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1 **Transcriptional expression levels of chicken collectins are affected by avian**
2 **influenza A virus inoculation.**

3
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15 **Abstract**

16 Mammalian collectins have been found to play an important role in the defense against influenza
17 A virus H9N2 inoculation, but for chicken collectins this has not yet been clarified. The aim of
18 this study was to determine the effect of avian influenza A virus (AIV) inoculation on collectin
19 gene expression in the respiratory tract of chickens and whether this was affected by age. For this
20 purpose 1- and 4-week-old chickens were inoculated intratracheally with PBS or H9N2 AIV.
21 Chickens were killed at 0, 8, 16 and 24 h post inoculation and trachea and lung were harvested
22 for analysis. Viral RNA expression and mRNA expression of chicken collectins 1 and 2 (cCL-1
23 and cCL-2), chicken lung lectin (cLL) and chicken surfactant protein A (cSP-A) were determined
24 using real-time quantitative RT-PCR. In lung, a decrease in mRNA expression of cCL-2, cLL and
25 cSP-A after inoculation with H9N2 was seen in both 1- and 4-week-old birds, although at
26 different time points, while in trachea changes were only seen in 4-week-old birds and expression
27 was increased. Moreover, collectin expression correlated with viral RNA expression in lung of 1-
28 week-old birds. These results suggest that both age and location in the respiratory tract affect
29 changes in collectin mRNA expression after inoculation with H9N2 and indicate a possible role
30 for collectins in the host response to AIV in the respiratory tract of chickens.

31

32 **Keywords:**

33 avian influenza virus; immune response; C-type lectin; innate immunity

34

35 **1. Introduction**

36 The first line of defense against invading pathogens in the respiratory tract is provided by
37 the innate immune system. This phylogenetically ancient system does not require previous
38 exposure to a pathogen and acts within hours after the first encounter. Especially in the early

39 stages of life, the host is highly dependent on innate immunity for protection because the adaptive
40 immune system is not fully developed yet. An important group of effector molecules of the
41 innate immune system comprises collectins, proteins characterized by a collagen- and a C-type
42 lectin domain. These proteins are pattern recognition molecules that can bind and neutralize a
43 wide array of pathogens including bacteria and viruses (reviewed by Hogenkamp et al., 2007).

44 For mammalian collectins, several *in vitro* studies have described a strong neutralizing
45 activity against influenza A virus (Hartshorn et al., 1997; Van Eijk et al., 2003; White et al.,
46 2008). Collectins are thought to bind carbohydrate residues present on influenza hemagglutinin
47 and neuraminidase, thereby aggregating viral particles and preventing invasion of host cells
48 (Reading et al., 2007). In addition, a highly reduced clearance of influenza virus was observed in
49 mice lacking either surfactant protein A or surfactant protein D, implicating the importance of
50 these mammalian collectins in defense against influenza virus inoculations *in vivo* (Sano et al.,
51 2005). In chicken, five collectins have been described thus far, which are mannan binding lectin
52 (Laursen et al., 1998), surfactant protein A (cSP-A) and chicken collectins 1, 2 and 3 (cCL-1-3)
53 (Hogenkamp et al., 2006). In addition, a chicken lung lectin (cLL) with high sequence homology
54 to cSP-A was reported. Whether chicken collectins can protect against influenza A virus
55 inoculation has not been clarified, but cLL has been shown to possess *in vitro* hemagglutination
56 inhibiting activity (Hogenkamp et al., 2008).

57 Avian influenza virus (AIV) enters the respiratory epithelium using sialic acid receptors linked to
58 galactose by an α -2,3 linkage, although the pattern of receptor distribution in chickens is less
59 defined than in mammals (Wan and Perez, 2006). H9N2 AIV has been shown to have a
60 preference for infecting the upper part of the respiratory tract (Nili and Asasi, 2002). After spray
61 inoculation, viral replication was detected at 24 hours post inoculation in both trachea and lung of
62 chickens. Virus was first detected in the epithelium of the trachea, intrapulmonary bronchus and

63 in adjacent parabronchi, and thereafter spread to other parabronchi deeper in the lung (Reemers et
64 al., 2009).

