The role of disease risk and life history in the immune function of larks in different environments

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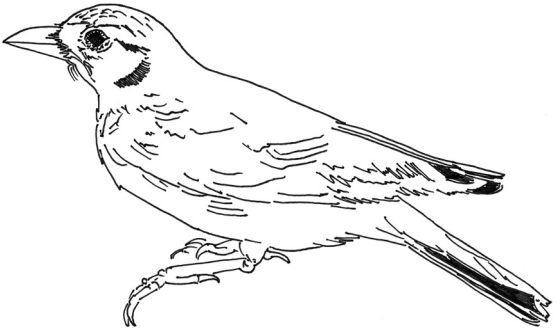

CHAPTER 7

A simple assay for measurement of ovotransferrin – a marker of inflammation and infection in birds

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Abstract

Ovotransferrin is an acute phase protein with iron-binding and immunomodulatory functions. In poultry, ovotransferrin levels increase in response to inflammation or infection, but little is known about responses in wild bird species. We present a simple assay for the determination of ovotransferrin-like activity in the plasma of wild birds. The assay uses very small sample volumes, works with previously-frozen plasma, is inexpensive to run, and requires only standard laboratory equipment and a spectrophotometer. Importantly, the assay does not require species-specific antibodies, making it applicable to a wide variety of species and particularly useful in comparative studies of immune function. We detected significant variation in ovotransferrin concentrations among 22 bird species. Ovotransferrin concentrations were significantly repeatable among individuals, and concentrations increased significantly in response to a lipopolysaccharide challenge. Within but not among species, concentrations of ovotransferrin were significantly and positively correlated with concentrations of haptoglobin, another acute phase protein that also binds iron. Differences in concentrations of acute phase proteins might reflect broader differences in immune strategies and responses to infection. Measuring ovotransferrin in addition to haptoglobin therefore provides fresh insights into differences in immunological defences among populations and species. This assay will serve as a useful addition to the existing arsenal of field-friendly assays that have been developed for addressing questions in ecological immunology.

Introduction

For ecologists interested in studying the immune function of free-living animals, a key advancement in methodology was the introduction of field-friendly assays that could be carried out using small volumes of blood or plasma and that did not require species-specific antibodies or reagents (Matson, Ricklefs and Klasing 2005; Tieleman *et al.* 2005; Matson 2006; Millet *et al.* 2007). These assays enable researchers to study the immune function of individuals that may be captured only once and about which little or nothing may be known of their current health status. Here we present a new addition to the immunoecologists' tool kit: an assay for measuring plasma concentrations of ovotransferrin, an acute phase protein in birds.

The transferrins are a group of iron-binding glycoproteins present in vertebrates and invertebrates (Lambert *et al.* 2005). In birds transferrin proteins occur as two forms, both called ovotransferrin, that are products of the same gene expressed in different tissues (Thibodeau, Lee and Palmiter 1978). Serum ovotransferrin is made in the liver and circulated in the blood. Egg-white ovotransferrin (formerly conalbumin) is synthesised in the oviduct (Superti *et al.* 2007) and deposited in the albumen of eggs. In eggs ovotransferrin constitutes a major component of albumen (Burley and Vadehra 1989) and aids in antimicrobial defence of the developing embryo. As products of the same gene, both forms of ovotransferrin have the same amino acid sequence and protein structure. They differ only in the composition of attached carbohydrate side groups (Lee, McKnight and Palmiter 1980; Jacquinot *et al.* 1994).

Transferrins can sequester iron with high affinity; each molecule has the ability to bind two Fe^{3+} ions (Aisen 1998). This property underlies their important function as iron transport proteins. It also helps explain the defensive role that ovotransferrin plays in birds. By binding free iron, an essential nutrient for bacterial growth (Skaar 2010), ovotransferrin limits infection by both gram-positive and gram-negative bacteria (Valenti *et al.* 1983; Abdallah and Chahine 1999; Superti *et al.* 2007). Ovotransferrin also contains a bactericidal domain that functions independently of the protein's iron-binding properties (Ibrahim *et al.* 1998). This domain causes selective ion efflux through bacterial membranes, which can also lead to bacteriostasis (Ibrahim, Sugimoto and Aoki 2000; Aguilera, Quiros and Fierro 2003). The protein exhibits antiviral (Giansanti *et al.* 2002; Giansanti *et al.* 2007) and antifungal activities (Valenti *et al.* 1985) and ovotransferrin also has an immunomodulatory role in birds (Xie *et al.* 2002b).

