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Short communication

Circulating extracellular miR-22, miR-155, and miR-365 as candidate biomarkers to assess transport-related stress in turkey (*Meleagris gallopavo*).

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Short title: Evaluation of circulating miRNA in stressed turkey.

Abstract

MicroRNA (miRNA) have been identified in circulating blood and might have the potential to be used as biomarkers for several pathophysiological conditions. To identify microRNA that are altered following stress events, turkeys (*Meleagris gallopavo*) were subjected to two hours of road transportation. The expression levels of five circulating microRNA, namely miR-22, miR-155-5p, miR-181a-3p, miR-204 and miR-365-3p, were detected and assessed by qPCR using TaqMan® probes, as potential biomarkers of stress. The areas under the receiver operating characteristic curves (AUROC) were then used to evaluate the diagnostic performance of miRNA.

A panel of three stress-responsive miRNA, miR-22, miR-155 and miR-365 were identified; their expression levels were significantly higher after road transportation and the AUC were 0.763, 0.71 and 0.704, respectively. Combining the three miRNA a specificity similar to the one found for the three miRNA separately was found. The AUC of the weighted average of the three microRNA was 0.763.

This preliminary study suggests that the expression levels of circulating miR-22, miR-155 and miR-365 are increased during transport-related stress and that they may have diagnostic value to discriminate between stressed- and unstressed animals.

Keywords: circulating miRNA, turkey, stress, welfare, biomarkers.

Implications

Accurate determination of stress in farm animals is critical, since a quantitative measure of stress-related parameters is difficulte. Besides ethical-related issues, the impact of animal welfare has a direct repercussionon meat quality and quantity, therefore it is of major economic relevance for food industry. Currently solid and standard protocols to assess turkey welfare lack. The present study highlights the possible use of molecular biomarkers to quantitatively assess stress in turkey. Three candidate biomarkers have been identified in serum of stressed turkey and they may be useful to discriminate between stressed and unstressed animals.

Introduction

Welfare is a multidimensional concept that embraces absence of suffering, high levels of biological functioning and the potential for animals to have positive experience. Besides ethical-related issues, the current importance of animal welfare has a direct impact on meat quality and quantity (Terlouw *et al.*, 2008), therefore is also of major economic concern for the turkey industry. Although solid and standard welfare protocols exist for poultry, the Welfare Quality®(2009), Assessment Protocol for Poultry, report that these protocols cannot be applied successfully to turkey species (http://www.welfarequality.net/everyone/45630/9/0/22). Traditional methods include behavioral observation and few quantifiable parameters, such as cortisol among the others, although their use is still debated (Marchewka *et al.*, 2013).

The knowledge of how welfare management tools, previously applied to other species, can be applied also to turkeys is unclear, and this prevents a correct quantification of the effects of management practices on turkey productivity and welfare.

63 The tandard protocols to assess animal welfare are often incomplete or unsuitable, since
64 they differ in the thresholds set to differentiate high vs. poor welfare, and/or in the way the
65 information is integrated to form an overall evaluation judgement (Botreau *et al.*, 2007).

66 MicroRNA (miRNA) are small non-coding RNA that regulate post-transcriptionally
67 gene expression, playing key roles in regulating immune response. The modulation of
68 miRNA expression is an early response to stressful conditions. Extracellular miRNA can
69 be easily extracted from body fluids. Therefore, circulating miRNA are among the most
70 promising clinical biomarkers for the diagnosis of a variety of diseases and stress
71 disorders in humans (Andersen *et al.*, 2014).

72 The aim of the present study was to a) ascertain whether transport-related stress
73 modulates the expression of circulating miRNA and b) to investigate the potential use of
74 differentially expressed miRNA as biomarkers to measure transport-related stress. The
75 study was carried out by measuring by quantitative PCR those miRNA that were
76 previously demonstrated to be related to stress events and immune defenses in chicken,
77 namely miR-22, miR-155, miR-181a, miR-204 and miR-365 (Ahanda *et al.*, 2014). Road
78 transportation was selected as stress model. The practices related to road transport, which
79 is regarded as one of the most stressful events in the turkeys'lifetime (Marchewka *et al.*,
80 2013), included catching, loading, transport, unloading and final feed deprivation until
81 slaughtering..

