgM in serum throughout the experimental period. At 5 weeks of age Bovans had a significantly higher amount of IgM than Hellevad and Hisex ($P < 0.01$).

Specific antibody titres. As expected, maternal antibodies for IBDV, IBV, and NDV (Figure 3) were observed for Bovans and Hisex whereas, for the Hellevad, only maternal antibodies specific for NDV were observed. Maternal antibody levels were diminutive by 3 weeks of age. An increase in IBDV-specific antibody titres was observed after the two GUMBORO vaccines given at 3 and 5 weeks of age. The ranking of IBDV-specific antibody levels after 7 weeks of age was as follows: Bovans > Hisex > Hellevad ($P < 0.05$).

The IBV-specific antibody titres were low at 3 weeks of age for all three chicken lines and this was followed by an increase at 5 weeks of age ($P < 0.01$). After this week, the IBV-specific antibody titres for the Bovans and Hellevad chickens were unchanged for the rest of the experiment, whereas a decrease was observed for the Hisex chickens from 5 to 16 weeks of age ($P < 0.05$).

289 The NDV-specific maternal antibody titres reached minimum at 5 weeks of age for all three
290 chicken lines. This was followed by an increase in NDV-specific antibody titres until 16
291 weeks of age. There was no significant difference between the lines.

292 *Flow cytometric assessment of lymphocyte subsets*

293 An absolute count flow cytometric protocol using a no-lyse no-wash method, as described
294 earlier (Kjaerup et al., 2014a; Seliger et al., 2012), was used for quantifying leukocyte
295 subsets in peripheral blood.

296 The numbers of monocytes and heterophils in whole peripheral blood were identified and
297 results are shown in Figure 4. The number of monocytes in the Hellevad and Hisex chickens
298 remained invariable throughout the experiment. Contrary to this, the number of monocytes
299 in the Bovans chickens started at the same level as the other two chicken lines, followed by
300 an increase until 5 weeks of age to above the levels of the other chicken lines ($P < 0.05$).
301 Finally, the numbers decreased, but at 15 weeks of age the number of monocytes was still
302 higher for the Bovans than the Hisex chickens ($P < 0.05$). The number of heterophils in the
303 Hellevad and Hisex chickens remained invariable throughout the experiment. The number
304 of heterophils in the Bovans chickens was the same as the other chicken lines at 1 week of
305 age. Hereafter the Bovans had a higher number of heterophils than the other two lines ($P <$
306 0.05). From 3 until 5 weeks of age the number of heterophils in the Bovans chickens
307 increased ($P < 0.01$) followed by a decrease again from 5 until 7 weeks of age ($P < 0.01$).

308 The T cell subsets were identified and the results are presented in Figure 5. For the $\text{TCR}\gamma\delta^+$
309 $\text{CD8}\alpha^-$ T cells, no significant difference was observed between the three chicken lines
310 except at 15 weeks of age where the number of cells was significantly higher for the Bovans
311 than for the Hellevad chickens ($P < 0.01$). For the $\text{TCR}\gamma\delta^+$ $\text{CD8}\alpha^+$ T cells a significant

