

# Altered gene expression of selected matrix metalloproteinase system proteins in the broiler chicken gastrointestinal tract during post-hatch development and coccidia infection<sup>1</sup>

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**ABSTRACT** Matrix metalloproteinases (MMPs) are a family of proteases, that can process extracellular matrix (ECM) components and non-ECM molecules. MMPs can also function intracellularly in proteolytic and nonproteolytic functions. The participation of MMPs in the remodeling of the chicken gastrointestinal tract is largely unknown. The aim of the present study was to examine 1) the early neonatal developmental changes and effect of delayed access to feed immediately post-hatch (PH) and 2) the effect of *Eimeria* infection on mRNA expression of selected MMPs, their tissue inhibitors (TIMPs), and a disintegrin and metalloproteinase (ADAM) metalloproteinase with thrombospondin type 1 motif 8 (ADAMTSS8) in the gastrointestinal tract of chicken. Protein localization of MMPs and TIMPs was also carried out in the normal ileal wall at -48, 24, and 336 h relative to hatch using immunofluorescence. In experiment 1, newly hatched Ross 708 chicks received feed and water immediately PH or were subjected to 48 h delayed access to feed. Chickens were sampled at -48, 0, 4, 24, 48, 72, 96, 144, 192, 240, 288, and 336 h PH. Ileum

was collected for investigation of gene expression or fixed in paraformaldehyde for immunofluorescence. In experiments 2 and 3, Ross 708 male broilers were infected, at 21 d of age with *Eimeria maxima* or *E. acervulina* or sham-infected with water. Intestinal tissues were collected at 7 and 10 d postinfection for gene expression analysis. In general, mRNA expression patterns of all examined genes showed downregulation during the first 2 wk PH and were not affected by delay in feed access. These development-dependent changes in expression and tissue-dependent localization in the ileum of selected MMPs and TIMPs indicate that these molecules participate in the remodeling of chicken intestinal tissues during PH development. Increased expression of MMP-7 and MMP-9 transcripts in the intestine of *Eimeria* infected birds suggests an important role for these enzymes in the process of tissue remodeling and destruction in pathological conditions. The findings of this study are important for understanding the relationship between the expression of the MMP system and intestinal development, as well its role in gastrointestinal infection and subsequent recovery.

**Key words:** MMPs, TIMPs, development, coccidiosis, chicken

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## INTRODUCTION

In the commercial broiler production systems, chickens are removed from the hatcher at once when most chicks have hatched (de Gouw et al., 2017). They have no access to water and feed up to 72 h post-hatch (PH) (Careghi et al., 2005; van de Ven et al., 2009; Willemsen et al., 2010). It has been shown that

prolonged lack of access to feed PH results in delay of gastrointestinal tract (GIT) development (de Jong et al., 2017), decrease in crypt depth and percentage of proliferating cells in crypts, depression in villi surface, changes in morphology of the microvilli, and alterations in GIT function (Uni et al., 1998; Geyra et al., 2001; Richards et al., 2010; Proszkowiec-Weglarz et al., 2019, 2020b; Qu et al., 2021). Timely feeding shortly after hatch, therefore, is critical for broiler chicken GIT development and overall health. Little is known about the effect of delayed feed PH on the GIT wall architecture associated with extracellular matrix (ECM) turnover in broiler chickens and the focus of this work was to investigate the expression of genes encoding MMPs during the development of chicks subjected to delay in feed immediately PH.

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The ECM plays an important part in maintaining tissue integrity and establishing the physiological stage-specific GIT microenvironment that allows or restricts access of various molecules to the GIT cells. Hence, GIT development, function, and health require extensive remodeling of the ECM constituents carried out by various proteolytic enzymes including matrix metalloproteinases (MMPs) (Visse and Nagase, 2003; Nagase et al., 2006), disintegrin and metalloproteinases (ADAMs) (Seals and Courtneidge, 2003), and ADAMs with thrombospondin motifs (ADAMTS) (Russell et al., 2015). The family of MMPs is divided into several subgroups: collagenases (MMP-1, MMP-8, MMP-13, MMP-18), gelatinases (MMP-2, MMP-9), matrilysins (MMP-7, MMP-26), stromelysins (MMP-3, MMP-10, MMP-11), membrane-anchored MMPs (e.g., MMP-14, MMP-16), and other non-classified MMPs (Murphy and Nagase, 2008; Jobin et al., 2017). The expression and activity of MMPs are regulated by a number of mechanisms (Madzharova et al., 2019; Bassiouni et al., 2021; Hrabia, 2021) including inhibition by specific tissue inhibitors of MMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) which act selectively on different MMPs. For example, TIMP-3 preferentially binds to MMP-9, while TIMP-2 has inhibitory activity toward MMP-2 (Sternlicht and Werb, 2001; Murphy, 2011; Yamamoto et al., 2015). MMPs are capable of processing ECM components and non-ECM molecules such as growth factors, receptors, signaling proteins, cytokines, and chemokines (Apte and Parks, 2015) as well as functioning intracellularly, where they have both proteolytic and nonproteolytic functions (e.g., can act as transcription and transduction factors) (Jobin et al., 2017; Young et al., 2019; Bassiouni et al., 2021). Thus, these zinc-dependent proteinases can contribute to extracellular and cellular events in physiological and pathological processes. MMP-2 and MMP-9 are the most widespread MMPs in tissues including GIT (Derkacz et al., 2021; Maronek et al., 2021). They share similar proteolytic activity and process a number of ECM molecules including native type IV, V and IX collagens, laminin, and fibronectin; however, MMP-2 degrades native collagen types I, II and III in a similar manner to collagenases, but MMP-9 does not. The MMP-7, an important matrilysin, degrades not only ECM constituents such as collagen IV, laminin and elastin, but also possesses the ability to cleave molecules that are matrix bound or present on the cell surface including the Fas ligand, growth factors, and cytokines. Thus, may control cell-extracellular matrix interactions and growth factors bioavailability (Patterson et al., 2001; Page-McCaw et al., 2007; Hrabia, 2021). Participation of MMPs in ECM turnover and in modulation of cell function in different types of tissues (Jobin et al., 2017; Young et al., 2019; Bassiouni et al., 2021; Hrabia, 2021), including the intestine (O'Sullivan et al., 2015; Maronek et al., 2021) is well known. However, in birds, there is limited information available, related to MMP involvement in physiological or pathological processes in the GIT. Elevated activity of MMPs, has been observed in necrotic intestinal tissue of broilers infected with

*Clostridium perfringens*, which causes avian necrotic enteritis (Olkowski et al., 2006). Moreover, MMP-7 was one of the most highly induced genes (4,000-fold) in the cecum of newly hatched chickens during the first 10 days following *Salmonella enteritica* infection (Rychlik et al., 2014). In turn, challenge of broilers with *Eimeria* and different *C. perfringens* strains, independent of their pathogenicity, reduced the expression of MMPs proteins in the jejunum, in comparison to *Eimeria*-only infected control chickens (Van Damme et al., 2020).

Several species of *Eimeria* cause coccidiosis, one of the most common diseases in the modern poultry industry. These parasites infect and develop in different regions of the chicken intestine where they exert different pathological effects, including destruction of epithelial cells, disruption of tissue integrity, fluid loss and malabsorption of nutrients, changes in mucosa permeability, inflammation of the intestinal wall, or villar destruction (Chapman, 2014). For example *Eimeria acervulina* infects the duodenum extending to the mid-intestine, and *E. maxima* infects the mid-intestine extending to the posterior intestine (Joyner, 1978).

Considering the fact that MMPs are crucial in maintaining intestinal ECM homeostasis and that chronic inflammatory diseases of the GIT, pathological process involving extensive mucosal degradation and tissue remodeling, are associated with increased expression of several MMPs and decreased capacity of TIMPs to inhibit their action (Liabakk et al., 1996; Murray et al., 1998; Kirkegaard et al., 2004; O'Sullivan et al., 2015; Maronek et al., 2021), we hypothesized that MMPs are involved in the normal and disrupted PH development of the chicken GIT as well as abnormal intestinal tissue remodeling during coccidiosis. Accordingly, the aim of the present study was to examine 1) the developmental changes and effect of delayed access to feed immediately PH and 2) the effect of *Eimeria* infection on mRNA expression of selected MMPs (MMP-2, MMP-7, and MMP-9), their tissue inhibitors (TIMP-2 and TIMP-3), and ADAMTS8 in the intestine of broiler chickens. Additionally, selected MMP and TIMP proteins were localized in the ileal wall of broiler chickens collected –48 h before and 24 h and 336 h PH by immunofluorescence.

