Characterisation of basal expression of selected cytokines in the liver, spleen, and respiratory, reproductive and intestinal tract of hens

M. Kolesarova, V. Spisakova, M. Matulova, M. Crhanova, F. Sisak, I. Rychlik

Veterinary Research Institute, Brno, Czech Republic

ABSTRACT: In this study we investigated the basal expression of chicken IFNγ, IL-18, IL-1β, LITAF, IL-12p35, IL-12p40, iNOS, IL-17, IL-15, IL-6, IL-8 and IL-22 in the ileum, caecum, colon, liver, spleen, lungs, blood and magnum of hens using real time PCR. We show that IL-18, LITAF and IL-15 were highly expressed in most tissues while IL-6 and IL-17 were usually expressed at a low level. IL-17 and IL-22 were expressed mainly in the lungs and intestinal tract and both subunits of IL-12 were produced in the liver. Unusually high expression of iNOS was observed in the blood which may explain the rapid increase in iNOS at inflamed sites in chickens. The second unexpected expression profile was that of IL-18 and IL-6 in magnum. These two cytokines were highly expressed in magnum in the absence of any infection pointing to an important, though as yet uncharacterised, physiological role in the reproductive tract of hens.

Keywords: cytokine; poultry; organ; tissue

Cytokines and chemokines play an important role in communication among different cells of the immune system as well as between cells from the immune system and non-immune cells. Although cytokines are mostly associated with control of the host's immune response to different foreign antigens (Berndt et al., 2007; Shaughnessy et al., 2009), they also contribute to processes not directly associated with infection, e.g., remodelling of the reproductive system in molting hens (Sundaresan et al., 2007, 2008) or control of the menstrual cycle in women (Laird et al., 1994). Tight control of their expression is therefore critical for both immune response to infection and maintenance of internal body homeostasis.

Due to the limited availability of specific antibodies against the cytokines of farm animals but the availability of their gene sequences, quantitative real-time PCR is often used to characterise their expression at the transcriptional level. Although the expression of avian cytokines in various tissues of hens has been characterised in many papers over the last few years, most papers reported on comparative experiments targeted at a limited number of tissues (Swaggerty et al., 2004; Withanage et al., 2005; Berndt et al., 2007). Consequently, most of the studies presented only fold inductions, i.e., the ratio between the expression of the cytokine gene in experimental and control groups and cytokine basal expressions in multiple tissues have been character-

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ised only marginally (Collado-Romero et al., 2010). Whilst the presentation of results in this manner can be useful for particular aspects, it also holds several pitfalls. Firstly, it can provide misleading results if the gene of interest is expressed at a low level (nearly approaching zero) in the control group. In such a case, when dividing by a very low number approaching zero, artificially high upregulation can be reported. Conversely, if a particular cytokine has a high level of basal expression, its upregulation upon stimulation can hardly result in fold increases truly reflective of its upregulation. For example, some cytokines may be expressed at a significantly lower absolute level even after a 100 fold upregulation when compared with a cytokine with a 1000 times higher basal expression responding to the same signal by fivefold induction. Finally, the fold level of up or down regulation does not allow a comparison of how different organs and tissues contribute to the total expression of the cytokine of interest within the host body.

In our previous studies we also characterised cytokine gene expression using fold inductions (Crhanova et al., 2011; Pavlova et al., 2011). However, we have noted that the basal expression in control groups differed considerably based on the type of tissue used for the analysis. In this study we were therefore interested in the basal expression of selected chicken cytokines in different organs of hens being aware of the fact that the basal expression may differ slightly in different chicken breeds (Swaggerty et al., 2004; Redmond et al., 2009). Interestingly, for three cytokines, iNOS, IL-18 and IL-6, we observed rather unexpected, tissue-dependent expression profiles. Even in the absence of any infection, iNOS was highly expressed in the blood and IL-6 and IL-18 were highly expressed in the reproductive tract.