312 difference between the chicken lines was observed at 1 and 5 weeks of age. At 1 week of
 313 age the number of cells was higher for the Hisex than for the Bovans chickens ($P < 0.05$)
 314 and at 5 weeks of age the number of cells for the Hisex chickens was higher than for both
 315 the Bovans and the Hellevad chickens ($P < 0.01$). For both $\text{TCR}\gamma\delta^+$ T cell subsets, an
 316 increase in the number of cells was observed for all chicken lines from 1 to 15 weeks of age.
 317 Significant differences were also observed for the $\text{TCR}\gamma\delta^-$ T cells. The number of double
 318 positive (DP) T cells ($\text{CD8}\alpha^+ \text{CD4}^+$) was significantly higher for the Hisex chickens
 319 throughout the experiment than for the other chicken lines ($P < 0.01$). At 5 weeks of age the
 320 number of $\text{TCR}\gamma\delta^-$ DP T cells in Hisex peaked. This peak in the number of cells at 5 weeks
 321 of age was unregistered for the Bovans and Hellevad chickens. For the $\text{TCR}\gamma\delta^- \text{CD8}\alpha^+ \text{CD4}^-$
 322 and the $\text{TCR}\gamma\delta^- \text{CD4}^+ \text{CD8}\alpha^-$ T cells an increase in the number of cells was observed for all
 323 chicken lines from 1 to 15 weeks of age. For the $\text{TCR}\gamma\delta^- \text{CD8}\alpha^+ \text{CD4}^-$ T cells, the number of
 324 cells differed significantly between Bovans and Hisex chickens at 1 week of age ($P < 0.05$),
 325 between Bovans and Hellevad chickens at 3 weeks of age ($P < 0.01$) and between all three
 326 chicken lines at 15 weeks of age ($P < 0.05$) with the following order Hellevad > Bovans >
 327 Hisex. For the $\text{TCR}\gamma\delta^- \text{CD4}^+ \text{CD8}\alpha^-$ T cells, the number of cells differed significantly between
 328 Hisex and Hellevad chickens at 5 weeks of age ($P < 0.01$) and between Bovans and the
 329 other two chicken lines at 15 weeks of age ($P < 0.05$).
 330 The numbers of B cells in whole peripheral blood were identified and results are shown in
 331 Figure 5. For the B cells an increase from 1 to 3 weeks of age was observed for all three
 332 chicken lines. Thereafter, a decrease followed by another increase was observed until 15
 333 weeks of age. The first increase in the number of B cells was most pronounced for the

334 Bovans chickens ($P < 0.01$). At 15 weeks of age the Hisex chickens had a lower number of
335 B cells than the two other chicken lines ($P < 0.05$).

336 **Discussion**

337 Genetic selection of poultry has resulted in several different breeds and lines, both within
338 broiler and layer chickens. Several studies indicate a relationship between performance
339 traits and immune responses (Gross et al., 1980; Siegel et al., 1982; Sumners et al., 2012).
340 The immune responses are also reported to vary between broilers and layers, with broilers
341 being more specialised in innate immune responses and layers being more specialised
342 adaptive humoral and cellular immune responses according to a study of systemically
343 administered antigen TNP-KLH (Koenen et al., 2002). Within the layers a higher body weight
344 (gain) has been correlated with lower immune responses (Gross et al., 1980; Parmentier et
345 al., 1996; Siegel et al., 1982). However, in the present study, the Hellevad chickens had a
346 higher body weight than both Bovans and Hisex chickens from 5 weeks of age and onwards.
347 Furthermore, the line strains originating from a cross between brown and white parents
348 (Bovans and Hellevad) had a higher body weight at 11 and 17 weeks of age than the line
349 strain originating only from white parents (Hisex). A previous study observed that brown
350 hens were heavier than white hens from 23 weeks of age and onwards (Kucukyilmaz et al.,
351 2012). A Danish line described to be particularly robust and useful for organic and free-
352 range production is the Hellevad chicken, with results showing a lower mortality rate within
353 the Hellevad chickens compared to the other chicken lines as described in the study of
354 Seges P/S (2005) (Seges P/S, 2005). The higher body weight for the Hellevad chickens
355 was, however, uncorrelated with a lower immune response with respect to the SP ratio
356 against NAb (Figure 1), total IgY and IgM concentrations (Figure 2) or virus-specific antibody

357 titres (Figure 3) Nor was any association between body weight and absolute number of
358 leucocyte cells (Figure 6 and Figure 5) observed in the current study.

359 High levels of the so-called Nabs have been suggested to be related to disease resistance
360 (Parmentier et al., 2004). High Nab levels have been suggested to enhance antigen-specific
361 antibody responses or the maturation of the immune system in chickens (Lammers et al.,
362 2004). A relation between natural competence in the form of NAb detected by binding to
363 KLH and the mortality has previously been suggested (Star et al., 2007). Furthermore, the
364 importance of Nabs for development of adaptive immunity has been indicated by a study
365 in knock-out mice (BALB/c) where the absence of NAb resulted in a delayed specific T-
366 dependent response (Baumgarth et al., 1999). At 1 week of age the Hellevad chickens have
367 a higher SP ratio (Figure 1) than the other chicken lines. However, this difference was
368 eliminated between the Hellevad chickens and the Hisex at the end of the experiment.

