Short Communication

The effect of transport stress on turkey (*Meleagris gallopavo*) liver acute phase proteins gene expression

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Abstract

The aim of this study was to investigate the effects of transport-related stress on the liver gene expression of four acute phase proteins (APP), namely α_1-acid glycoprotein (AGP), C-Reactive Protein (CRP), Serum Amyloid A (SAA) and PIT54, in turkeys (*Melegaris gallopavo*). A group of seven BUT BIG 6 commercial hens was subjected to a two-hour long road transportation and the quantitative gene expression of APP in the liver was compared to that of a non transported control group. The expression of AGP and CRP mRNA was found to be increased in animals slaughtered after road transport. The presence of AGP protein was also confirmed by immunohistochemistry and Western Blotting. The results of this study showed that road-transport may induce the mRNA expression of immune related proteins. The finding that AGP and CRP can be upregulated during transport could suggest their use as for the assessment of turkey welfare during transport.

Keywords: Acute phase proteins; Animal welfare; *Meleagris gallopavo*; Turkey; Road transportation stress.

Road transportation is a necessary practice that livestock encounters and includes the assembly and loading of animals at their place of origin, their confinement on a moving or stationary vehicle without food, water and rest, the unloading and the lairage at their final destination (Schwartzkopf-Genswein et al., 2012). Road transport is regarded as one of the most stressful events in the turkeys' lifetime (Marchewka et al., 2013). Most studies focused on the impact of the transport-related stress on economical losses due to injuries and/or the increased death on arrival (DOA) rate (Petracci et al., 2006). Beside this economical perspective, there is also an ethical aspect that has to be addressed. The identification of suitable biomarkers of stress is of paramount importance in order to develop handling and transport procedures that minimize the stress level in animals.

Acute Phase Proteins (APP) are a class of proteins whose plasma concentration is modified during inflammation, cancer, trauma and non-inflammatory stress (Murata, 2007). Major APP in poultry include alpha1-acid glycoprotein (AGP), C-Reactive Protein (CRP), Serum Amyloid A (SAA) and PIT54 (O'Reilly and Eckersall, 2014). From a clinical perspective, APP also provide useful biomarkers to detect the early onset of diseases or to assess welfare. A recent study (Sherlock et al., 2012) determined the differential hepatic gene expression in broilers in response to combined stressors (feed withdrawal, catching and transport), identifying 733 genes which were differently expressed but the use of APPs as biomarkers for transport stress in poultry has not been adequately addressed so far.

Although turkey (*Meleagris gallopavo*) farming is economically relevant, no information regarding APP is available for this species. There is also very limited information on the welfare of turkeys during transport

The aim of this study is to fill this gap by investigating the expression of APP in turkey liver at gene level and, where specific antibodies are available, at protein level. In the second part of the study, the gene expression modification of APP mRNA was

investigated.as a preliminary step to determine if APPs could be considered as possible biomarkers in assessing welfare in turkeys,

Liver samples were collected during routine slaughtering procedures from eight BUT BIG 6 breeding line female turkeys and either snap frozen into liquid nitrogen and stored at −80ºC (for gene expression analyses) or fixed in 10% buffered formalin for immunohistochemistry.

The nucleotide sequences of four APP, namely AGP, CRP, SAA and PIT54, were identified among the sequences available in NCBI (Table 1, Supplementary). Primers were designed on turkey sequences available in NCBI by using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0) preventing possible secondary structures with the mfold Web Server (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) and ensuring the specificity of the sequence by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo).

Total RNA extraction and reverse transcription was carried out as previously described (Lecchi et al., 2012).

A single band with the expected size in agarose gel electrophoresis confirmed gene specific amplification and the results revealed that liver can physiologically express the four APP (Fig. 1a).