82

83 **Material and Methods**

84 *Sample collection*

85 Blood was collected from sixteen clinically healthy 105 day-old turkeys by branchial
86 vein venipuncture using serum collection tubes during routine disease testing. After a 2-

hours road-transportation, further blood samples were collected during routine slaughtering process from the neck vessels cut by the automatic processing killer. Road-transport was carried out according to EU procedures for animal transport (Council Regulation (EC) No 1/2005 of 22 December 2004 on the protection of animals during transport and related operations and amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No 1255/97). Serum was stored at -80°C until RNA extraction.

MiRNA extraction and real-time quantitative PCR

Total RNA was extracted using miRNeasy Serum/Plasma Kit (Qiagen, catalog number 217184). Serum was thawed on ice and centrifuged at 3000 g for 5 min at 4°C. An aliquot of 150 μl per sample was transferred to a new tube and 1 ml of Qiazol was added. The *Caenorhabditis elegans* miRNA cel-miR-39 (Qiagen, catalog number 219610) was used as synthetic spike-in control due to lack of sequence homology to avian miRNA. After an incubation at room temperature for 5 min, 3.75 μl (25 fmol final concentration) of spike-in control was added and the samples vortexed to ensure complete mixing. The RNA extraction was then carried out according to manufacturer's instruction. Total RNA concentration and quality were validated as ratio A_{260}/A_{280} by NanoDrop ND-1000 UV–vis spectrophotometer (NanoDrop Technologies Inc). The reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, catalog number 4366596) using miRNA-specific stem-loop RT primers as according to manufacturer's instructions. Reverse transcription reactions were performed in 15 μl volume reactions containing 1.5 μl 10X miRNA RT buffer, 1 μl MultiScribe reverse transcriptase (50 U/μl), 0.30 μl 100 mM dNTP mix, 0.19 μl RNase Inhibitor (20 U/μl), 6 μl of custom RT primer pool and 3.01 μl of nuclease-free water. The custom RT primer pool was prepared combining 10 μl of each individual 5X RT primer in a final volume of 1000 μl;

111 the final concentration of each primer in the RT primer pool was 0.05X each. Three μl
112 serum RNA were added to each RT reaction. RT reaction mixture were incubated on ice
113 for 5 minutes, 16°C for 30 minutes, 42°C for 30 minutes and then 85°C for 5 minutes.

114 The qPCR experiments were designed following MIQE guidelines. Small RNA
115 TaqMan assays were performed according to manufacturer's instruction. The selected
116 primer/probe assays (Life Technologies) included cel-miR-39-3p (assay ID000200), hsa-
117 miR-22 (assay ID398), hsa-miR-181a-3p (assay ID516), hsa-miR-155-5p (assayID479),
118 hsa-miR-204 (assay ID508), hsa-miR-365-3p (assay ID1020). Quantitative reactions were
119 performed in duplicate in scaled-down (12 μl) reaction volumes using 6 μl TaqMAN 2X
120 Universal Master Mix II (Applied Biosystems, catalog number 4440044), 0.6 μl miRNA
121 specific TaqMan Assay 20X and 1μl of the RT product per reaction on Eco Real Time PCR
122 detection System (Illumina). The standard cycling program was 50°C for 2 min, 95°C for
123 10 min and 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Data were normalized
124 relative to the expression of cel-miR-39. MiRNA expression levels are presented in terms
125 of fold change normalized to cel-miR-39 expression using the formula $2^{-\Delta\Delta Cq}$.