369 The Bovans were found to have statistically significantly lower MBL serum concentrations
370 (Figure 1) than the other two chicken lines, except at 1 and 5 weeks of age. This difference
371 in MBL serum concentrations has been observed between chicken lines previously and is
372 in accordance with a study by Norup et al. (2009) (Norup et al., 2009) where variations were
373 found in MBL serum concentration between the two lines ISA Brown (6.57 µg/mL) and
374 Hellevad chickens (18.81 µg/mL). Also, 14 other chicken lines representing both layers and
375 broilers have been observed to differ in basal MBL serum concentrations owing to genetic
376 variation (Laursen et al., 1998). The 14 different strains consisted of congenic and hybrid
377 White Leghorns, Red Jungle Fowls, Danish Landrace and New Hampshire-White Leghorn
378 hybrids. An increase was observed during the growth period for the Hellevad and Hisex
379 chickens ending with higher MBL serum concentrations at the end of the experiment (16
380 weeks of age). An age-dependent increase has previously been observed for chickens

381 (Norup et al., 2009). Contrary to this, the MBL serum concentrations for the Bovans chickens
382 were the same in the beginning and in the end of the experiment interrupted by an acute
383 phase-like response at 5 weeks of age. This response may be triggered by the GUMBORO
384 vaccine given at weeks 3 and 4. High MBL concentrations have been found beneficial in
385 human infants making them less susceptible to diseases (Israels et al., 2012; Luo et al.,
386 2014). Studies have shown that different levels of MBL serum concentrations in chickens
387 may have an impact on disease severity after infections with pathogens such as infectious
388 bronchitis virus (Juul-Madsen et al., 2003; Kjaerup et al., 2014b), *Salmonella* Infantis (Ulrich-
389 Lynge et al., 2015), *Escherichia coli* (Norup et al., 2009), and *Pasteurella multocida* (Schou
390 et al., 2010).

391 Heterophils, the avian counterpart of mammalian neutrophils, are the dominating
392 granulocytes in the circulating blood of chickens (Maxwell and Robertson, 1998) and they
393 are rapidly recruited to the site of infection (Genovese et al., 2013). Thus, an increased
394 number of heterophils may be a sign of an inflammatory response. In the present study, an
395 increased number of heterophils were observed in the Bovans chickens from 1 to 5 weeks
396 of age, but not in the other chicken lines, indicating that the Bovans chickens may react
397 strongly to live attenuated vaccines. In chicken flocks, successful vaccine responses are
398 often monitored by demonstrating an increase in antibodies within a few days after
399 vaccination (Sharma, 1999). The antibodies monitored during the current study were total
400 IgY and IgM concentrations in serum as well as vaccine-specific antibody titres (Figure 2
401 and Figure 3). The total IgY concentration of the Bovans chickens was significantly higher
402 than in both the Hellevad and Hisex chickens throughout the experiment, except at 1 week
403 of age, and so was the IBDV-specific Ig titre. The total IgM concentrations of the Bovans
404 chickens were increased at week 5 and were significantly higher than in both the Hellevad

405 and Hisex chickens where no increase was observed at week 5. Together with the observed
406 results for MBL, monocytes and heterophils at week 5, this could be indications of an
407 inflammatory response in the Bovans chickens not seen in the Hellevad or the Hisex
408 chickens.

409 Even though the presence of DP T cells in both peripheral blood and tissues of chickens is
410 known to be genetically determined (Luhtala et al., 1997), their function in the chicken is still
411 unclear. In humans and pigs, their roles have been suggested to be memory T cells
412 (Zuckermann, 1999), participating among other things in the antiviral secondary immune
413 response (Nascimbeni et al., 2004; Overgaard et al., 2015). In the present study, a large
414 variation in the number of DP T cells was found both between the three lines, but also
415 between individuals within each line. This is consistent with a previous study that found a
416 variation in DP T cells ranging from 20 to 40 % of peripheral blood lymphocytes with only
417 five out of 40 animals having the DP T cells (Luhtala et al., 1997). In conclusion, the current
418 study showed considerable differences in various immune parameters between three
419 chicken lines used in European organic production. How the differences observed in growth
420 and immune parameters may influence general chicken health and protection against
421 infection needs to be studied further. Provided that relevant phenotypes can be associated
422 with genomic markers, this may pave the way for selective breeding for immune competence

423 **Conflict of interest statement**

424 The authors declare that they have no conflicts of interest.

425 **Acknowledgement**

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429 diseases” (POLY-REID)(grant number 10-093534) granted by the Danish Council for
430 Strategic Research, the Danish Poultry Council, The Hatchery Hellevad, and Cobb-
431 Vantress.