65 The aim of the current work was to determine changes in gene expression of chicken
66 collectins upon AIV inoculation in the respiratory tract of 1- and 4-week-old chickens and the
67 effect of age on collectin gene expression after AIV inoculation.

68

69 **2. Materials and methods**

70 **2.1. Inoculation model**

71 AIV, subtype H9N2, isolate A/Chicken/United Arab Emirates/99 was kindly provided by Intervet
72 Schering-Plough Animal Health, Boxmeer, The Netherlands. The experiment was carried out
73 according to protocols approved by the Animal Experiment Committee of Utrecht University
74 (The Netherlands). One-week and four-week-old Lohmann Brown layer chickens were
75 inoculated intratracheally with either 0.1 ml PBS or $10^{7.7}$ EID₅₀ H9N2. Before inoculation 5
76 control chickens per age were killed and at 8, 16 and 24 h post inoculation (p.i.) 5 control and 5
77 H9N2 inoculated chickens per age were killed. The cranial part of the lung containing the
78 intrapulmonary bronchus and the upper trachea were used for analysis.

79

80 **2.2. Real-time quantitative RT-PCR (qRT-PCR)**

81 Lung and trachea were homogenized and total RNA was isolated using the RNeasy Mini Kit and
82 DNase treated (Qiagen Benelux B.V., Venlo, The Netherlands) as described previously (Reemers
83 et al., 2009). cDNA was generated with reverse transcription using iScript cDNA Synthesis Kit
84 (Biorad Laboratories B.V., The Netherlands).

85 Detection of GAPDH, H9 hemagglutinin (HA) and 28S products was performed as described by
86 Reemers et al. (2009). Real-time qRT-PCR was performed for cCL-1, cCL-2, cLL and cSP-A

87 using primers and probes depicted in Table 1 according to the following cycle protocol: 2 min at
88 50°C, 10 min at 95°C (denaturation); 40 cycles: 15 s at 95°C and 60 s at 60°C. GAPDH was used
89 as a reference gene for correction of viral HA RNA expression and 28S as reference gene for
90 collectin mRNA expression. Corrections for variation in RNA preparation and sampling were
91 performed as previously described (Reemers et al., 2009). Results are expressed in terms of the
92 threshold cycle value (Ct) and given as corrected 40-Ct values.

93

94 2.3. Statistical analysis

95 Significance between H9N2 inoculated and control samples and between age groups within a
96 time point was determined with an ANOVA. Significance between time points within age groups
97 and treatment groups was determined with an ANOVA and Tukey post-hoc test. Correlation
98 between viral RNA and collectin mRNA expression was based on the Pearson correlation
99 coefficient (r) and determined using SPSS 15.0 software. Data were expressed as means with
100 standard error of the mean (SEM). A p -value < 0.05 was considered significant.

101

102 3. Results

103 3.1. Viral RNA expression in lung and trachea

104 Viral RNA expression was detected in lung and trachea of all H9N2 inoculated birds (Fig. 1).
105 There was no significant difference in viral RNA levels in lung between 1- and 4-week-old birds
106 within a time point or between time points within an age group. In trachea there was no
107 significant difference between 1-week and 4-week-old birds within a time point. Only the decline
108 of viral RNA expression in trachea of 1-week-old birds at 24 h p.i. was significantly different
109 from the expression at 16 h p.i.

110

111 3.2. Collectin mRNA expression in lung and trachea

112 Expression of cCL-1, cCL-2, cLL and cSP-A mRNA was determined in individual birds at all
113 time points in both lung and trachea (Fig. 2 and 3). cCL-2, cLL and cSP-A mRNA levels in lung
114 of H9N2 inoculated birds were generally lower compared to control birds. In 1-week-old birds,
115 cCL-2, cLL and cSP-A mRNA levels were significantly decreased after H9N2 inoculation at 16 h
116 p.i. In 4-week-old birds, there was a significant decrease in mRNA levels of cCL-2 at 8 h, cSP-A
117 at 16 h and cLL at 24 h p.i. between lung of H9N2 inoculated and control birds.

