

1 **doi:10.1017/S1751731115003043**

2 Short communication

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

5
6 C. Lecchi ^{1*}, A. T. Marques ¹, M. Redegalli ¹, S. Meani ², L. J. Vinco ², V. Bronzo ³
7 and F. Ceciliani ¹

8
9 ¹ *Department of Veterinary Science and Public Health, Università degli Studi di*
10 *Milano, Via Celoria 10, 20133 Milan, Italy*

11 ² *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via*
12 *Bianchi 9, 25124 Brescia, Italy*

13 ³ *Department of Health, Animal Science and Food Safety, Università degli Studi di*
14 *Milano, Via Celoria 10, 20133 Milan, Italy*

15
16 Corresponding author. Cristina Lecchi. E-mail: cristina.lecchi@unimi.it

17 Short title: Evaluation of circulating miRNA in stressed turkey.

18

19 **Abstract**

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

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

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

36

37

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

39 **Implications**

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

48

49 **Introduction**

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

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

63 The standard 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-

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

93 *MiRNA extraction and real-time quantitative PCR*

94 Total RNA was extracted using miRNeasy Serum/Plasma Kit (Qiagen, catalog
95 number 217184). Serum was thawed on ice and centrifuged at 3000 g for 5 min at 4°C. An
96 aliquot of 150 µl per sample was transferred to a new tube and 1 ml of Qiazol was added.
97 The *Caenorhabditis elegans* miRNA cel-miR-39 (Qiagen, catalog number 219610) was
98 used as synthetic spike-in control due to lack of sequence homology to avian miRNA. After
99 an incubation at room temperature for 5 min, 3.75 µl (25 fmol final concentration) of spike-
100 in control was added and the samples vortexed to ensure complete mixing. The RNA
101 extraction was then carried out according to manufacturer's instruction. Total RNA
102 concentration and quality were validated as ratio A_{260}/A_{280} by NanoDrop ND-1000 UV-vis
103 spectrophotometer (NanoDrop Technologies Inc). The reverse transcription was
104 performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems,
105 catalog number 4366596) using miRNA-specific stem-loop RT primers as according to
106 manufacturer's instructions. Reverse transcription reactions were performed in 15 µl
107 volume reactions containing 1.5 µl 10X miRNA RT buffer, 1 µl MultiScribe reverse
108 transcriptase (50 U/µl), 0.30 µl 100 mM dNTP mix, 0.19 µl RNase Inhibitor (20 U/µl), 6 µl of
109 custom RT primer pool and 3.01 µl of nuclease-free water. The custom RT primer pool
110 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 μ
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 μ l) reaction volumes using 6 μ l TaqMAN 2X
120 Universal Master Mix II (Applied Biosystems, catalog number 4440044), 0.6 μ l miRNA
121 specific TaqMan Assay 20X and 1 μ l 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

136 **Results**

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

139 The comparative analysis demonstrated that three circulating miRNA, namely miR-
140 22, miR-155 and miR-365, were differentially expressed in serum samples collected after
141 road transportation if compared with those collected before transportation from the same
142 animal. In detail, the levels of miR-22, miR-155 and miR-365 were significantly higher after
143 road transportation (all $P \leq 0.05$) (Fig. 1).

144 The median expression levels of miR-22, miR-155 and miR-365 were 0.90 (range,
145 0.22 to 7.90), 0.97 (range, 0.43 to 2.23) and 0.66 (range, 0.27 to 1) before transportation,
146 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)
147 after transportation, respectively.

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

149 In the second part of the study we explored the potential use of the three miRNA
150 that were found to be differentially regulated as biomarker predictors of transport-related
151 stress. Receiver Operating Characteristic (ROC) analysis was used to estimate the
152 diagnostic value of miR-22, miR-155 and miR-365 alone, or in combination. The ROC
153 analysis was carried out by plotting the true positive (sensitivity) versus false positive (1-
154 specificity). Cut-off points were set in order to maximize the sum of sensitivity and
155 specificity; the cut off points for miR-22, miR-155 and miR-365 were 1.31, 1.48 and 1.59,
156 respectively. The diagnostic accuracy of miR-22, miR-155 and miR-365, as measured by
157 the area under the curve (AUC), was 0.763 (95% CI 0.586-0.941), 0.710 (95% CI 0.512-
158 0.908) and 0.704 (95% CI 0.497-0.912), respectively (Fig. 2). Further statistical analysis
159 was performed considering the weighted average relative quantification (RQ) values of the
160 three stressed-related miRNA (Fig. 3A). The median expression levels were 1.02 (range,
161 0.62 to 1.38) and 1.97 (range, 1.73 to 6.26) before and after transportation, respectively.

