

# Serum and acute phase protein changes in laying hens, infested with poultry red mite

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**ABSTRACT** The poultry red mite (PRM) is one of the most economically important ectoparasites of laying hens globally. This mite can have significant deleterious effects on its fowl host including distress, anemia, reduced egg production, and reduced egg quality. This study was conducted to evaluate the influence of PRM on the serum protein profile in laying hens and its effect on the acute phase proteins (APPs) to assess their potential as biomarkers for mite infestation.

Three APPs: alpha-1 acid glycoprotein (AGP), serum amyloid-A (SAA), and ceruloplasmin (CP) were measured in serum samples collected from laying hens at 12 and 17 wk of age, and then for up to 4 mo after a challenge with PRM (starting at 18.5 wk of age). The serum protein profile (SDS-PAGE/nanoflow HPLC electrospray tandem mass spectrometry) and concentration of individual serum proteins (SDS-PAGE-band densitometry) were also compared.

Post challenge there was a positive correlation ( $r = 0.489$ ;  $P < 0.004$ ) between the levels of SAA and the PRM numbers. The levels of SAA steadily increased after the PRM challenge and were significantly different than the pre-challenge levels at 28, 32, and 36 wk of age ( $P < 0.01$ ). The PRM numbers also peaked around 31-33 wk of age. The results for AGP and CP in comparison were inconsistent. Proteomics revealed the presence of 2 high molecular weight proteins in the serum between 12 and 17 wk of age. These were identified as Apolipoprotein-B and Vitellogenin-2, and their increase was commensurate with the onset of lay. No other major differences were detected in the protein profiles of blood sera collected pre and post challenge. We conclude that SAA could be used as a useful biomarker to monitor PRM infestation in commercial poultry flocks and that PRM infestation does not disrupt the production of the major proteins in the serum that are associated with egg formation.

**Key words:** acute phase proteins, poultry red mite, *Dermanyssus gallinae*, proteomics, laying hen

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## INTRODUCTION

One of the most important ectoparasites affecting the global poultry industry is the poultry red mite (PRM) (*Dermanyssus gallinae*) which is a haematophagous ectoparasite of domestic poultry and wild birds (Kristofik et al., 1996; Brannstrom et al., 2008). PRM reside off-host in the structure and furniture of poultry housing and emerge in darkness to feed on the hens every 2-4 d (Maurer et al., 1988). Serious health problems appeared when the PRM infestation level reaches 150,000 to 200,000 mites per bird (Kilpinen, 2005). PRM causes a significant deleterious effect on its avian host such as a high level of psychogenic stress (Kowalski and Sokol,

2009), decrease in egg production, anemia, blood staining of eggs, and an increase in mortality rate (Chauve, 1998; Kilpinen, 2005). Traditionally, PRM control often relies upon synthetic acaricide spraying of poultry houses and equipment; however, resistance against these compounds has been reported to limit efficacy (Beugnet et al., 1997; Sparagano et al., 2014). The number of acaricides licensed for PRM treatment is severely limited in the European Union (Flochlay et al., 2017), but there is evidence of illegal use of banned acaricides (Marangi et al., 2012), including detection of Fipronil residues in eggs from laying flocks in several EU countries (Anonymus, 2017).

The use of acute phase proteins (APPs), as physiological markers for assessment of overall health and welfare, has potential for diagnosis and prognosis in veterinary medicine (Ceron et al., 2005; Cray et al., 2009; Ceciliani et al., 2012), thus allowing the more rational and targeted use of drugs and treatments. APPs

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are classified into positive and negative according to their response increasing or decreasing after stimulation respectively (Chamanza et al., 1999). The positive type can be sub-divided into major, moderate, and minor protein classes depending on their response magnitude (Ceron et al., 2005). Alpha-1 acid glycoprotein (AGP) is a moderate positive APP in most species. The main function of AGP is to act as a natural anti-inflammatory factor inhibiting neutrophil activation and increasing the secretion of IL-1 receptor antagonists by macrophages (Murata et al., 2004). This has a clearance function in removing bacterial lipopolysaccharide by binding and neutralizing its toxicity (Murata et al., 2004) and it also acts as a plasma transport protein (Cecilian and Pocacqua, 2007). AGP is one of the most widely measured APPs in chickens owing to the commercial availability of easy to use and chicken-specific ELISA kits (O'Reilly and Eckersall, 2014; Peebles et al., 2014; Koppenol et al., 2015). Another APP with known beneficial biological functions is Ceruloplasmin (CP). Like AGP, CP is a moderate positive APP in chickens (Georgieva et al., 2010). The main function of CP is to transfer and store copper within the body, and it also has anti-oxidative functions (Floris et al., 2000). *In vivo* studies have shown that CP has the ability to oxidize the Fe<sup>2+</sup> ion, preventing the iron inducing oxidation effect (Floris et al., 2000). Serum amyloid-A (SAA) is a major positive APP in chickens (Alasonyalilar et al., 2006). The main function of SAA is to modify the transport and metabolism of lipoprotein in the acute phase response (APR) including, specifically, cholesterol transport to damaged tissues. This is essential for repair and reconstruction of cell membranes and to clear lipid debris formed by bacteria and damaged tissues (O'Reilly and Eckersall, 2014). SAA also has a modulatory role to inhibit pyrexia and pro-inflammatory events during the APR (Shainkin-kestenbaum et al., 1991; Uhlar and Whitehead, 1999). In this study, we investigated if the levels of AGP, CP, and SAA change in response to a PRM infestation in laying hens.

