Short Communication

Investigation of peripheral blood responses in chickens infested with *Dermanyssus gallinae*

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Running head: *D. gallinae* elevates CCL4 and B2M levels

Abbreviations

PRM, poultry red mite; NIE, non-infested experimental chickens; PBMCs, peripheral blood mononuclear cells; NIC, non-infested commercial chickens; b.e., before exposure to PRMs; p.e., post-exposure to PRMs; qPCR, quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; CCL4, C-C chemokine ligand 4; B2M, β2 microglobulin; FPKM, fragments per kilobase of exon per million reads mapped.

The invasion of blood-sucking ectoparasites, especially poultry red mites (*Dermanyssus gallinae,* PRMs), northern fowl mites (*Ornithonyssus sylviarum*, NFMs), and tropical fowl mites (*Ornithonyssus bursa*) is a global threat to the poultry industry. Mass infestation by these mites causes various health problems in chickens, especially laying hens, including anaemia and hyposthenia, lowered feed conversion, and a decrease in egg quality and production, thereby resulting in considerable economic losses to the poultry industry [1]. Among these mite species, PRMs are prevalent worldwide and the most devastating agents for the poultry industry, particularly in European countries and Asian countries, including Japan [1]. In addition to the direct damage induced by blood sucking, PRMs can transmit several pathogens including avian influenza virus and *Salmonella gallinarum* [2, 3]. Current preventive strategies against PRMs are mainly dependent on the use of acaricides; however, due to the development of acaricide resistance, controlling PRMs has become extremely difficult [4]. Therefore, controlling PRMs is crucial for the poultry industry.

Various host responses including haemostasis, complement activation, and inflammatory responses, are induced in response to infestation with haematophagous ectoparasites, such as infestations by tick species [5]. Proinflammatory chemokines are secreted to recruit inflammatory cells to the sites of infestation [6], whereas various immunosuppressants secreted in tick saliva can suppress the immunity of the host, help to evade its immune and haemostatic responses, and facilitate the undisturbed blood sucking process [7, 8]. Thus, several reactions, both advantageous

and disadvantageous to haematophagous ectoparasites, are induced in the infested host.

Among the heamatophagous mites on poultry farms, it was previously reported that PRMs potentially modulate host immunological states [9, 10]; for example, PRM infestations reduce γ-globulin levels in the blood and are correlated with decreased egg production [9]. Another report indicated that concentrations of corticosterone and β-globulin are significantly elevated in the blood of PRM-infested hens compared to that in non-infested animals, with the blood levels of corticosterone negatively correlated with the egg yield [11]. Additionally, several studies demonstrated that mass infestation by PRMs impairs responses to vaccination and reduces antibody production [10, 12]. However, studies on the impacts of blood sucking by avian mites on host physiological states are limited, and host responses against infestation have not been fully elucidated. Therefore, to shed light on the host reactions against infestation by avian mites in detail, in the present study, we focused on PRMs that are highly prevalent in Japan, and the gene expression profile in peripheral blood cells isolated from hens infested with PRMs was evaluated.

To comprehensively investigate the highly expressed genes in peripheral blood cells from hens infested with PRMs, we first analysed the expression intensities of chicken genes in blood-fed PRMs, which included the hen blood cells, using RNA-Seq. RNA-Seq was performed using total RNA isolated from blood-fed PRMs as previously reported [13], and the obtained data were used to analyse the expression of chicken genes. The obtained reads were mapped to the genome sequence

of the chicken (*Gallus gallus*, NCBI: txid9031) and expression analysis was performed as previously described [13]. All procedures were conducted at the Hokkaido System Science Co. (Hokkaido, Japan). Among the transcripts exhibiting high expression, genes related to haemoglobin synthesis (haemoglobin subunits; *HBA1*, *HBBA*, and *HBM*) and iron storage (ferritin heavy chain; *FTH1*) were identified (Table 1), suggesting the anaemic status of the infested chickens, as previously reported [11], and compensatory erythropoiesis. Regarding stress responses, the gene encoding the heat-shock-related 70-kDa protein 2 (*HSPA2*) was also found to be highly expressed (Table 1), which may reflect the stress induced by PRM infestation [11]. To clarify whether the expression of these genes was upregulated in response to PRM infestation, the mRNA levels were examined and compared in blood samples obtained from chickens housed in PRM-contaminated and clean farms. Blood samples were collected in heparinised tubes from non-infested chickens raised at the Field Science Center for Northern Biosphere, Hokkaido University (non-infested experimental [NIE] chickens) and from a PRM-contaminated egg-laying farm in Japan (infested chickens). Blood samples were transported to the laboratory, and plasma and peripheral blood mononuclear cells (PBMCs) were isolated within 2 days of blood collection. PBMCs were isolated by density gradient centrifugation using Percoll solution (GE Healthcare UK Ltd., Little Chalfont, UK). Total RNA was extracted from isolated PBMCs using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer instructions. The primers used for the analyses are listed in Supplementary table 1, and quantitative