In some organisms, ovotransferrin is known to be an acute phase protein (APP). Often produced in the liver, APPs increase (i.e. positive APP) or decrease (i.e. negative APP) in concentration during acute phase responses (Gruys *et al.* 2005). Because these changes can stem from inflammation, infection, poor nutrition or disease, APPs can be used as non-specific markers of these processes but

not as indicators of specific diseases. In fact, several APPs, including transferrins, are currently used as markers of health in humans and animals (Ritchie *et al.* 1999; Cray, Zaias and Altman 2009). In chickens serum ovotransferrin is a positive APP (Hallquist and Klasing 1994; Tohjo *et al.* 1995; Chamanza *et al.* 1999; Xie *et al.* 2002a; Rath *et al.* 2009), while in mammals transferrins are classed as negative APPs (Gruys *et al.* 2005). Chicken ovotransferrin is a moderate APP (one- to ten-fold increase in circulating concentration in response to a trigger; Cray, Zaias and Altman 2009), and remains elevated as long as inflammation persists (Rath *et al.* 2009; Xie *et al.* 2009). Moderate APPs generally show prolonged increases and relatively slow declines, and may be particularly associated with chronic inflammatory processes (Cray, Zaias and Altman 2009).

In this paper we describe a simple assay for quantifying ovotransferrin in avian plasma samples. We explored the applicability of the assay by analysing samples from 22 species of birds. We also tested the ability of the assay to detect changes in ovotransferrin concentrations in response to inflammation by comparing plasma samples that were collected before and after lipopolysaccharide injection. Finally, we examined the correlation between concentrations of ovotransferrin and haptoglobin, another iron-binding positive APP in birds. Variation in the response of similar-functioning proteins to infection may provide new insights into differing strategies of immune defence among species, and highlight otherwise hidden differences in how infections are dealt with. Overall, our study identifies the utility of measuring ovotransferrin for answering questions in ecological immunology.

Materials and methods

Assay principle

We used a modified version of the assay described and verified by Yamanishi *et al.* (2002). This assay measures total iron-binding capacity – the maximum amount of iron necessary to saturate all the ovotransferrin in a sample. This correlates very well with ovotransferrin concentration, as determined by comparison with immunological measurement of serum transferrin (Gambino *et al.* 1997; Yamanishi *et al.* 2002). A similar version of the assay has been used previously to measure concentrations of ovotransferrin in egg albumen (Shawkey *et al.* 2008; D'Alba *et al.* 2010). The assay consists of three reaction steps. First, ovotransferrin in the sample is saturated with ferric iron (Fe^{3+}) under alkaline conditions. Then, the unbound excess iron is reduced to Fe^{2+} by addition of ascorbic acid, and this Fe^{2+} becomes inactivated by forming coloured complexes with the chromogen FerroZine. Finally, the ovotransferrin-bound Fe^{3+} is dissociated under acidic conditions. This newly released Fe^{3+} allows further formation of the coloured Fe^{2+}-FerroZine complex. The associated increase in absorbance of the reaction mixture due to this additional formation of the coloured complex is monitored over time.

Assay procedure

Each well of a 96-well microplate was filled with 50 μl of reagent one (300 mM Tris, 150 mM sodium hydrogen carbonate, 4.2 g l^{-1} Triton X-100, pH 8.4) containing a 1:250 dilution of iron-standard solution (1000 mg l^{-1}). Ten microlitres of plasma samples or ovotransferrin standards (conalbumin from chicken egg white, C0755, Sigma-Aldrich, St Louis, Missouri, USA) were added to duplicate wells in the plate. The standards were prepared in reagent one (without iron-standard solution) for a standard curve ranging from 1.0–20.0 mg ml^{-1}. The assay plate was placed in a spectrophotometric microplate reader (VersaMax, Molecular Devices, Sunnyvale, California, US). In the reader, the plate was shaken for 10 seconds to mix the well contents and then incubated for 5 minutes at 36°C. Following incubation, initial 'pre-read' absorbances were recorded at 570 nm (primary wavelength) and 660 nm (reference wavelength) to later account for any differences among the plasma samples and between the coloured plasma samples and the colourless standards. Ten microlitres of reagent two (50 mM Tris, 32.6 mM L-ascorbic acid, 10 mM FerroZine, pH 4.0) were then added to each well. The contents were mixed again for 10 s and left to incubate for 5 minutes at 36°C in the plate reader. Finally, 20 μl of reagent three (600 mM citric acid, 25.6 mM thiourea) were added to each well, the contents were mixed for 3 seconds, and absorbances were first recorded immediately after mixing (t = 0). Absorbance at 570 and 660 nm were recorded again at six minutes (t = 6). The microplate reader and all reagents were warmed to 36°C prior to use in the assay. All chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA).