432 Table 1: Recommendation for vaccination program for layers in Denmark. Chickens in the
433 experiment were vaccinated in accordance with the recommendations of the Knowledge
434 Centre for Agriculture, Denmark. The vaccines were acquired at MSD Animal Health,
435 Denmark.

436 Table 2: Fertility and hatchability of eggs purchased from Top Æg ApS, Denmark (Bovans
437 and Hisex) and from the Hellevad Hatchery, Denmark (Hellevad) incubated under the same
438 conditions at the Dept. of Animal Science, Aarhus University.

439 Table 3: Chicken weight and relative organ weight in the three chicken lines. Relative organ
440 weight was measured at the termination of the experiment when the chickens were 15
441 weeks of age. Results are shown as mean values $\pm$ SEM. The letters *a*, *b* and *c* indicate
442 statistically significant differences ($P < 0.05$) between the chicken lines within weeks or
443 within an organ.

444 Figure 1: Natural antibodies (Nabs) isotype IgY against KLH and MBL concentration in
445 serum as measured by ELISA. Results for KLH are shown as mean values of SP ratio based
446 on the OD-values obtained for the 1:90 dilutions $\pm$ SEM, and results for MBL concentration
447 are shown as mean values $\pm$ SEM. $n=11-12$ for all chicken lines. α indicates a significant
448 difference ($P < 0.05$) between the Bovans and the Hellevad chickens. $\alpha\alpha$ indicates a
449 significant difference ($P < 0.01$) between the Bovans and the Hellevad chickens. # indicates
450 a significant difference ($P < 0.05$) between the Hellevad and Hisex chickens. ## indicates a

451 significant difference ($P < 0.01$) between the Hellevad and Hisex chickens. †† indicates a
452 significant difference ($P < 0.01$) between the Bovans and the Hisex chickens. Statistical
453 analyses of KLH data were performed on log transformed values of the SP ratio.

454 Figure 2: Serum concentrations of total IgY and IgM during the experimental period as
455 measured by ELISA. Results are shown as mean values $\pm$ SEM. $n=11-12$ for all chicken
456 lines. * indicates significant differences ($P < 0.05$) between all three chicken lines. $\alpha\alpha$
457 indicates a significant difference ($P < 0.01$) between the Bovans and the Hellevad chickens.
458 †† indicates a significant difference ($P < 0.01$) between the Bovans and the Hisex chickens.

459 Figure 3: The specific antibody titres against IBDV, IBV and NDV during the experimental
460 period as measured by ELISA. Results are shown as mean values $\pm$ SEM. $n=11-12$ for all
461 chicken lines. * indicates significant differences ($P < 0.05$) between all three chicken lines.
462 ** indicates significant differences ($P < 0.01$) between all three chicken lines. $\alpha\alpha$ indicates a
463 significant difference ($P < 0.01$) between the Bovans and the Hellevad chickens. # indicates
464 a significant difference ($P < 0.05$) between the Hellevad and Hisex chickens. † indicates a
465 significant difference ($P < 0.05$) between the Bovans and the Hisex chickens. †† indicates a
466 significant difference ($P < 0.01$) between the Bovans and the Hisex chickens.

467 Figure 4: Monocytes and heterophils in peripheral blood during the experimental period.
468 Staining and gating of cell populations were performed according to Supplementary Figure
469 S1A. The absolute counts of monocytes are shown in the upper row, and the absolute counts
470 of heterophils are shown in the lower row. Results are shown as mean values $\pm$ SEM. $n=11-$
471 12 for all chicken lines. α indicates a significant difference ($P < 0.05$) between the Bovans
472 and the Hellevad chickens. $\alpha\alpha$ indicates a significant difference ($P < 0.01$) between the
473 Bovans and the Hellevad chickens. † indicates a significant difference ($P < 0.01$) between

474 the Bovans and the Hisex chickens. †† indicates a significant difference ($P < 0.01$) between
475 the Bovans and the Hisex chickens.