78 PCR (qPCR) was performed as previously described [14]. *β-actin* was amplified as an internal
79 control [14]. The mRNA expression of *HBA1* and *HBBA* was significantly higher in infested
80 chickens than in NIE chickens. In addition, *HSPA*2 and *FTH1*expression in infested chickens tended
81 to be higher than that in NIE chickens, although no statistical significance was observed (Fig. 1).
82 These results further indicate that infestation by PRMs induces anaemic and stress responses in
83 chickens.

84 Concerning molecules related to host immune reactions, high expression of *CCL4*, encoding C-C
85 chemokine ligand 4, which is involved in the recruitment of immune cells including monocytes [15],
86 and *B2M*, encoding a light chain of the major histocompatibility complex, β2 microglobulin [16],
87 was also observed (Table 1). Although the transcripts of other cytokines and chemokines were also
88 detected, their fragments per kilobase of exon per million reads mapped (FPKM) values were lower
89 than the limit of quantification (FPKM = 0; Supplementary table 2). Thus, in addition to the
90 parameters indicating anaemia and stress responses, the expression of *CCL4* and *B2M* among
91 immune-related molecules seemed to be particularly elevated in the peripheral blood of PRM-
92 infested chickens.

93 Next, the mRNA and protein levels of *CCL4* and *B2M* were assessed and compared in blood
94 samples obtained from NIE chickens and infested chickens. qPCR was performed using the primers
95 listed in Supplementary table 1. The expression of *CCL4* in infested chickens was significantly

96 higher than that in NIE chickens (Fig. 2A), suggesting upregulated *CCL4* expression in response to
97 PRM infestation. Additionally, *B2M* expression tended to be upregulated in infested chickens,
98 although no significant differences were observed (Fig. 2A). Next, protein levels of CCL4 and B2M
99 were assessed. Plasma was isolated by centrifugation from the blood collected from NIE and
100 infested chickens, and the concentrations of CCL4 and B2M were measured by enzyme-linked
101 immunosorbent assay (ELISA) using commercial kits, a chicken CCL4 Do-It-Yourself ELISA kit
102 (Kingfisher Biotech Inc., Saint Paul, MN, USA) and a chicken B2M ELISA kit (LSBio, Seattle,
103 WA, USA), respectively, according to manufacturer instructions. NIE chickens used in this study
104 were not vaccinated, whereas chickens from commercial farms were vaccinated to prevent several
105 infectious diseases (such as Marek's disease, avian infectious bronchitis, Newcastle disease,
106 mycoplasmosis, and fowl pox, among others). Therefore, to determine the influence of other
107 extrinsic factors including vaccination, serum samples were obtained from a PRM-free commercial
108 egg-laying farm in Japan (non-infested commercial (NIC) chickens) and also subjected to the
109 analysis. Consistent with the results of gene expression analyses, significantly higher concentrations
110 of CCL4 and B2M were detected in the plasma of infested chickens than that in NIE and NIC
111 chickens, with CCL4 being undetectable in NIE chickens (Fig. 2B). These findings confirmed the
112 increased levels of CCL4 and B2M in peripheral blood from PRM-infested chickens. To further
113 confirm that PRM infestation upregulates the expressions of CCL4 and B2M, blood samples were

collected from the same flock from another poultry farm in Japan before and after the transfer from the breeding farm to the production farm, and these samples were designated as b.e. (before exposure to PRMs) and p.e. (post-exposure to PRMs), respectively, and the concentrations of CCL4 and B2M in the b.e. and p.e. plasma samples were also examined. The concentrations of CCL4 and B2M were significantly higher in p.e. samples than those in b.e. samples (Fig. 2C). Collectively, CCL4 and B2M expression seemed to be upregulated by PRM-infestation.