118 Although expression in lung was mostly decreased after H9N2 inoculation, collectin mRNA
119 expression in trachea was mostly increased in H9N2 inoculated birds. The cCL-1 mRNA level
120 was significantly higher at 16 h p.i. in H9N2 inoculated birds compared to control birds in
121 trachea of both 1- and 4-week-old birds. In 4-week-old birds, cCL-2, cLL and cSP-A mRNA
122 levels were significantly increased at 8 h p.i. after H9N2 inoculation. All observed changes were
123 between 1 and 2 Ct values corresponding to a 2-4 fold change in mRNA levels.

124

125 3.3. Correlations between viral RNA and collectin mRNA expression in lung and trachea

126 First the correlation between viral RNA and collectin mRNA expression was determined per age
127 group for all collectins in lung and trachea over time. If a correlation is found this means that
128 viral RNA expression and collectin mRNA expression correlate at every time point. There was no
129 significant correlation between viral RNA expression and mRNA expression of any of the
130 collectins in lung and trachea of 4-week-old birds over time. No significant correlation was seen
131 in trachea of 1-week-old birds for any of the collectins, however in lung there was a significant
132 strong negative correlation over time between viral RNA expression and mRNA expression of
133 cCL-2 ($r=-0.668$, $p=0.007$), cLL ($r=-0.655$, $p=0.008$) and cSP-A ($r=-0.657$, $p=0.008$).

134 Although no correlation was found between viral RNA and collectin mRNA expression in
135 trachea of 4-week-old birds over time, there was a significant difference in collectin mRNA
136 expression after H9N2 inoculation at certain time points. Only cCL-2 mRNA expression
137 correlated with viral RNA expression at 8 h p.i.

138

139 **4. Discussion**

140 The aim of this study was to determine changes in chicken collectin gene expression due to AIV
141 inoculation in the chicken respiratory tract and whether this was affected by age. Collectins have
142 only relatively recently been described in the chicken and their role in the innate immune
143 response is largely unknown. Based on sequence homology and gene expression profiles, two
144 chicken homologues for SP-A were found: cSP-A and cLL. However, the chicken genome did
145 not seem to contain a homologue for the second pulmonary collectin SP-D. In addition, chicken
146 collectins 1, 2 and 3 were found which resemble the mammalian collectin liver 1 (CL-L1)
147 (Ohtani et al., 1999), collectin kidney 1 (CL-K1) (Keshi et al., 2006) and collectin placenta 1
148 (CL-P1) (Ohtani et al., 2001). The biological roles of CL-L1 and CL-K1 have not been
149 determined yet, leaving no indication for a possible role of chicken collectins cCL-1 and cCL-2.
150 However, both cCL-1 and cCL-2 are expressed in the chicken respiratory tract (Hogenkamp et
151 al., 2007). The cCL-3 homologue CL-P1 is different from other collectins in the way that it is a
152 type II membrane protein. It acts as a scavenger receptor on endothelial cells, while all other
153 collectins are soluble effector molecules involved in direct neutralization of pathogens. A role
154 for cCL-3 in direct neutralization of viruses in the lung is therefore unlikely.

155 Based on this similarity to mammalian collectins and the location of expression in the chicken we
156 investigated the changes in mRNA expression of cSP-A, cLL, cCL-1 and cCL-2 after an H9N2
157 AIV inoculation in the chicken respiratory tract. In the lung of 1 week-old birds, a down