In addition to APPs, there has been growing interest in the use of advanced proteomic analysis to assess changes in the serum proteome of poultry and other livestock in order to identify additional biomarkers of disease (Almeida et al., 2015). In commercial layers, the onset of lay requires a major change in the physiology of the hen, with nutrients such as lipid and protein being required in copious amounts for egg production. With the exception of immunoglobins, synthesis of most of the major egg yolk proteins (Vitellogenins and VLDLs) takes place in the liver predominantly under the regulation of estrogen and to a lesser extent androgens. These egg yolk precursors are then transported in the blood to the ovary where they are further processed to phosvitin, lipovitellin, triglycerides, cholesterol, and phospholipids within the developing oocyte (Johnson 2015). To date, there have been few investigations of the alteration this causes in the serum proteome of hens as

they approach sexual maturity. Liou et al. (2007), however, did report that Vitellogenin and Apolipoprotein-A1 changed dramatically at peak egg production relative to initial egg production and went on to demonstrate an association between these proteins and egg production in different groups of Taiwan red-feathered country chickens. In the current study, we wanted to evaluate if a PRM challenge disrupts the transport of these and other major proteins in the blood serum required for egg production.

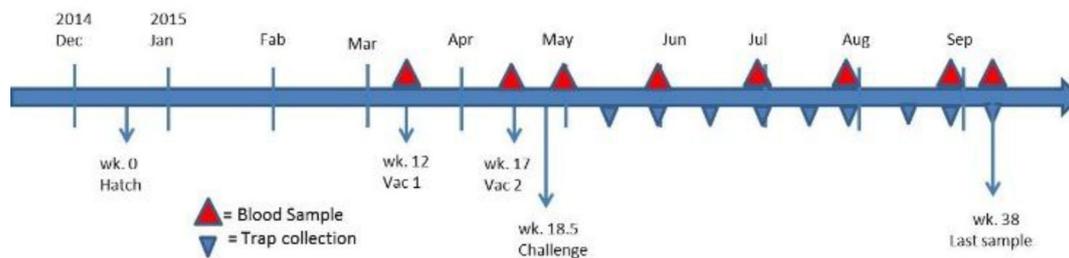
The aims of this study were therefore (i) to investigate the APP response over the time course of an experimental PRM infestation in laying hens, (ii) to demonstrate the major changes in serum proteome following the onset of lay, and (iii) to evaluate if a PRM infestation significantly alters the serum protein profile of laying hens.

## MATERIALS AND METHODS

### Treatment and Sampling

This study was performed using additional data collected from the control hens which were part of a vaccine field trial carried out by the Moredun Research Institute (Bartley et al., 2017). The hens were Lohmann Brown Hens (n = 384) and as the "controls", each hen had received 2 injections of a 0.5 mL dose of a vaccine placebo of Montanide ISA70VG adjuvant (SEPPIC, Terrassa Bellini, Paris) formulated in 7:3 ratio with 10 mM Tris-HCl; 0.5 M NaCl, pH 7.4 at both 12 and 17 wk of age. At the time of the 2nd placebo vaccine injection (week 17), the birds were placed in cages, with 4 birds per cage. At 18.5 wk of age, all 96 cages were subsequently infested with PRM (10,000 live mites per cage). This infestation rate was used so that the experimental cages were not immediately overwhelmed with mites (Bartley et al., 2017). Every 2 wk for 4.5 mo post challenge (Figure 1), the PRM populations were estimated using a trapping and counting procedure (Bartley et al., 2017). Plastic ADAS Mite Monitor traps (ADAS Ltd, Oxon, UK) were fixed to the egg collection tray at the front of each cage. After 24 h, the mites in the traps were collected into individually marked plastic containers containing 70% (v/v) ethanol and counted.

Blood samples were collected at (i) placebo vaccination 1 (pre-PRM challenge, week 12); (ii) placebo vaccination 2 (pre-PRM challenge, week 17); (iii) 5 d post PRM challenge (week 19); (iv) at times which coincided with alternate PRM trap evaluations viz. Weeks 23, 27, 31, 36 and 38 post PRM challenge (Figure 1). At each sampling time point, blood was collected from 8 randomly selected birds by bleeding directly from the wing vein into a non-heparinised tube. The blood (1 mL) was then allowed to clot at 4°C for 24 h and the sera were obtained following centrifugation at 3,000 g for 10 min. Sera were stored at -20°C, until required for further analysis.



**Figure 1.** Study timeline. The timeline shows when placebo vaccinations were administered and when the poultry red mite (PRM) challenge took place. The timings of PRM Trap counts (below the line) and serum collections (above the line) are also indicated.

The PRM experiment was performed under the terms and conditions of UK Home Office licenses (PPL 60/4324), and the experimental design was ratified by the ethics committee of the Moredun Research Institute.