476 Figure 5: T cell subsets and B cells in peripheral blood during the experimental period.
477 Staining and gating of B and T cell populations were performed according to Supplementary
478 Figure S1A and B, respectively. The absolute counts of TCR $\gamma\delta$ + cells and B cells are shown
479 in the left column. The absolute counts of TCR $\gamma\delta$ +CD8 α - cells are shown in the upper row,
480 the absolute counts of TCR $\gamma\delta$ +CD8 α + cells are shown in the middle row, and the B cells are
481 shown in the lower row. The absolute counts of TCR $\gamma\delta$ - cells are shown in the right column.
482 The absolute counts of TCR $\gamma\delta$ -CD8 α +CD4+ cells (DP) are shown in the upper row, the
483 absolute counts of TCR $\gamma\delta$ -CD8 α +CD4- cells are shown in the middle row, and the absolute
484 counts of TCR $\gamma\delta$ -CD8 α -CD4+ cells are shown in lower row. Results are shown as mean
485 values $\pm$ SEM. n=11-12 for all chicken lines. * indicates significant differences ($P < 0.05$)
486 between all three chicken lines. α indicates a significant difference ($P < 0.05$) between the
487 Bovans and the Hellevad chickens. $\alpha\alpha$ indicates a significant difference ($P < 0.01$) between
488 the Bovans and the Hellevad chickens. ## indicates a significant difference ($P < 0.01$)
489 between the Hellevad and Hisex chickens. † indicates a significant difference ($P < 0.01$)
490 between the Bovans and the Hisex chickens. †† indicates a significant difference ($P < 0.01$)
491 between the Bovans and the Hisex chickens.

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615

Comparison of growth performance and immune parameters of three commercial chicken lines used in organic production

R.B. Kjærup, H.R. Juul-Madsen, L.R. Norup, P. Sørensen and T.S. Dalgaard

Figure 1

Comparison of growth performance and immune parameters of three commercial chicken lines used in organic production

R.B. Kjærup, H.R. Juul-Madsen, L.R. Norup, P. Sørensen and T.S. Dalgaard

Figure 2

Comparison of growth performance and immune parameters of three commercial chicken lines used in organic production

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Figure 3

Comparison of growth performance and immune parameters of three commercial chicken lines used in organic production

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Figure 4

Comparison of growth performance and immune parameters of three commercial chicken lines used in organic production

R.B. Kjærup, H.R. Juul-Madsen, L.R. Norup, P. Sørensen and T.S. Dalgaard

Figure 5

Table 1:

Age [weeks]	Vaccination against	Vaccine	Application
0	IB + Mareks disease	Nobilis® IB Ma5 Vet. and Nobilis Rismavac (MSD Animal Health, Denmark)	Coarse spray
1	Coccidiose	Paracox® -8 vet. (MSD Animal Health, Denmark)	Orally in drinking water
2	IB	Nobilis® IB 4-91 (MSD Animal Health, Denmark)	Coarse spray
3	Gumboro disease	Nobilis® Gumboro 228E (MSD Animal Health, Denmark)	Orally in drinking water
4	ND	Nobilis® ND C2 (MSD Animal Health, Denmark)	Coarse spray
4	Gumboro disease	Nobilis® Gumboro 228E (MSD Animal Health, Denmark)	Orally in drinking water
8	IB	Nobilis® IB Ma5 Vet. (MSD Animal Health, Denmark)	Coarse spray
10	ND	Nobilis® ND C2 (MSD Animal Health, Denmark)	Coarse spray
13	IB	Nobilis® IB 4-91 (MSD Animal Health, Denmark)	Coarse spray

Table 2

Chicken line	Number of eggs	Number of fertilised eggs	Number of hatched eggs	Fertilisation [%]	Hatching [%]
Hellevad	100	100	91	100	91
Hisex	120	108	102	90	94
Bovans	120	96	84	80	88

Table 3

	Chicken line		
	Bovans	Hellevad	Hisex
Chicken weight¹ [g]			
1 week of age	81.1 ± 0.6	88.9 ± 0.9	79.7 ± 0.8
3 weeks of age	210.4 ± 2.3	228.2 ± 2.4	208.7 ± 2.2
5 weeks of age	379.2 ± 5.5 ^a	425.1 ± 4.5 ^b	387.0 ± 4.0 ^a
7 weeks of age	618.5 ± 9.1 ^a	662.3 ± 7.2 ^b	613.9 ± 5.5 ^a
11 weeks of age	1050.4 ± 16.1 ^a	1088.2 ± 12.1 ^b	979.0 ± 8.6 ^c
17 weeks of age	1509.4 ± 22.5 ^a	1583.1 ± 17.4 ^b	1421.8 ± 15.2 ^c
Relative organ weight² [%]			
Heart	0.46 ± 0.13	0.44 ± 0.13	0.45 ± 0.14
Liver	1.69 ± 0.49 ^a	1.65 ± 0.48 ^a	2.38 ± 0.72 ^b
Spleen	0.15 ± 0.04	0.14 ± 0.04	0.13 ± 0.04