Absorbance values were used to calculate ovotransferrin concentrations. First, we corrected for initial differences in absorbance values among samples and the standards. We subtracted well-specific 'pre-read' absorbances at 570 and 660 nm from both the t = 0 and the t = 6 read at the corresponding wavelength. Then, we normalised all absorbance values by subtracting the reference wavelength (600 nm) absorbances from the primary wavelength (570 nm) absorbances at both time points. Finally, we determined the change in absorbance (ΔA) due to release of Fe^{3+} from the ovotransferrin and additional formation of the coloured Fe^{2+}-FerroZine complex. For each well we subtracted the normalised absorbance value at the start of the assay (t = 0) from the normalised absorbance value at the end of the assay (t = 6; i.e. $\Delta A = A_{570\text{-}660}\text{end} - A_{570\text{-}660}\text{start}$). A standard curve, which related ΔA and ovotransferrin concentration of the standards, was plotted. This curve was used to calculate ovotransferrin concentration of the samples (in mg ml^{-1}) based on their ΔA. If sample volume was sufficient, then a sample was run in duplicate, and the mean concentration was used in further analyses.

To account for potential among-assay variation (i.e. plate-to-plate differences), we included a standard sample in duplicate on all plates. Logistical limitations at our lab prevented us from using a plasma standard as intended. Instead the inter-plate standard was made from equal parts by weight of the albumen from three

chicken eggs. We calculated the mean ovotransferrin concentration of this standard over all assay plates. Then we determined a correction factor for each plate by dividing the among-plate mean by the within-plate mean. Ovotransferrin concentrations of each sample on a plate were multiplied by the plate-specific correction factor to give a plate-corrected concentration. We report plate-corrected values, unless otherwise stated.

Experimental samples

We assayed five sets of plasma samples. The first set, which was used to examine assay variation, comprised 40 samples collected from adult homing pigeons (*Columba livia*, 8 males, 12 females) that were each sampled twice, four months apart (June and September 2008; 2 samples per bird). All of these samples were run in duplicate.

The second set consisted of plasma from 16 of these same pigeons (8 males, 8 females) and was used to estimate the repeatability of ovotransferrin concentration as an individual trait. The samples were collected on a monthly basis between November 2007 and February 2008. We considered this to be a physiologically quiescent period since the birds were not breeding or moulting (see Versteegh *et al.* (2008), for a similar approach to testing repeatability of physiological traits). When sampled, none of the birds showed signs of disease or were receiving medical or experimental treatment.

The third set was used to investigate the effect of sample storage duration on ovotransferrin concentration and also to examine species variation in ovotransferrin concentration. It comprised 222 plasma samples from 22 bird species (Table 7.1) that were collected for various projects within the authors' research group. All samples came from adult birds that were either captured using standard techniques (mist-netting or clap traps) or maintained in captivity. None had experienced any experimental manipulations and all were apparently healthy and disease-free at the time of sampling.

The fourth set of samples was used to test the effect of age class on ovotransferrin concentration. Plasma samples from 10 adults and 20 chicks from 12 broods were collected from Woodlarks (*Lullula arborea*).

The fifth and final set consisted of plasma from 8 pigeons (4 males, 4 females) and 5 red knots (*Calidris canutus islandica*, 2 males, 3 females) that received an intraperitoneal injection of lipopolysaccharide (LPS; L7261; Sigma-Aldrich). We used these samples to test the effect of the LPS injection, which simulates a bacterial challenge, on ovotransferrin concentration. Apart from the LPS injection, these birds were from un-manipulated control groups in other experiments (see Buehler *et al.* (2009) and van de Crommenacker *et al.* (2010), for full details). Samples were collected from the pigeons immediately before, and 18 hours after LPS injection. For the red knots the post-LPS sample was collected one week after the baseline sample, and 17 hours post-injection.

In every case plasma was separated from whole blood by centrifugation within one hour of collection, and all samples were stored at –20°C or –80°C prior to being assayed. None of the samples showed signs of gross haemolysis (i.e. an obvious deep-red colour indicating contamination with free haem). Since the ovotransferrin assay relies on the binding of iron, excess haem from lysed erythrocytes can interfere with the assay, leading to abnormally high readings.

