Breeding phased dependent oxidative balance in a small High Arctic seabird, the little auk

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Abstract

Reproduction is a demanding phase of bird's life cycle and imposes various physiological challenges. Increasing oxidative stress (OS) has been proposed as one of the costs associated with reproduction. In this study, we investigate the level of OS in a small Arctic seabird, the little auk Alle alle in relation to sex and phase of breeding (incubation, chick-rearing). We also examine whether OS is related to the birds leucocyte profile. We expected increase in OS with the progress of breeding period (due to increasing energetic demands) and higher values in females (due to high initial investments in production of a large egg). Surprisingly, we found higher OS during incubation compared to chick-rearing period, suggesting that incubation is a highly demanding reproductive phase in terms of oxidative balance. We suggests that those changes may be attributed to changes in hormone levels affecting oxidative status. Also, in contrasts to our expectations, we did not find sex differences in OS throughout the studied periods of breeding. Finally, we found positive relationships between OS level and haematological parameters: heterophils/lymphocytes ratio, number of leucocytes and lymphocytes per 10 000 erythrocytes, suggesting the effect of the oxidative stress on the immunological system.

Keywords: dovekie, reproductive investments, stress level

Introduction

Reproduction is a demanding phase of avian life cycle and imposes a number of physiological challenges. One of the costs associated with the reproduction is an increased rate of oxidative damage, i.e. oxidative stress (OS; Alonso-Alvarez et al. 2004, Costantini 2008, Metcalfe and Monaghan 2013, Cram et al. 2015a). Oxidative damage leads to cell senescence, decreased organs and decreased overall organism performance (Costantini 2008). OS depends on a complex balance between pro-oxidant production. It is expressed by presence of reactive oxygen metabolites (ROMs), being an outcome of the oxidative damage caused by reactive oxygen species (ROS, i.e. chemically reactive chemical species containing oxygen), and anti-oxidant mechanisms including repair systems (plasma anti-oxidant capacity, OXY) (Finkel and Holbrook 2000; Halliwell and Gutteridge 2007). Total antioxidant barrier is sophisticated detoxifying system based on endogenous and exogenous anti-oxidants, i.e. substances that significantly delay or inhibit oxidation of biological macromolecules (Halliwell and Gutteridge 2007). According to the 'oxidative cost of reproduction' hypothesis (Alonso-Alvarez et al. 2004) resources allocated to reproduction are no longer available to protect the animal against OS (Wiersma et al. 2004, Alonso-Alvarez et al. 2004, Ołdakowski et al. 2012, Costantini et al. 2016). Thus, high reproduction investment are expected to result in somatic deterioration, and indeed there is a growing number of evidence supporting the hypothesis. For example, zebra finches (Taeniopygia guttata) responded to artificially increased breeding effort (brood size enlargement) by increase in their blood antioxidants level (Alonso-Alvarez et al. 2004). Although the mechanisms of somatic deterioration caused by the oxidative stress are rather unclear, a recent meta-analysis suggests that it is a physiological cost associated with the immune response (Constatini and Møller 2009). Although the other way round, an experimental study on the common kestrel Falco tinnunculus shows that birds pay oxidative cost while being immuno-stimulated (with increased levels of ROMs, and decreased OXY; Costantini and Dell'Omo 2006).

Avian immune system bases on various interacting cells, tissues and proteins (Janeway et al. 2004). Some immune cells kill pathogens with pro-oxidant compounds. A multi-component enzyme complex in phagocytes (macrophages, eosinophils, heterophils) and lymphocytes produces ROS serving as important agents in pathogens killing. However, ROS may also damage host tissues, especially during chronic inflammation what results in elevated OS level (Sorci and Faivre 2009, Costantini and Møller 2009).

If breeding effort results in oxidative stress, sexual difference in a breeding effort should be manifested in difference in oxidative stress level. Also, owing to the basic sex differences in hormones levels, one may expect different physiological effects of reproduction on males and females. However, recent meta-analysis found no evidence for significant sex differences in oxidative balance in fish, birds, and mammals (Costantini 2018). Nevertheless, sex-dependent oxidative stress has rarely been examined, and more studies on various taxonomical and ecological groups are needed to fully comprehend the issue of sex-dependent oxidative stress.