### ELISA Assay

The chicken-specific ELISA assays for AGP, SAA, and CP were obtained from Life Diagnostics Inc, (West Chester, USA). Each assay was performed according to the manufacturer's instructions using conditions previously validated (Kaab et al., 2017). Serum was diluted 1:10000 for AGP, 1:20 for SAA, and 1:40000 for CP. Each individual serum sample ( $n = 8$ ) from each sampling point ( $n = 8$ ) was analyzed in duplicate.

### Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis

For each of the 8 sampling times, equal aliquots of sera ( $n = 8$ ) were pooled for SDS-PAGE using the method described in Thomas et al. (2016).

### Nanoflow HPLC Electrospray Tandem Mass Spectrometry

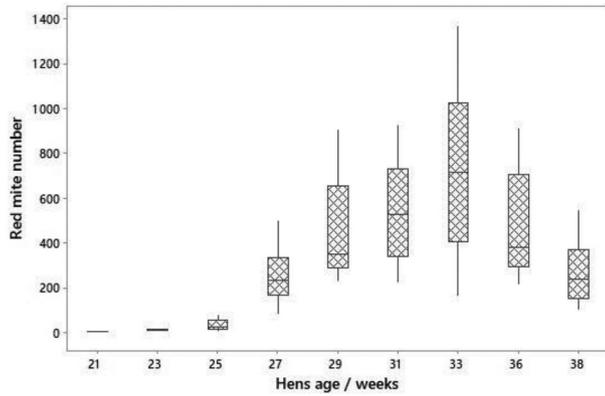
Eleven selected protein bands were excised manually by scalpel and placed in individual vials to be subjected to in-gel digestion for protein extraction prior to identification via mass spectrometry analysis. Gel pieces were washed with 100 mM ammonium bicarbonate for 30 min and then for a further hour with 100 mM ammonium bicarbonate in 50% (v/v) acetonitrile. After each wash, all solvent was discarded. Gel plugs were then dehydrated with 100% acetonitrile for 10 min prior to solvent being removed and dried completely by vacuum centrifugation. Dry gel pieces were then rehydrated with 10  $\mu$ L trypsin at a concentration of 20 ng/ $\mu$ L in 25 mM ammonium bicarbonate (Cat No. V5111, Promega, Madison, WI, USA) and proteins allowed to digest overnight at 37°C. This liquid was transferred to a fresh tube (first extract), and gel pieces washed for 10 min with 10  $\mu$ L of 50% acetonitrile. This wash was pooled with the first extract, and the tryptic peptides dried.

Proteins were identified using nanoflow HPLC electrospray tandem mass spectrometry at Glasgow Polyomics. Peptides were solubilized in 2% acetonitrile with 0.1% trifluoroacetic acid and fractionated on a nanoflow uHPLC system (Thermo Scientific RSLCnano) before analysis by electrospray ionization (Collgros et al., 2013) mass spectrometry on an Amazon Speed ion trap MS/MS (Bruker Daltonics). Peptide separation was performed on a Pepmap C18 reversed phase column (Thermo Scientific). Peptides were desalted and concentrated for 4 min on a C18 trap column followed by an acetonitrile gradient (in 0.1% v/v formic acid) (3.2-32% v/v 4-27 min, 32-80% v/v 27-36 min, held at 80% v/v 36-41 min and re-equilibrated at 3.2%) for a total time of 45 min. A fixed solvent flow rate of 0.3  $\mu$ L/min was used for the analytical column. The trap column solvent flow was fixed at 25  $\mu$ L/min using 2% acetonitrile with 0.1% v/v trifluoroacetic acid. Mass spectrometric (MS) analysis was performed using a continuous duty cycle of survey MS scan followed by up to 10 MS/MS analyses of the most abundant peptides, choosing the most intense multiply charged ions with dynamic exclusion for 120 s and with the FDR set at 0.01.

MS data were processed using Data Analysis software (Bruker) and the automated Matrix Science Mascot Daemon server. Protein identifications were assigned using the Mascot search engine to interrogate protein sequences in the NCBI Genbank database, allowing a mass tolerance of 0.4 Da for both MS and MS/MS analyses and with the Mascot score set off as 100.

### Protein Band Densitometry Measurements

To determine the protein concentration in each electrophoretic band of interest and to account for individual bird variation, serum samples from 4 individual birds at each time point were run on 1D SDS-PAGE gels. The concentration of each band was then estimated by protein band densitometry using ImageJ software (<https://imagej.nih.gov/ij/>), and expressed as a mean percentage of the total lane protein. The mean percentage for each sample ( $n = 4$  birds) were then converted to mg/mL by calculating the quantity of the band of interest as a proportion of the density of the



**Figure 2.** Poultry red mite (PRM) trap count data. PRM numbers recorded in traps ( $n = 92$ ) at consecutive sampling time points following a PRM challenge with 10,000 PRM at 18.5 wk of age. The highest level of PRM infestation was observed at 31-33 wk of age which corresponds to late July and early August. Data presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles.

whole lane, where 100% equals 2 mg/mL (the original concentration of the loaded samples).