CCL4 is produced by various cell types including epithelial cells, platelets, and lymphocytes, and recruits immune cells, such as natural killer cells, T cells, and monocytes, to mount the inflammatory response [13, 14]. B2M, in turn, was reported to act as an initiator of inflammation [17]. Therefore, our data suggest that blood sucking by PRMs induces an inflammatory response at the sites of infestation. The upregulation of both CCL4 and B2M might be stimulated by mechanical skin injury caused by the penetration of the mouthparts of PRMs, as reported in tick infestation [5]. However, the existence of other ectoparasites, including the NFMs, could induce similar reactions. The PRM-contaminated farm included in this study did not have other haematophagous ectoparasites detectable by eye, such as ticks or lice, except for small mites. It is noteworthy that among the small mites present across poultry houses, PRMs are the predominant small mite species prevalent in Japan; the prevalence of NFMs has also been reported across poultry farms in Japan. Moreover, the majority of small mites identified herein based cytochrome c oxidase subunit 1 (*COX1*) gene

sequencing in the contaminated farm were confirmed to be PRMs [18]. Moreover, this study demonstrated that in chickens from the same flock, plasma CCL4 and B2M concentrations were higher in samples post-PRM exposure compared to those before exposure, thereby confirming that RPM infestation could induce the upregulation of CCL4 and B2M in the peripheral blood of infested chickens. In addition, in the present study, blood samples of infested chickens from a PRM-contaminated farm were collected on a monthly basis for 2 years; this showed that chickens raised at farms heavily contaminated with PRMs might present with the chronic upregulation of CCL4 and B2M.

In summary, these RNA-Seq data suggest that PRM infestation induces anaemia, promotes erythropoiesis, and triggers stress-induced and immune responses in chickens. Among the immune-related molecules, CCL4 and B2M mRNA and protein levels were elevated in PRM-infested chickens. Thus, our RNA-Seq data could be a clue to understand the molecular mechanisms of pathogenesis and the modulation of host immunity caused by PRM infestation. However, despite the higher expression of these mediators of inflammation, infested chickens tested in this study did not show severe skin lesions or dermatitis. Moreover, PRM-contaminated farms were threatened by PRMs for more than a 2-year period, implying that PRMs are capable of suppressing the host inflammatory responses. Several studies demonstrated that haematophagous parasites, including ticks, secrete various effector molecules in their saliva to modulate the host physiological states,

especially immune responses including inflammation, thereby contributing to the maintenance and transmission of the pathogen [6, 19]. Therefore, PRMs might also secrete immunosuppressants in their saliva to maintain undisturbed blood feeding. Further experiments, such as an analysis of inflammatory responses in skin samples of infested chickens, are required to elucidate the complex immune reactions in chickens triggered by PRM infestation.

Ethics statement

Blood samples used in this study were collected upon obtaining informed consent from farm owners. Sample collection was carried out in accordance with the Procedures and Guidelines ruled by the Ethics Committee of the Faculty of Veterinary Medicine, Hokkaido University.

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CRediT authorship contribution statement

Sotaro Fujisawa: Methodology, Investigation, Writing - original draft, Funding acquisition. **Shiro Murata:** Methodology, Investigation, Writing - review & editing, Resources, Project administration, Funding acquisition. **Takumi Sato:** Investigation, Resources, Writing - review & editing. **Eiji Oishi:** Investigation, Resources, Writing - review & editing. **Akira Taneno:** Resources, Writing - review & editing. **Satoru Konnai:** Resources, Writing - review & editing. **Kazuhiko Ohashi:** Resources, Writing - review & editing, Funding acquisition.

Declaration of interest

TS, EO, and AT are employed by Vaxxinova Japan K.K, Tokyo, Japan. The other authors have no conflicts of interest to declare.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Appendix A. Supplemental data

The following are the supplemental data to this article:

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Table 1. Top 30 chicken-derived transcripts in blood-fed poultry red mites (PRMs).