In the second part of the study, the possible impact of transport-related stress on the expression of APP genes was investigated. 2 groups of seven 92 day-old clinically healthy BUT BIG 6 female turkeys. The status of the animals was verified following a clinical examinations of the animals and the absence of evident pathologic lesions in the carcasses. The first group was slaughtered and sampled in an abattoir located on site (control group – non transported animals) , the second group of seven animals received the same treatment after a 2 hour transport (transport-stressed animals). The transport of turkeys was carried out on a truck that was authorized by Italian Health Institutions following the Council

Regulation (EC) No 1/2005 of 22 December 2004 on the protection of animals during transport and related operations. Animal were transported in plastic coop 1 meter width x 60 cm length x 39"L x 40 cm high. Temperature ranged from 20 to 22° C. Both groups underwent the same handling procedure (namely catching and caging and the same slaughtering procedure), with the exception of road-transport.

The mRNA abundance of the four APP gene was then measured by quantitative PCR after normalization of the sample using the geometric mean of reference genes (GAPDH, RPL4 and YWHAZ), which were selected based on previous studies on chicken (Yang et al., 2013; Yue et al., 2010) (Table 1 Supplementary). In order to evaluate the PCR efficiency using a relative standard curve, dilution series were prepared by performing fourfold serial dilution starting from the pooled sample composed by liver cDNA of five non-transported animals. Each sample was tested in duplicate. No-RT controls were performed by omitting reverse transcription and no template controls were conducted by adding nuclease free water. The MIQE guidelines were followed (Bustin et al., 2009).

Statistical analysis was performed using a General Linear Model procedure of SAS (SAS version 9.2 Inst. Inc., Cary, NC). Least significant difference was used to compare mean values. Statistical significance was accepted at p value ≤ 0.05.

Results are presented in Fig. 1b. Liver AGP and CRP mRNA abundance was found to be statistically significantly increased three fold in animals after two-hour transport, as compared with not transported ones (p = 0.0205 and 0.0109, respectively). The findings presented in this paper identify AGP as one of the major acute-phase proteins in turkey, at least for what at least concerning stress measurement after road transport. Our hypothesis is supported by previous studies in chicken, which demonstrated that measurement of AGP titration could provide useful information on both health and welfare (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).

AGP is one of the most widely measured APPs in chicken (O'Reilly and Eckersall, 2014) and its expression has been found to be related with depressive disorders and stress in humans (Healy et al, 1991). AGP belongs to the family of lipocalins, e.g binding proteins which also fulfil immune-modulatory function, including inhibition of phagocytosis (Lecchi et al., 2013), neutrophil degranulation, chemotaxis and respiratory burst (Lecchi et al., 2008; Miranda-Ribera et al., 2010; Rinaldi et al., 2008) and monocyte apoptosis (Ceciliani et al., 2007a). The immunomodulatory activity of AGP is focused on dampening the side effects of inflammation. Liver function is strongly influenced by circulating glucocorticoid levels, and gene array studies have shown that hepatic response follows acute stress in chicken (Desert et al, 2008, Sherlock et al, 2012). On the background that corticosteroids can upregulate AGP gene expression (Ceciliani and Pocacqua, 2007b), we may therefore speculate that the overexpression of AGP in liver is induced by the increase of cortisol concentration induced by transport-related procedures.

Among the other APP included in the present study, only liver CRP mRNA was shown to be upregulated in road transported animals as compared to controls. 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) and none in turkey. Although Sohail et al., (2010) found that CRP serum concentrations increased in chicken exposed to heat stress conditions and a very recent study correlated CRP concentration with the intensity of inflammatory reaction after *S. typhimurium* LPS treatment (Rauber et al., 2014), the use of CRP as an APP in chicken is yet to be established. The present results are encouraging, and provide for the first time clues that CRP is related to road transportation stress in turkey.

Neither PIT54 nor SAA liver mRNA abundance was modified after road transport, confirming in turkey what has already been demonstrated in chicken (Georgieva et al., 2010), i.e. that PIT54 and SAA are of little use as biomarkers for transport stress, since they probably need a more intense challenge that that provide in this study.