### Statistical Analysis

A Mann-Whitney Test (Minitab 17.1.0) was used to examine the effect of PRM infestation on each APP by comparing serum samples taken prior to the PRM challenge (weeks 12 and 17) with those taken following the challenge (weeks 19, 23, 27, 31, 36, and 38).

Spearman's correlation was used to determine the relationship between levels of PRM infestation on specific APPs levels (GraphPad Prism v.5 software). All statements of significance are based on testing at  $P \leq 0.05$ .

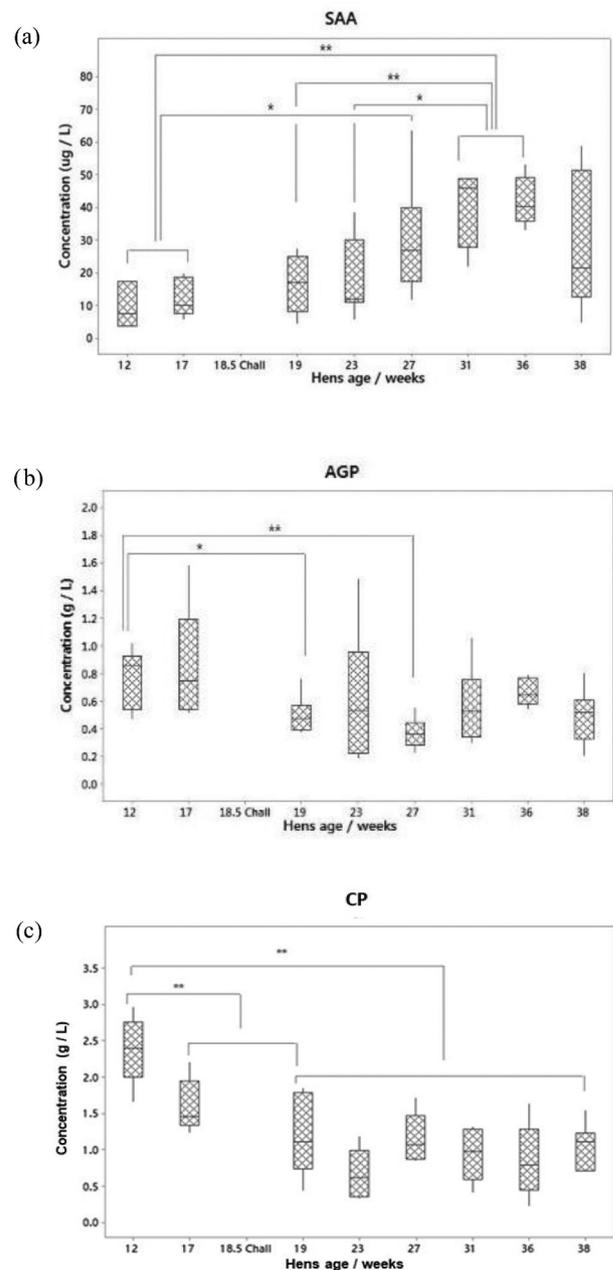
## RESULTS

### PRM Infestation Levels

The mean number of PRM per trap is presented in Figure 2. Low numbers of PRM were recorded in the first traps at week 21 and 23. Following this initial lag period, the PRM population expanded and the number of PRMs caught in the traps increased sharply and peaked in weeks 31-33 (Figure 2). Following the peak, the PRM numbers decreased in weeks 36 (late August) and 38 (September).

### Serum Concentration of APPs and their Relationship to PRM Infestation Rates

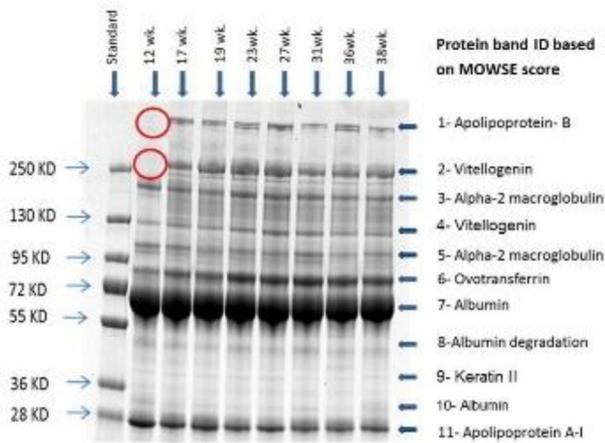
**Serum Amyloid-A.** There was a significant increase in the concentration of SAA in serum obtained post infestation at 27 ( $P < 0.05$ ), 31 and 36 weeks ( $P < 0.01$ ) compared with pre-infestation (weeks 12 and 17) levels (Figure 3a). The pattern of change in the mean SAA levels closely mirrored the mean PRM levels of



**Figure 3.** Acute phase proteins pre and post poultry red mite (PRM) challenge. SAA (a), AGP (b), and CP (c) levels in blood serum pre and post PRM challenge at 18.5 wk of age. Data presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles. Horizontal bars indicate statistical differences between groups (\*\* =  $P \leq 0.01$ , \* =  $P \leq 0.05$ ).

infestation (Figure 2). To investigate this relationship further, data where the PRM infestation levels could be directly matched to a blood sample from the same cage were identified and analyzed using Spearman's correlation. This revealed a moderate positive correlation ( $r = 0.489$ ;  $P < 0.004$ ) between SAA levels and the numbers of PRM.