162 The predicted probability of being discriminated as stressed-animals from the logit model
163 based on the three miRNA [$\text{logit} = (0.065 \times \text{expression level of miR-22}) + (0.562 \times$
164 $\text{expression level of miR-155}) + (1.395 \times \text{expression level of miR-365})$] was used to
165 construct a ROC curve (Fig. 3B). The AUC for the combined miRNA was 0.763 (95% CI
166 0.557-0.906).

167

168 **Discussion**

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

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

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

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

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

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

206

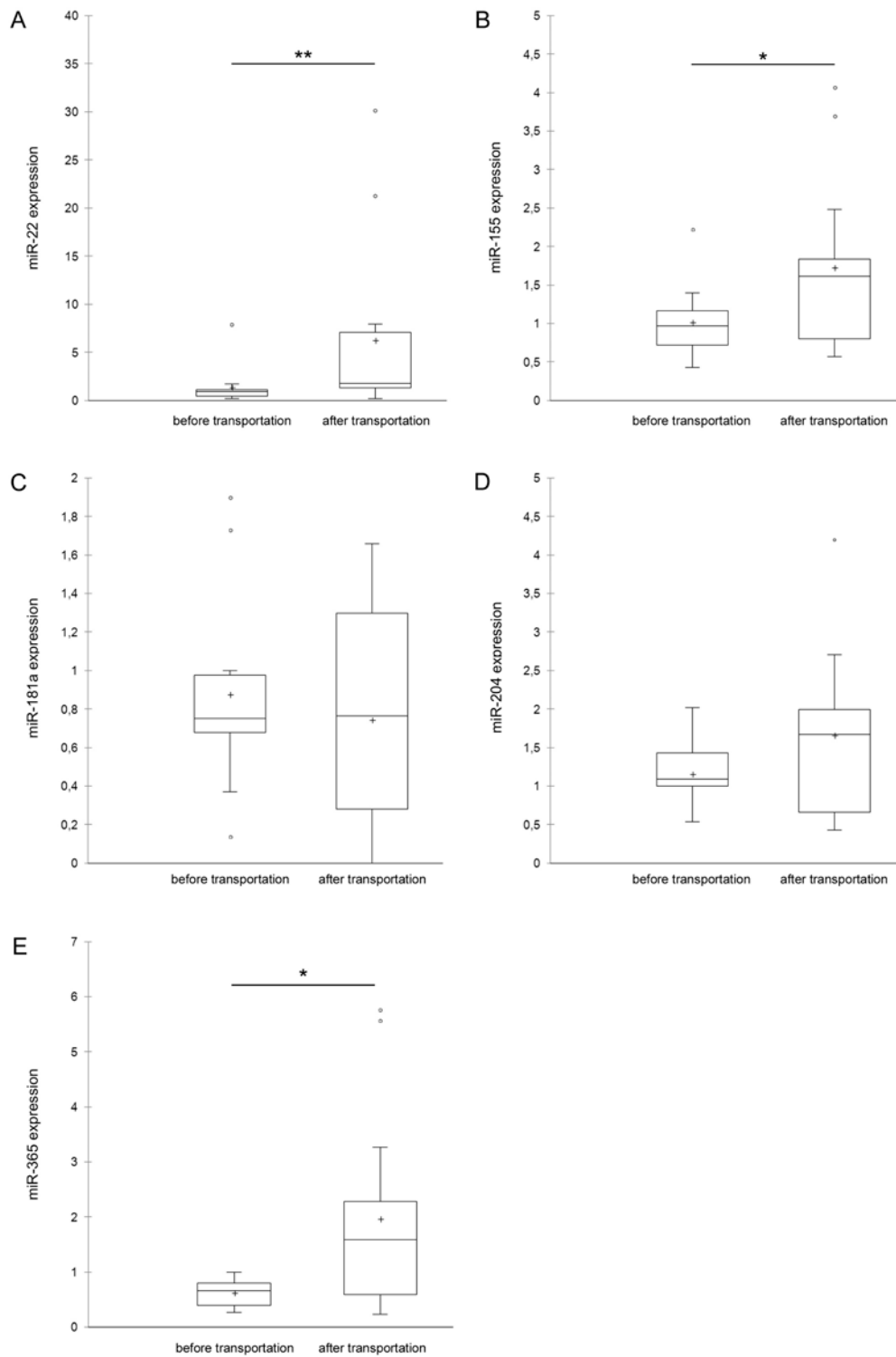
207 **Acknowledgements**

208 This work was partially supported by grant from the Italian Ministry of Health (grant
209 number: RC IZS VE 7/2012).

210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239

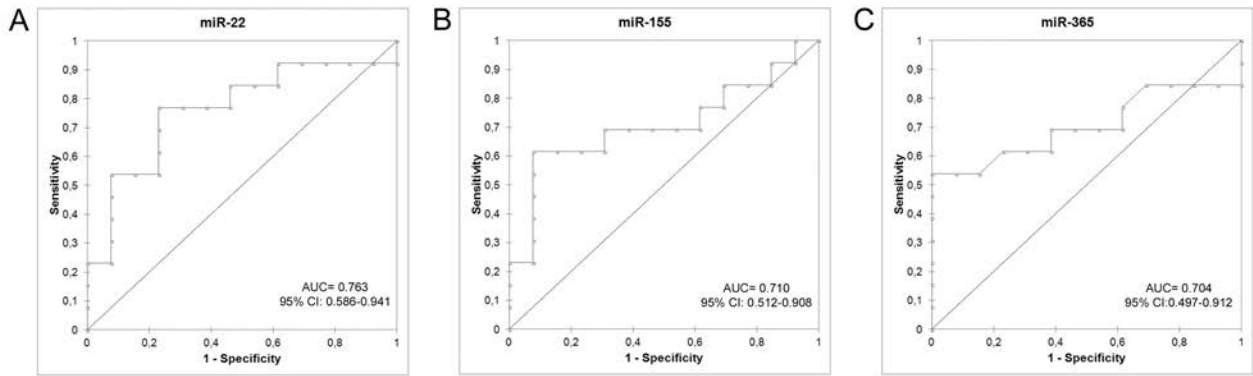
References

- Ahanda ML, Zerjal T, Dhorne-Pollet S, Rau A, Cooksey A and Giuffra E 2014. Impact of the genetic background on the composition of the chicken plasma MiRNome in response to a stress. *PLoS One* 9: e114598.
- Andersen HH, Duroux M and Gazerani P 2014. MicroRNAs as modulators and biomarkers of inflammatory and neuropathic pain conditions. *Neurobiology of Disease* 71: 159-168.