MATERIAL AND METHODS

Animals

Nine Lohmann Brown egg-laying hens from a commercial egg-producing company were used in this study. Three hens were sacrificed and analysed when 30 weeks old, additional three hens were analysed when 33 weeks old and the remaining three hens were analysed when 50 weeks old. The hens of different age from the same farm were included to minimise the risk of a subclinical infection at a particular period of life which could influence cytokine expression. Before the onset of lay, the birds were vaccinated against Marek's disease, Newcastle disease, coccidiosis, infectious bursitis, infectious bronchitis, avian encephalomyelitis, salmonellosis and egg drop syndrome. The last vaccination was administered when the birds were 16 weeks old. Except for these vaccinations, the flock was not administered antibiotics nor it was subjected to any infection.

Quantitative real-time PCR

Tissue samples (ileum, caecum, colon, liver, spleen, lungs and magnum of the oviduct) taken during necropsies were cut into approx. 20 mg pieces which were immediately placed into RNALater and stored at –20 °C prior to RNA purification. After storage, a single tissue fragment was transferred into 1 ml of TRI Reagent (Molecular Research Center, Cincinnati, USA) and homogenized using zirconia silica beads (BioSpec Products) in a MagNA Lyser (Roche). To separate phases, 50 µl of 4-bromanisole was added, the whole content of the tube was centrifuged and the upper aqueous phase was collected for RNA purification using the RNAeasy kit according to the instructions of the manufacturer (Qiagen). In addition, peripheral blood from the vena jugularis dextra was taken by puncture prior to the necropsy and RNA was immediately purified from total blood using TRI Reagent® RT – Blood following the recommendations of the manufacturer (Molecular Research Center, Cincinnati, USA). The purity and concentration of RNA was determined spectrophotometrically and 1 µg of RNA was immediately reverse transcribed using M-MLV reverse transcriptase (Invitrogen) and oligo-dT primers. The resulting cDNA was 10× diluted in sterile distilled water and used as a template in real-time PCR or stored at –20 °C until use. The expression of 12 cytokines or immune relevant proteins (IFNγ, IL-18, IL-1β, LITAF, IL-12p35, IL-12p40, iNOS, IL-17, IL-15, IL-6, IL-8 (also called chCXCLi2) and IL-22) was determined. The expression levels of three house-keeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TATA box binding protein (TBP) and ubiquitin (UB), were used for data normalisation. Real-time PCR was performed in microplates using a LightCycler II (Roche) and QuantiTect SYBR Green PCR Kit (Qiagen). Each sample was subjected to real-time PCR in duplicate and the mean values of the duplicates were used

for subsequent analysis. The Ct values of genes of interest were normalised to an average Ct value of the house-keeping genes (ΔCt) and the relative expression of each representative was calculated as $2^{-\Delta Ct}$. These expression levels were then used for comparative data analysis. All primers used in the real-time PCR in this study are listed in Table 1.

Data analysis

In all figures, average values ± standard deviation are shown. Expression levels of particular cytokines in individual organs were compared by ANOVA followed by a post hoc Tukey's test using SPSS v.14.0 statistical software.

Table 1. List of primers used for chicken cytokine mRNA quantification

Primer	Sequence 5'–3'	Reference
IL-1βFor	GAAGTGCTTCGTGCTGGAGT	Crhanova et al., 2011
IL-1βRev	ACTGGCATCTGCCCAGTTC	
IL-6For	GCTACAGCACAAAGCACCTG	this study
IL-6Rev	GACTTCAGATTGGCGAGGAG	
IL-8For	ATGAACGGCAAGCTTGGAGCT	Rychlik et al., 2009
IL-8Rev	GCAGCTCATTCCCCATCTT	
IL-12p35For	AAGGGACTCAACTGCTCCAG	this study
IL-12p35Rev	TCTTCAAGGGTGCACTCAAA	
IL-12p40For	TGGTCCACGCTTTGCAGAT	Berndt et al., 2007
IL-12p40Rev	AAGGTTAAGGCGTGGCTTCTTA	
IL-15For	TGGAGCTGATCAAGACATCTG	this study
IL-15For	CATTACAGGTTCCTGGCATTC	
IL-17For	TATCAGCAAACGCTCACTGG	Crhanova et al. 2011
IL-17Rev	AGTTCACGCACCTGGAATG	
IL-18For	ACGTGGCAGCTTTTGAAGAT	Rychlik et al., 2009
IL-18Rev	GCGGTGGTTTTGTAACAGTG	
IL-22For	CAGACTCATCGGTCAGCAAA	Crhanova et al. 2011
IL-22Rev	GGTACCTCTCCTTGGCCTCT	
IFNγFor	GCCGCACATCAAACACATATCT	Berndt et al., 2007
IFNγRev	TGAGACTGGCTCCTTTTCCTT	
LITAFFor	AATTTGCAGGCTGTTTCTGC	this study
LITAFRev	TATGAAGGTGGTGCAGATGG	
iNOSFor	GAACAGCCAGCTCATCCGATA	Berndt et al., 2007
iNOSRev	CCCAAGCTCAATGCACAACTT	
GAPDHFor	CCTGCATCTGCCCATTT	De Boever et al., 2008
GAPDHRev	GGCACGCCATCACTATC	
TBPFor	TAGCCCGATGATGCCGTAT	Li et al., 2005
TBPRev	GTTCCCTGTGTCGCTTGC	
UBFor	GGGATGCAGATCTTCGTGAAA	De Boever et al., 2008
UBRev	CTTGCCAGCAAAGATCAACCTT	