**Alpha-1 Acid Glycoprotein.** There was no consistent change in the serum AGP levels pre and post PRM challenge. Levels of serum AGP prior to infestation



**Figure 4.** SDS-PAGE of pooled serum pre and post poultry red mite challenge. Electrophoretic profiles of pooled serum samples corresponding to different sampling time points. Two high molecular weight bands are missing in the serum sampled at week 12 (red circles). Subtle differences in the density of the other protein bands can also be observed. The protein identity of each band based on the MOWSE score is indicated (further details are provided in Table 1 and in the supplementary data).

(week 12) were significantly higher than at week 19 ( $P < 0.05$ ) and 27 ( $P < 0.01$ ). AGP levels following the PRM challenge were not significantly different than that observed at 17 wk (Figure 3b). In addition, there was no correlation between the AGP concentration and PRM infestation levels ( $r = 0.257$ ;  $P < 0.6$ ).

**Ceruloplasmin.** Serum concentrations of CP at 12 wk were significantly higher ( $P < 0.05$ ) than at most time points post-infestation, with the exception of week 17 (also a pre-challenge time point) (Figure 3c). CP levels were not correlated with PRM infestation level ( $r = 0.328$ ;  $P < 0.3$ ).

### SDS-PAGE and Identification of the Major Serum Protein Components

SDS-PAGE separation of the pooled serum samples from all sampling points are compared in Figure 4 and the top 3 proteins in terms of MOWSE score for each band are given in Table 1 with full details of the proteomic analysis provided in the supplementary data. Two high molecular weight (MWt) bands of approximately 300 kDa (band 1) and 250 kDa (band 2) were absent in the 12-wk sample but present in all of the other samples. The 300 kDa protein (band 1) corresponded to *Gallus gallus* Apolipoprotein-B precursor (gi—113206052) with coverage 40%. The 250 kDa protein (band 2) corresponded to *Gallus gallus* Vitellogenin-2 (gi—71896765) with coverage of 66%. Bands 3 and 5 with MWt of ~200 and 100 kDa both corresponded to the same protein (Alpha-2 macroglobulin like protein). Ovotransferrin, Albumin, Apolipoprotein A-IV, and Apolipoprotein A-I were amongst the other proteins identified.

### Protein Densitometry Measurements

Figure 5 compares the relative concentration of proteins in band 1-11 between 12 and 17 wk of age for 4 individual hens selected at random at each time point. As well as there being a significant increase in Apolipoprotein-B (MWt ~300 kDa;  $P = 0.007$ ) and Vitellogenin-2 (MWt ~250 kDa;  $P = 0.007$ ), there was a significant decrease in Alpha-2 macroglobulin's (~200 kDa, band number 3;  $P = 0.007$ ) and ~100 kDa, band number 5;  $P = 0.007$ ) and Apolipoprotein A-1 (28 kDa;  $P = 0.003$ ) between 12 and 17 wk of age.

Protein densitometry measurements carried out on serum from 4 individual hens at each sampling time point pre and post PRM challenge revealed that Apolipoprotein-B levels increased 22.6-fold (from 0.008 to 0.181 mg/mL;  $P \leq 0.01$ ) and Vitellogenin-2 increased 100-fold (from 0.002 to 0.20 mg/mL;  $P \leq 0.01$ ) between 12 and 23 wk of age and remained high throughout the study period (Table 2). Meanwhile, Apolipoprotein A-I (band 11) decreased by 2.1-fold (from 0.193 to 0.09 mg/mL,  $P < 0.05$ ) at 23 wk compared to week 12. Other protein bands were also compared but no statistically significant differences were observed other than that for Alpha-2 macroglobulin (band 3 and 5) between 12 and 17 wk, as previously reported (data not presented).

### DISCUSSION

Infestation of laying hens with PRM resulted in an APP response which was characterized by SAA concentrations being 6.25-fold higher in the blood sera at 31 and 36 wk compared to week 17 pre-infestation levels ( $P < 0.01$ ). Mite infestation has been reported to cause a significant increase in SAA in other species. For example, experimental infestation of sheep with the sheep scab mite (*Psoroptes ovis*) led to a significant elevation of SAA ( $P \leq 0.001$ ) compared to pre-infestation levels (Wells et al., 2013). A similar SAA response has also been reported in the case of mange infestation (*Sarcoptes scabiei*) in the Alpine ibex (Rahman et al., 2010), with an SAA peak occurring at week 4-5 post-infestation. The serum concentration of SAA in the current study was moderately correlated to the PRM infestation levels ( $r = 0.489$ ;  $P < 0.004$ ), suggesting that exposure to PRM may trigger an APP response probably mediated by cytokines released by the cells of the innate immune system in response to the mites feeding (Kowalski and Sokol, 2009). However, less than 50% of the variation in PRM can be predicted from the relationship between SAA and PRM. This is not unexpected as there are many other factors which can affect the numbers of PRM including the environmental conditions (Sparagano et al., 2014), the feeding behavior of the mites and the acquired as well as innate immune responses of the host. APPs can also respond to increased corticosterone (Zulkifli et al., 2014), which has previously been shown to rise in response

**Table 1.** Proteomic identification of the proteins contained in bands 1-11 excised from a 1D-SDS-PAGE gel of pooled serum obtained from hens (n = 8) at 17 wk of age.