- Botreau R, Bonde M, Butterworth A, Perny P, Bracke MB, Capdeville J and Veissier I 2007. Aggregation of measures to produce an overall assessment of animal welfare. Part 1: a review of existing methods. *Animal* 1: 1179-1187.
- Elton TS, Selemon H, Elton SM and Parinandi NL 2013. Regulation of the MIR155 host gene in physiological and pathological processes. *Gene* 532: 1-12.
- Kocerha J, Dwivedi Y and Brennand KJ 2015. Noncoding RNAs and neurobehavioral mechanisms in psychiatric disease. *Molecular Psychiatry* 20:677-684.
- Liang X, Liu Y, Mei S, Zhang M, Xin J, Zhang Y and Yang R 2015. MicroRNA-22 impairs anti-tumor ability of dendritic cells by targeting p38. *PLoS One* 10(3):e0121510.
- Marchewka J, Watanabe TTN, Ferrante V and Estevez I 2013. Review of the social and environmental factors affecting the behavior and welfare of turkeys (*Meleagris gallopavo*). *Poultry Science* 92: 1467-1473.
- Pineiro M, Pineiro C, Carpintero R, Morales J, Campbell FM, Eckersall PD, Toussaint MJM and Lampreave F 2007. Characterisation of the pig acute phase protein response to road transport. *Veterinary Journal* 173: 669-674.
- Terlouw EM, Arnould C, Auperin B, Berri C, Le Bihan-Duval E, Deiss V, Lefèvre F, Lensink BJ and Mounier L 2008. Pre-slaughter conditions, animal stress and welfare: current status and possible future research. *Animal* 2:1501-1517.
- Zhou M, Liu W, Ma S, Cao H, Peng X, Guo L, Zhou X, Zheng L, Guo L, Wan M, Shi W, He Y, Lu C, Jiang L, Ou C, Guo Y, Ding Z 2013. A novel onco-miR-365 induces cutaneous squamous cell carcinoma. *34(7):1653-1659*.



240
 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.