## MATERIALS AND METHODS

### *Animals and Experimental Protocols*

All animal procedures used in experiments 1–3 were approved by the Institutional Animal Care and Use Committee of the Beltsville Agricultural Research Center (BARC).

**Experiment 1** The experiment was performed as described previously (Proszkowiec-Weglarz et al., 2019, 2020b). Fertile Ross 708 broiler chicken eggs (250 eggs ranging from 49.1 to 67.9 g with mean egg weight of  $57.3 \pm 0.23$  g, 33 wk breeder flock age) were obtained from a local hatchery (Perdue Hatchery, Hurlock, MD) and incubated under standard conditions (37.5°C and 60% humidity). All birds used in this experiment were hatched



**Table 1.** Gene-specific primers used for the analysis of mRNA levels using quantitative real-time RT-PCR<sup>a</sup>.

Gene <sup>b</sup>	GenBank accession No. <sup>c</sup>	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp)
ADAMTS8	XM_015298130	ACTGGACAAGTGTGGGGTGT	CGCTGCTTGATGTCCAATGTT	138
Beta2-microglobulin	Z48921	TGGAGCACAGACCTGAAG	TTTGCCGTCATACCAGAAGT	161
Beta-actin	X0082	TTCTTTTGGCTTGACTCA	GCGTTTCGCTCCAACATGTT	88
GAPDH	NM_204305	AGCCATTCTCCACCTTTGAT	AGTCCACAACACGGTTGCTGTAT	112
MMP-2	NM_204420	AGGAAAACCCACAGGTCCTC	CCCAGTACTCATTTCCTGCAA	125
MMP-7	NM_001006278	GATGGAAGAGGTGGCACATT	TCATGAGCAGCAACAAGGAA	134
MMP-9	NM_204667	ATGCCTGCATGGAGAAGAAC	CCTGATTTCCAGAAGGACGA	106
TIMP-2	NM_204298	CCAGAAGAAGAGCCTCAACC	CCAGTCTGTCCAGAGGCACT	115
TIMP-3	NM_205487	ATGAGAAATGGGACCGACTG	CATTCTTGAGGTGGCAAAG	123
Ubiquitin	X02650	GGGATGCAGATCTTCGTGAAA	CTTGCCAGCAAAGATCAACCTT	146

<sup>a</sup>All primers used for expression analysis were design using primer3 program (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>; Untergasser et al., 2007).

<sup>b</sup>Abbreviations of the gene names are defined in text.

<sup>c</sup>Reference chicken gene sequences that contain the corresponding PCR products list.

PCR amplicon of appropriate size was amplified in each reaction. Primer sequences were designed using Primer3 software (Rozen and Skaletsky, 2000; Untergasser et al., 2007) for MMP-2, MMP-7, and MMP-9, TIMP-2 and TIMP-3, and ADAMTS8 and are listed in Table 1. Primer efficiency was confirmed to be at an acceptable level. The obtained data were normalized to the geometric mean (Vandesompele et al., 2002) of four housekeeping genes ( $\beta$ -actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Ubiquitin, and  $\beta$ 2-microglobulin (B2M)) in Experiment 1, three housekeeping genes ( $\beta$ -actin; GAPDH, B2M) in Experiment 2 and in jejunum of Experiment 3, while in duodenum of Experiment 3,  $\beta$ -actin was used as housekeeping gene. Selection of housekeeping genes were performed for each experiment and each tissue examined separately, to obtain the most uniformly expressed housekeeping gene or geometric mean. After normalization, the data were transformed using the equation  $2^{-Ct}$ , where Ct represents the fractional cycle number when the amount of amplified product reached a fixed threshold for fluorescence. The data were analyzed and are presented as fold changes relative to the e19 (–48 h) group set to 100% or to the C birds of D7 group set to 100%.

## Evaluation of MMP and TIMP Protein Localization by Immunofluorescence

Immunofluorescence assay for MMP-2, MMP-7, MMP-9, TIMP-2, and TIMP-3 protein was performed according to Hrabia et al. (Hrabia et al., 2018; Hrabia et al., 2019) with small modifications. Briefly, after deparaffinization and rehydration, ileal tissue sections (6  $\mu$ m thickness) were subjected to heat-induced epitope retrieval by microwaving in 0.01M citric buffer (pH 6.0) and nonspecific binding of the secondary

antibody was blocked by treatment with 5% (v:v; 10 min) normal goat serum in Tris-buffered saline with 0.1% v:v Tween 20 (TBST, pH 7.4). Sections were then incubated overnight (4°C) in the presence of respective primary polyclonal rabbit antibodies against MMP-2, MMP-7, MMP-9, and TIMP-3 or mouse monoclonal antibody anti-TIMP-2 (Table 2), followed by incubation for 90 min with the fluorescent secondary antibody DyLight 594 anti-rabbit IgG (H + L; 1:150; Vector Laboratories, Burlingame, CA) or anti-mouse IgG (H + L; Invitrogen, Rockford, IL) and mounted with VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (Vector Laboratories, Inc.). Specificity of TIMP-2 and MMP-2 antibodies was checked by the manufacturers and recommended for chicken. Specificity of anti-MMP-2, -MMP-7, -MMP-9, and -TIMP-3 antibodies was checked by Western blot and reported in previous studies in chickens (Lesniak-Walentyn and Hrabia, 2016; Hrabia et al., 2018, 2019; Hrabia, 2021). Negative control slides were prepared by replacement of the primary antibody with non-immune rabbit or mouse IgG. Analysis of the sections was performed using an Axio Scope.A1 fluorescent microscope and photographed with an AxioCam 503 color camera and ZEN 2.3 pro software (Carl Zeiss, Germany). The intensity of the immunoreactivity was estimated subjectively as strong, moderate, weak, and very weak. Micrographs show blue fluorescence representing DAPI staining of cell nuclei, red fluorescence representing immunopositive reaction specific for MMPs or TIMPs, and merge of blue and red fluorescence.

## Statistical Analysis

Gene expression data were analyzed by two-way ANOVA using the general linear models (GLM) procedure of the Statistical Analysis System (SAS) software

**Table 2.** Primary antibodies used for immunofluorescence (IF).

Antibody <sup>1</sup>	Serum	Host species	Dilution	Catalog no	Vendor
Anti-MMP-2	5% NGS	Rabbit	1:150	ab37150	Abcam, Cambridge, UK
Anti-MMP-7	5% NGS	Rabbit	1:50	sc-30071	Santa Cruz Biotechnology, CA, USA
Anti-MMP-9	5% NGS	Rabbit	1:150	ab38898	Abcam, Cambridge, UK
Anti-TIMP-2	5% NGS	Mouse	1:100	635401	BioLegend, San Diego, CA, USA
Anti-TIMP-3	5% NGS	Rabbit	1:100	ab85926	Abcam, Cambridge, UK

<sup>1</sup>Protein abbreviations are deciphered in the text of the manuscript. NGS, normal goat serum.



**Table 3.** The effect of delay access to feed post-hatch (treatment) on MMP, TIMP, and ADAMTS8 gene expression in the ileum during development (age).

Gene <sup>1</sup>	Pr > F		
	Treatment	Age	Treatment × Age
MMP-2	0.995	<0.001	0.998
MMP-7	0.849	<0.001	0.689
MMP-9	0.842	<0.001	0.941
TIMP-2	0.852	<0.001	0.828
TIMP-3	0.941	<0.001	0.684
ADAMTS8	0.862	<0.001	0.236

<sup>1</sup>Gene abbreviations are deciphered in the text of the manuscript.