126 *Statistical analysis*

127 Normality of the distribution of each of the miRNA variables was assessed using
128 Shapiro-Wilk test. Since data were not normally distributed a non parametric method, the
129 Wilcoxon test, was used in the analysis of differences in miRNA expression. A receiver
130 operating characteristic (ROC) curve was used to determine the sensitivity and specificity
131 of the assay in discriminating between pre- and post-transported animals. The area under
132 the curve (AUC) for the ROC curves was calculated. Statistical analysis was performed
133 using XLSTAT for Windows (Addinsoft, New York, U.S.A.) and MedCalc 14.0 (MedCalc
134 Software bvba, Ostend, Belgium).

135

Results

miR-22, miR-155 and miR-365 levels are elevated in the blood serum of stressed turkeys

The comparative analysis demonstrated that three circulating miRNA, namely miR-22, miR-155 and miR-365, were differentially expressed in serum samples collected after road transportation if compared with those collected before transportation from the same animal. In detail, the levels of miR-22, miR-155 and miR-365 were significantly higher after road transportation (all $P \leq 0.05$) (Fig. 1).

The median expression levels of miR-22, miR-155 and miR-365 were 0.90 (range, 0.22 to 7.90), 0.97 (range, 0.43 to 2.23) and 0.66 (range, 0.27 to 1) before transportation, and 1.81 (range, 0.19 to 30.13), 1.61 (range, 0.57 to 4.07) and 1.59 (range, 0.23 to 5.76) after transportation, respectively.

Diagnostic performance of miR-22, miR-155 and miR-365

In the second part of the study we explored the potential use of the three miRNA that were found to be differentially regulated as biomarker predictors of transport-related stress. Receiver Operating Characteristic (ROC) analysis was used to estimate the diagnostic value of miR-22, miR-155 and miR-365 alone, or in combination. The ROC analysis was carried out by plotting the true positive (sensitivity) versus false positive (1-specificity). Cut-off points were set in order to maximize the sum of sensitivity and specificity; the cut off points for miR-22, miR-155 and miR-365 were 1.31, 1.48 and 1.59, respectively. The diagnostic accuracy of miR-22, miR-155 and miR-365, as measured by the area under the curve (AUC), was 0.763 (95% CI 0.586-0.941), 0.710 (95% CI 0.512-0.908) and 0.704 (95% CI 0.497-0.912), respectively (Fig. 2). Further statistical analysis was performed considering the weighted average relative quantification (RQ) values of the three stressed-related miRNA (Fig. 3A). The median expression levels were 1.02 (range, 0.62 to 1.38) and 1.97 (range, 1.73 to 6.26) before and after transportation, respectively.

The predicted probability of being discriminated as stressed-animals from the logit model based on the three miRNA [logit= (0.065 x expression level of miR-22) + (0.562 x expression level of miR-155) + (1.395 x expression level of miR-365)] was used to construct a ROC curve (Fig. 3B). The AUC for the combined miRNA was 0.763 (95% CI 0.557-0.906).

Discussion

Accurate determination of stress in poultry, and farm animals in general, is critical, since it is often difficult to measure quantitatively stress-related parameters. Beside the use of behavioral scoring system, biochemical parameters have been proposed to provide a broad assessment of animal welfare including corticosteroids and acute phase proteins (Pineiro *et al.*, 2007; Marchewka *et al.*, 2013). MiRNA act as regulators of gene expression during many different pathophysiological pathways, including those involved in neuropsychiatric disorders and stress (Kocerha *et al.*, 2015), moreover a recent study reported that miRNome is capable of quickly reacting to feed deprivation stress in chicken (Ahanda *et al.*, 2014).

The hypothesis of this study was that circulating miRNA could provide a useful source of biomarkers for objective measurements of animal welfare. This hypothesis was validated by demonstrating that three miRNA were significantly upregulated during road transport in turkeys. Given their involvement in the modulation of immune response, the present results suggest that transport-related procedures may interfere with the immune status of the turkey by modifying the gene expression level of immune-related miRNA.