¹n=35 (Bovans and Hellevad) or n=34 (Hisex); ²n=12 (Bovans and Hellevad) or n=11 (Hisex)

Comparison of growth performance and immune parameters of three commercial chicken lines used in organic production

R.B. Kjærup, H.R. Juul-Madsen, L.R. Norup, P. Sørensen and T.S. Dalgaard

Supplementary Table S1 Vaccination program followed by the parents of the Bovans, Hellevad and Hisex chickens. IB: infectious bronchitis; ND: New Castle disease; AE: avian encephalomyelitis; CAV: chicken anaemia virus; EDS: egg drop syndrome.

Age (weeks)	Vaccination against	Vaccine	Parents of chicken line
0	IB	Nobilis® IB Ma5 Vet. (MSD Animal Health, Denmark)	Bovans Hisex
0	Coccidiose	Paracox® -8 vet. (MSD Animal Health, Denmark)	Bovans Hisex
2	IB	Nobilis® IB 4-91 (MSD Animal Health, Denmark)	Bovans Hisex
3	ND	Nobilis® ND C2 (MSD Animal Health, Denmark)	Bovans Hisex Hellevad
4	Gumboro disease	Nobilis® Gumboro D78 (MSD Animal Health, Denmark)	Bovans Hisex
7	IB	Nobilis® IB 4-91 (MSD Animal Health, Denmark)	Bovans Hisex
9	ND	Nobilis® ND C2 (MSD Animal Health, Denmark)	Bovans Hisex Hellevad
12	ND	Nobilis® IB Ma5 Vet. (MSD Animal Health, Denmark)	Bovans Hisex
13	AE	AviPro® AE (Lohmann Animal Health)	Bovans Hisex
15	CAV	Nobilis® CAV p4 (MSD Animal Health, Denmark)	Bovans Hisex
16	ND	Nobilis® ND C2 (MSD Animal Health, Denmark)	Hellevad
17	IB ND EDS	Nobilis® IBmulti-ND-EDS	Bovans Hisex

Comparison of growth performance and immune parameters of three commercial chicken lines used in organic production

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Supplementary Table S2 MHC genotypes of the chicken lines. The MHC genotypes were determined using the LEI025 microsatellite locus by a PCR-based fragment analysis. – indicates not found in the chicken line

Chicken line	MHC genotype											
	254/246	254/254	254/332	254/349	254/507	291/246	291/291	291/445	332/332	332/393	332/507	393/507
Hellevad	-	-	1	-	7	-	-	-	1	1	-	-
Hisex	-	-	-	-	-	1	9	1	-	-	-	1
Bovans	2	1	5	2	-	-	-	-	1	-	1	-

Supplementary Figure S1: Detection of leucocytes in whole blood using a no-lyse no-wash flow cytometry procedure. A) Multiple-colour gating strategy for B cells, monocytes and heterophils. The gating strategy is shown for the identification of B cells, monocytes and heterophils through singlet gating. Heparin-stabilised blood was stained with anti-CD45-PerCP-Cy5.5, anti-Bu-1-FITC and anti-Monocyte/Macrophage-R-PE. B) Multiple-colour gating strategy for T-cell subsets. The gating strategy is shown for the identification of T-cell subsets through singlet gating. Heparin-stabilised blood was stained with anti-CD3-SPRD, anti-TCRgd-FITC, anti-CD4-RPE and anti-CD8 α -Cy5. The PERFECT-COUNT MicrospheresTM were identified by FITC/PE gating (R1) and FITC/PerCP-Cy gating (R2).

Highlights

- Commercial chicken breeds popular in organic production was compared
- Hellevad chickens showed higher body weight from weeks 5-17 of age
- Hellevad and Hisex chickens had 2-fold higher MBL serum concentration than Bovans
- The humoral response to IBDV and IBV vaccines varied largely between breeds
- The absolute numbers of circulating leukocytes varied largely between breeds