To the best of our knowledge, no antibody specific for turkey CRP has also far been reported in literature. Attention was thus focused on AGP only, the presence of which in liver was confirmed by Western Blotting and immunohistochemistry by using an anti-bovine AGP antibody (Ceciliani et al., 2007) that was shown to cross-react with a most prominent band with a MW of 55–65 kDa (Fig.2B). Immunolocalization in liver was carried out as previously described (Rahman et al. 2015). In hepatocytes (Fig. 2A), anti-AGP positive intracytoplasmic immunolabeling was multifocally detected. Positive signal was not diffuse and small groups of positive hepatocytes were scattered throughout the liver parenchyma, without any evident pattern. In order to rule out any un-specific cross-reactivity, blocking of the anti-bovine AGP antibody specific sites was carried out by incubating an aliquot of antibody with a purified bovine AGP (Rahman et al., 2015). The mixture was then utilized as primary antibody on Western Blotting membrane or on FFPE liver. Neither turkey nor bovine reactive bands' or tissue immunostaining was detected, thus confirming that all bands visualized were derived from AGP (Fig. 2B).

In conclusion, the present study provides for the first time information on acute-phase proteins in turkey, suggesting that, on the background of mRNA abundance data, AGP and CRP expression is related to road transportation stress in turkey. Although encouraging, these results must be considered as preliminary, and needed to be confirmed by the measurement in serum of the respective transcript product, e.g. the AGP and CRP proteins, in order to confirm that its serum concentration can provide a suitable measure of turkey welfare during road-transport.

Conflict of interest declaration

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. Moreover, none of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Table 1 Supplementary. List of the genes under study and their respective primers sequences, accession numbers, fragment lengths, PCR efficiency and regression coefficient (r^2).

Gene	GenBank	Primer Forward (5'-3')	Primer Reverse (5'-3')	Length(bp)	PCR efficiency (r^2)
AGP	XM_003211214	TCCCTGCCGAAATAGACAAC	TCCTTCATCTCAGCCATGTG	103	95.6% (r^2) - 0.999
SAA	XM_003206257.1	TGCTTCGTGTTGCTCTCCAT	CATGTCCCGGTATGCTCTCC	123	100.2% (r^2) - 0.998
PIT54	XM_003202017	GCCAGTGCAATTTGTTCAGA	TCCCGTAAATCCCAGTTGTC	146	95.6% (r^2) - 0.999
CRP	EU106581.1	ATCCCATGCTCAACTTCACC	CCGACGTAGAAGCGGTACTC	145	105.8% (r^2) - 0.99
GAPDH	GQ184819.1	GATCCCTTCATCGACCTGAA	ACAGTGCCCTTGAAGTGTCC	77	94.7% (r^2) - 0.998
RPL4	XM_003209573.1	TGTTTGCCCCAACCAAGACT	TCCTCAATGCGGTGACCTTT	136	102% (r^2) - 0.998
YWHAZ	XM_003205203.1	TTCCCTTGCAAAAACGGCTT	TTCAGCTTCGTCTCCTTGGG	148	97.4% (r^2) - 0.998

Primers were designed on turkey sequences available in NCBI using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0) preventing possible secondary structures with the mfold Web Server (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) and ensuring the specificity of the sequence by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo). The same primers were used for qualitative and quantitative PCR.
AGP, α1-acid glycoprotein; SAA, Serum Amyloid A; CRP, C-Reactive Protein (CRP); PIT54, Scavenger receptor cysteine-rich domain-containing protein LOC284297 homolog; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPL4, Ribosomal protein L4; low density lipoprotein receptor-related protein 10; YWHAZ, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta

Figure Legends

Fig. 1: the liver gene expression of AGP, SAA, PIT54 and CRP Acute Phase Proteins

Fig. 1a. PCR amplification of AGP, SAA, PIT54 and CRP in liver turkey mRNA separated by agarose gel electrophoresis (1.6%) stained with ethidium bromide. Non-template reaction was used as negative control. PCR products were visualized on 1.6% agarose gel stained with ethidium bromide.

