



# Differentially expressed marker genes and glycogen levels in pectoralis major of Ross308 broilers with wooden breast syndrome indicates stress, inflammation and hypoxic conditions

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## ABSTRACT

The occurrence of wooden breast (WB) in broiler production is increasing, but onset of its development is only described in part. In this study, we determined the regulation of marker genes related to oxidative stress in Ross308 broilers categorized as no-, mild- or severe-WB, on days 21 and 30 of production. The biochemical parameters, lactate dehydrogenase and pro- and macro-glycogen, were also determined. On day 21, breast meat from birds affected severely by WB had increased mRNA abundances of heat-shock protein 70, heme-oxygenase 1, cyclooxygenase 2, tumor necrosis factor 1, and hypoxia inducible factors as well as higher pH and lower dry matter contents. On day 30, breast meat from both mild and severely affected birds had increased mRNA for heme oxygenase 1, lactate dehydrogenase, and hypoxia inducible factor. Moreover, pro- and micro-glycogen, as well as the total pool of glycogen, were decreased compared with the non-WB birds. In conclusion, this study indicates oxidative stress, inflammation and hypoxic conditions in WB.

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## 1. Introduction

Modern broiler production has led to intensive genetic selection and optimization of feeding, resulting in increased growth rate and breast yield (i.e. breast filet makes up a higher percentage of total weight). For example, the Ross308 broiler line has, in an 11 year period (2001–2012), reduced the time taken to achieve a body weight of 2.2 kg by 8 days (from 43 to 35 days) (Petracci et al., 2015). Similarly, the breast yield in Ross308 broilers has increased from 15.8% in 2001 to 21.1% in 2012 (Petracci et al., 2015). In parallel, adverse effects at the tissue level are emerging, which are thought to be attributable to such optimization practices. Phenomena such as “white striping” (superficial white striations) and “wooden breast” (WB) (pale and bulging areas) are more common features of large breast muscle (pectoralis major) in rapidly growing broiler strains, as reviewed by Petracci et al. (2019).

WB is a phenomenon where the *M. pectoralis major* is characterized by pale and bulging areas of substantial hardness (Petracci et al., 2015), which have implications for the quality of fresh and processed products. This muscle tissue abnormality is detected phenotypically by palpation (Dalgaard et al., 2018; Sihvo et al., 2014). Further characterization of the

WB myopathy, at the morphological level, has revealed muscle fiber necrosis, fibrosis, and muscle fiber regeneration (Sihvo et al., 2014; Velleman & Clark, 2015) as well as infiltration of excess adipose tissue into the breast muscle (Petracci et al., 2013). However, there is only limited knowledge about the development of WB.

In WB-affected tissue, the number of fibers is reduced and the fibers are variable in size, rounded and separated, or replaced, by connective tissue (Sihvo et al., 2014). Also, infiltration of the tissue by inflammatory cells, including heterophils and macrophages, has been observed (Sihvo et al., 2014; Velleman & Clark, 2015). Investigations have also indicated that oxidative stress, including acute phase response signaling (e.g. heat shock proteins; HSPs), is present as well as indicators for hypoxia and muscle fiber repair (e.g. fibroblast growth factors and myosin content) (Mutryn et al., 2015). Tissue alterations are evident and indicators for WB have been reported to be more severe in tissue distant from blood vessels, i.e. superficial layer of the breast muscle (Soglia et al., 2017).

Thus, we hypothesize that ischemia/hypoxia and cellular stress have a distinct role in the development of WB and related abnormalities and this study aimed to determine whether markers of these events are mobilized through altered genes expression in 21-day old chickens.

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## 2. Material and methods

### 2.1. Animals and sampling

Ross308 broilers were reared at a commercial farm in Denmark. On days 21 and 30, post-hatching, 200 birds were selected (out of a total of 25,000) and killed by cervical dislocation.

Upon receiving the birds, the pectoralis major was removed and scored as no (WB0), mild (WB1) or severe (WB2) wooden breast, as described by Dalgaard et al. (2018). Within the 200 birds collected, all WB1 (8 on day 21 and 14 on day 30) and WB2 (8 on day 21 and 9 on day 30) were included, while a number (18 on day 21 and 17 on day 30) of WB0 were also selected for further analysis. Tissue samples (1 sample of approximately 5 g/animal), from the center of the cranial (thick) region of the *M. pectoralis major*, were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further analysis.

### 2.2. Biochemical analyses

For determination of pH and dry matter, samples were freeze-dried (CoolSafe ScanVac, Labogene, Allerød, Denmark) and dry matter content calculated as the difference in weight before and after. Subsequently, the samples (1 sample of approximately 150 mg/animal), were homogenized in 145 mM KCl, 10 mM NaCl and 5 mM Na-iodoacetate (pH 7.0) in a ratio of 1:20 (sample weigh: buffer volume) and pH measured using a pH electrode (InLab Surface, Mettler Toledo, Glostrup, Denmark).