In particular, miR-155 is physiologically expressed at low levels in B and T cells,

macrophages, dendritic cells, and progenitor/stem cell populations, and is upregulated after their activation by immune stimuli, eading to modulation of humoral and innate cell-mediated immune responses (Elton *et al.*, 2013). MiR-22 is one of the very few ubiquitously expressed microRNAs, and is likely to be involved in buffering cellular activities that are common to the vast majority cells. Among others activities, it is involved in the hematopoiesis process, in the down regulation of IL6 and in the differentiation of Th17 cells (Liang *et al.*, 2015).

The role of miR-365 is still not well understood. MiR-365 expression has been so far associated to cancer development and its progression (Zhou et al., 2013). The finding that miR-365 may be related to transport-stress confirms what has been recently reported by Ahanda *et al.* (2014), who demonstrated that miR-365 family members are present in plasma and red blood, but not white blood cell, and their expression is modulated by food deprivation stress.

In conclusion, this preliminary study highlighted that transport-related procedures are capable of modifying expression of immune-related miRNA, providing for the first time a molecular link between stress and immune defenses in turkey species. In the second part of this investigation, we demonstrated by ROC analysis that the combined panel of three miRNAs may be useful to discriminate between transport stressed- and unstressed animals. In order to confirm the diagnostic value of these candidate miRNA, and develop a minimally invasive screening tool for assessing turkey welfare, further studies on a higher number of samples and different transport conditions are required.

Acknowledgements

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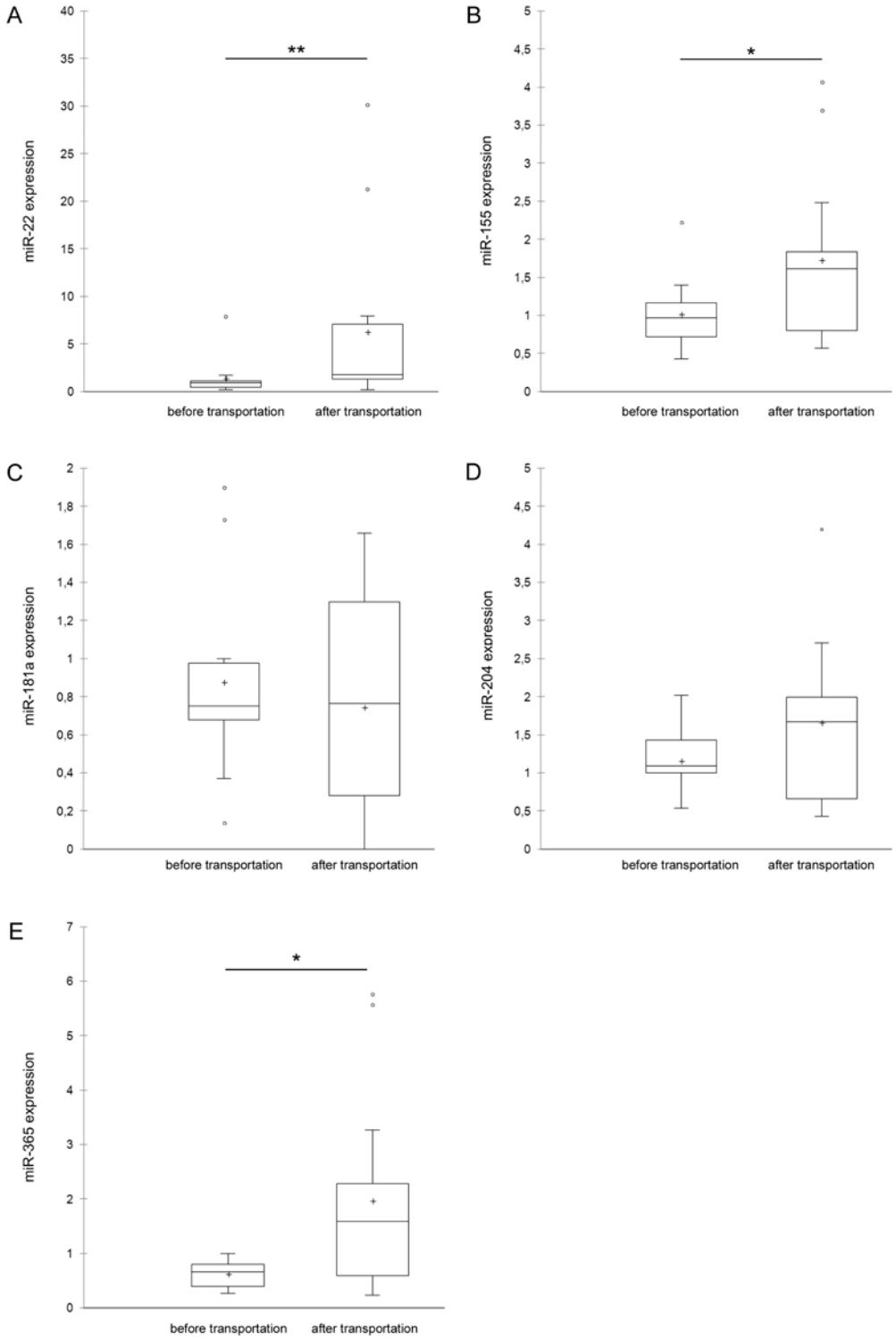