RESULTS

Gene expression of cytokines in adult hens

There were no significant differences in gene expression in hens of different ages and we therefore averaged the expression levels of all nine hens. IL-15, LITAF and IL-18 were the cytokines with the highest expression in the majority of tissues. On the other hand, IL-6 and IL-17 exhibited the lowest expression in hen tissue; despite this, IL-6 exhibited exceptionally high expression in magnum of oviduct (see below).

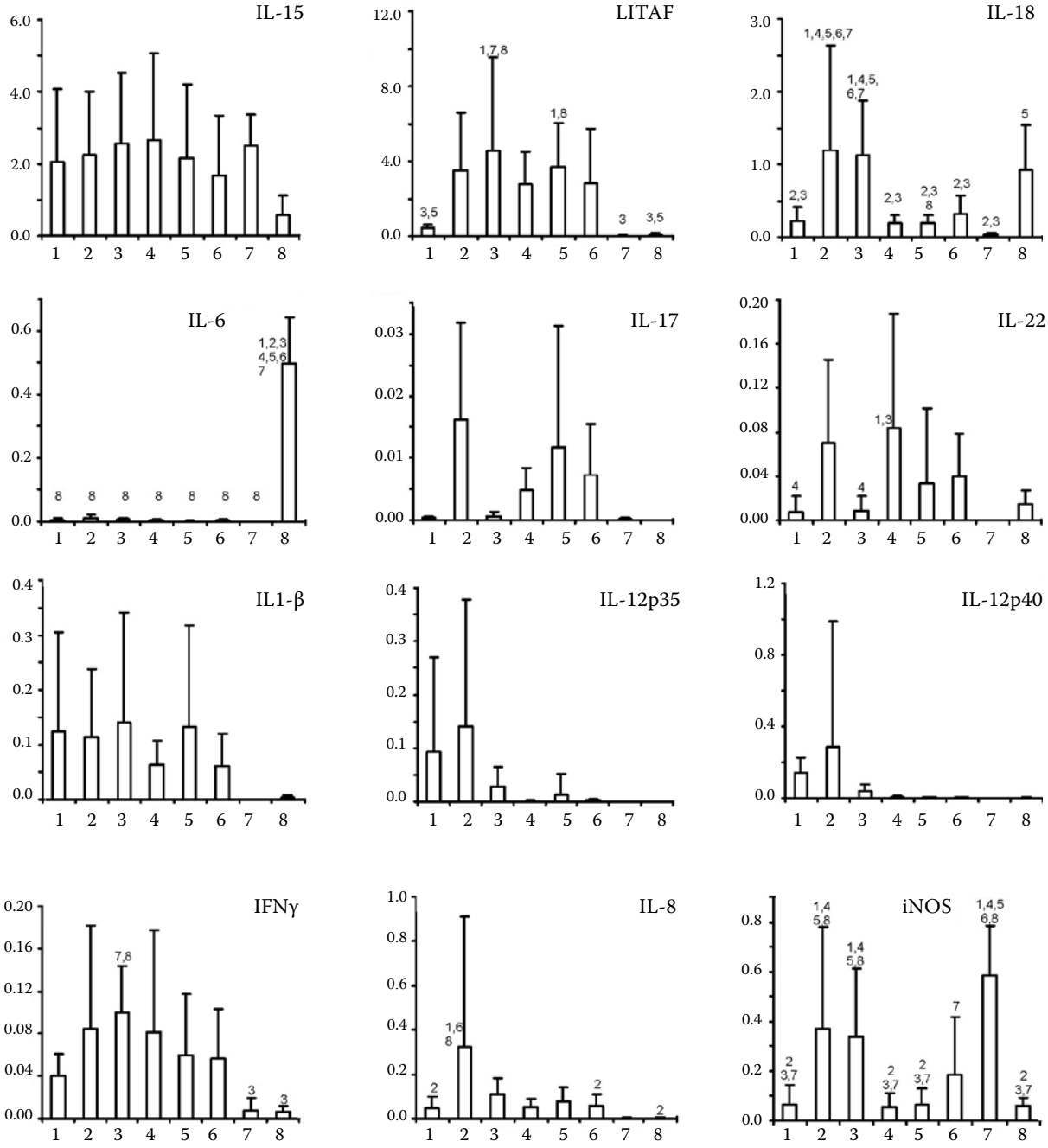

Figure 1. Contribution of different organs of adult hens to the total expression of individual cytokines in adult hens. The Y-axis shows relative expression of individual cytokines after their normalisation to the average expression of three house-keeping genes (mean from nine hens ± SD). Numbers at the columns inside the graph mark the organs (see their identification numbers on the X-axis) with significantly different ($P < 0.05$) expressions of a particular cytokine. For expression levels of individual cytokines, mind also the Y-axis range

1 = liver, 2 = lungs, 3 = spleen, 4 = ileum, 5 = caecum, 6 = colon, 7 = blood, 8 = magnum

IL-1β was expressed similarly in all hen organs and it was impossible to determine a major production site of this cytokine in the absence of any infection. IL-22 was produced at a significantly higher level in the ileum when compared with the liver or spleen and an increased expression of IL-22 was also recorded in other parts of the intestinal tract and lungs although expression levels in these organs did not differ significantly from the other organs. Expression patterns of IL-17 were similar to that of IL-22 although also in this case the differences in the expression levels in different organs were not significant. LITAF was expressed at low levels in liver, blood and magnum. The major production sites for IL-18 were in the lungs and spleen. iNOS was expressed mainly in the lungs, spleen and blood and INFγ was expressed at a low level in the liver, blood and magnum. IL-8 and IL-6 were the two cytokines with dominant expression in a particular organ. Lungs were the major production site of IL-8 and IL-6 was expressed exclusively in the magnum (Figure 1).

Cytokine expression profiles within different tissues

Next, we analysed the cytokine expression profiles in different organs. The lungs followed by the spleen were the two organs in the hen's body with the highest cytokine expression in the absence of any infection. Moderate expression of different cy-

