RESPONSE TO ROAD TRANSPORTATION IN TURKEY *(MELEAGRIS GALLOPAVO):* THE ACUTE PHASE PROTEIN EXPRESSION IN LIVER AND ADIPOSE TISSUE.

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Summary
Road transportation is one of the most stressful events during the turkeys' lifetime and is associated with economic losses. Beside their use as biomarkers of inflammation, acute phase proteins (APP) have been also used as biomarkers of animal welfare, including stress due to transport, but no information is available in turkey species.
The aim of the present study was to evaluate whether the gene expression of four APP, namely α1-acid glycoprotein (AGP), C-Reactive Protein (CRP), Serum Amyloid A (SAA) and PIT54, as potential indicators of transport stress in turkey (*Meleagris gallopavo*), by qualitative and quantitative real time (qPCR) in liver and adipose tissue. Fourteen healthy animals were divided into two groups: a group subject to road transport and a control group not subject to road transport.
The expression of AGP and CRP mRNA was found to be increased in animals slaughtered after road transport. AGP mRNA expression was increased in both liver and adipose tissue, and identified as one of the major stress indicators . The presence of AGP protein in liver and adipose tissue was also confirmed by immunohistochemistry. CRP mRNA expression was found to be increased in liver alone. The results of this study suggest that AGP may serve as biomarker of stress to evaluate the transporting conditions in turkeys.

INTRODUCTION

Animal welfare is of major concern in animal productions, in order to produce safe and quality food. Road transportation is an inevitable practice that animals encounter in the livestock industry and represents a critical phase in animal meat production (Schwartzkopf-Genswein et al., 2012).
Recently, it has been suggested that acute phase proteins (APPs) may also represent useful indicators of animal welfare, such as stress caused by road transport (Giannetto et al., 2011; Pineiro et al., 2007), suggesting their potential use as a parameter to evaluate stress.
Although APPs are produced mainly in liver, they can be produced also in adipose tissue (Sauerwein et al., 2014). Adipose tissue is a loose connective tissue which, beside its role in regulating metabolism and homeostasis, is also related to innate immunity. Moreover, during a stressful condition, the activation of the sympatho-adrenal and hypothalamic-pituitary-adrenal axis leads in turn to the activation of adipose tissue

metabolism (Chrousos, 2000), thus making adipose tissue to extremely reactive and sensitive to stress.
While APPs have been studied in mammals, no information about APPs and its relationship with adipose tissue in poultry has been made available so far. In this study we investigated if road transport alters the gene expression of major APPs in liver and adipose tissue of turkey (*Meleagris gallopavo*), with the final aim to find markers of transport related stress.

MATERIAL AND METHODS

Samples collection

Liver and adipose tissue samples were obtained during routinely slaughtering procedures. A group of seven animals were slaughtered on farm (control group) and a group of seven animals were slaughtered after 2 hours-transportation (stressed animals). Portions of tissues were removed immediately after slaughtering and collected in liquid nitrogen and stored at −80ºC. Samples for immunohistochemistry were fixed in 10% buffered formalin. Samples were collected during the routine slaughtering procedures.

Primers design, RNA extraction and cDNA synthesis

Four APPs were selected (AGP, CRP, SAA and PIT54) based on previous studies on chicken. Three housekeeping genes were selected (GAPDH, RPL4 and YWHAZ) based on previous studies and literature. Total RNA was isolated from liver and adipose tissue using Trizol standard protocol (Invitrogen). RNA was treated with DNase (Fermentas) and the first-strand cDNA synthesis was carried out using iScript cDNA synthesis kit (BioRad).

Qualitative and quantitative mRNA expression

The same primers were used in qualitative and quantitative PCR. Each sample was tested in duplicate. The thermal profile for qPCR was 50ºC for 2 min, 95ºC for 10 min, 40 cycles of 95ºC for 8s and 60ºC for 20s. Conditions for melting curve construction were 95ºC for 5s, decreasing to 55ºC for 5s and increasing to 95ºC for 5s. Results were compared using the Δ-Δ Cq method.

Western blot analysis

Samples for Western blot analysis were prepared from aliquots of 50–100 mg tissues. Aliquots with different concentrations were separated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotted onto nitrocellulose membrane. The membranes were immunolabelled for the presence of AGP using bovine AGP antibody and immunoreactive bands were visualized by enhanced chemiluminescence (ECL) using Immobilon Western Chemiluminescence substrate (Millipore). Bovine adipose tissue and AGP purified from bovine serum were used as positive controls.