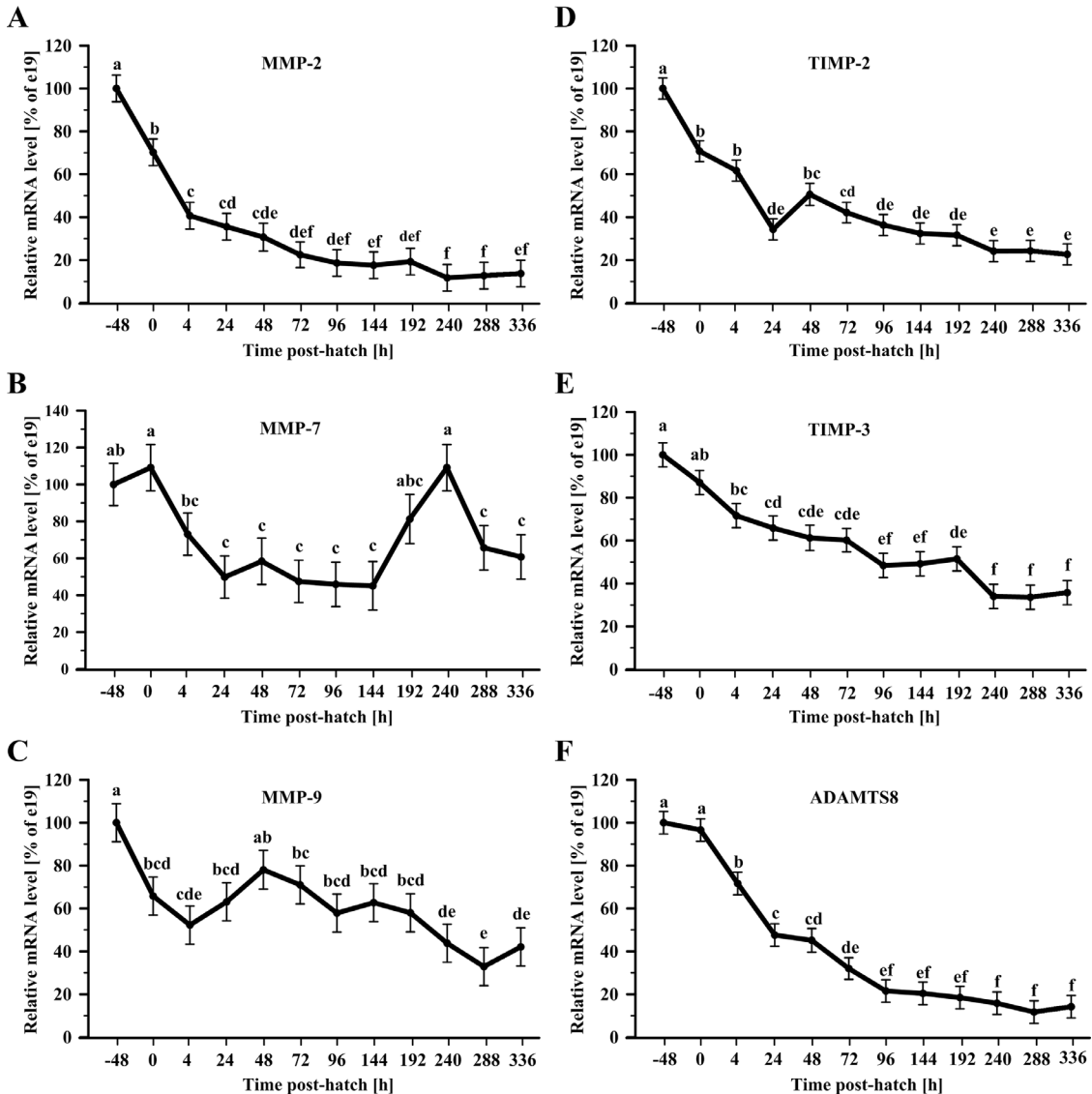
v9.4 (SAS Institute, Cary, NC). Least significant differences were used to determine which means differed significantly. Each sampled bird (n = 6 replicates per treatment) was considered as an experimental unit. Time (age in experiment 1, and D7 or D10 in experiment 2 and 3), treatment, and their interaction were set as the fixed effects in all analysis. Main effects were

not analyzed separately if the interaction between them was significant. For all analysis significance was set at  $P < 0.05$ .

**RESULTS**

**Gene Expression in the Ileum During Neonatal Development. Effect of Delayed Feeding Post-hatch (Experiment 1)**

The effect of delayed access to feed PH (treatment) on MMP, TIMP, and ADAMTS8 gene expression in the ileum during development (age) is summarized in Table 3. There was no significant treatment effect or age × treatment interaction for all examined mRNA expression. The developmental expression patterns of MMP, TIMP, and ADAMTS8 genes in the ileum are presented in Figure 1. All 6 genes were significantly ( $P < 0.001$ ) affected by age with overall decrease in expression during PH development. MMP-2, TIMP-2, and TIMP-3



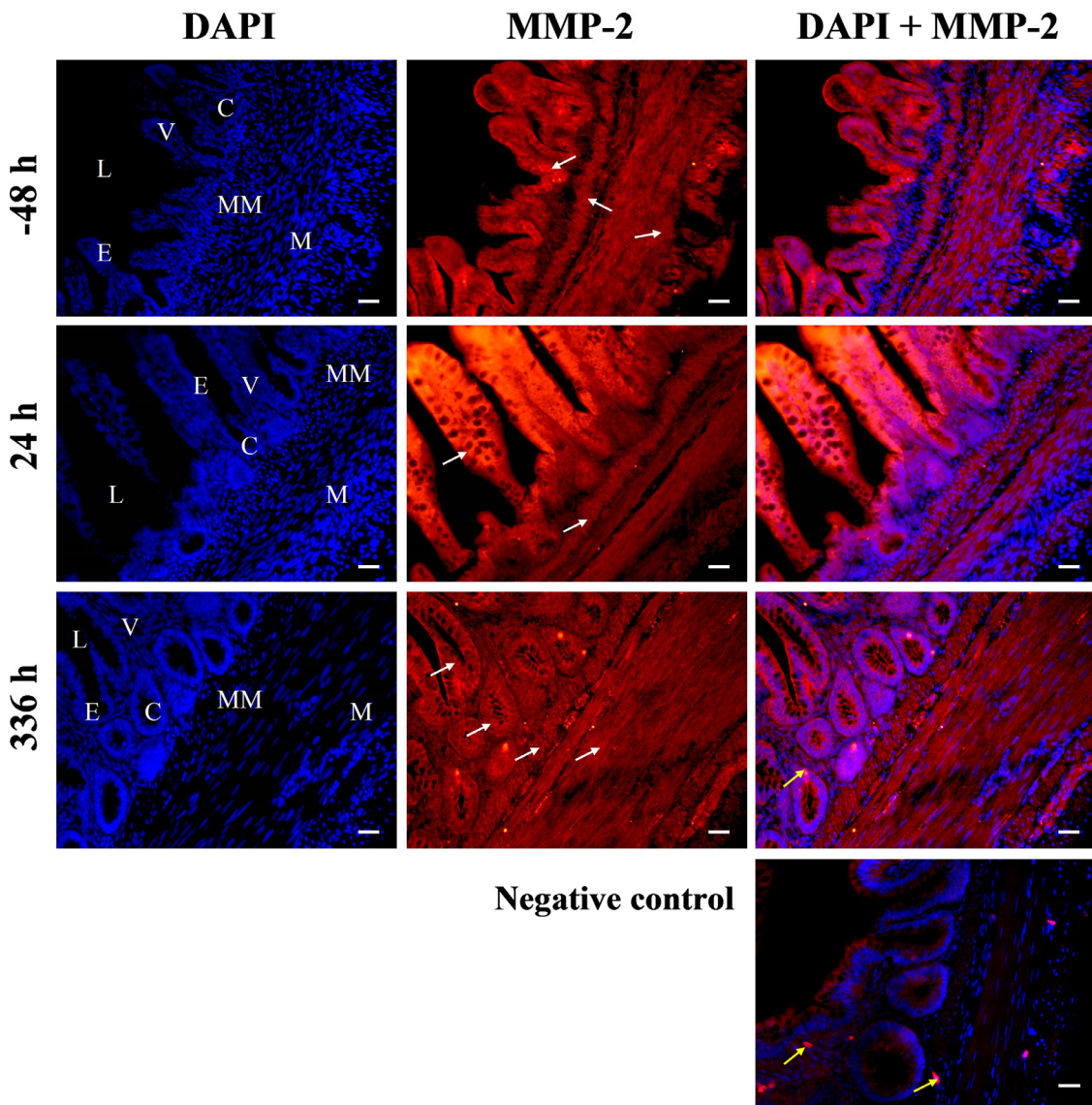
**Figure 1.** Effect of age (development) on ileal levels of mRNA for MMP-2 (A), MMP-7 (B), MMP-9 (C), TIMP-2 (D), TIMP-3 (E), and ADAMTS8 (F). The expression level of e19 (-48 h) birds was set to 100% and the other values are presented as % of the -48 h data. Each value represents mean ± SEM of six birds. Different letters denote statistically significant ( $P < 0.05$ ) differences between means. Abbreviations: ADAMTS8, ADAM metalloproteinase with thrombospondin type 1 motif 8; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

as well as ADAMTS8 transcript levels were downregulated during development, showing the lowest mRNA levels during the second week PH (Figures 1A, D–F). MMP-7 mRNA expression sharply decreased between –48 h and 24 h PH and was followed by a constant level until 144 h, which then increased at 240 h PH, followed by decrease (Figure 1B). A sharp decrease in MMP-9 mRNA level was noted from –48 h until 4 h PH, then an increase until 48 h, followed by a gradual decrease, with the lowest level observed at 288 h PH (Figure 1C).