Rank	Gene ID	Gene name	FPKM value
1	*HBA1*	Haemoglobin subunit alpha 1	6679.91
2	*HBBA*	Haemoglobin beta, subunit A	2055.28
3	*ATP6*	ATP synthase F0 subunit 6	1308.59
4	*COII*	Cytochrome c oxidase subunit 2	1017.79
5	*GABARAP*	GABA type A receptor-associated protein	788.364
6	*FTH1*	Ferritin heavy chain 1	770.677
7	*CCL4*	C-C motif chemokine ligand 4	514.071
8	*ND1*	NADH dehydrogenase subunit 1	482.681
9	*COX3*	Cytochrome c oxidase subunit 3	441.171
10	*B2M*	Beta-2-microgloblin	372.985
11	*COX7B*	Cytochrome c oxidase subunit 7B	341
12	*HBM*	Haemoglobin subunit alpha-D	332.525
13	*LAMTOR5*	Late endosomal/lysosomal adaptor and MAPK and MTOR activator 5	331.576
14	*BF1*	MHC class I alpha chain 1	308.629
15	*ITM2B*	Integral membrane protein 2B	302.773
16	*COX1*	Cytochrome c oxidase subunit 1	294.16
17	*C26H6orf106*	Uncharacterized	284.683
18	*RHOA*	RhoA	270.931
19	*PEX16*	Peroxisomal membrane protein PEX16	250.568
20	*UBB*	Polyubiquitin-B	236.877
21	*CHURC1*	Protein Churchill	231.939
22	*CTNNBIP1*	ICAT domain-containing protein	204.974
23	*NFYB*	Nuclear transcription factor Y subunit beta	185.559
24	*C1D*	Nuclear nucleic acid-binding protein C1D	174.626
25	*EIF5*	Eukaryotic translation initiation factor 5	159.976
26	*EIF1*	Eukaryotic translation initiation factor 1	130.353
27	*WDFY2*	WD repeat and FYVE domain-containing protein 2	120.008
28	*KLHL28*	Kelch-like protein 28	119.365
29	*LSM14A*	LSM14A mRNA processing body assembly factor	111.907
30	*HSPA2*	Heat shock-related 70-kDa protein 2	107.311

Spliceosomal and ribosomal RNA genes are not included.

Figure captions

Figure 1. Gene expression of anaemia- and stress-related molecules in poultry red mite (PRM)-non-infested and infested chickens.

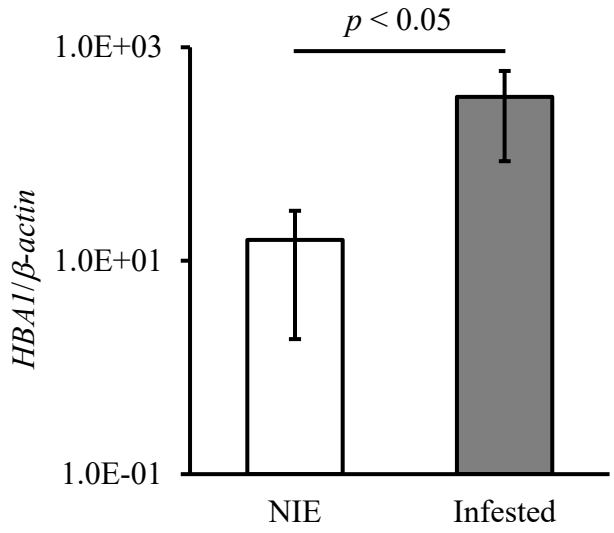
The mRNA expression of *HBA1*, *HBBA*, *FTH1*, and *HSPA2* in peripheral blood mononuclear cells collected from non-infested experimental (NIE) chickens raised at a PRM-free farm (n =11) and infested chickens from a PRM-contaminated commercial laying hen farm (n = 12) were examined by quantitative real-time polymerase chain reaction. To generate standard curves for quantification, serial dilutions of the plasmids harbouring the sequences for *HBA1*, *HBBA*, *FTH1*, *HSPA2*, and *β-actin* were used. The expression of each target was presented as the ratios obtained by dividing the concentrations of target mRNA by those of *β-actin* mRNA. Data are represented as the mean ± standard error of the mean. Statistical significance was determined by the Mann-Whitney *U* test.

Figure 2. CCL4 and B2M levels in poultry red mite (PRM)-non-infested and infested chickens.