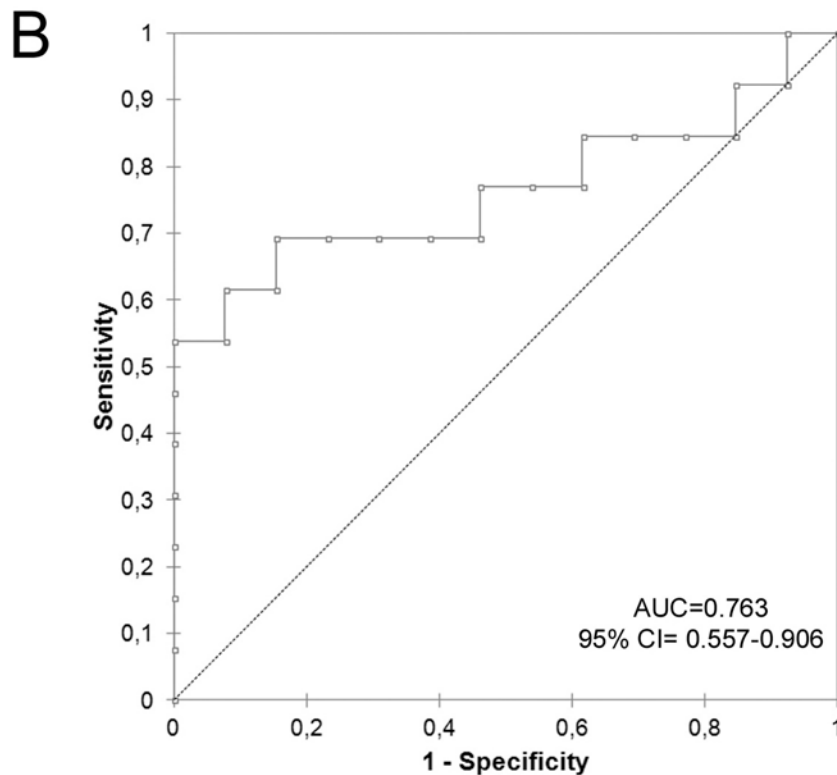
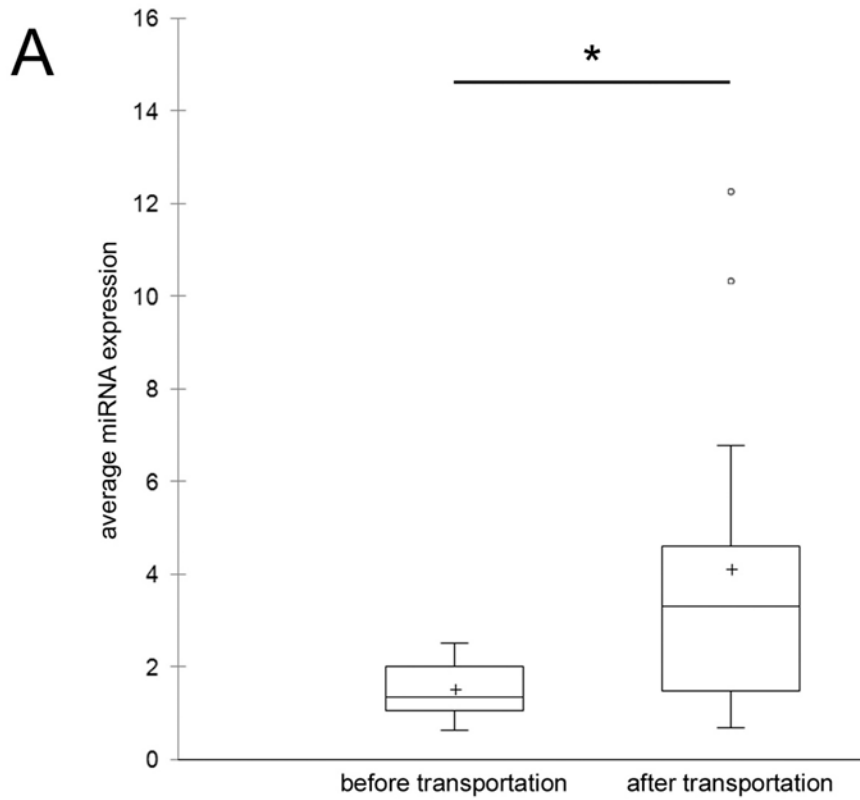


246

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

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

251



252

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. * $P < 0.05$.