### **Immunofluorescent Localization of MMPs and TIMPs in the Ileum During Neonatal Development (Experiment 1)**

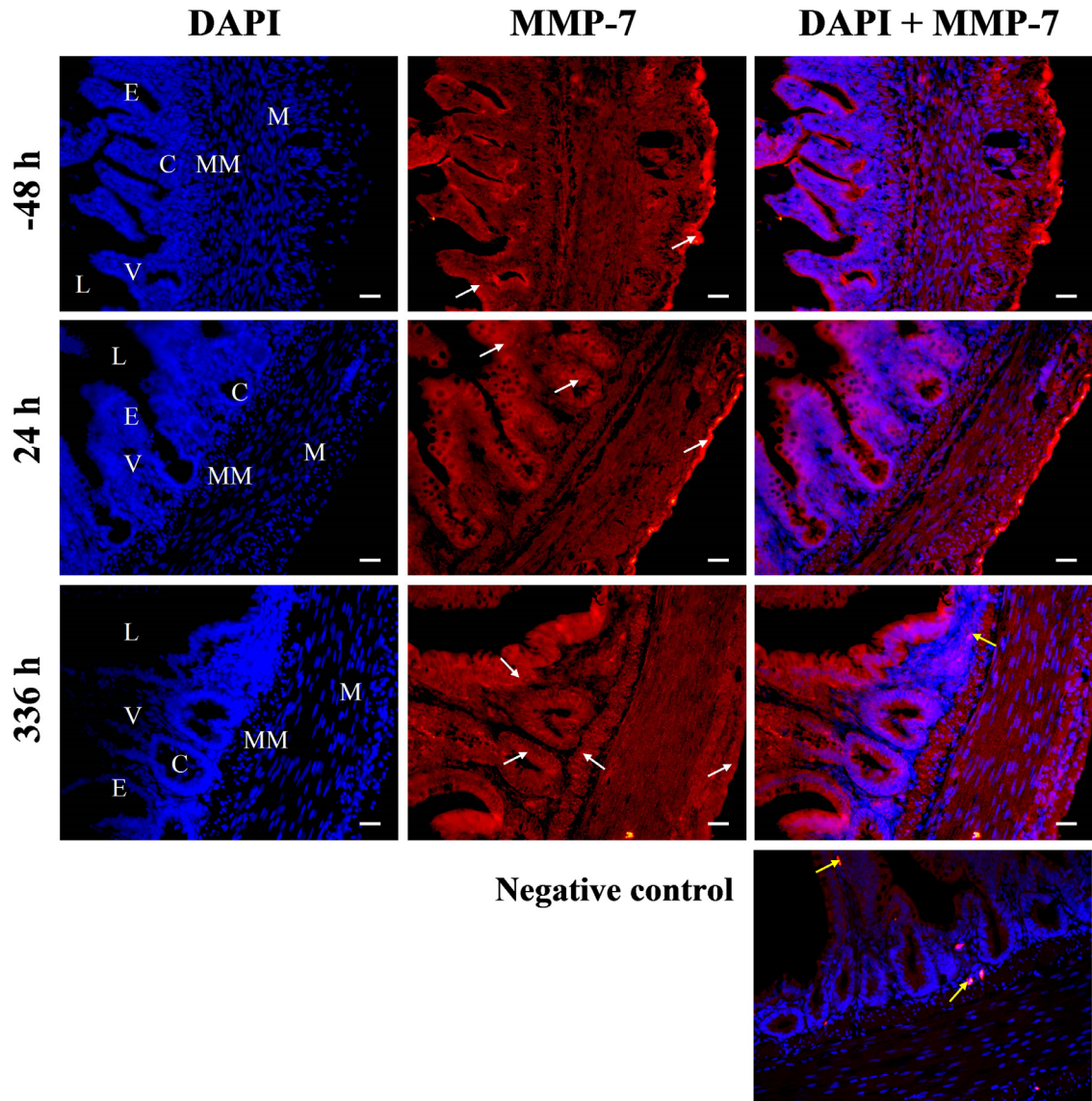
Specific immunoreactivity for MMPs and TIMPs was found in the wall of the ileum of chicks before (e19) and after hatching (24 h and 336 h). Localization of MMPs

and TIMPs is demonstrated in Figures 2–6. Owing to limited availability of commercial antibodies for chicken proteins, we were not able to localize the ADAMTS8 protein. Distinct cell- and tissue-specific patterns of the localization of MMPs and TIMPs were observed during development. The intensity of fluorescence of MMP-2 protein was strong in the luminal and crypt epithelium, and moderate in the muscularis mucosa and smooth muscles (Figure 2). Moderate immunoreactivity for MMP-7 protein was localized predominantly on the apical side of the luminal and crypt epithelium, and strong in the surface epithelium. Weak immunoreactivity for MMP-7 was localized in the muscles (Figure 3). Strong staining for MMP-9 protein was clearly present in the connective tissue, mainly around the blood vessels, muscle fibers, and in the basement membrane of the luminal and crypt epithelium (Figure 4). Moderate immunoreactivity for TIMP-2 was observed in the epithelium and weak in the muscularis mucosa and muscles (Figure 5).



**Figure 2.** Immunolocalization of MMP-2 protein in the ileum of broiler chickens collected –48 h before and 24 h and 336 h post-hatch. White arrows indicate immunopositive reaction (red fluorescence). DAPI staining (blue) shows nucleus only. Negative control section incubated without primary antibody did not exhibit positive staining, excluding red blood cells which always show nonspecific fluorescence (yellow arrows). Scale bar = 20  $\mu$ m. Abbreviations: C, crypt; E, luminal epithelium; L, intestinal lumen; M, smooth muscles; MM, muscularis mucosa; SE, surface epithelium; V, villi.





