Acta Biologica Hungarica 61(4), pp. 391–400 (2010)
DOI: 10.1556/ABiol.61.2010.4.3

DOES BREEDING STATUS INFLUENCE HAEMATOLOGY AND BLOOD BIOCHEMISTRY OF YELLOW-LEGGED GULLS?

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(Received: December 2, 2009; accepted: WWW)

We compared the haematological and biochemical values within a population of yellow-legged gulls *(Larus michahellis)* in the Chafarinas Islands (Northern Africa), in non-breeding (February) and breeding (May) animals. We collected blood samples from 51 adults. We found that according to the haematological data, there was a significant variation in haemoglobin content, and a higher proportion of heterophils, thrombocytes, and *Haemoproteus* infection in breeding individuals with a lower level in basophils. Blood biochemistry showed a higher level in plasmatic proteins, calcium, phosphorus, thiobarbituric acid-reactive substances and alkaline phosphatase as well as alanine aminotransferase activity in breeding animals while cholesterol and phospholipid levels showed a lower level. There was also an effect of sex in triglycerides, albumin, thiobarbituric acid-reactive substances and alkaline phosphatase activity. Hence, the haematological and blood chemistry values of yellow-legged gulls showed some differences between breeding and non-breeding individuals as well as between sexes.

Keywords: Haematology – blood biochemistry – yellow-legged gulls – breeding condition – sex

INTRODUCTION

Description of haematological and biochemistry values is a tool commonly used to assess the health of wild animals. A wide range of these traits have been described for many groups such as chickens [20], spoonbills [13], vultures [35], penguins [36, 38], fulmars [15], and gulls [6]. Many studies have been carried out in gulls examining changes in blood values in relation to age [3], fasting and recovery processes [2, 4, 22] incidence of *Haemoproteus* infection [32], and exposure to pollutants [5]. There is strong evidence for the biological stress induced by reproduction in birds [14, 30] and therefore, a change in the hematological and biochemistry values would be expected during this time.

There are very few published accounts of haematological and biochemistry values comparing breeding and non-breeding individuals of the same species [15]. The objective of this study was to present baseline measurements from yellow-legged

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gulls collected in the breeding colony of the Chafarinas Islands (Northern Africa) and examine potential differences between the data obtained from breeding to non-breeding animals.

MATERIAL AND METHODS

A total number of 51 adults of *Larus michahellis* were captured for banding in February 2007 (non-breeding group) (8 males and 15 females) and by nest traps in April 2007 (breeding group) (14 males and 14 females), in the Chafarinas Islands (Melilla, Spain: 35º 1' N, 3º 46' 35'' E) (see 32 for further details of the sampling area).

Blood (5 ml) was obtained by venipuncture from the brachial vein of the gulls, using disposable needles (25G) and plastic syringes, and was carefully transferred to collecting tubes containing EDTA (1.5 mg/ml) and heparin for haematology and biochemistry, respectively. The blood collection tubes were kept cool on ice and transported to the laboratory where cell counting and haematological analyses were performed between two to four hours after blood sampling.

Haematology analysis

In the laboratory, packed cell volume (PCV) was determined by microhematocrit centrifugation and haemoglobin concentration (Hb) was determined by a cyanmethaemoglobin method following lysate centrifugation [40]. Mean cell volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated using standard formulae [6]. Aliquots of blood were diluted (200 and 50 times for red and white cell counts, respectively) in haematological pipettes with Natt-Herrick's solution [28]. Total red blood cell (RBC) and white blood cell (WBC) counts were determined in a Neubauer chamber.

Blood smears were fixed in methanol and stained with commercial Giemsa stain diluted 1 : 4 (v/v) in phosphate buffer, pH 6.80, for 1 hour. Identification and counting of leucocytes and thrombocytes was done under a light microscope using an oil immersion lens (×100). At least 200 white cells were counted in each sample to establish cell ratio [25]. The number of thrombocytes was obtained by an estimation method which consists of counting the number of thrombocytes in five fields submerged and applying the following formula [8]: Estimated number of thrombocytes (thrombocytes/µl) = (mean number of thrombocytes in five fields × 3,500,000) × 10^{-3}.

The intensity of RBC parasites (*Haemoproteus sp.*) was established by counting the number of infected erythrocytes in 40 fields, thus on the basis of 4,000 red blood cells observed per sample [32].