Immunohistochemistry studies

Sections of liver and adipose tissue were mounted on poly-lysine-coated slides. The

sections were deparaffinized in xylene, rehydrated with ethanol and the endogenous peroxidase activity was blocked with H_2O_2 in methanol. Antigen retrieval was performed by heating the slides in a pressure cooker in citrate buffer solution. The nonspecific protein-binding was blocked with normal goat serum and the slides were incubated with the same primary antibody used for Western blot. The sections were treated with PolyView mouse/rabbit nanopolymer detection reagents (Enzo Life Sciences, Inc.), developed with the chromogen, 3-amino-9-ethylcarbazole (Vector Laboratories) and counterstained with Mayer hematoxylin.

RESULTS

In this work, we have identified by mRNA expression analyses four major APPs– AGP, CRP, SAA and PIT54 produced in turkey livers. Adipose tissue can also produce a small quantity of these APPs in a non-stressed animal, which surprisingly increased after exposure to transport-related stress.

Quantitative PCR revealed that liver AGP and CRP mRNA increased with a statistical significance in animals slaughtered after road transport. On the contrary, the mRNA abundance of PIT54 and SAA did not show any statistically significant difference after road transportation.

Adipose tissue AGP mRNA increased with a statistical significance in animals slaughtered after road transport. Unlike liver, adipose tissue PIT54 seems to be more expressed during road transportation with a positive trend. CRP and SAA have shown a slight increase in transported animals but the results are not significant.

The second part of the experiment was focused on proteins. We demonstrated that bovine anti-AGP cross-reacted with the equivalent turkey protein. Immunohistochemistry was done to detect the precise AGP location in liver and adipose tissue. In adipose tissue sections, anti-AGP stained an elevated number of adipocytes. Immunostaining was localized at the periphery of adipocyte cytoplasm and lining cell borders. Liver also stained positively.

DISCUSSION

The present study provides for the first time information on acute phase proteins in turkeys. We demonstrated that two-hours-long road transportation is able to modify APP mRNA expression in liver. In particular, AGP and CRP mRNA was overexpressed after road transportation stress. Remarkably, we found that adipose tissue was also reactive to road transportation, since AGP mRNA also increased in transported animals.

AGP seems to be the most relevant APP in turkey and the AGP protein presence was confirmed by Western Blot and immunohistochemistry. Western Blot electropherogram clearly identified in adipose tissue a 55-66 kDa band, with the same molecular weight of the corresponding bovine protein (Rahman et al., 2015).

Our hypothesis is supported by previous studies in other poultry species, which demonstrated that the measurement of AGP may provide useful information ont both health and welfare in chickens (Salamano et al., 2010). The serum concentration of AGP was also significantly increased in organically produced broilers as

compared to conventionally produced ones (Tuyttens et al., 2008).
Among the other APPs included in the present study, only liver CRP mRNA was shown to be upregulated (3.5-folds higher) in road transported animals. CRP is a major APP in humans and dogs and frequently used in veterinary field (Eckersall and Bell, 2010), but little evidence is available in poultry (O'Reilly and Eckersall, 2014). Neither PIT54 nor SAA liver mRNA abundance was modified after road transport.
The PIT54 is homologous to the scavenger receptor cysteine-rich family of proteins and has been identified as the major haemoglobin-binding protein, corresponding to mammalian haptoglobin, (Wicher and Fries, 2006)me="No Spacing" w:uiPriority="1" w:qFormat="1"/><w:lsdException w:name="Light Shading" w:uiP. The present findings thus confirm in turkey that only adipose tissue PIT54 mRNA is overexpressed following transport.
SAA may be used as markers of stress in other species (Lomborg et al., 2008; Soler et al., 2013) but in the current study no statistical increase of SAA mRNA was observed, neither in liver nor in adipose tissue.

CONCLUSION

In conclusion, we demonstrated that APP expression is influenced by road transport and may be explored as a suitable biomarker of stress transport. We suggest that, on the background of mRNA abundance data, the concentration of AGP and CRP might be modified in turkey after transport by road and may provide useful clinical indicators of stress in poultry. We also demonstrated that adipose tissue is capable to mount a local acute phase response during this stress condition. The present results represent the first step to develop turkey specific assays.

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