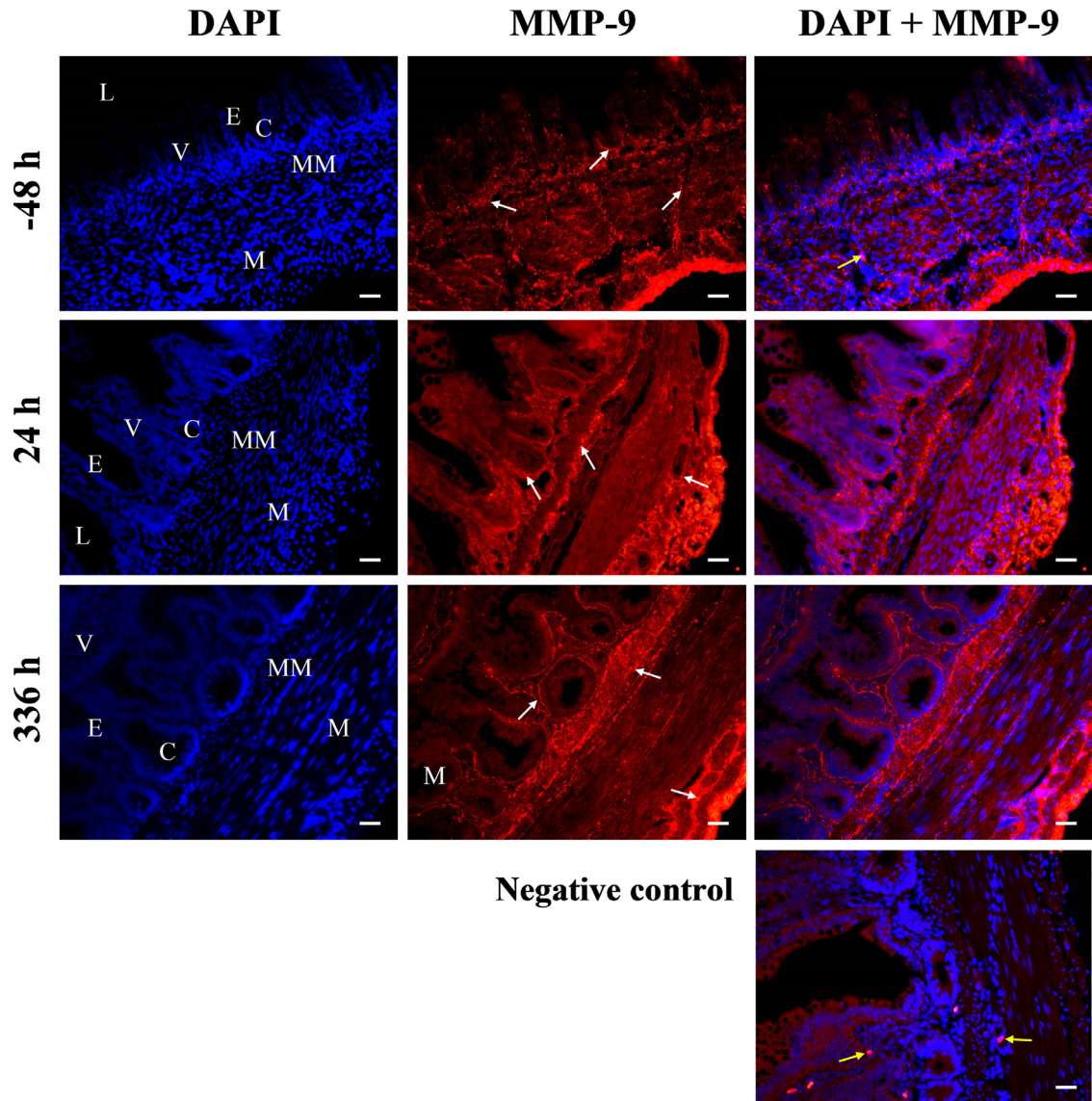
**Figure 3.** Immunolocalization of MMP-7 protein in the ileum of broiler chickens collected  $-48$  h before and 24 h and 336 h post-hatch. Explanations and abbreviations as in Figure 2.

Moderate immunopositive reaction for TIMP-3 protein was detected in the epithelium, muscularis mucosa, and muscles. In e19 embryos the intensity of staining in the muscles was stronger than in the epithelium, and in 24 h and 336 h chicks more intense reaction was in the epithelium than in the muscles (Figure 6). When primary antibody was omitted, no staining was present (negative control in Figures 2–6).

### **Effect of *Eimeria maxima* Infection on the Gene Expression of MMPs, TIMPs, and ADAMTS8 (Experiment 2)**

Effect of *E. maxima* infection on MMP, TIMP, and ADAMTS8 mRNA expression in the jejunum, ileum and cecum at D7 and D10 PI is shown in Figure 7. For the relative mRNA levels of MMP-2, there was significant interaction between treatment and time PI ( $P = 0.013$ ) in the cecum. Chickens in the IF group had significantly lower MMP-2 transcript level at D7 in

comparison to C birds, and the level tended to increase at D10. Expression level of MMP-2 was not affected by time PI, treatment or their interaction in the jejunum and ileum (Figure 7A). The mRNA levels of MMP-7 were only impacted by infection in the jejunum ( $P = 0.003$ ) and ileum ( $P = 0.019$ ) with higher mRNA levels in IF birds with significant differences in the cecum (Figure 7B). MMP-9 transcript abundance was influenced by treatment in the jejunum ( $P = 0.015$ ), ileum ( $P < 0.001$ ), and cecum ( $P = 0.004$ ), with significantly higher MMP-9 levels in the IF groups (Figure 7C). For TIMP-2 in the jejunum, there was interaction between treatment and time PI ( $P = 0.016$ ) on the mRNA levels. The mRNA levels in the IF group at D7 were higher in comparison to the C group and both treatment groups at D10. The TIMP-2 gene expression in the ileum was affected by treatment ( $P = 0.003$ ), with higher abundance in IF groups. In the cecum, there was significant interaction between treatment and time PI ( $P = 0.022$ ), with higher TIMP-2 mRNA levels in the IF group at D10 in comparison to



**Figure 4.** Immunolocalization of MMP-9 protein in the ileum of broiler chickens collected  $-48$  h before and 24 h and 336 h post-hatch. Explanations and abbreviations as in Figure 2.

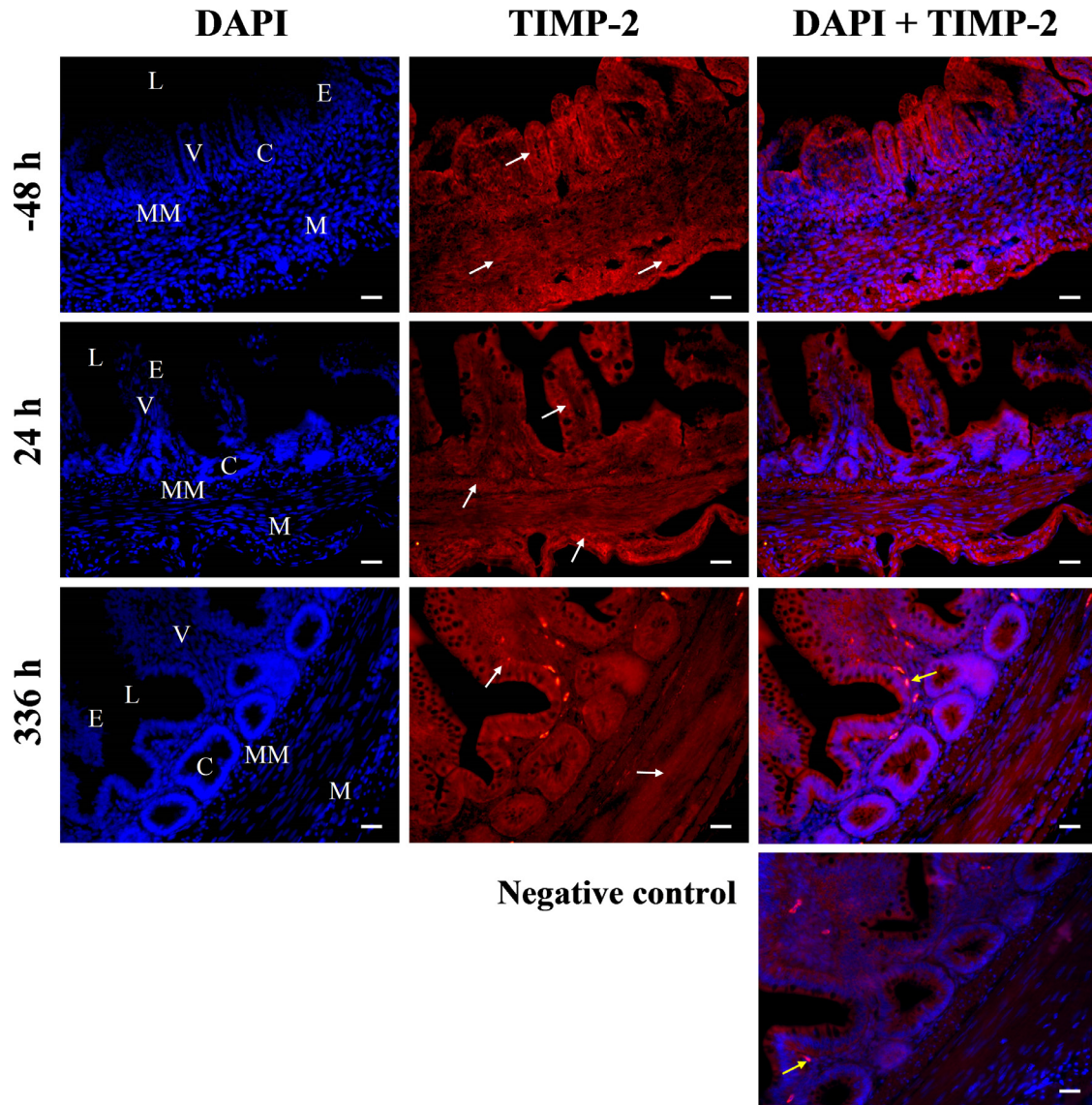
the C group at the same time PI (Figure 7D). The mRNA level of TIMP-3 was affected by treatment ( $P = 0.009$ ) in the jejunum, where IF birds had lower mRNA expression in comparison to C birds. In the ileum and cecum TIMP-3 mRNA levels were not affected by *Eimeria* infection (Figure 7E). For ADAMTS8 mRNA expression, in the jejunum, there were no impact of time, infection or interaction, while in the ileum there was a significant effect of time ( $P < 0.001$ ) with higher mRNA levels at D10, and in the cecum significant interaction between infection and time PI ( $P = 0.029$ ) was observed, with significantly lower mRNA level in the IF group at D7 in comparison to D10 (Figure 7F).