Band	Protein [species]	Accession number	Mass	MOWSE Score	emPAI	pI	No. Unique Peptides	Sequence Coverage (%)
1	<b>Apolipoprotein B</b> precursor [Gallus gallus]	<u>gi-113206052</u>	524519	3838	0.83	8.51	105	40%
	Vitellogenin [Gallus gallus]	<u>gi-212881</u>	206862	345	0.18	9.22	11	17%
	Apolipoprotein B	<u>gi-114013</u>	50873	230	0.43	8.22	6	32%
2	<b>Vitellogenin-2</b> precursor [Gallus gallus]	<u>gi-71896765</u>	206732	3299	1.53	9.23	57	51%
	<i>Vitellogenin-2-like, partial</i> [Gallus gallus]	<u>gi-971443652</u>	34967	1073	2.32	8.95	13	66%
	Vitellogenin-1 precursor [Gallus gallus]	<u>gi-52138705</u>	212608	791	0.47	9.16	27	23%
3	<b>Alha-2-macroglobulin</b> [Gallus gallus]	<u>gi-971373602</u>	131990	952	0.78	6.19	24	35%
	<i>Alpha-2-macroglobulin-like</i> [Gallus gallus]	<u>gi-971373692</u>	166154	884	0.5	5.56	19	20%
	Vitellogenin, [Gallus gallus]	<u>gi-212881</u>	206862	823	0.42	9.22	24	29%
4	<b>Vitellogenin-2</b> precursor [Gallus gallus]	<u>gi-71896765</u>	206732	785	0.42	9.23	22	28%
	Complement C3 precursor [Gallus gallus]	<u>gi-45382303</u>	185510	500	0.32	6.69	17	14%
	Vitellogenin-1 precursor [Gallus gallus]	<u>gi-52138705</u>	212608	182	0.11	9.16	7	12%
5	<b>Alha-2-macroglobulin-like protein 1</b> [Gallus gallus]	<u>gi-971434955</u>	164547	743	0.59	5.98	24	39%
	Vitellogenin [Gallus gallus]	<u>gi-212881</u>	206862	551	0.25	9.22	14	20%
	Vitellogenin, partial [Gallus gallus]	<u>gi-50582493</u>	163656	483	0.27	9.51	12	20%
6	<b>Chain A Ovotransferrin</b>	<u>gi-83754919</u>	77518	1338	1.88	6.7	25	69%
	Ovotransferrin Precursor	<u>gi-1351295</u>	79551	1338	1.8	6.85	25	63%
	Ovotransferrin BC type [Gallus gallus]	<u>gi-71274079</u>	79588	1302	1.7	7.08	24	66%
7	<b>Serum Albumin</b> precursor [Gallus gallus]	<u>gi-766944282</u>	71841	3889	6.56	5.51	36	87%
	Unnamed protein product, partial [Gallus gallus]	<u>gi-63524</u>	54462	422	0.56	6.84	8	31%
	Complement C3 precursor [Gallus gallus]	<u>gi-45382303</u>	185510	349	0.2	6.69	10	13%
8	<b>Serum albumin Precursor</b>	<u>gi-113575</u>	71868	593	0.46	5.51	8	48%
	Unnamed protein product, partial [Gallus gallus]	<u>gi-63524</u>	54462	273	0.32	6.84	5	20%
	Complement C3 precursor [Gallus gallus]	<u>gi-45382303</u>	185510	182	0.1	6.69	6	5%
9	<b>Keratin, type II cytoskeletal 8</b> [Gallus gallus]	<u>gi-971446176</u>	53214	292	0.19	5.36	3	11%
	Keratin 6A [Gallus gallus]	<u>gi-762207165</u>	57371	258	0.17	8.48	3	8%
	Serum albumin Precursor	<u>gi-113575</u>	71868	243	0.34	5.51	7	26%
10	<b>Serum albumin Precursor</b>	<u>gi-113575</u>	71868	357	0.66	5.51	11	40%
	<i>keratin, type II cytoskeletal 8</i> [Gallus gallus]	<u>gi-971446176</u>	53214	111	0.06	5.36	1	5%
11	<b>Apolipoprotein AI</b> [Gallus gallus]	<u>gi-227016</u>	28790	885	4.83	5.45	16	89%
	Apolipoprotein A-I [Gallus gallus]	<u>gi-211159</u>	30673	885	4.24	5.58	16	78%
	Immunoglobulin lambda chain [Gallus gallus]	<u>gi-1536804</u>	10883	192	0.69	4.72	2	38%

For each band the top three protein hits based on the MOlecular Weight SEArch (MOWSE) scores are presented. Characters in italics represent proteins predicted from known genomic sequences. (See supplementary data for the full proteomic data set.)