Table 7.1. List of species assayed for plasma ovotransferrin concentration

Order and Family	Species	Sexes	*n*	Origin
Charadriformes				
Scolopacidae	Ruff *Philomachus pugnax*	1 F, 16 M	17	Netherlands
	Red Knot *Calidris canutus islandica* [Hp]	6 F, 4 M	10	Netherlands [C]
Glareolidae	Cream-coloured Courser *Cursorius cursor* [Hp]	1 M, 4 NA[1]	5	Saudi Arabia
Columbiformes				
Columbidae	Namaqua Dove *Oena capensis* [Hp]	1 F, 2 M	3	Saudi Arabia
	Homing pigeon *Columba livia*[2] [Hp]	8 F, 8 M	16	Netherlands [C]
Passeriforme				
Laniidae	Great Grey Shrike *Lanius excubitor* [Hp]	3 F, 4 M, 3 NA	10	Saudi Arabia
Alaudidae	Rufous-naped Lark *Mirafra africana* [Hp]	3 F, 1 M	4	Kenya
	Bar-tailed Desert Lark *Ammomanes cincturus* [Hp]	5 F, 12 M	17	Saudi Arabia
	Hoopoe Lark *Alaemon alaudipes* [Hp]	16 F, 19 M	35	Saudi Arabia
	Bimaculated Lark *Melanocorypha bimaculata* [Hp]	10 F, 1 M	11	Afghanistan
	Calandra Lark *Melanocorypha calandra* [Hp]	4 F, 4 M	8	Afghanistan
	Red-capped Lark *Calandrella cinerea* [Hp]	3 F, 4 M	7	Kenya
	Dunn's Lark *Eremalauda dunni* [Hp]	2 F, 11 M	13	Saudi Arabia
	Crested Lark *Galerida cristata* [Hp]	9 F, 6 M	15	Saudi Arabia
	Woodlark *Lullula arborea* [Hp]	3 F, 7 M	10	Netherlands
Muscicapidae	European Stonechat *Saxicola rubicola*	1 F, 2 M	3	Austria [C]
	African Stonechat *Saxicola torquata*	3 F, 2 M	5	Kenya [C]
	Desert Wheatear *Oenanthe deserti* [Hp]	3 F, 2 M	5	Saudi Arabia
	Isabelline Wheatear *Oenanthe isabellina* [Hp]	NA	4	Saudi Arabia
Turdidae	Eastern Bluebird *Sialia sialis* [Hp]	5 F, 8 M	13	USA
Sturnidae	European Starling *Sturnus vulgaris* [Hp]	NA	7	Netherlands
Passeridae	House Sparrow *Passer domesticus* [Hp]	3 F, 1 M	4	Saudi Arabia

[1] NA – sex unknown; [2] Mean of November 2007-February 2008 values (sample set two, see Materials and Methods); [C] Maintained in captivity; [Hp] Used in the analysis of the correlation between ovotransferrin and haptoglobin.

Haptoglobin

Like the transferrins, haptoglobin and its functional equivalents are haem-binding APPs found in many taxa, including birds (Delers, Strecker and Engler 1988; Matson *et al.* 2006; Matson 2006). Generally, these proteins are absent or circulate at low levels in the blood, but concentrations increase during the acute phase response. Elevated levels can therefore indicate an immune response (Dobryszycka 1997; Quaye 2008). We determined haptoglobin concentrations using a commercially-available functional assay that measures the haem-binding capacity of plasma (TP801; Tri-Delta Diagnostics, NJ, USA). We followed the manufacturer's instructions for the 'manual method'.

Statistical analyses

We used linear models to test the effects of various factors on ovotransferrin concentration. Where repeated measures were involved (repeated measures per individual or per species) we used general linear mixed models (glmm) with the relevant term included as a random effect. To test the correlation between ovotransferrin and haptoglobin we used within-subject centreing (van de Pol and Wright 2009). This allowed us to distinguish between the contributions of within-species and among-species variation. We calculated haptoglobin species means (to account for among species variation) and individual deviations from these means (to account for within-species variation). Then, we ran a mixed model with species as a random effect, haptoglobin species means and individual deviations as fixed terms, and ovotransferrin concentration as the response variable. The residuals of statistical models were examined graphically for normality and homogeneity of variance and met these assumptions in every case. Statistical analyses were performed using R, version 2.10.1 (R Development Core Team 2009).

Assay variation

Within- and among-assay variation was calculated using ovotransferrin concentrations (not plate-corrected) of 40 pigeon samples (sample set one) spread over 16 plates. Each sample was run in duplicate on two of the 16 different plates. That is, each sample was run four times in total over two plates.

In the case of three samples, one of the within-plate duplicates did not work, leaving 97 instances where two values were available for the same sample within a plate. Within-assay variation was calculated from the mean and standard deviation of each of these 97 intra-plate duplicates. We summarise the within-assay variation by reporting the mean, minimum and maximum values of these standard deviations (SD) and of the associated standard errors (SE, $n = 2$ wells) and coefficients of variation (CV).

Among-assay variation was determined by first calculating plate-specific mean ovotransferrin concentrations for each sample (i.e. mean of the duplicates on a plate). Inter-plate means and SDs were then calculated based on pairs of plate-

specific means that originated from identical samples. We summarise among-assay variation by reporting the mean, minimum and maximum values of the inter-plate SD, SE (n = 2 plates) and CV.

Individual repeatability

We used sample set two to estimate the repeatability of ovotransferrin concentration as an individual trait. We calculated repeatability according to Lessells and Boag (1987; repeatability = among-individual variance / within-individual variance + among-individual variance). Since samples were run on 13 different assay plates without regard to sample month, we used plate-corrected values to account for among-plate variation. Within- and among-individual variances were obtained from a mixed model containing only the intercept and with bird identity as a random effect. For comparison we also tested mixed models containing the fixed terms sex, month, and all their permutations. Statistical significance was evaluated using a log-likelihood ratio test. Standard errors for repeatability values were calculated according to Becker (1984). Within-individual CV was calculated as the mean of all the individual CVs, ascertained by first calculating means and SDs per individual (n = 4 monthly samples per individual). Among-individual CV was determined by using the mean ovotransferrin concentration per individual, and then deriving CV from the overall mean and SD of these individual means. Thus, both within- and among-individual variation subsumed within- and among-assay variation.