Biochemical analyses

Plasma and cells were separated as soon as possible by centrifuging at 3000g for 5 min and plasma was stored at –20 °C until processing. All biochemical parameters were assayed with the commercial kits SPINREACT (BIO ANALITICA S.L., MADRID). Plasma protein was assayed by colorimetric test with Biuret; albumin by a Bromocresol test; glucose by colorimetric-enzymatic test with Trinder; cholesterol, triglycerides and phospholipids by colorimetric-enzymatic tests based on Trinder's reaction; phosphorus by a Phosphomolibdate test; calcium by test based on Cresolftaleine; zinc by colorimetric test color 5 Br-PAPS; magnesium by a Calmagita test; iron by a TPTZ tripiridile-triazine test; uric acid was assayed according to the colorimetric-enzymatic method of Uricase-PAP; Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) by a colorimetric Reitman-Frankel test; creatine kinase (CK) was assayed through a kinetic test by N-acetylcysteine (NAC) activated and alkaline phosphatase (ALP) was assayed by a kinetic test based on the measurement of p-nitrophenol. Finally, lipid peroxidation was estimated in blood samples by colorimetric method based on thiobarbituric acid reaction substances (TBARS) [29].

Statistical analyses

In order to test differences in haematological and biochemical values between breeders and non-breeders, we formulated linear mixed models (GLMMs) using SAS statistical software (SAS 1989–96 Institute Inc., Cary, NC, USA). We analysed the relationship between each haematological and biochemical trait (response variable), sex and breeding status (fixed factors) and their interaction. When variables did not present a normal distribution (Lillefors $P < 0.05$), we checked residuals from the models. In all cases, residuals showed a normal distribution (Lillefors $P > 0.05$), for which the use of GLMMs was suitable. All analyses were two-tailed. Means ± Standard deviation are given.

RESULTS

Haematological data are presented in Table 1 by sex and breeding status. There was a significant effect of sex and breeding condition on haemoglobin content, ($F = 6.20$; $p < 0.05$) whereas no changes were found in PCV, MCH, MCHC, MCV, RBC and WBC counts. In the blood smear analyses, breeding gulls had a significantly higher proportion of heterophils ($F = 7.88$, $p < 0.01$), thrombocytes ($F = 4.38$; $p < 0.05$) and *Haemoproteus sp.* infection ($F = 19.62$, $p < 0.01$) than non-breeders while their percentage of basophils decreased significantly ($F = 23.43$, $p < 0.01$).

Table 2 shows the blood biochemistry values obtained from yellow-legged gulls. No changes were found in uric acid levels. Males showed significantly lower levels

Table 1
Combined haematological values by sex and breeding status of *Larus michahellis*