#### **Effect of *Eimeria acervulina* Infection on the Gene Expression of MMPs, TIMPs, and ADAMTS8 (Experiment 3)**

Effect of *E. acervulina* infection on MMP, TIMP, and ADAMTS8 gene expression in the duodenum and

jejunum is shown in Figure 8. MMP-2 mRNA abundance was only affected by treatment in both tissues, duodenum ( $P = 0.01$ ) and jejunum ( $P = 0.024$ ), with lower MMP-2 transcript levels in IF birds (Figure 8A). MMP-7 mRNA expression in the duodenum was affected by treatment, but the effect was dependent on time PI (significant interaction,  $P = 0.037$ ) with IF birds characterized by significantly higher mRNA level at D7 PI in comparison to C birds at D7 or both treatment groups at D10 (Figure 8B). In the jejunum, MMP-7 mRNA level was only affected by infection ( $P = 0.002$ ) with higher mRNA level in infected birds (Figure 8B). MMP-9 mRNA levels were higher ( $P = 0.006$ ) in IF birds at both time points PI in the duodenum, while in the jejunum the IF effect depended on the time PI ( $P = 0.023$ ), with significantly higher mRNA level in IF birds at D10 in comparison to C birds or both groups at D7 (Figure 8C). There was no significant effect of *Eimeria* infection on TIMP-2 mRNA level in both tissues, duodenum and jejunum (Figure 8D). TIMP-3 mRNA levels were impacted by treatment in both, duodenum





**Figure 5.** Immunolocalization of TIMP-2 protein in the ileum of broiler chickens collected  $-48$  h before and  $24$  h and  $336$  h post-hatch. Explanations and abbreviations as in [Figure 2](#).

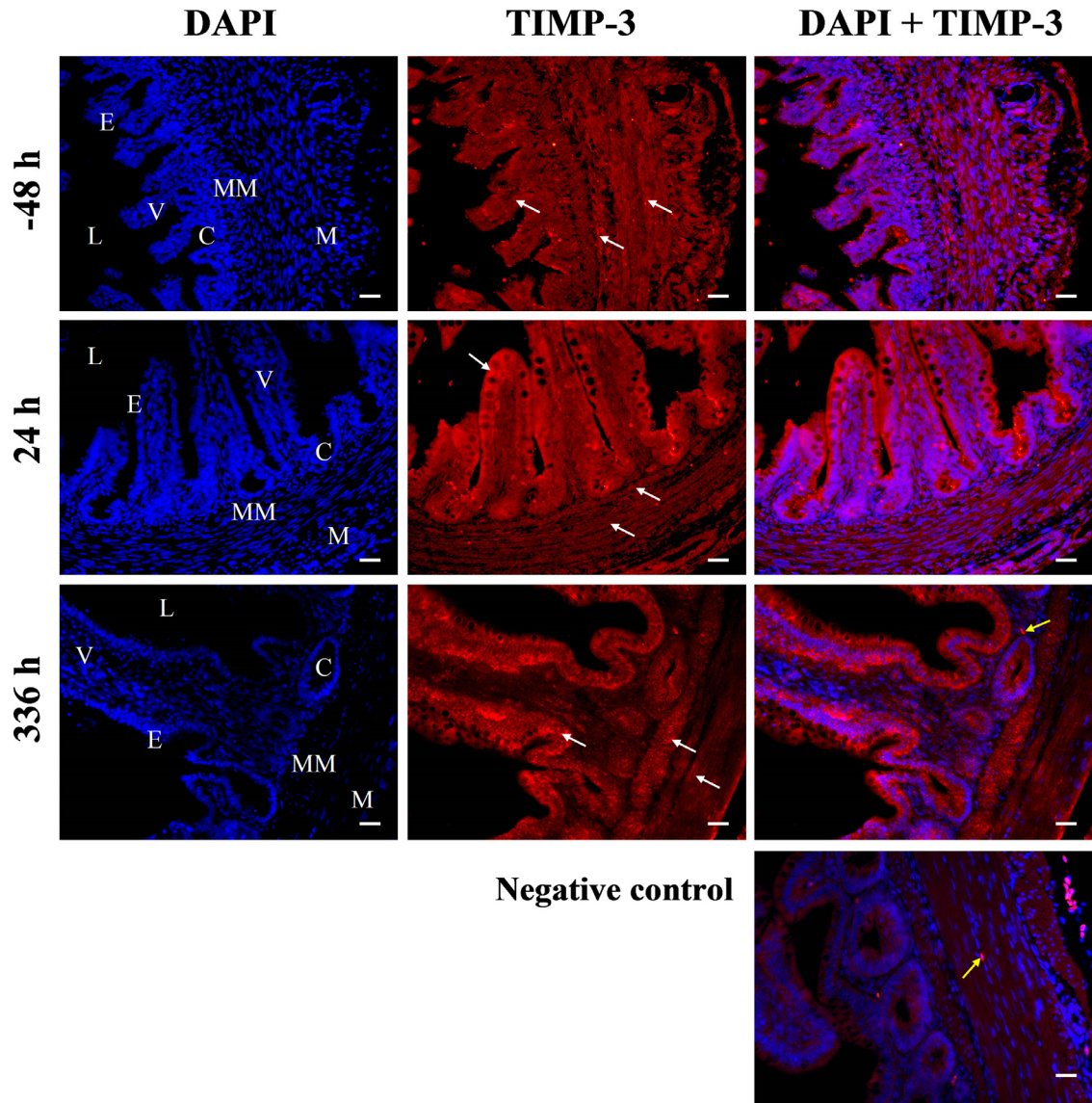
( $P < 0.001$ ) and jejunum ( $P = 0.001$ ) with significantly lower levels in IF groups than in C groups ([Figure 8E](#)). For ADAMTS8, C birds had significantly higher ( $P = 0.046$ ) mRNA expression in comparison to IF birds in duodenum, while ADAMTS8 mRNA expression was not significantly affected in jejunum. A trend toward lower ADAMTS8 mRNA levels was observed (treatment,  $P = 0.052$ ) in IF groups ([Figure 8F](#)).

## DISCUSSION

In the first experiment, we reported the expression of MMP-2, MMP-7, MMP-9, TIMP-2, TIMP-3, and ADAMTS8 at the mRNA level in the ileal tissue during early PH development of normal chicks and those disrupted by delayed access to feed. Proteins encoded by these genes are crucial in ECM turnover, which in turn, is responsible for regulation of cellular physiology and orchestration of environment for cell proliferation, differentiation, apoptosis, adhesion, and migration. Proper

ECM remodeling is therefore essential for tissue growth and morphogenesis.

In general, mRNA expression patterns of all examined genes showed downregulation during the first 2 wk PH, with the most prominent drop of the mRNA expression at 4 or 24 h PH. Only MMP-7 mRNA expression increased around d 10 PH, newly differentiated cells during development, especially the luminal epithelium cells. During the first days PH, the small intestine undergoes tremendous morphological and functional changes related to digestion and nutrient absorption, and gastrointestinal barrier development ([Uni et al., 1998](#); [Proszkowiec-Weglarz et al., 2020b](#)). Hence, decrease in mRNA expression of examined MMP system elements may be attributable to maturational processes in the GIT including cell differentiation. Previously, [Proszkowiec-Weglarz et al. \(2020b\)](#) found that among mRNAs in which expression is down regulated immediately PH in the ileum are several genes encoding tight junction proteins that have important functions in gut barrier maintenance and intestinal permeability. Epithelial