158 regulation was observed for cLL, cSP-A and cCL-2 mRNA, at 16 h p.i. This time point
159 significantly correlated to the observed peak in viral load in the lung. In addition the expression
160 of cCL-2 and cSP-A mRNA was down regulated in 4-week-old birds at 8 and 16 h p.i., but no
161 correlation to viral RNA expression was found. This indicates that expression of cSP-A, cLL and
162 cCL-2 mRNA expression is affected by H9N2 inoculation early after inoculation and in 1-week-
163 old birds is directly related to viral RNA expression in lung. Interestingly, in the trachea, all
164 significant changes due to H9N2 inoculation were related to up regulation of collectin mRNA
165 expression, suggesting host collectin responses after H9N2 inoculation are site specific. cCL-1
166 was up regulated at 16 h p.i. in trachea of both 1- and 4-week-old birds, while cCL-1 mRNA
167 expression was not affected by H9N2 inoculation in lung. This suggests that changes of cCL-1
168 mRNA expression after H9N2 inoculation are dependent on the location in the respiratory tract.
169 cCL-2 expression was affected by H9N2 inoculation in 4-week-old birds at 8 h p.i. in both lung
170 and trachea, although down regulated in lung and up regulated in trachea, indicating that cCL-2
171 mRNA expression changes time specifically at 8 h p.i. after H9N2 inoculation. In lung, changes
172 in mRNA expression of cCL-2, cLL and cSP-A after H9N2 inoculation were seen in both 1- and
173 4-week-old birds, although at different time points, while in trachea changes were only seen in 4-
174 week-old birds. This suggests that both age and location in the respiratory tract affect changes in
175 collectin mRNA expression after H9N2 inoculation, though one has to keep in mind that changes
176 found at early time points are caused by the virus inoculum whereas at 24 h.p.i. viral replication
177 might affect the outcome. In mammals site specific expression and secretion of collectins in the
178 airways has been reported (Coalson et al., 1998; Rooney, 2001). Furthermore, down regulation of
179 SP-A gene expression through p38 MAPK and PI-3 kinase pathways have been described in lung
180 epithelial cells (Miakotina et al., 2002a; Miakotina et al., 2002b). Epithelial cells in the lung
181 differ from tracheal ciliated epithelial cells supporting the possibility of site specific collectin

182 expression after H9N2 inoculation in the chicken. Whether similar signalling pathways are
183 involved in regulation of chicken collectins needs to be addressed to clarify our observed tissue
184 specific changes of these collectins.

185 Without knowing the exact function of chicken (col)lectins in innate defense , it is difficult to
186 relate the observed changes in gene expression to a biological effect. In mammals, an up
187 regulation of collectins has frequently been observed in both bacterial and viral infections
188 (Murray et al., 2002; Grubor et al., 2004), which is usually interpreted as a direct response of the
189 host to increase its defense against the incoming pathogens. A down regulation of collectins upon
190 infection is less frequently reported, but could reflect a survival strategy of the pathogen to
191 overcome the host's hostile environment, as has been described for other innate immune proteins
192 such as Toll like receptors and defensins (Wang et al., 2000; Shin et al., 2007). However, whether
193 this applies to AIV in the chicken lung and why the altered expression of chicken (col)lectins is
194 tissue specific is not clear at this moment and requires further investigation.

195 In conclusion, the results obtained in this study show that mRNA expression of chicken collectins
196 cCL-1, cCL-2, and cSP-A and cLL are affected by H9N2 AIV inoculation. The effect is tissue
197 specific, showing up regulated mRNA expression in the trachea and down regulation in the lung.
198 Furthermore, changes in collectin mRNA expression are age specific, showing differential gene
199 expression in 1- and 4-week old birds. These observed changes in collectin mRNA expression
200 implicate that chicken collectins can play an important role in innate defense against viral
201 infection, especially in neonates. More research is needed to relate the changes in collectin
202 mRNA expression after H9N2 AIV inoculation to a biological function and clarify the function
203 of collectins within the innate response to infection.

204

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210

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- 269

270 **Table captions**

271 **Table 1**

272 Real-time qRT-PCR primers and probes.

273

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274 **Figure captions**

275 **Fig. 1.** Viral RNA expression in 1-week and 4-week-old H9N2 inoculated birds at 8, 16 and 24 h
276 p.i. in lung and trachea determined with real-time qRT-PCR. * Indicates significant difference in
277 viral RNA expression between 16 and 24 h p.i. in 1-week-old birds ($p < 0.05$).