Analysis of glycogen content and lactate dehydrogenase (LDH) activity was carried out as described by Oksbjerg et al. (2019).

### 2.3. RNA extraction and RT-PCR

Total RNA was extracted from approx. 20 mg tissue using the Trizol method, according to the manufacturer's protocol (Sigma-Aldrich, Søborg, Denmark). Following conversion to cDNA, using an iScript kit (Bio-Rad, Copenhagen, Denmark), real-time PCR was performed, as described by Rasmussen et al. (2011). Ct-values were normalized to Ct-values for 60S acidic ribosomal protein P0 (RPLP0) before converting these to fold-changes using the  $\Delta\Delta\text{Ct}$  method (Pfaffl, 2001). Ct-values for RPLP0 were not different between the wooden breast categories within day (for data see Supplementary data, Fig. 1).

Design of the TaqMan primers and probe sets was achieved using Primer Express software and chicken-specific gene-sequences (<https://www.ncbi.nlm.nih.gov/Genbank/>), and custom-made by LGC Biosearch Technologies (Riskov, Denmark). Specificities of the primers and probes were verified using BLAST. Primers and probes are given in Supplementary data (Supplementary data, Table 1).

### 2.4. Statistics

Data are presented as mean  $\pm$  standard error of the mean. One-way ANOVA was used to evaluate the effect of WB category within day. If an overall effect was observed, Tukey's post hoc test was used to determine differences between groups. If the equally variance test failed, data were log<sub>10</sub> transformed before ANOVA. For all tests,  $p < 0.05$  was regarded as significant. Statistical tests were performed in SigmaPlot 11.0 (Systat Software, San Jose, California, USA).

## 3. Results and discussion

The aim of this study was to investigate the biochemical characteristics and mRNA contents of selected marker-genes in broilers categorized as no-, mild- and severe-WB on days 21 and 30 post-hatching. We included 34 birds on day 21 and 40 on day 30, reared by a commercial producer, and scored WB according to Dalgaard et al. (2018).

### 3.1. Biochemical properties

On day 21, we categorized 8 broilers with mild WB and 8 with severe WB. We observed higher pH and reduced dry matter contents in the pectoralis major of birds affected severely by WB compared with no- and mild-WB. There were no differences in glycogen contents and LDH activities on day 21 (Table 1).

On day 30, we categorized 14 broilers with mild WB and 9 with severe WB. Samples were not significantly different for pH and dry matter contents compared with those from birds with no symptoms of WB. Glycogen contents (both pro and macro) were lower in samples from mild and severely-affected birds compared to those without WB. LDH activities were lower in mild and severely affected birds compared to those without WB, while no statistically significant difference was observed between the two WB categories. A metabolomics study, conducted on the same birds, revealed that WB-affected muscles had reduced carnosine contents (Sundekilde et al., 2017), which corroborated the proposed association between carnosine content and glycolytic activity (Aristoy & Toldra, 1998).

Total glycogen can be split into two fractions, pro-glycogen and macro-glycogen, based on protein content and acid solubility. Macro-glycogen ( $< 104$  kDa) is acid-soluble and has a high carbohydrate to protein ratio while the acid-insoluble pro-glycogen (up to 400 kDa) contain less carbohydrate in relation to protein (Rosenvold et al., 2002; Sterten et al., 2010). For anaerobic metabolism, pro-glycogen is metabolized in favor of macro-glycogen (Sterten et al., 2010), while macro-glycogen is used primarily during aerobic metabolism. As oxidative stress is associated with WB-affected tissue (Mutryn et al., 2015), the balance between these glycogen pools might be altered. However, our results showed that lower total glycogen concentrations originated from reduced amounts of both of pro- and macro-glycogen pools. Reduced glycogen in WB-affected muscle is, thus, likely to reflect overall reduced muscle fiber content, which is supported by reduced dry matter contents and LDH activities. Reduced muscle fiber content in WB-affected tissue is evident from studies reporting replacement of fibers with connective tissue (Dalgaard et al., 2018; Sihvo et al., 2014) as well as reduced protein content and increased collagen in the superficial region of WB-affected muscle (Baldi et al., 2019).

**Table 1**

Biochemical characteristics of pectoralis muscle of broilers with no, mild or severe wooden breast on day 21 and day 30.