(A) The mRNA expression of *CCL4* and *B2M* in peripheral blood mononuclear cells collected from non-infested experimental (NIE) chickens raised at a PRM-free farm (n = 11) and infested chickens from a PRM-contaminated commercial laying hen's farm (n = 16) were examined by quantitative real-time polymerase chain reaction. To generate standard curves for quantification, serial dilutions of the plasmids harbouring the sequences for *CCL4*, *B2M*, or *β-actin* were used. *CCL4* and *B2M*

mRNA expression levels were presented as the ratios obtained by dividing the concentrations of *CCL4* or *B2M* mRNA by those of *β-actin* mRNA. Data are represented as mean ± standard error of the mean. Statistical significance was determined by the Mann-Whitney *U* test. (B) The concentrations of CCL4 and B2M in the plasma of NIE chickens, non-infested commercial (NIC) chickens raised at a PRM-free laying hen farm, and infested chickens from a PRM-contaminated commercial laying hen farm were determined by enzyme-linked immunosorbent assay. CCL4 analysed samples: n = 13, 8, and 14 from NIE, NIC, and infested chickens, respectively. B2M analysed samples: n = 5, 8, and 9 from NIE, NIC, and infested chickens, respectively. Statistical differences were determined by Mann-Whitney *U* test (CCL4) and Steel-Dwass test (B2M). White circles indicate samples with values lower than the limit of quantification. N.D.: Not detected. (C) Concentrations of CCL4 and B2M in plasma samples collected from chickens before transfer to productive farm (before exposure to PRMs, n = 8) and infested chickens after transfer to productive farm (post-exposure to PRMs, n = 8) were determined by enzyme-linked immunosorbent assay. Statistical significance was determined by the Mann-Whitney *U* test. White circles indicate samples that were lower than the limit of quantification.