278
279 **Fig. 2.** cCL-1, cCL-2, cLL and cSP-A mRNA expression in lung of 1- and 4-week-old birds at 0,
280 8, 16 and 24 h p.i. determined with real-time qRT-PCR. * Indicates significant difference in
281 collectin mRNA expression between control and H9N2 inoculated birds ($p < 0.05$).

282
283 **Fig. 3.** cCL-1, cCL-2, cLL and cSP-A mRNA expression in trachea of 1- and 4-week-old birds at
284 0, 8, 16 and 24 h p.i. determined with real-time qRT-PCR. * Indicates significant difference in
285 collectin mRNA expression between control and H9N2 inoculated birds ($p < 0.05$).

Fig. 1

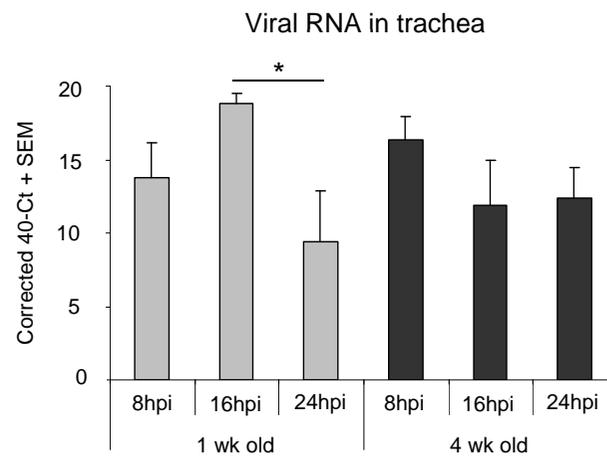
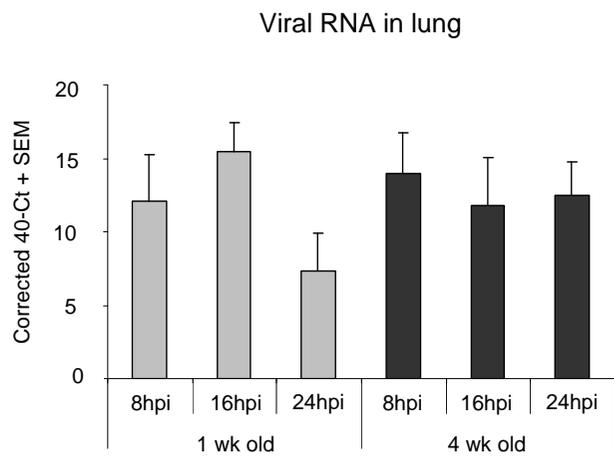