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241 Figure 1. Circulating stress-related miRNA levels of individual turkeys.

242 Serum samples were collected from sixteen turkeys before and after road-transportation and were
243 analyzed for the presence of stress-related miRNA. Levels of (A) miR-22, (B) miR-155, (C) miR-
244 181a, (D) miR-204 and (E) miR-365 levels. The black lines mark the medians. * P< 0.05; ** P<
245 0.01.

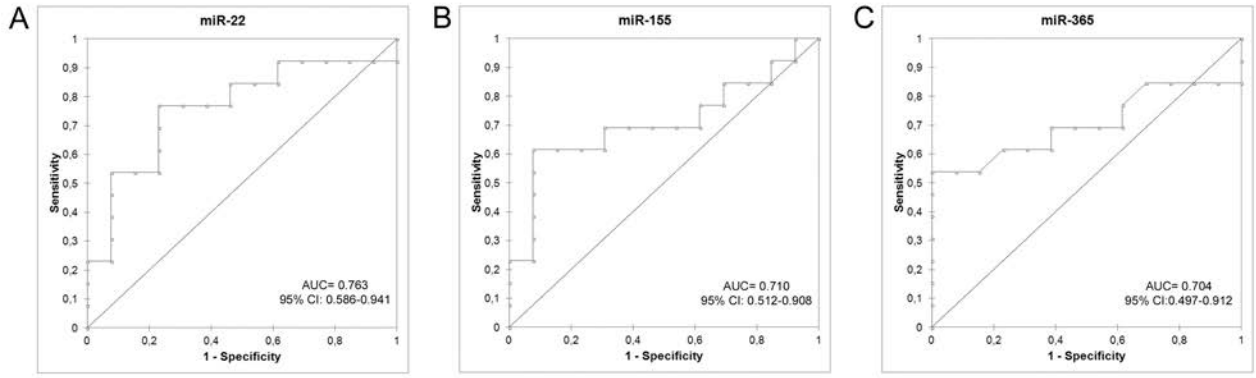
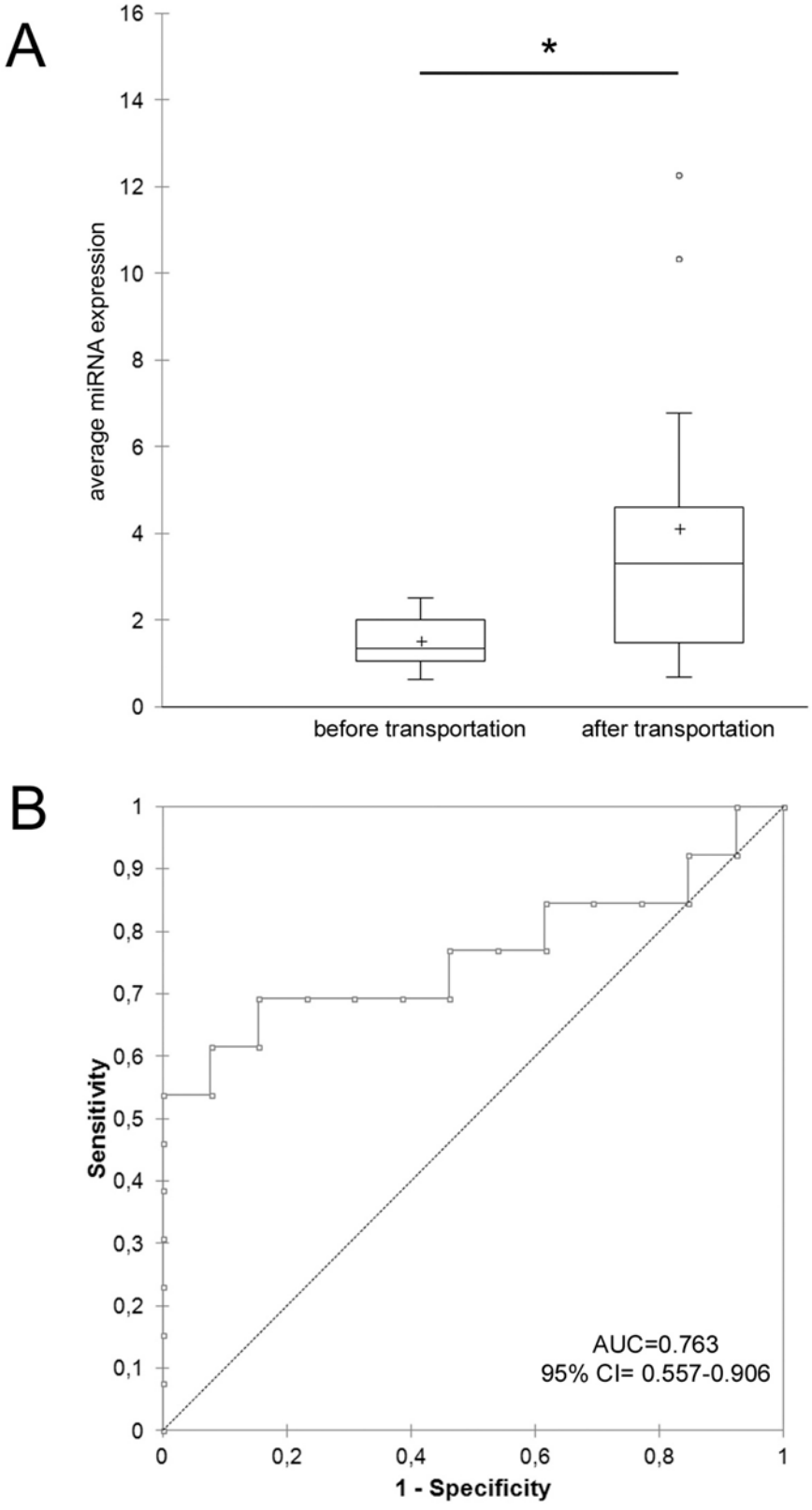


Figure 2. Receiver-operator characteristics (ROC) curve analysis of candidate stress-related miRNA.

ROC plots for (A) miR-22, (B) miR-155 and (C) miR-365 were used to differentiate stressed from not-stressed animals. AUC, area under the curve; CI, confidence interval.



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253 Figure 3. The average expression of the three candidate stress-related miRNA.

254 (A) The weighted average relative quantification (RQ) values of the three candidate stress-related
255 miRNA of individual turkeys. (B) ROC curve analysis was constructed using the logit model. AUC,
256 area under the curve; CI, confidence interval. The black lines mark the medians. * P< 0.05.