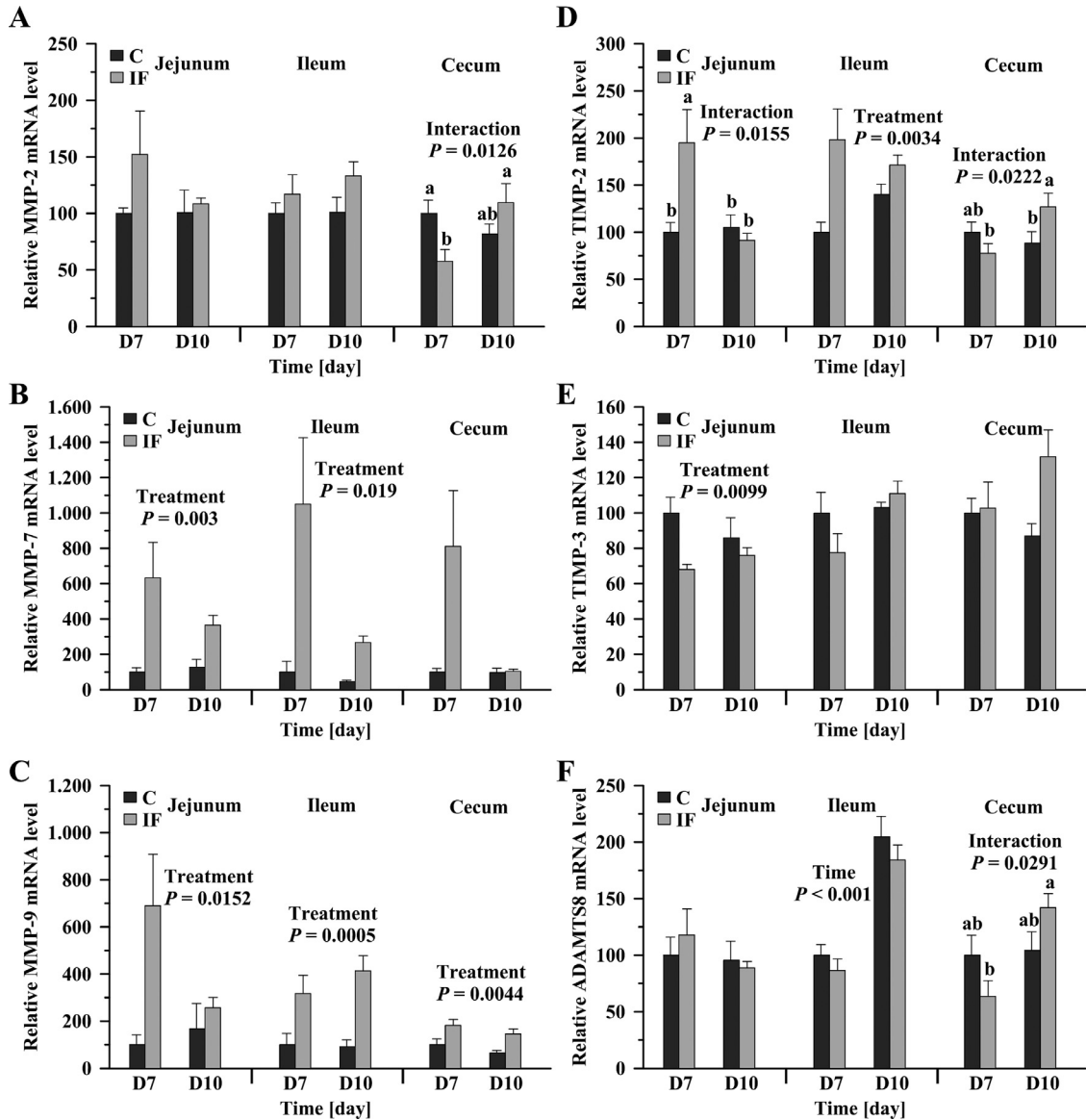
**Figure 6.** Immunolocalization of TIMP-3 protein in the ileum of broiler chickens collected  $-48$  h before and 24 h and 336 h post-hatch. Explanations and abbreviations as in Figure 2.

barrier integrity is essential in supporting intestinal homeostasis, and MMPs are also involved in modulation of the epithelial barrier in the gut (O'Sullivan et al., 2015), via direct or indirect regulation of epithelial barrier gene expression, including mucin 2, claudin-1, or zona occludens protein 1 (Garg et al., 2006; Liu et al., 2013; Pope et al., 2014). Moreover, it has been shown recently that MMP-9 at clinically relevant concentrations caused an increase in intestinal epithelial tight junction permeability *in vitro* (using filter-grown Caco-2 monolayers) and *in vivo* (utilizing mouse small intestine recycling perfusion) (Al-Sadi et al., 2019, 2021). It is plausible that in the developing GIT of chickens there is also interplay between MMPs and proteins forming epithelial barrier. Although, delay in feed access for the first 48 h PH only slightly affected tight junction-related gene expression, we did not observe any impact of this factor on examined MMP, TIMP, or ADAMTS8 mRNA expression. In this study, we were not able to examine the amounts of MMPs, TIMPs and ADAMTS8 proteins

or MMP activity, however, it cannot be ruled out that delay in feeding immediately PH influences protein expression or MMP activities in the chicken intestine.

Furthermore, an important finding of the study was the determination of specific immunoreactivity for selected MMP and TIMP proteins in the wall of the ileum of chickens before ( $-48$  h) and after hatch (24 h and 336 h). Distinct cell- and tissue-specific patterns of localization of MMP and TIMP proteins highlight specific roles of MMPs in the ileal wall. These results correspond with localization/cellular sources of MMPs and TIMPs in the GIT of other animals including humans (O'Sullivan et al., 2015) and further support suggestion that MMPs/TIMPs are involved in chicken GIT development and function. Role of MMP-2 protein in the chicken ileum, that is localized inside and outside cells of the luminal and crypt epithelium as well as, with lower intensity, in the muscularis mucosa and smooth muscles, may involve, among others, degradation of ECM components and maintenance of the epithelial barrier function





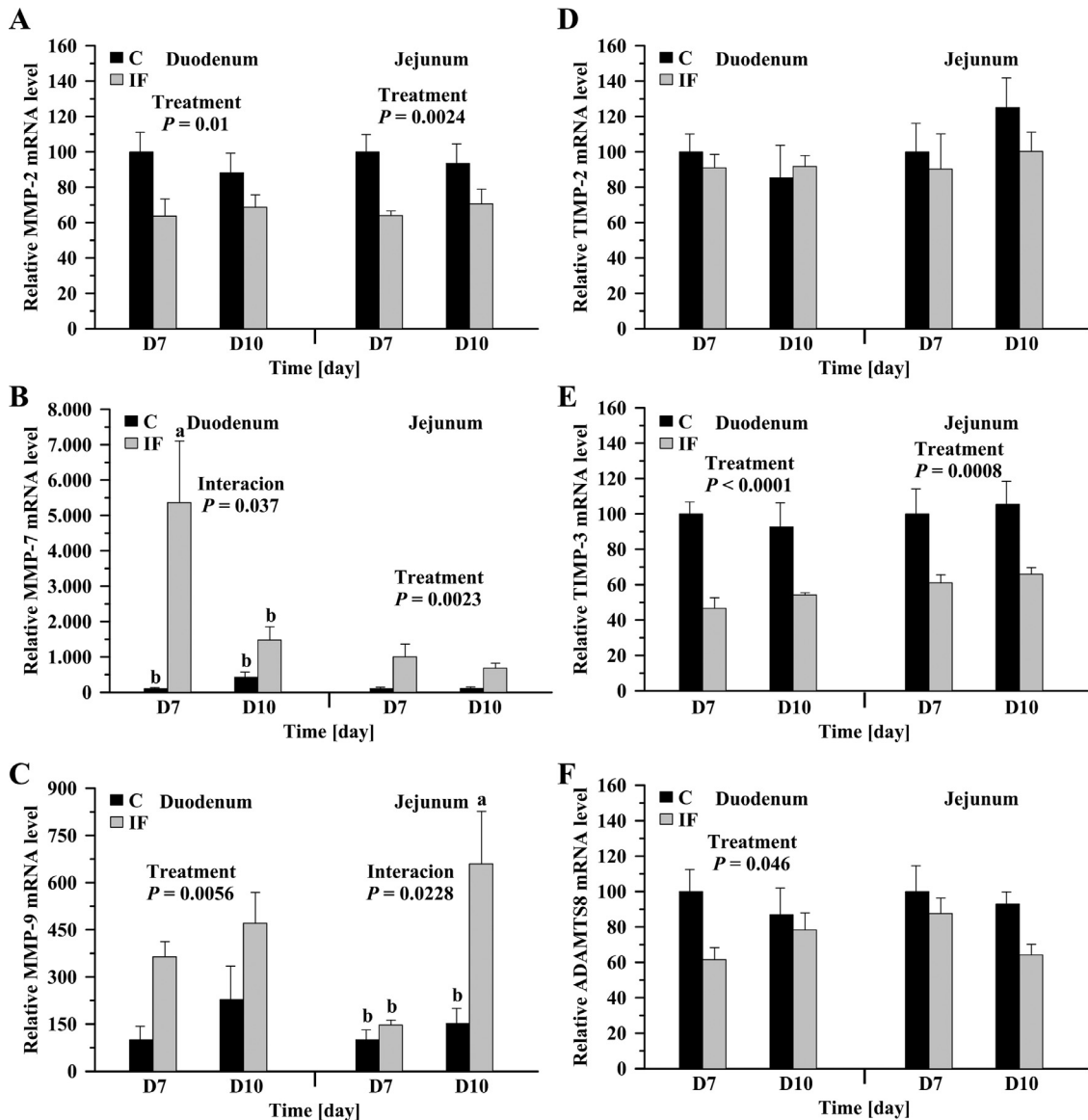
**Figure 7.** Effect of *Eimeria maxima* infection (treatment) on MMP-2 (A), MMP-7 (B), MMP-9 (C), TIMP-2 (D), TIMP-3 (E), and ADAMTS8 (F) mRNA level in chicken jejunum, ileum and cecum at 7 (D7) or 10 (D10) day post-infection (PI). The expression level of control (C) birds of D7 was set to 100% and the other values are presented as % of C D7 data. Each value represents mean  $\pm$  SEM of six birds. Different letters denote statistically significant ( $P < 0.05$ ) differences between means within particular ileal tissue. Abbreviations: ADAMTS8, ADAM metalloproteinase with thrombospondin type 1 motif 8; IF, birds infected with *Eimeria maxima* oocysts; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