Fig. 2

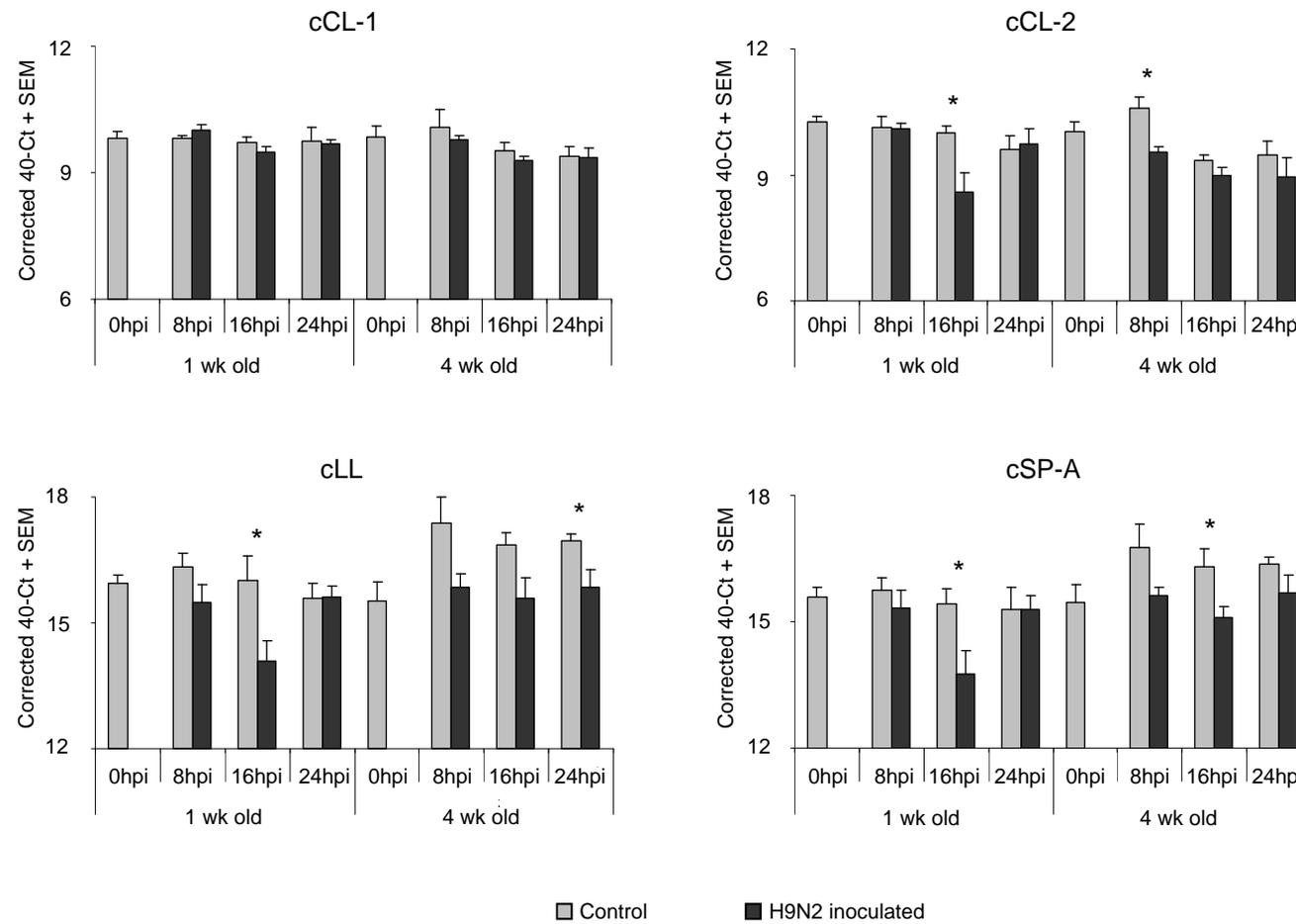
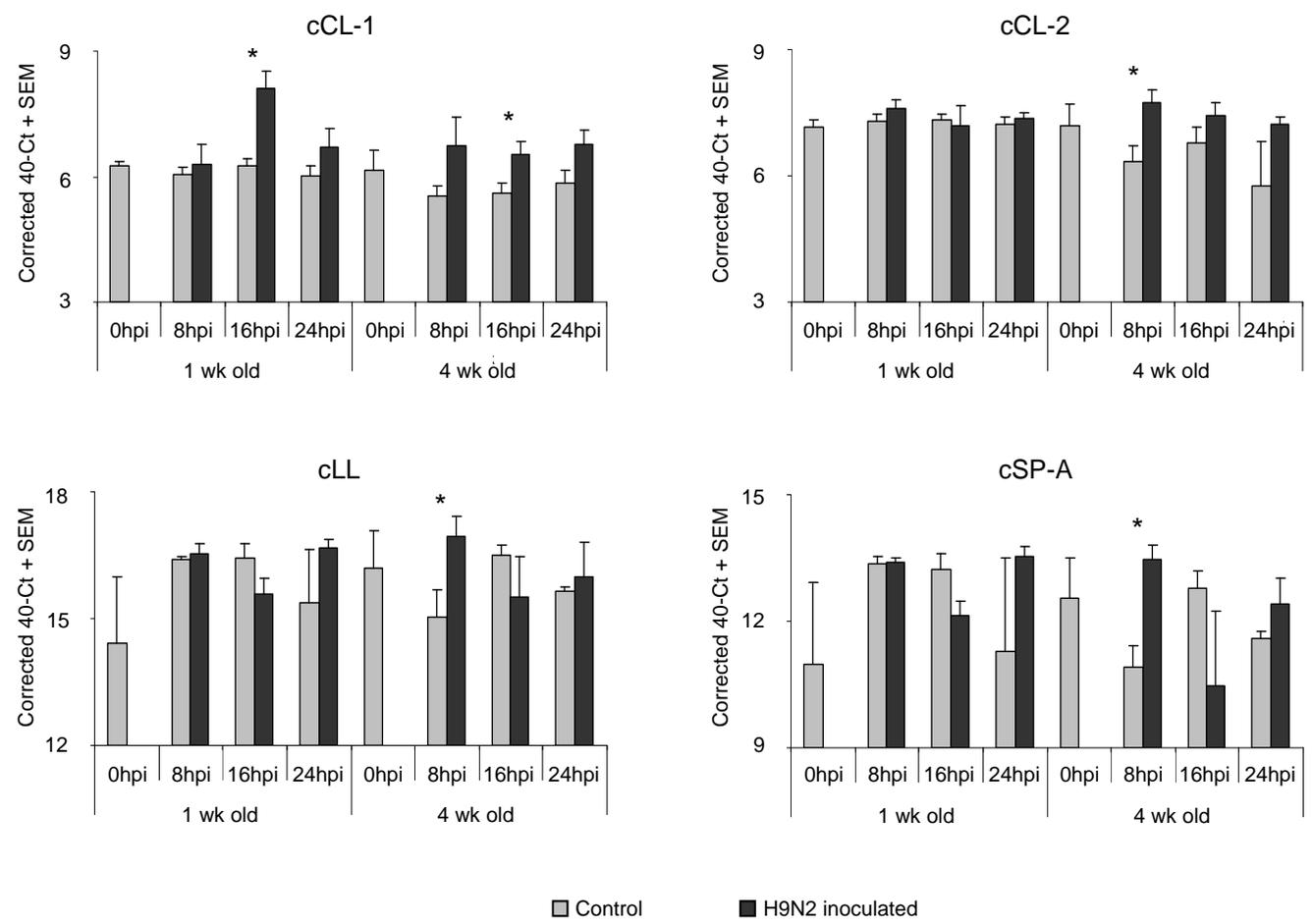