Parameter	Non-breeding group		Breeding group	
	Males	Females	Males	Females
Hematocrite (%)	42.23±0.85 (39–45.85)	40.92±0.70 (36–45.70)	42.77±1.16 (30.49–47.37)	41.38±0.56 (38.92–44.76)
Haemoglobin (g/dL)	10.08±0.37 (8.93–11.61)	10.56±0.25 (8.54–12.21)	11.89±0.44[c] (9.38–15.48)	10.08±0.57 (6.32–12.98)
RBC ($\times10^6/\mu L$)	3.79±0.09 (3.58–4.01)	3.50±0.1 (3.35–3.92)	3.73±0.08 (3.35–4.22)	3.44±0.18 (2.60–4.18)
WBC ($\times10^3/\mu L$)			10.54±0.42 (8.50–14.30)	10.65±0.80 (7.40–17.40)
MCV (fL)	114.69±5.47 (102.64–128.07)	118.21±3.15 (109.80–128.27)	115.59±4.29 (85.41–139.97)	124.64±7.08 (99.14–170.96)
MCH (pg)	29.15±1.37 (26.13–32.43)	30.30±0.95 (27.57–32.61)	32.00±1.15 (24.94–38.37)	30.82±2.84 (16.42–49.92)
MCHC (g/100 mL)	23.87±0.74 (20.98–27.44)	26.06±0.76 (19.96–31.63)	28.12±1.34 (21.27–40.91)	24.35±1.31 (16.24–30.09)
Lymphocytes (%)	51.97±1.89 (49.23–57.41)	52.83±1.30 (47.22–59.10)	50.83±1.66 (41.67–60.75)	54.55±2.24 (43.00–70.00)
Heterophils (%)	26.87±1.40 (23.85–30.56)	27.81±1.07 (24–33.33)	34.26±1.39[aa] (27.10–45.37)	31.55±2.17[aa] (11.67–39.52)
H/L	0.51±0.01 (0.48–0.53)	0.53±0.03 (0.42–0.65)	0.65±0.11 (0.25–0.78)	0.61±0.06 (0.17–0.91)
Basophils (%)	12.96±1.56 (8.33–14.96)	11.72±0.59 (9.00–14.00)	7.18±1.09[aa] (1.96–16.51)	6.90±0.76[aa] (2.84–12.00)
Eosinophils (%)	4.46±0.93 (2.78–7.1)	4.22±0.70 (1.00–8.00)	3.80±0.36 (1.83–6.48)	3.96±0.47 (1.080–6.73)
Monocytes (%)	3.76±1.64 (0.93–8.46)	3.42±0.69 (0.00–6.67)	3.73±0.90 (0.68–13.94)	3.21±0.29 (1.80–5.00)
Trombocytes ($n\times10^3/\mu L$)	9.93±3.24 (5.15–19.41)	11.12±1.40 (5.32–17.22)	28.35±7.31[a] (4.69–91.80)	21.65±5.52[a] (1.35–73.59)
Haemoproteus ($n/4\times10^3$RBC)	3.98±1.95 (0.30–6.95)	5.16±1.18 (1.50–7.72)	15.78±1.88[aa] (4.65–25.43)	12.74±1.82[aa] (2.50–23.40)

Mean ± Standard deviation and range in parenthesis are given.
[a]$p<0.05$; [aa]$p<0.01$ vs. non breeding group.
[c]$p<0.05$ differences by breeding and sex status.

of triglycerides ($F=5.66$, $p<0.05$) and albumin ($F=5.74$, $p<0.05$) than females. Breeding birds had higher plasmatic proteins (and subsequently, a significantly decrease in albumin/protein ratio) than non-breeders ($F=34.78$, $p<0.01$). Moreover, there was a statistically significant decrease in cholesterol ($F=14.84$, $p<0.01$) and phospholipids ($F=9.65$, $p<0.01$) levels in the plasma of breeding gulls when compared with non-breeding gulls.

Table 2
Values of biochemical parameters of *Larus michahellis* by sex and breeding status

	Non-breeding group		Breeding group	
	Male (n=8)	Female (n=15)	Male (n=14)	Female (n=14)
Albumin (g/L)	17.7±0.7 (15.2–21.7)	18.5±0.5[b] (14.9–21.7)	16.4±0.8 (12.3–22.8)	20.1±1.6[b] (15.3–29.8)
Proteins (g/L)	30.1±2.6 (19.3–43.9)	29.3±1.4 (19.6–38.0)	40.0±1.4 (31.1–48.7)	42.7±3.2 (33.6–66.0)
A/P	0.61±0.04 (0.46–0.78)	0.63±0.03 (0.51–0.88)	0.42±0.03[aa] (0.27–0.60)	0.47±0.05[aa] (0.28–0.70)
Glucose (mmol/L)			14.06±1.08 (7.18–19.01)	13.41±1.59 (5.90–19.95)
Triglycerides (mmol/L)	0.78±0.05 (0.63–0.99)	1.03±0.18[b] (0.47–3.37)	0.66±0.06 (0.37–1.09)	1.09±0.10[b] (0.55–1.59)
Phospholipids (mmol/L)	7.19±0.45 (5.97–9.53)	7.44±0.47 (5.30–12.50)	3.61±0.41[aa] (2.22–7.08)	4.49±0.56[aa] (2.83–6.57)
Cholesterol (mmol/L)	8.11±1.12 (4.47–13.81)	7.87±0.59 (5.78–14.65)	5.94±0.24[aa] (4.45–7.89)	5.74±0.28[aa] (4.67–7.24)
Uric acid (µmol/L)	384.24±99.93 (129.67–835.1)	298.59±55.94 (88.03–994.51)	366.99±51.15 (34.50–610.86)	471.68±68.99 (212.94–989.15)

Mean ± Standard deviation and range in parenthesis are given.
[b] $p < 0.05$ vs. males; [aa] $p < 0.01$ vs. non-breeding group.