Results

Assay variation, individual repeatability, and effects of sample storage duration

Within- and among-assay variation (sample set one) is shown in Table 7.2. Mean within-assay CV equalled 0.15 and mean among-assay CV was 0.24. The inter-plate repeats (used to calculate among-assay variation) were run in two batches approximately one month apart, but with most of the same reagents, so there are many possible sources of variation. This might account for why among-assay variation is higher than within-assay variation. Nonetheless, with practice and when plates are analyzed in short succession, variation can be reduced to almost zero, as the minimum CV values in Table 7.2 demonstrate. For comparison, Table 7.2 also shows the assay variation calculated using the chicken albumen pool used to correct for plate-to-plate variation.

Individual repeatability, based on four measures per bird (sample set two) and with a mixed model containing only the intercept and no fixed effects, equalled 0.32 and was highly significant (P = 0.007; Table 7.3). Thus, ovotransferrin concentration can be considered a distinctive trait of individuals, at least during the

Table 7.2. Within-and among-assay variation

Variation	Sample	*n*	Mean	SD			SE			CV		
				mean	min	max	mean	min	max	mean	min	max
Within-assay*	Pigeon plasma	97 repeats	5.93	0.77	0.00	5.20	0.55	0.00	3.68	0.15	0.00	1.14
Among-assay*		50 plate pairs	5.96	1.33	0.02	4.51	0.94	0.02	3.19	0.24	0.01	0.70
Within-assay	Chicken albumen	18 repeats	15.11	1.35	0.04	3.34	0.96	0.03	2.36	0.09	0.00	0.24
Among-assay		18 plates	15.11	1.59			0.38			0.11		

*Samples were spread over 16 different plates.

winter. Sex significantly affected ovotransferrin concentration, with males having higher concentrations than females (mean ovotransferrin concentration: males 5.05 mg ml^{-1}; females 3.69 mg ml^{-1}; $F_{1, 14} = 10.03$, $P = 0.007$). The interaction of sex by month was not significant ($F_{3, 42} = 0.08$, $P = 0.969$), and there was no effect of month on ovotransferrin concentration ($F_{3, 45} = 0.55$, $P = 0.650$). Including sex in the model decreased the repeatability (by decreasing among individual variation) and made it no longer significant ($R = 0.16$, $P = 0.160$).

To examine the effect of sample storage on ovotransferrin concentration we used 206 samples of 21 species from sample set one. These samples had been stored frozen (at least –20°C) for between 431 and 2174 days (approximately 1–6 years) before being assayed. The results of a mixed model containing species as a random effect and sample age as fixed effect indicated that there was no effect of storage time on ovotransferrin concentration (sample age $F_{1, 184} = 2.16$, $P = 0.143$).

Table 7.3. Repeatability of ovotransferrin as an individual trait

Repeatability *r*	SE	χ^2	*P*	Within-individual CV	Among-individual CV
0.32	0.14	7.28	<0.001	0.25	0.25

Effects of species, sex, and age class

Species variation in ovotransferrin concentrations was tested using values from 22 species (sample set one), which encompassed nine families in three orders (Table 7.1). Since we were initially uncertain about the importance of accounting for variation in ovotransferrin concentrations due to sex, we first tested this effect using all individuals of known sex. A mixed model with species as a random factor showed no significant differences in ovotransferrin concentrations between males and females ($F_{1, 183} = 0.89$, $P = 0.347$, $n = 204$ individuals from 20 species). Ovotransferrin concentrations varied significantly among species, regardless of whether sex and the species by sex interaction were included (species $F_{19, 183} = 2.74$, $P < 0.001$; sex $F_{1, 183} = 0.49$, $P = 0.484$; species*sex $F_{18, 165} = 13.77$, $P = 0.915$, $n = 204$ individuals from 20 species) or excluded ($F_{21, 200} = 2.62$, $P < 0.001$ $n = 222$ individuals from 22 species; Fig. 7.1).

To test the effect of age class on ovotransferrin concentration we used samples collected from Woodlarks (*Lullula arborea*; sample set two). Because some individuals were related (i.e. chicks or adults from the same nest), we used a mixed model with age class as a fixed effect and nest identity as a random effect. The mean ovotransferrin concentration of chicks (9.80 mg ml^{-1}) was slightly higher than that of adults (9.42 mg ml^{-1}), but this difference was not significant ($F_{1, 9} = 0.025$, $P = 0.877$).