Fig. 3



247 **Table 1**

248 Real-time qRT-PCR primers and probes.

Target	Probe or primer ^a	sequence (5'-3')	Accession No. ^b
cCL-1	F	5'- ATTGTCAAAGAAGAGAAGAATTACAGAG -3'	DQ129668
	R	5'- GAGGAGATGTAATCAGCAAGCAG -3'	
	Probe	5'- (FAM) – CGTTGGTCACCTCATCTTTAGGCATGGC – (BHQ) - 3'	
cCL-2	F	5'- GGGAGCCCAACAATGCCTATG -3'	DQ129669
	R	5'- GTAATATGACATGCAACATCATTCCAC -3'	
	Probe	5'- (FAM) – TGCCACCATCTCCACACAGTCCTCCTC – (BHQ) -3'	
cLL	F	5'- CTTACAAGGGAAGATAACAAAGTCTGG -3'	DQ129667
	R	5'- CATTCTTGGAGATGCAATACACC -3'	
	Probe	5'- (FAM) - CTCCTGGCACATTTTCACCGTAGCATGG – (BHQ) - 3'	
cSP-A	F	5'- GGAATGACAGAAGGTGCAATCAG -3'	AF411083
	R	5'- GCAATGTTGAGTTTATTAGCTACAAATG -3'	
	Probe	5'- (FAM) – CCGCCTTGTTGTCTGCCAGTTTTAGTGC – (BHQ) - 3'	

249 ^a F, forward; R, reverse.250 ^b Genomic DNA sequence.