(Garg et al., 2006) and intercellular junctions. MMP-2, similarly to MMP-7 and MMP-9, is able to cleave the gap junction protein, connexin 43 (Jobin et al., 2017). Moreover, this proteinase exhibits a proangiogenic role in different tissues including intestinal tissues (Bergers et al., 2000; Lee et al., 2005), therefore, it may participate in the development of blood vessels in the GIT of growing chickens. In turn, the function of MMP-7 localized predominantly in the apical side of the luminal and crypt epithelium, in the surface epithelium, and in less abundance in the muscles, may be linked to multiple molecule bioavailability and cell-extracellular matrix interactions. The correlation of MMPs with TIMP expression is key to the balance of MMP activity, therefore, we localized TIMP-2 (with a high affinity to MMP-2) and TIMP-3 (binds preferentially to MMP-9 and can also inhibit some members of the ADAM and ADAMTS

family) in the ileal wall. It was observed that localizations of these inhibitors in large part, correspond with their target MMP locations, highlighting the involvement of TIMP-2 and TIMP-3 in the control of MMP and/or ADAMTS activity resulting in tissue remodeling of the chicken intestine. It should be noted, that TIMPs are multifunctional molecules and in addition to MMP inhibition, TIMPs participate in different biological processes including angiogenesis and cell apoptosis, proliferation and differentiation (Chirco et al., 2006; Peng et al., 2015). It cannot be ruled out that TIMPs could play a similar role during the growth/maturation of the GIT in birds.

Several MMPs are upregulated in inflammatory conditions including inflammatory bowel disease. During this pathological process in the GIT, their roles are associated with regulation of epithelial barrier function,





**Figure 8.** Effect of *Eimeria acervulina* infection (treatment) on MMP-2 (A), MMP-7 (B), MMP-9 (C), TIMP-2 (D), TIMP-3 (E), and ADAMTS8 (F) mRNA level in chicken duodenum and jejunum at 7 (D7) or 10 (D10) day post-infection (PI). The expression level of control (C) birds of D7 was set to 100% and the other values are presented as % of C D7 data. Each value represents mean  $\pm$  SEM of six birds. Different letters denote statistically significant ( $P < 0.05$ ) differences between means within particular ileal tissue. Abbreviations: ADAMTS8, ADAM metalloproteinase with thrombospondin type 1 motif 8; IF, birds infected with *Eimeria acervulina* oocysts; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

immune response, angiogenesis, fibrosis, and wound healing (O'Sullivan et al., 2015; Maronek et al., 2021). Thus, an important observation of the second portion of this study was the increased mRNA expression of MMP-7 in jejunum and ileum, and MMP-9 in jejunum, ileum and cecum, in conjunction with decreased expression of TIMP-3 mRNA in jejunum of broiler chickens at D7 and D10 PI infected with *E. maxima*. In contrast, MMP-2 and ADAMTS8 transcript levels were decreased at D7 PI but not significantly different at D10 PI in comparison to C birds. These changes were accompanied by increased levels of TIMP-2 mRNA at D7 PI in jejunum, in ileum at both examined time points, and at D10 PI in cecum. Similar to effects of *E. maxima*, *E. acervulina* infection caused an increase in MMP-7 and MMP-9 mRNA expression and a decrease in TIMP-3 transcript

levels in duodenum and jejunum, as well as a decrease in MMP-2 and ADAMTS8 mRNA levels and no changes in TIMP-2 mRNA. TIMPs form a reversible complex with MMPs in a 1:1 ratio, therefore, a balance between MMP and TIMP is crucial for the activity of MMP. Thus, the observed increased expression of MMPs simultaneously with lower expression of TIMP, raising the possibility of increased MMP-to-TIMP ratio, promoting MMP activity. In general, our results are in line with those previously observed in inflamed intestines, where expression levels of numerous MMPs are elevated (Olkowski et al., 2006; O'Sullivan et al., 2015; Maronek et al., 2021). However, our results regarding MMP-2 expression, differ from those previously reported. In contrast to our findings, Olkowski et al. (2006) observed elevated activity of gelatinases with molecular weight of 72 and 62 kDa

corresponding to pro-MMP-2 and active MMP-2, respectively, in the intestinal tissue homogenates from jejunum and ileum of broiler chickens challenged with *Clostridium perfringens*. Moreover, these authors detected more intense immunoreactivity for MMP-2 protein in the disrupted villi of infected birds compared to healthy controls. The discrepancies between our findings and those of [Olkowski et al. \(2006\)](#), regarding to MMP-2 expression, may be attributable to different pathogens causing intestinal disease, the degree of tissue damage, and/or measured parameters (MMP-2 mRNA vs. MMP-2 protein/activity). The observed downregulation of MMP-2 mRNA expression in our study may be linked to protection of the luminal epithelium from excessive damage in *Eimeria*-challenged chickens. Taken together, our results indicated that *E. maxima* and *acervulina* challenge affects gene expression of some MMPs or TIMPs, whose products may lead to degradation of ECM components, alterations in tight junctions and/or epithelial barrier disruption in the wall of broiler intestine. An imbalance between the expression of MMPs and associated TIMPs, may be one of the mechanisms responsible for dysfunction of the intestine caused by *Eimeria* infection.

There are no available reports examining the effect of *Eimeria* or other infections on the ADAMTS8 expression/activity in the avian intestine. This enzyme, a member of ADAMTS family, possesses anti-angiogenic properties ([Dunn et al., 2006](#)) can inhibit cell proliferation, migration and invasion and promote apoptosis ([Zhao et al., 2018](#); [Zhang et al., 2020](#)), therefore, some alterations in ADAMTS8 mRNA expression observed in this study may be related to the modulation of mentioned processes in the intestinal sections of *Eimeria*-challenged chickens.

In conclusion, the degree of ileal development-dependent alterations in expression and tissue-dependent localization of chosen MMPs and TIMPs suggest that these proteins are involved in the complex orchestration of chicken intestinal tissue turnover during PH development. Increase in MMP-7 and MMP-9 mRNA abundances in the intestine of *Eimeria* infected birds suggest an important role for these proteases in tissue remodeling and destruction in pathological conditions. In addition, the results obtained indicate that augmented levels of MMP-7 and MMP-9 mRNA might serve as markers of mucosa damage in the intestine of *Eimeria*-challenged chickens. The novel findings of this study are important in understanding the relationship between the expression of the MMP system in intestinal development, health, and function in birds. Further investigation evaluating MMP/TIMP protein levels and MMP activity at different physiological and pathological states of the chicken gut are warranted.

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## DISCLOSURES

The authors declare no conflicts of interest.

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