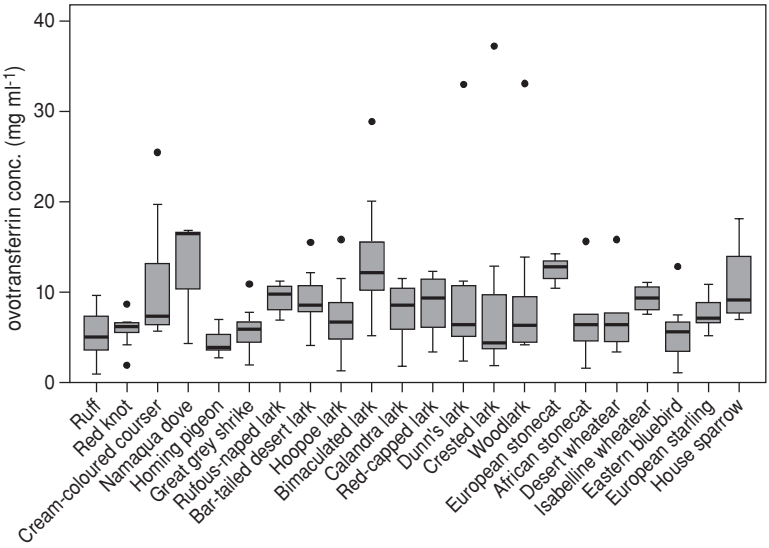

Figure 7.1. Box plot illustrating species variation in ovotransferrin concentration. Boxes encompass all data points between the 25th and 75th percentiles (interquartile range, IQR). Thick bars in boxes indicate the median data value. 'Whiskers' indicate either the minimum or maximum data value, or 1.5 times the IQR (approximately two standard deviations), whichever is smaller. Data points outside this range ('outliers') are plotted individually as black dots.

Effect of simulated bacterial infection

The effect of a simulated bacterial infection on ovotransferrin concentration was tested in homing pigeons and red knots that were injected with LPS (sample set five). Specifically, we examined the within-individual changes in ovotransferrin concentration between baseline and LPS-response samples. We ran a mixed model containing the terms treatment (baseline or LPS response), species, their interaction, and the random factor of individual identity to account for the repeated measures design. The interaction term was non-significant (treatment* species, $F_{1, 11} = 0.70$, $P = 0.420$) and the two species also did not differ in their response to LPS (species, $F_{1, 11} = 0.12$, $P = 0.731$). The effect of LPS injection on ovotransferrin concentration was highly significant (treatment, $F_{1, 12} = 9.73$, $P = 0.009$): response samples had higher ovotransferrin concentrations than baseline samples (Fig. 7.2).

Correlation between ovotransferrin and haptoglobin

We tested the correlation between ovotransferrin and haptoglobin, another iron-binding APP, using within-subject centreing (van de Pol and Wright 2009). Analysing values of 192 individuals from 19 species (sample set one; Table 7.1),

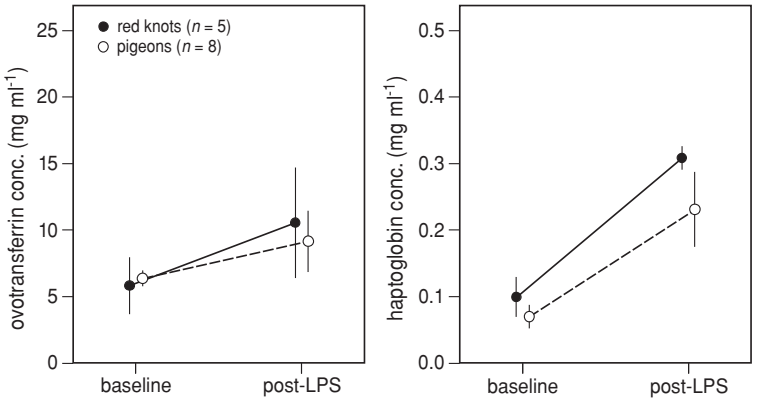

Figure 7.2. Effect of LPS injection on ovotransferrin (left) and haptoglobin (right) plasma concentrations, in homing pigeons and red knots. The relative scaling of the y-axis in each graph is the same, to highlight that the magnitude of the change in haptoglobin concentration in response to LPS is greater than that of ovotransferrin. Error bars indicate 2x standard errors.

we found a positive relationship between concentrations of haptoglobin and ovotransferrin. At the within-species level the correlation was highly significant ($F_{1, 172} = 11.42$, $P < 0.001$) while at the among-species level this was not the case ($F_{1, 17} = 1.22$, $P = 0.284$).