Fig. 1b. Relative expression of AGP, PIT54, SAA and CRP in liver of non transported and transported turkey studied by qPCR. The results were normalized using the geometric mean of reference genes (GAPDH, YWHAZ and RPL4). Data are means ± SE of seven animals.. Quantitative reactions (qPCR) were performed in 12 µL of Eva Green Supermix (BioRad), 250 nM of AGP and PIT54 primers, 300 nM of SAA and CRP primers and 400 nM of GAPDH, YWHAZ and RPL4 primers, on Eco Real-Time PCR System (Illumina). The thermal profile was the same for each target gene: 95 °C for 90 s, 50 cycles of 95 °C for 15 s and 60 °C for 60 s; for melting curve construction, 55 °C for 60 s and 80 cycles starting to 55 °C and increasing 0.5 °C each 10 s.

Fig. 2.

Fig 2 a. Immunolocalization of AGP in turkey. Section of liver. Immunohistochemical staining anti-AGP (standard ABC method, AEC chromogen). A positive, intracytoplasmic, finely granular immunostaining (red staining) is visible in a moderate number of hepatocytes. Bar 20 micrometers.

Fig 2b. The detection of turkey AGP Western Blotting in liver tissue. Left panel: anti-bovine AGP primary antibody. Right panel: anti-bovine AGP primary antibody after blocking specific sites with purified bovine AGP (Rahman et al., 2015).

Table 1. List of the genes under study and their respective primers sequences, accession numbers, fragment lengths, PCR efficiency and regression coefficient (r^2).

Gene	GenBank	Primer Forward (5'-3')	Primer Reverse (5'-3')	Length(bp)	PCR efficiency (r^2)[a]
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SAA	XM_003206257.1	TGCTTCGTGTTGCTCTCCAT	CATGTCCCGGTATGCTCTCC	123	100.2% (r^2) - 0.998
PIT54	XM_003202017	GCCAGTGCAATTTGTTCAGA	TCCCGTAAATCCCAGTTGTC	146	95.6% (r^2) - 0.999
CRP	EU106581.1	ATCCCATGCTCAACTTCACC	CCGACGTAGAAGCGGTACTC	145	105.8% (r^2) - 0.99
GAPDH	GQ184819.1	GATCCCTTCATCGACCTGAA	ACAGTGCCCTTGAAGTGTCC	77	94.7% (r^2) - 0.998
RPL4	XM_003209573.1	TGTTTGCCCCAACCAAGACT	TCCTCAATGCGGTGACCTTT	136	102% (r^2) - 0.998
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Figure 1

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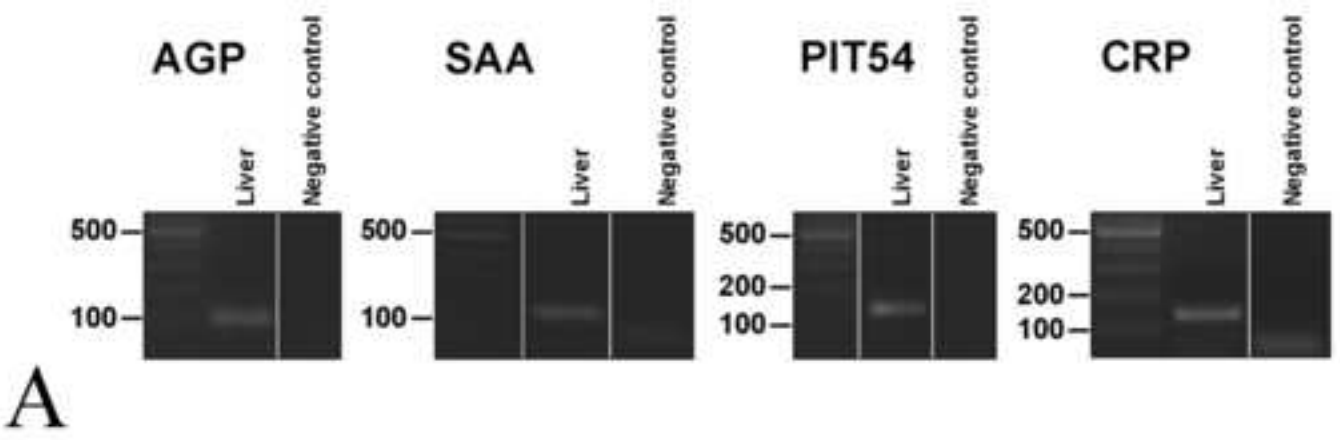