	Wooden breast score		
	No	Mild	Severe
<i>Day 21</i>			
pH	5.96 $\pm$ 0.15 a	5.96 $\pm$ 0.16 a	6.16 $\pm$ 0.19 b
Dry matter (%)	24.15 $\pm$ 1.68 a	24.12 $\pm$ 1.16 a	21.85 $\pm$ 1.40 b
Glycogen, pro ( $\mu\text{mol/g}$ )	12.52 $\pm$ 3.22	11.75 $\pm$ 1.19	10.71 $\pm$ 3.68
Glycogen, macro ( $\mu\text{mol/g}$ )	7.52 $\pm$ 2.88	5.99 $\pm$ 1.19	6.38 $\pm$ 1.22
Total glycogen ( $\mu\text{mol/g}$ )	20.04 $\pm$ 5.00	17.74 $\pm$ 2.60	17.09 $\pm$ 4.60
LDH ( $\mu\text{mol/g}$ )	1868.99 $\pm$ 320.39	1831.28 $\pm$ 277.37	1825.25 $\pm$ 657.89
<i>Day 30</i>			
pH	5.98 $\pm$ 0.21	6.08 $\pm$ 0.15	6.14 $\pm$ 0.22
Dry matter, %	23.19 $\pm$ 3.18	22.44 $\pm$ 1.69	21.42 $\pm$ 2.88
Glycogen, pro ( $\mu\text{mol/g}$ )	12.41 $\pm$ 3.56 a	8.95 $\pm$ 3.28 b	7.66 $\pm$ 1.89 b
Glycogen, macro ( $\mu\text{mol/g}$ )	6.72 $\pm$ 1.16 a	5.04 $\pm$ 2.27 b	4.74 $\pm$ 1.90 b
Total glycogen ( $\mu\text{mol/g}$ )	19.13 $\pm$ 4.15 a	13.99 $\pm$ 4.68 b	12.40 $\pm$ 3.54 b
LDH ( $\mu\text{mol/g}$ )	1833.49 $\pm$ 380.47 a	1220.23 $\pm$ 389.58 b	1089.26 $\pm$ 332.26 b

Data are mean  $\pm$  standard deviation. Numbers no sharing subscription are statistical different from each other within days.

### 3.2. Expression of selected marker genes

On day 21, mRNA contents of HSP-70, HO-1, COX-2, TNF-1s and HIF were elevated in samples from birds categorized as severe WB compared with those from birds with no WB. We observed no differences in VEGF- $\alpha$ , LDH, BCL-2, and IL-1 $\beta$  (Table 2).

On day 30, samples from severely affected birds had greater mRNA contents for HSP-70, HO-1, COX-2, IL-1 $\beta$ , TNF-1, and HIF. No differences were observed in mRNA contents of VEGF $\alpha$  and BCL-2. LDH mRNA contents were lower in samples from mild- and severely affected birds compared with samples from birds with no symptoms of WB. No difference between the two WB categories was observed.

HSP-70 belongs to a family of heat shock proteins and is induced in response to physiological stress (Evans et al., 2010). HSP-70 act as chaperone proteins, stabilizing and guiding protein folding, and removing damaged proteins (Jolly & Morimoto, 2000). We observed increased HSP70 contents in samples from severely affected birds at both days 21 and 30, suggesting increased cellular stress is associated with WB. Samples from birds affected only mildly had intermediate concentrations of HSP-70 mRNA, suggesting lower levels of cellular stress. However, the HSP70 mRNA from mildly affected birds did not significantly differ from either the non-affected birds nor from the severely affected birds.

Similarly, HO-1 is a heme-metabolizing enzyme that is induced by oxidative stress (Morse & Choi, 2002). WB-affected tissue had an increased content of HO-1 mRNA at day 30 but, at day 21, only samples from severely affected birds had increased HO-1 mRNA contents. This confirms that WB is associated with oxidative stress also in Ross308.

Inflammation is often associated with elevated COX-2 expression (Turini & DuBois, 2002). COX-2 enzymes drive conversion of arachidonic acid to prostaglandins, which have pro-inflammatory properties, which is why COX-2 induction is used as a marker for inflammation. In this study, increased COX-2 mRNA was associated with WB, suggesting inflammatory processed, which was further supported by increased amounts of mRNA for IL-1 $\beta$  and TNF- $\alpha$  in samples from birds with WB.

Development of WB has been suggested to be induced by hypoxia, either limited oxygen availability and/or by impaired local blood supply. Hence, as markers of these events, we also analyzed mRNA contents of VEGF- $\alpha$  and HIF. Angiogenesis is important for supply of oxygen locally, via the bloodstream, and is controlled by the transcription factor VEGF- $\alpha$

(Murukesh et al., 2010). In 2018, it was shown that numbers of micro vessel are lower in WB-affected muscle (Sihvo et al., 2018).