We also used the pigeon and red knot data (sample set five) to assess whether the changes in ovotransferrin concentration following LPS injection were matched by changes in haptoglobin concentration. As previously reported, both pigeons (van de Crommenacker *et al.* 2010) and red knots (Buehler *et al.* 2009) significantly increase haptoglobin concentration in response to LPS injection (Fig. 7.2). We ran a linear model containing the terms haptoglobin change (i.e. the intra-individual change in haptoglobin concentration between baseline and post-LPS samples), species, and the interaction of these two terms. The intra-individual change in ovotransferrin concentration between baseline and post-LPS samples was used as the response variable. All terms were non-significant (all $P > 0.132$), suggesting that haptoglobin response to LPS cannot be used to predict ovotransferrin response to LPS.

Discussion

The method we describe for assaying ovotransferrin concentrations in avian plasma samples is a valuable new tool for addressing questions about the ecology and evolution of immune function. It will also be useful for monitoring animal health

and welfare. Specifically, our study identified five important attributes of ovotransferrin and its quantification that highlight the assay's value. First, within populations, ovotransferrin concentrations are repeatable. Second, concentrations differ significantly among species (Fig. 7.1). Third, ovotransferrin concentration increases in response to a simulated bacterial challenge (Fig. 7.2). Fourth, within species ovotransferrin concentrations are correlated with concentrations of another APP haptoglobin, but among species these two indices appear to be independent of each other. Fifth, the assay has practical and cost advantages that make it applicable to a wide variety of studies. The combination of these attributes results in a valuable new immune index in birds that provides useful information about differences in immune defences among species.

Ovotransferrin concentrations were repeatable

In our flock of pigeons ovotransferrin concentrations were significantly repeatable. As such, ovotransferrin concentration appears to reflect a consistent aspect of an individual's physiology, at least during the winter, and can be considered an individual-bound trait. Individual-bound traits are interesting to ecologists because they can be meaningfully correlated among individuals. Moreover, repeatability represents a maximum limit for heritability (Falconer and Mackay 1996). By definition, APP concentrations vary in response to inflammation or infection, so the significant repeatability of ovotransferrin during winter supports the notion that this season might be a physiologically quiescent period. However, this must be confirmed by comparing repeatabilities among seasons.

Accounting for differences between males and females decreased among-individual variation, which led to a reduced and non-significant repeatability. Differences in health, which were not quantified in this study, could also affect repeatability. Non-synchronous changes (i.e. those affecting one or a few individuals, but not all, on a particular sampling day) would lead to a deflated repeatability by increasing within-individual variance. Synchronous changes in health (i.e. those affecting the whole flock) were controlled for however, by including the term 'month' in the model. If health effects are left unaccounted, as will likely be the case in many studies of wild birds, the calculated repeatability should likely be viewed as a low estimate. Other differences, like season or age, which did not vary within or among our pigeons, can affect immune indices (Nelson and Demas 1996; Cichoń, Sendecka and Gustafsson 2003; Lozano and Lank 2003; Buehler *et al.* 2008) and might affect ovotransferrin concentrations. These effects should also be considered when calculating repeatability.

Compared with other immunological traits measured over a similar time frame (i.e. months rather than days or weeks), ovotransferrin repeatability is high. Repeatability of serum proteins, including APPs, was 0.26 in captive greenfinches (*Carduelis chloris*) that were sampled twice over four months (Hõrak *et al.* 2002). In red knots sampled over an entire annual cycle, repeatabilities of several

leukocyte and plasma parameters were mostly below 0.20 (Buehler *et al.* 2008). It should be noted however, that repeatabilities are population measures; we cannot discount the possibility that ovotransferrin repeatability may differ among populations or species.

Ovotransferrin concentrations differed among species

Ovotransferrin concentrations differed significantly among species (Fig. 7.1), and this effect could not be attributed to differences in sample storage time. This finding makes measurement of ovotransferrin particularly useful in comparative studies among species, for example those investigating links between immune function and life history, species distribution, or other 'species-bound' traits (e.g. Tieleman *et al.* 2005; Matson 2006; Buehler, Tieleman and Piersma 2009).

In contrast to the differences among species, we found no overall differences in ovotransferrin concentrations between the sexes. When tested using a different, single-species dataset however (sample set one, instead of sample set three), male pigeons had significantly higher ovotransferrin concentrations than females. Combined, these results hint that the effect of sex may act in opposite directions in different groups (e.g. seasons, populations). The interaction between species and sex was not significant, but other interactions (e.g. sex by season) remain unexplored. We also found no difference in ovotransferrin concentration between age classes, but whether this finding is particular to the species we tested or applies more generally is uncertain. Data on age-related changes in APPs in non-human species is limited, and further research in this area is warranted.

Ovotransferrin concentration increased after an immune challenge

Ovotransferrin concentration increased significantly in response to a simulated bacterial challenge in two unrelated species (Fig. 7.2). This result confirmed that, in contrast to mammals, ovotransferrin is a positive APP in birds (Hallquist and Klasing 1994; Tohjo *et al.* 1995; Chamanza *et al.* 1999; Xie *et al.* 2002a; Rath *et al.* 2009). LPS-injected chickens also exhibit elevated levels of serum ovotransferrin and liver mRNA for the protein (Hallquist and Klasing 1994). Interestingly, these same LPS-injected chickens exhibit reduced levels of egg white ovotransferrin mRNA in the oviduct. These results allude to a potential trade-off between circulating ovotransferrin and the ovotransferrin deposited in eggs.