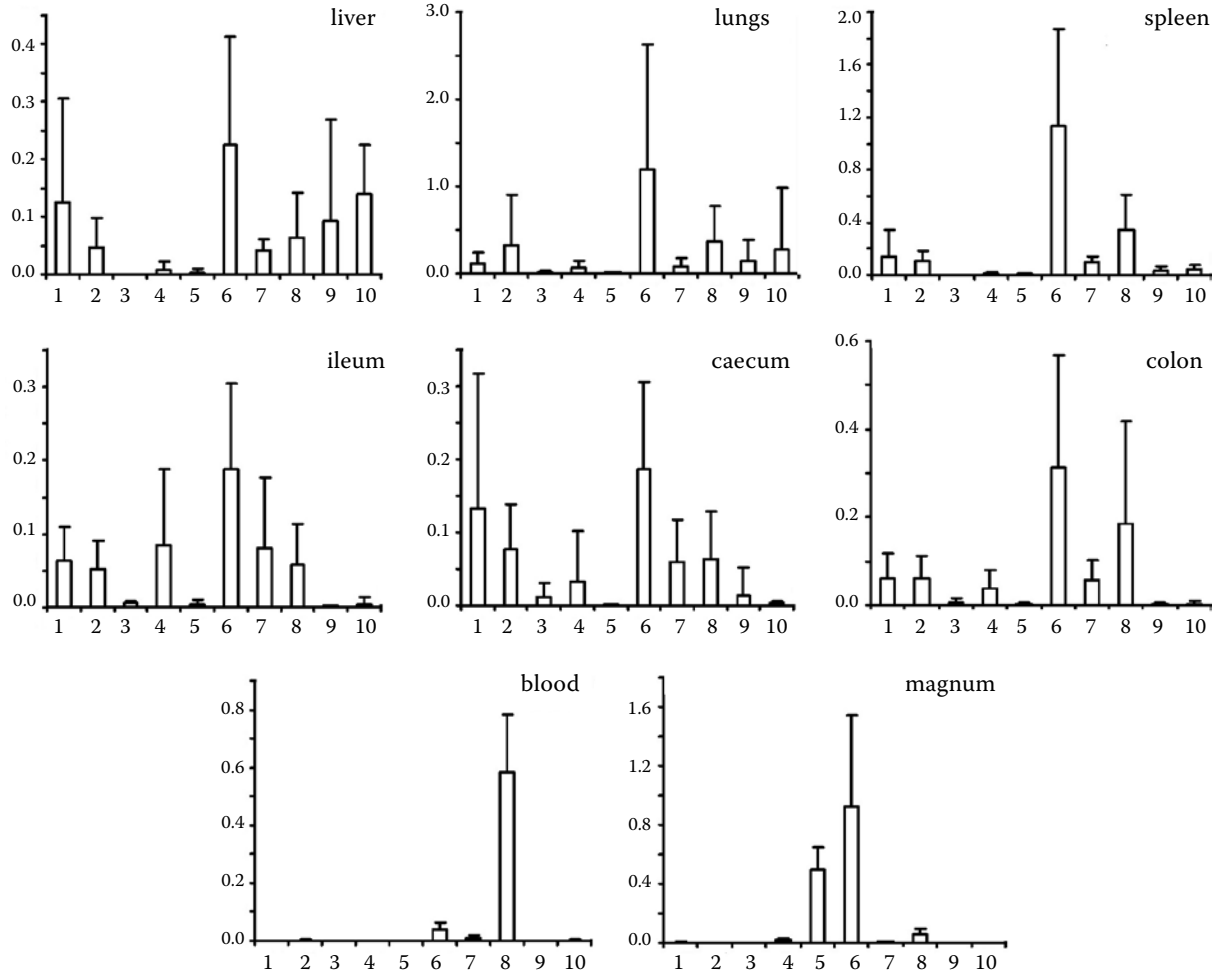

Figure 2. Expression of different cytokines in individual organs of adult hens. The Y-axis shows the relative expression of individual cytokines after their normalisation to the average expression of three house-keeping genes (mean from nine hens ± SD). Data for IL-15 and LITAF are not shown because their expression was too high and the Y-axis scale would not allow the expression levels of the remaining cytokines to be accurately seen (see Figure 1 and the Y-axis scaling for the expression profiles of IL-15 and LITAF)

1 = IL-1β; 2 = IL-8; 3 = IL-17; 4 = IL-22; 5 = IL-6; 6 = IL-18; 7 = IFNγ; 8 = iNOS; 9 = IL-12p35; 10 = IL-12p40

tokines could be observed in all parts of the intestinal tract and liver. Except for two cases, blood and magnum represented the organs with the lowest cytokine expression. The first exception was the expression of iNOS in the blood. The expression of iNOS in the blood was nearly as high as the level of the expression of house-keeping genes while the expression of the remaining cytokines was very low, on the border of the detection limit of RT-PCR. The second exception was the expression of both IL-18 and IL-6 in magnum. Although the majority of cytokines were not expressed in magnum, a high level of expression was observed for IL-18 and IL-6. In addition, IL-6 was not expressed in any other organ besides the reproductive tract (Figure 2).