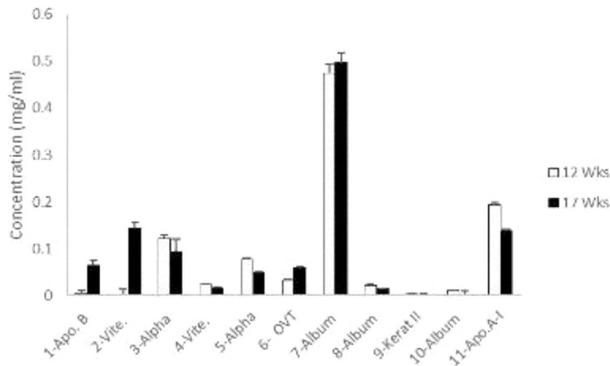
to PRM infestation in laying hens (Kowalski et al., 2006). Monitoring SAA levels could therefore be valuable as an aid to assessing the PRM infestation level and the welfare of laying hens in poultry farms and as a means to monitor the innate immune response mediated via pro-inflammatory cytokines (Baumann and Gauldie, 1994, Jensen and Whitehead 1998; O'Reilly and Eckersall 2014)

Neither AGP nor CP levels increased in response to the PRM infestation. The pro-inflammatory cytokine stimulation of the APR was therefore relatively moderate as it did not stimulate the production of these 2 APPs. In other species, SAA is one of the most sensitive major APPs to stimulation (Ceron et al., 2005; Ceciliani et al., 2012). Consequently, a similar immunological trend might be true for laying hens. In future studies, it would be useful to monitor the changes in pro-inflammatory cytokines such as interleukin 1 and interleukin 6, to confirm that only a moderate cytokine response had been stimulated.

The serum concentrations of AGP and CP fell in the early stages of our study (Figure 3). Such subtle

changes may reflect an age-related alteration in these 2 APPs; age-related differences in APPs have been reported in other species such as in pig (Christoffersen et al., 2015), but until now have not been reported in laying hens. The fall in CP between 12 and 19 wk of age, for example, may be associated with the onset of sexual maturity and oogenesis. Oocyte development requires the presence of various elements, including copper that binds specifically to egg proteins such as Ovotransferrin (Johnson, 2015). CP contains over 95% of the copper found in the plasma (Martínez-Subiela et al., 2007). The high demand for copper-associated follicle development at the onset of lay therefore provides a plausible explanation for the observed reduction in serum CP levels in our study.

Additional changes in the serum proteome were investigated in 4 individual samples from each time point using SDS-PAGE gel electrophoresis and protein densitometry. Both Apolipoprotein-B (MWt 300 kDa) and Vitellogenin-2 (MWt 250 kDa) were significantly increased by 17 wk and reached a peak by 23 wk which is just in advance of peak production for Lohmann



**Figure 5.** Protein band densitometry measurements at 12 and 17 wk. Mean  $\pm$  standard deviation protein band densitometry measurements (mg/ml) for  $n = 4$  individual hens at 12 and 17 wk of age. Significant differences were observed for Apolipoprotein-B (band 1-Apo-B;  $P = 0.007$ ), Vitellogenin-2 (band 2-Vite.;  $P = 0.007$ ), Alpha 2-macroglobulin (band 3-Alpha;  $P = 0.007$  and 5-Alpha;  $P = 0.007$ ), and Apolipoprotein A-I (band 11-Apo. A-I;  $P = 0.003$ ).

**Table 2.** Protein band densitometry measurements for bands 1, 2, and 11 pre and post PRM challenge.

Age (weeks)	Apolipoprotein B (band 1)	Vitellogenin-2 (band 2)	Apolipoprotein-A-1 (band 11)
12	0.008 $\pm$ 0.007 <sup>a</sup>	0.002 $\pm$ 0.003 <sup>a</sup>	0.193 $\pm$ 0.007 <sup>a</sup>
17	0.117 $\pm$ 0.031 <sup>b</sup>	0.143 $\pm$ 0.019 <sup>b</sup>	0.138 $\pm$ 0.015 <sup>b</sup>
19	0.115 $\pm$ 0.031 <sup>b,c</sup>	0.139 $\pm$ 0.031 <sup>b,c</sup>	0.115 $\pm$ 0.009 <sup>b</sup>
23	0.181 $\pm$ 0.025 <sup>c</sup>	0.201 $\pm$ 0.004 <sup>c</sup>	0.098 $\pm$ 0.010 <sup>b</sup>
27	0.166 $\pm$ 0.011 <sup>b,c</sup>	0.171 $\pm$ 0.007 <sup>b,c</sup>	0.111 $\pm$ 0.006 <sup>b</sup>
31	0.137 $\pm$ 0.028 <sup>b,c</sup>	0.141 $\pm$ 0.027 <sup>b,c</sup>	0.090 $\pm$ 0.036 <sup>b</sup>
36	0.132 $\pm$ 0.015 <sup>b,c</sup>	0.137 $\pm$ 0.016 <sup>b,c</sup>	0.087 $\pm$ 0.008 <sup>b</sup>
38	0.131 $\pm$ 0.017 <sup>b,c</sup>	0.143 $\pm$ 0.030 <sup>b,c</sup>	0.095 $\pm$ 0.009 <sup>b</sup>

Comparison of protein band densitometry measurements (mg/ml) for Apolipoprotein B, Vitellogenin-2, and Apolipoprotein-A-1 in serum sampled pre (12 and 17 wk) and post PRM challenge (19-38 wk).