Figure 1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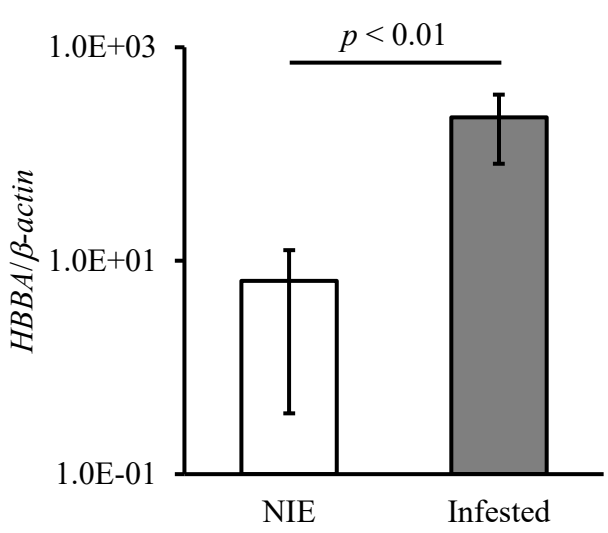
1.0E+03
1.0E+01
1.0E-01
HBA1/β-actin
NIE
Infested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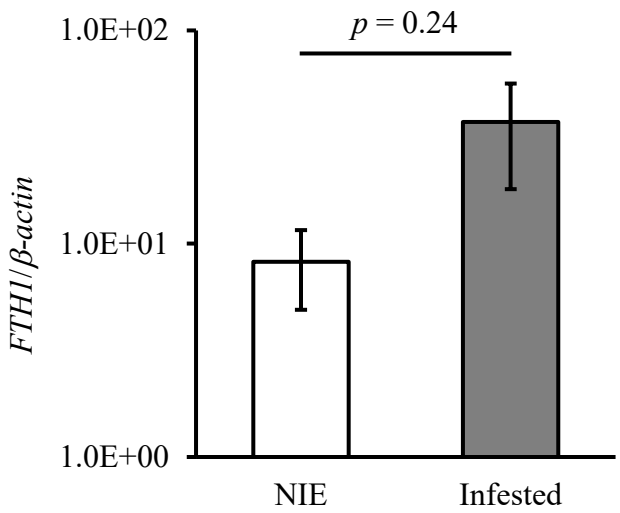
1.0E+03
1.0E+01
1.0E-01
HBBA/β-actin
NIE
Infested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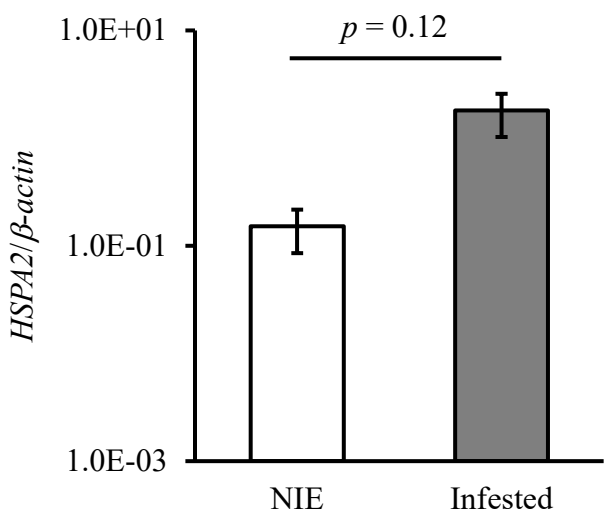
FTH1/β-actin
NIE
Infested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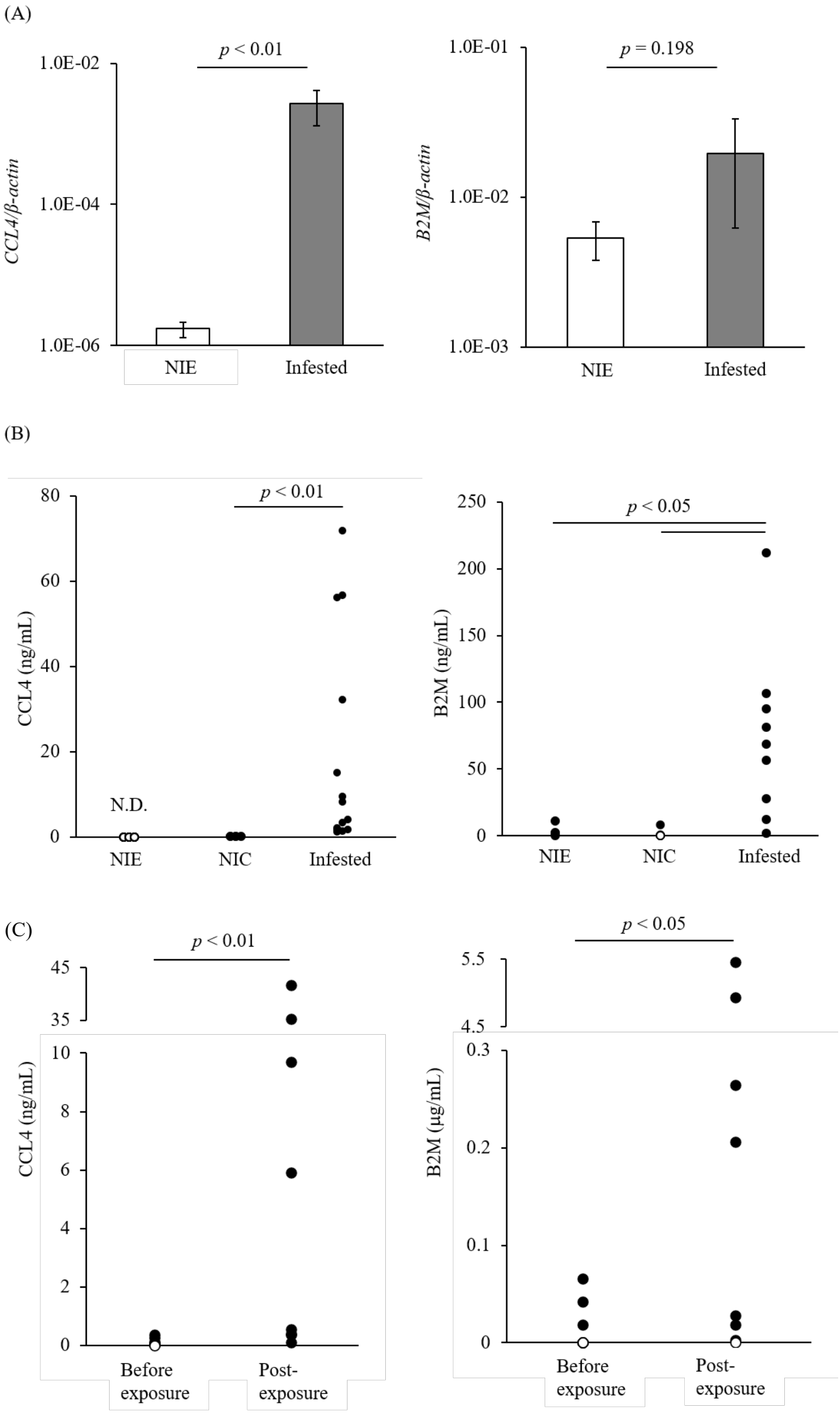
1.0E-01
1.0E-03
HSPA2/β-actin
NIE
Infested