DISCUSSION

When analysing the data from the RT-PCR assays, we first verified that the expression profiles in birds of different ages were similar, thus excluding any infection occurring in the flock which could influence the determination of basal expression levels (not shown). Next, we considered that there might be some inter-breed differences (Swaggerty et al., 2004). However, using our limited data which shows similar expression profiles also in the ISA Brown chicken line, it is likely that the basic relationships in cytokine gene expression will be similar also in other breeds. Furthermore, the ISA Brown chickens were raised under SPF conditions, without any vaccination, indicating that the vaccination programme performed during the first 16 weeks of life of the Lohmann Brown hens did not influence the basal expression of cytokines in hens at the ages of 30, 33 and 50 weeks.

The majority of expression profiles fit the expected theoretical predictions based on results from other animal species. IL-12 was maximally expressed in liver, likely due to the presence of macrophage-like Kupffer cells (Takahashi et al., 1996). IL-17 was associated with mucosal surfaces of the intestinal tract and lungs, consistent with its role in Th17-dependent autoimmune mucosal diseases and the immune response to extracellular bacterial pathogens (Maloy, 2008; Curtis and Way, 2009). IL-22 was expressed at relatively low levels in all tissues consistent with its role in tissue protection during infection (Wolk et al., 2004). In mammals, major TNFα producers are recruited from activated mononuclear cells and macrophages (Henter et al., 1988; Cron et al., 1989; Pavlova et al., 2011). This is consistent with our findings that LITAF (a functional homologue of TNFα in chickens) was expressed highly in all mucosal tissues and spleen, but at very low levels in blood and magnum. The expression of IL-15 was also detected in several chicken tissues using standard RT PCR (Choi et al., 1999).

In two cases we observed rather unusual expression profiles. These included iNOS expression in blood and high IL-6 and IL-18 expression in the reproductive tract (magnum). Although we did not determine the iNOS producing cells, its high expression in the blood of non-infected hens may explain the rapid onset of iNOS expression at sites of inflammation, e.g., the caecum of chickens after oral infection with *Salmonella* (Berndt et al., 2007). Inflammation and vasodilatation triggered by the proinflammatory cytokines IL-1β and IL-8 may result in an influx of blood leukocytes constitutively expressing iNOS and this influx can cause an increase in iNOS expression. The apparent induction, however, need not be caused by a net increase in gene transcription but by an influx of cells constitutively expressing high levels of iNOS.

The reasons for the high expression of IL-18 and its biological function in magnum are unclear. It has been reported that this cytokine is upregulated during amniotic infection and its upregulation correlates with an increased risk of preterm delivery (Jacobsson et al., 2003). However, as we did not observe any signs of infection, we considered the notion that IL-18 expression was high in magnum due to the presence of an unknown infection as quite unlikely and instead, this cytokine may have a yet unknown role in the control of reproduction, similar to IL-6 in mammals (Robertson et al., 2010; Thomakos et al., 2010). Concerning IL-6, its expression in the reproductive tract of hens has been described (Sundaresan et al., 2007, 2008). Unfortunately, these studies were focused on the expression of IL-6 and other cytokines in the reproductive tract only and the authors did not therefore report on the expression of IL-6 in the reproductive tract relative to other tissues. Several papers described the presence of potential IL-6 and IL-18 producer such as macrophages, B- and T-lymphocytes in the reproductive tract of hens (Withanage et al., 1997; Barua et al., 1998b; Barua and Yoshimura, 1999). Moreover, oestrogen has been shown to increase the counts of B- and T-lymphocytes in the ovaries of hens (Barua et al.,

1998a; Barua and Yoshimura, 1999) but whether oestrogen or any other sex hormone specifically found in reproductive tract stimulates lymphocytes for IL-6 production is unknown. Despite this, our data indicate that due to the high expression of IL-6 specifically in the magnum, even in the absence of any infection, this cytokine must have an important reproductive tract-specific function. Moreover, since IL-6 and IL-18 are upregulated in response to infections, this may explain why certain infections interfere with reproduction and cause preterm deliveries or abortuses.

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Corresponding Author:

Ivan Rychlik, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic
Tel. +420 533 331 201, Fax +420 541 211 229, E-mail: rychlik@vri.cz