HIF is a transcription factor playing an important part in the response to low oxygen pressure. It is one of the main genes involved in the regulation of vascularization in local areas following ischemia and tumors (Ziello et al., 2007). Hence, its expression profile can be indicative of local hypoxia. We confirmed tissue hypoxia associated with HIF in WB-affected chickens at both 21 and 30 days. Induction was more pronounced at day 30 with both mild- and severe-WB affected compared with day 21 where only samples from severe-WB birds had significant amounts of HIF mRNA. Also, myofiber area per vessel tended to increase in samples from WB-affected chickens, indicating that WB might be triggered by decreased blood supply leading to tissue hypoxia (Sihvo et al., 2018). However, expression of VEGF- $\alpha$  was not affected at either day 21 or at day 30, which does not exclude the possibility that it was dysregulated at an earlier stage.

BCL-2 is a key regulator in the inhibition of signaling pathways for apoptosis and, interestingly, BCL-2 over-expression has been associated with increased myocyte proliferation (Limana et al., 2002). Given that muscular hypertrophy is pronounced in WB-affected birds, potentially, expression of BCL-2 could be important in its development. However, we observed no differences in BCL-2 expression in samples from birds affected by WB compared with those without symptoms.

In conclusion, this study showed that several genes related to oxidative stress were upregulated in WB-affected birds and changes in regulation were more pronounced at day 30, compared with 21, as well as in tissue scored as severe WB compared to mild WB. Apart from COX-2 day 21 no significant differences were observed between mild and severely affected birds on any of the markers at gene-level indicating that the severity of WB is not detectible at mRNA level. These findings support the idea that hypoxia is related to WB development already when the symptoms are only mild. However, the resulting oxidative stress did not alter the balance between pro-glycogen and macro-glycogen.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochms.2020.100001>.

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**Table 2**

Gen-expression of selected genes in the pectoralis muscle of broilers with no, mild or severe wooden breast on day 21 and day 30.

Gene	No	Mild	Severe
<i>Day 21</i>			
Heat shock protein 70	1.00 $\pm$ 0.29 a	1.15 $\pm$ 0.28 a,b	1.41 $\pm$ 0.33 b
Heme oxygenase 1	1.00 $\pm$ 0.66 a	1.29 $\pm$ 0.74 a,b	2.01 $\pm$ 0.67 b
Vascular endothelia growth factor $\alpha$	1.00 $\pm$ 0.26	0.87 $\pm$ 0.14	0.93 $\pm$ 0.17
Cyclooxygenase 2	1.00 $\pm$ 0.34 a	1.41 $\pm$ 1.83 a	1.63 $\pm$ 0.67 b
Lactate dehydrogenase	1.00 $\pm$ 0.26	0.84 $\pm$ 0.20	0.76 $\pm$ 0.20
B-cell lymphoma 2	1.00 $\pm$ 0.30	0.93 $\pm$ 0.18	1.05 $\pm$ 0.15
Interleukine-1 $\beta$	1.00 $\pm$ 0.70	1.44 $\pm$ 1.67	1.75 $\pm$ 0.69
Tumor necrosis factor 1	1.00 $\pm$ 0.52 a	1.29 $\pm$ 0.75 a,b	1.35 $\pm$ 0.21 b
Hypoxia inducible factor	1.00 $\pm$ 0.33 a	1.07 $\pm$ 0.32 a,b	1.37 $\pm$ 0.21 b
<i>Day 30</i>			
Heat shock protein 70	1.00 $\pm$ 0.37 a	1.05 $\pm$ 0.19 a,b	1.33 $\pm$ 0.21 b
Heme oxygenase 1	1.00 $\pm$ 0.69 a	1.83 $\pm$ 0.60 b	2.46 $\pm$ 0.73 b
Vascular endothelia growth factor $\alpha$	1.00 $\pm$ 0.38	0.97 $\pm$ 0.34	1.03 $\pm$ 0.35
Cyclooxygenase 2	1.00 $\pm$ 0.58 a	1.38 $\pm$ 0.69 a,b	1.67 $\pm$ 0.71 b
Lactate dehydrogenase	1.00 $\pm$ 0.36 a	0.54 $\pm$ 0.43 b	0.57 $\pm$ 0.18 b
B-cell lymphoma 2	1.00 $\pm$ 0.33	1.09 $\pm$ 0.29	1.36 $\pm$ 0.62
Interleukine-1 $\beta$	1.00 $\pm$ 0.74 a	1.55 $\pm$ 0.79 a,b	2.43 $\pm$ 1.86 b
Tumor necrosis factor 1	1.00 $\pm$ 0.59 a	1.43 $\pm$ 0.71 a,b	1.94 $\pm$ 1.03 b
Hypoxia inducible factor	1.00 $\pm$ 0.39 a	1.43 $\pm$ 0.40 b	1.76 $\pm$ 0.44 b

Data are mean  $\pm$  standard deviation. Numbers no sharing subscription are statistically different from each other within days.

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