Table 3
Values of plasmatic minerals of *Larus michahellis* by sex and breeding status

	Non-breeding group		Breeding group	
	Male (n=8)	Female (n=15)	Male (n=14)	Female (n=14)
Zinc (µmol/L)			25.37±1.14 (20.16–31.81)	25.61±2.32 (17.11–30.51)
Calcium (mmol/L)	1.87±0.06 (1.60–2.15)	2.04±0.08 (1.58–2.38)	2.23±0.06[aa] (1.93–2.47)	2.20±0.05[aa] (1.96–2.44)
Magnesium (mmol/L)	1.02±0.16 (0.55–1.97)	1.01±0.05 (0.77–1.36)	0.88±0.09 (0.43–1.34)	0.89±0.13 (0.35–1.42)
Iron (µmol/L)	19.43±2.88 (11.32–32.76)	17.87±1.44 (9.46–28.03)	20.15±4.14 (7.88–37.23)	12.22±5.17 (5.01–21.48)
Phosphorus (mmol/L)	0.46±0.07 (0.22–0.71)	0.49±0.08 (0.21–0.98)	0.70±0.09[aa] (0.30–1.32)	0.99±0.16[aa] (0.37–1.94)

Mean ± Standard deviation and range in parenthesis are given.
[aa] $p < 0.01$ vs. non breeding group.

Table 4
Values of TBARS and plasmatic enzyme activity of *Larus michahellis* by sex and breeding status

	Non-breeding group		Breeding group	
	Male (n=8)	Female (n=15)	Male (n=14)	Female (n=14)
TBARS (nmol MDA/ml)	27.68±2.84 (16.95–42.33)	41.58±4.28[b] (23.08–85.39)	63.65±4.24[aa] (24.79–87.53)	71.15±4.60[aa] (43.89–94.09)
ALP (IU/L)	65.90±4.47 (51.15–83.54)	56.22±4.87 (24.75–89.10)	116.19±9.61[aa] (51.70–150.71)	78.51±9.41[aa,b] (35.20–123.19)
AST (IU/L)	35.91±4.91 (25.45–49.09)	38.72±1.13 (34.85–43.94)	28.99±5.15 (7.63–57.52)	32.75±4.69 (14.56–55.03)
ALT (IU/L)	13.37±2.12 (8.62–18.82)	12.64±1.52 (7.02–23.42)	18.20±2.22[a] (10.87–40.40)	21.64±3.44[a] (5.71–32.27)
CK (IU/L)			182.50±31.01 (44.03–321.91)	110.31±24.30 (30.25–264.13)

Mean ± Standard deviation and range in parenthesis are given.
[a] $p<0.05$; [aa] $p<0.01$ vs. non breeding group.
[b] $p<0.05$ vs. males.

As shown in Table 3, there was a significant increase in plasmatic calcium ($F=11.89$, $p<0.01$) and phosphorus ($F=9.65$, $p<0.01$) associated with breeding condition. No changes were statistically significant in the rest of plasmatic minerals.

Breeding condition produced a statistically significant increase in ALT ($F=6.63$, $p<0.05$) and ALP ($F=17.57$, $p<0.01$) activities and in blood TBARS ($F=54.08$, $p<0.01$). Significant differences between sexes were also found in ALP activity ($F=8.67$, $p<0.01$) and blood TBARS ($F=5.57$, $p<0.05$) (Table 4).

DISCUSSION

In general, the mean haemoglobin values found for yellow-legged gulls in the Chafarinas Islands were consistent with those published for other gulls, although some of these studies refer to non-centrifuged samples [3, 6, 27]. There was a statistically significant interaction between sex and breeding condition, with males during the breeding period showing the highest levels of haemoglobin. Male gulls are larger than females; therefore higher levels in males are expected since there is a close relationship between this haematological parameter and body mass [16, 17].