<sup>a-c</sup>Mean  $\pm$  Standard deviation values within a column with different superscripts are significantly different ( $P \leq 0.05$ ).

brown layers. These results were obtained by densitometry of serum electrophoresis, a standard procedure in veterinary diagnostic laboratories (Eckersall, 2008), but which is not specific and it would be of value in future investigation to apply quantitative methods of greater specificity such as immunoassay or quantitative proteomics. However, the results obtained here indicate the large increases in Apolipoprotein-B and Vitellogenin-2 at week 17 and also the time of their maximum level in chicken serum. Both Apolipoprotein-B and Vitellogenin-2 are lipid-binding proteins, and are precursors of the major proteins of the yolk (Finn, 2007). At sexual maturation, and in response to rising levels of oestrogen, plasma levels of Vitellogenin have been shown to rise as production in the liver increases (Wallace, 1985; Johnson, 2015). In the ovary, Vitellogenin is taken up by the developing oocytes to form 2 lipovitellins, phosvitin by proteolytic cleavage mediated by Cathepsin D (Bourin et al., 2012). In addition, Vitellogenin (and albumin) is important in the transport of circulating non-diffusible protein bound calcium, which is important for eggshell formation (Johnson, 2015). Liou

et al. (2007) reported that Vitellogenin levels in the plasma were positively correlated to egg productivity in a local breed of chicken in Taiwan (TRFCC). It would be of interest to determine if a similar relationship exists in more highly selected breeds of layer chickens.

Oogenesis is also considered to be the main factor affecting the plasma levels of apolipoproteins and total lipid in chicken plasma, thus enabling efficient delivery of very low-density lipoproteins (VLDL) to the egg (Pinchasov et al., 1994). Apolipoprotein B is a major protein of VLDL. This protein undergoes proteolytic cleavage in the yolk into at least 9 protein fragments, the presence of some of which have been shown to contribute to the excellent emulsifying properties of egg yolk (Jolivet et al., 2008). Apolipoprotein A-I is involved in incorporating lipid into the yolk mass (Vieira et al., 1995), so the demand for this protein will also be greatest at peak production (Finn, 2007). Interestingly, Liou et al. (2007) found that the levels of Apolipoprotein A-I were negatively correlated to egg productivity in their study of a native Taiwan breed of chicken. Alpha-2 macroglobulin (bands 3 and 4, Figure 5) significantly decreased between 12 and 17 wk and then remained at a relatively constant level for the remainder of the experiment. This protein is a part of the innate immune system and functions to clear active proteases from tissue fluids (Armstrong and Quigley, 1999). Alpha-2 macroglobulin also known as Ovostatin has been isolated from the plasma of vertebrates as well as the egg white of birds and reptiles (Armstrong and Quigley, 1999; Rehman et al., 2013). A decrease in plasma levels of this protein at the onset of lay may be related to oviduct development and the increased demand for this protein to be incorporated into the forming egg. Previous studies on human plasma samples have reported that the MWt of Alpha-2 macroglobulin is 180 and 85 kDa (Harpel and Brower, 1983). In the current study, the MWts were  $\sim 200$  and  $\sim 100$  kDa (Figure 4). This could be related to differences in the degree of glycosylation, species variance, or methodology differences.

In relation to the third aim of this study, which was to determine if PRM significantly alters the serum protein profile of laying hens, we did not find any differences using SDS-PAGE which could be directly attributable to the PRM challenge (Figure 5). Changes in SAA were only observed using an ELISA which had the sensitivity to measure this low abundance protein. SAA has a low molecular weight (14 kDa) when it becomes dissociated from the high-density lipoprotein and is known to be a difficult protein to analyze using SDS-PAGE (Soler et al., 2013). This, together with SAA having a concentration in the  $\mu\text{g/L}$  range ( $>1,000$  lower than high abundance serum proteins such as albumin and IgG which have concentrations of 1-50 g/L), means that electrophoresis is not suitable for monitoring this acute phase and low abundance protein.

In conclusion, following a PRM challenge at 18.5 wk, the serum levels of SAA were significantly increased and

this was subsequently found to be positively correlated with the level of PRM infestation. Our proteomic investigation revealed a marked change in the levels of 2 major proteins viz. Apolipoprotein B and Vitellogenin-2 in the sera of hens between 12 and 17 wk of age. The serum proteome however did not appear to be significantly altered in the weeks following the PRM challenge. It may be that the levels of mite infestation were too low to stimulate a major APR or that the chronicity of the disease did not show a clear proteomic change as the hens quickly acclimatized to the stress of the infestation. Further work is needed to establish if serum proteins differ in high- and low-producing hens and to determine if PRM infestation alters the serum protein profiles in older birds.

## SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

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