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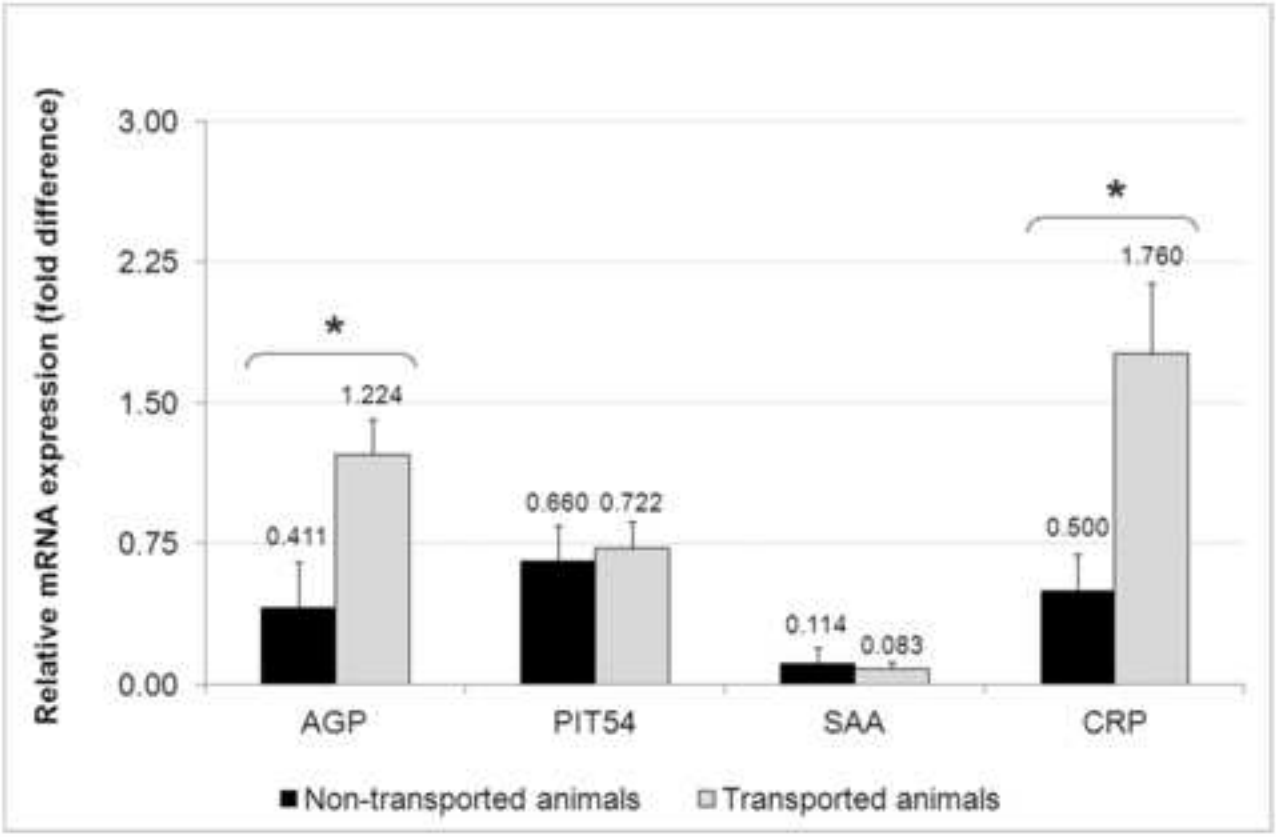

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Figure 2

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