Leukocyte profiles are particularly useful in the field of conservation physiology because they are altered by stress and can be directly related to stress hormone levels. Significant differences were found between breeding and non-breeding yellow-legged gulls in the differential white cell counts. Non-breeding birds had significantly lower heterophil proportion than breeding birds. This result was consistent with findings in other birds, such as chickens [20], penguins [38], fulmars [15] and gulls [6] which showed that birds under greater stress have high heterophil/lymphocyte (H/L) ratios,

partly due to the release of corticosterone and the corresponding effect of enhancing heterophils and decreasing lymphocytes. Thus, our data support the general idea that reproduction is stressful for birds [14].

All blood samples were infected with *Haemoproteus sp*., but there was a significant increase in breeding gulls. This fact has been described in the literature and it has been related to the immunosuppressive action of sexual hormones, increased exposure to parasite vectors or breeding effort [32]. However, blood parasites, such as *Haemoproteus sp.*, were not found to cause anaemia in their avian hosts [16]. To support this fact, in our samples no changes in any haematological parameter were found.

The number of thrombocytes increased significantly in the blood of breeding gulls. Phagocytic properties of thrombocytes toward bacteria and viruses have long been described in birds [7, 9, 10, 19, 24]. They have even been shown to internalize protozoa [11]. This fact could explain the simultaneous increase of *Haemoproteus sp.* Infection and thrombocytes during reproduction.

The estimation of plasmatic proteins has been recognized as a valuable test for evaluating the general nutritional state of a bird, and a decrease in plasmatic protein is indicative of malnutrition [1]. In our study, there was a significant increase of plasmatic proteins in breeding gulls, a fact probably related to periods of rapid growth or high metabolic rate [39].

Although the blood cholesterol range for *L. michahellis* fell well within the range for normal concentrations measured in a great number of avian species [18, 31], this is a variable heavily dependent on the state of nourishment and the nature of food consumed by the bird [22]. It has been described that plasma cholesterol is the best predictor of individual changes in body mass in yellow-legged gulls [4]. Thus, low cholesterol levels would indicate high consumption of energy stores related to breeding effort.

Plasma triglyceride levels are positively correlated to body fat content in several avian species, being proposed as fat indices [2]. The fact that we have not detected changes between breeding and non-breeding gulls might suggest that the free access to food available to these birds could prevent mobilization of fat reserves.

Calcium is an element which is usually present in concentrations that are very constant in blood [34]. High serum calcium, as breeding gulls show, might reflect the intense osteoblastic activity required for egg production and reproduction effort [37]. Although previously published data regarding phosphorus levels are relatively limited, this plasmatic mineral is related to bone metabolism and consequently, its increase in breeding gulls might be related, as is calcium, to reproductive effort [39].

Elevated ALP activity in birds is associated with increased osteoblastic activity such as skeletal growth and repair, egg production, or nutritional deficiencies [26]. It has been described that increases in calcium and phosphorus levels were correlated with ALP enhanced activity during the nestling period of red kites (*Milvus milvus*) [37]. This correlation was also found in our study. Moreover, calcium and phosphorus concentrations were higher for breeding females than for non-breeding females.

Indeed, during egg formation, females mobilize high quantities of triglycerides, calcium, phosphate and increase alkaline phosphatase activity [23, 33]. Therefore, higher levels of triglycerides, calcium and ALP activity are probably a consequence of the breeding condition of the females under study.

Significant differences in ALT activity were recorded in breeding gulls. This fact might reflect higher muscular activity, associated to reproductive effort [5]. Finally, the most widely used test for oxidative stress is the measurement of malondialdehyde (MDA), a product of lipid peroxidation, by the TBARS assay. In our study, breeding gulls showed almost two times of blood MDA levels than non-breeding birds, suggesting that reproduction might be also related to reactive oxygen species production increase [12].

Thus, the avian haematological and biochemical profile established for yellow-legged gulls in this study is an important contribution towards constituting a set of reference values that will assist in the diagnosis and monitoring of pathological and clinical factors detected in the Chafarinas Islands. Moreover, our results demonstrate that there is an important variation of haematological parameters and biochemistry values between breeding and non-breeding animals that must be accounted for in future studies and to assess the stress caused by reproduction

ACKNOWLEDGEMENTS

This research was funded by the Organismo Autónomo de Parques Nacionales. We thank Reserva Nacional de Caza de las Islas Chafarinas and its director and staff. The present research complies with the current laws of the country in which the experiments were performed.

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