In pigeons and red knots, both ovotransferrin and haptoglobin increased significantly in response to LPS (Fig. 7.2), but among individuals the changes were not significantly correlated. In terms of percent increase, the ovotransferrin response to LPS was smaller than the haptoglobin response in both species (Fig. 7.2). Differences in ovotransferrin and haptoglobin responses to infection have been previously reported. For example, chickens infected with *Escherichia coli* show significant increases in ovotransferrin and haptoglobin concentration, but chickens infected with the gastrointestinal protozoan *Eimeria tenella* show only a

significant increase in ovotransferrin (Rath *et al.* 2009; Georgieva *et al.* 2010). One study even contradicts the post-challenge increases in ovotransferrin that we and others observed: chickens infected with fowl typhoid (*Salmonella gallinarum*) show increased haptoglobin but decreased ovotransferrin (Garcia *et al.* 2009). This contradiction is particularly intriguing given that the LPS we used originated from the outer membrane of a strain of *Salmonella* (*S. enterica* serotype typhimurium). Regardless, greater or lesser reliance on a particular protein may reflect the nature of immune challenge or could indicate differences in how free iron is managed.

Correlations between iron-binding acute phase proteins

In a larger analysis of birds that were not experimentally challenged, we found that haptoglobin and ovotransferrin correlated at the individual level but not at the species level. This suggests that for comparisons among species, measuring concentrations of both ovotransferrin and haptoglobin provides more information than measuring only one of the proteins. Differences among species in the relationship between these two proteins might relate to broader immunological differences among species. For example, the extent to which a species relies on ovotransferrin, haptoglobin or both might provide insight into the relative importance of induced responses in general or of limiting free iron more specifically. Moreover, the reliance on one protein versus the other might have downstream immunological ramifications, as APPs can provide feedback to the inflammatory process (Janeway *et al.* 2004).

Within species, baseline concentrations of ovotransferrin and haptoglobin were significantly correlated. Thus, in studies of unchallenged individuals of a single species, measuring baseline concentrations of both proteins may be redundant. However, since inter-specific responses of different APPs can be challenge-specific (see above; Rath *et al.* 2009; Georgieva *et al.* 2010), we think that measuring both ovotransferrin and haptoglobin remains a useful endeavour. Any 'positive redundancy' identified by measuring both proteins can be advantageous when interpreting results. Veterinarians have highlighted the importance of using multiple APPs to monitor disease processes, and they suggest that multiple positive and negative APPs should be measured (Cerón *et al.* 2008). This suggestion complements the conclusions of ecoimmunologists regarding the importance of measuring multiple immune parameters when assessing immune status (Adamo 2004; Matson *et al.* 2006).

Advantages of measuring ovotransferrin

The ovotransferrin assay has several practical advantages that make it applicable to a wide variety of species and useful in a range of settings. The assay requires only a small volume of plasma (10 μl), meaning the assay can be safely applied to the smallest of birds. The assay does not require species-specific antibodies or

reagents, thereby avoiding problems with lack of cross-reactivity, which other researchers have encountered when using antibody-based methods and multiple species (Rath *et al.* 2009). In combination with the inter-specific variation in ovotransferrin concentration that we observed (Fig. 7.1), these qualities make the assay particularly suited to comparative studies of immune function among species. Furthermore, although we tested plasma from birds, we see no reason why the assay could not be used or adapted for different taxonomic classes. If doing so, care must be taken to understand whether ovotransferrin levels increase or decrease during the acute phase response, given the differences between mammals and birds (Cray, Zaias and Altman 2009).

The assay is also inexpensive to run. While it costs approximately 4 euro per sample for the reagents to measure haptoglobin concentration, the cost of reagents to measure ovotransferrin is less than 0.05 euro per sample. Given that both assays require similar equipment (pipettes, 96-well microplates, spectrophotometer) and processing time, this cost-saving can represent a considerable advantage of ovotransferrin quantification over the measurement of haptoglobin.

In conclusion, the assay described here provides a simple, reliable, and inexpensive means for measuring ovotransferrin concentrations in blood plasma. The assay detects significant species variation in ovotransferrin concentrations, and it can be used to assess changes in response to infection or inflammation. Within populations, ovotransferrin concentration is a repeatable, individual-bound trait. Within, but not among species, ovotransferrin concentrations correlate significantly with concentrations of another iron-binding APP, haptoglobin. The assay is ideally suited to comparative studies of immune function and health status. In combination with its field-friendly nature, these attributes make this ovotransferrin assay a new and valuable addition to the ecoimmunologists' toolkit.

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