Figure 2


(A)
p < 0.01
1.0E-02
1.0E-04
1.0E-06
CCL4/β-actin
NIE
Infested
p = 0.198
1.0E-01
1.0E-02
1.0E-03
B2M/β-actin
NIE
Infested
(B)
p < 0.01
80
60
40
20
0
CCL4 (ng/mL)
N.D.
NIE
NIC
Infested
p < 0.05
250
200
150
100
50
0
B2M (ng/mL)
NIE
NIC
Infested
(C)
p < 0.01
45
35
10
8
6
4
2
0
CCL4 (ng/mL)
Before exposure
Post-exposure
p < 0.05
5.5
4.5
0.3
0.2
0.1
0
B2M (μg/mL)
Before exposure
Post-exposure

Highlights

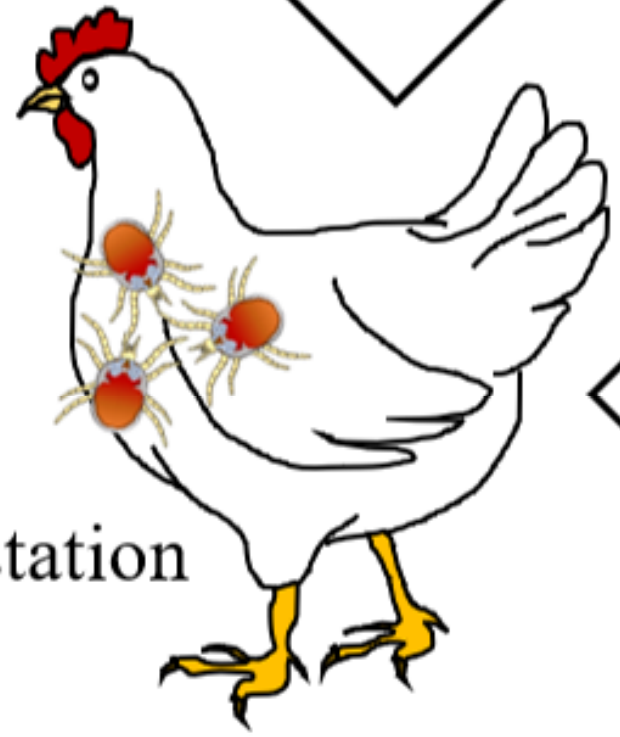
- Host reactions in peripheral blood to PRM-infestation were analyzed via RNA-seq.
- Chicken infestation with PRMs might promote erythropoiesis as an anemic response.
- PRM infestation seemed to induce stress-responses in chickens.
- The expression of CCL4 and B2M was significantly higher in PRM-infested chickens.
- We provided a clue to elucidate the complex host responses to PRM infestation.

Abstract

Among haematophagous ectoparasites that infest chickens, poultry red mite (*Dermanyssus gallinae*, PRM) is one of the most serious threats to poultry farms. Mass PRM infestation causes various health problems in chickens, resulting in significant productivity reduction in the poultry industry. Despite the efficiency of acaricides for controlling PRMs, the emergence of acaricide-resistant PRMs represents a challenging setback. Infestation with haematophagous ectoparasites, such as PRMs, induces inflammatory and haemostatic reactions in the host. Therefore, we aimed to explore the gene expression in chicken peripheral blood cells to elucidate host responses against PRM infestation in detail. RNA sequencing of blood-fed PRMs was performed, and the levels of the chicken-derived transcripts obtained from the ingested blood cells were analysed. Genes encoding haemoglobin subunits were found to be significantly more expressed, suggesting that PRM infestation causes anaemia in chickens. Additionally, the mRNA and plasma concentrations of C–C chemokine ligand 4 and β2 microglobulin among the immune-related molecules were found to be significantly higher in PRM-infested chickens compared with non-infested animals. These results suggest that PRM infestation induce inflammation in chicken. Further studies are warranted to better understand the influence of PRM infestation on the host physiological states, including immunity.

Keywords: chicken, *Dermanyssus gallinae*, ectoparasite, gene expression, immunity, poultry red mite, RNA sequencing.

- Erythropoiesis
  (Hemoglobin subunits ↑)
- Iron storage
  (FTH1 ↑)
- Stress response
  (HSPA2 ↑)



PRM infestation

· Modulation of immunity

- Chemotaxis of immune cells
  (CCL4 ↑)
- Initiation of inflammation
  (B2M ↑)