In this study we aim to investigate the level of oxidative stress in the little auk (or dovekie Alle alle) in relation to phase of breeding and sex. We also investigate how oxidative stress is related to birds leukocyte profiles. The studied species is a small, colonially breeding, monogamous alcid, with life-history traits, characteristic for seabirds: long-lived, with long and extensive bi-parental care over a single offspring (Harding et al. 2004, Wojczulanis-Jakubas et al. 2009, Wojczulanis-Jakubas and Jakubas 2012). Due to its small wing area and subsequent high wing loading, the little auk exhibits one of the highest mass-specific daily energy expenditures among seabirds (Gabrielsen et al. 1991). For this reason, little auk's foraging is believed to be costly (Gabrielsen et al. 1991, Konarzewski et al. 1993). Given all this, the little auk is a good model species to study changes in OS in the context of energy expenditures during the breeding period.

We sampled the birds during three consecutive breeding phases i.e. late incubation, mid and late chick rearing period, assuming an increase in energy expenditure along the three periods. Given the increasing energy requirements, we expected that OS level would be the lowest during the incubation, and highest at the late chick rearing. Although both little auk partners are equally involved in incubation and chick rearing, the female abandons the brood in the last week of the nesting period; the male stays with the offspring until fledging, and probably continues the care during post-fledging period at the sea (Stempniewicz 2001, Harding et al. 2004, Wojczulanis-Jakubas et al. 2009, Wojczulanis-Jakubas and Jakubas 2012). The female's brood desertion has been a subject of few studies, however, the reason of her earlier departure still remains unclear. One of the hypothesis is that the female ceases feeding due to her worse condition due to higher primary reproductive investment (female produces one large egg constituting ~20% of her body mass; Wojczulanis-Jakubas et al. 2012, 2014, Wojczulanis-Jakubas and Jakubas 2012). If so, she should also exhibit higher OS level, that in turn could trigger her to

abandon the brood. Consistently, we expected that little auk females would experience more pronounced changes in OS level than males during the three breeding periods. Since OS is the ratio of ROMs to OXY, increasing along with ROMs values and decreasing along the OXY (Costantini et al. 2006), we expected that ROMs and OXY would change in an opposite way in respect to each other. We also expected positive correlations between OS and four haematological parameters related to physiological stress and immune function. Of that we expected a positive correlation of OS with: a) the proportion of heterophils to lymphocytes (H/L); this is because H/L values positively correlates with corticosterone (stress hormone) level (Davis et al. 2008); b) the number of heterophils; this is because the number of heterophils increases during stress, trauma, and chronic bacterial infections; c) number of lymphocytes as the lymphocytes are responsible for T-cell-mediated immune response (Davis et al. 2008), and d) total number of leukocytes per 10 000 erythrocytes as that parameter suggests mobilization of the whole immune system (Alonso-Alvarez and Tella 2001), which would be expected in the condition of high OS. Consistently, we expected that ROMs will increase, and OXY decrease with all haematological variables.

1. Methods

2.1. **Study area and field work**

We conducted the study in the large little auk breeding colony in Hornsund (SW Spitsbergen; 77°00’ N 15°33’ E). We captured and blood sampled the birds three times in the breeding season 2015: during late incubation (3-4 days before median date of hatching in the colony), mid and late chick rearing periods (15 d and 21 d after hatching median date, respectively). We captured the birds directly in the nests during incubation, and using noose carpets exposed in the vicinity of nests during the chick rearing period. The breeding status of the birds during the incubation was confirmed by the presence of egg in the nest, and during the chick rearing period by the presence of food in gular pouch. In all nests of individuals sampled during incubation, the chicks hatched successfully. Given the high synchronization of breeding in 30 control nests (hatching dates ranging from 16 to 19 July), we assumed that all captured birds were at the same phenological phase of the

breeding. We ringed birds to control for multiple sampling of the same individual (although due to the study design all individuals were sampled only once). We took a small blood sample (100–150 μL) from the brachial vein of each individual using Na-heparinized micro-haematocrit-tubes. Since changes in body mass are often reported to accompany changes in OS level, and/or in response to an increase in breeding effort, to control the body mass (Totzke et al. 1999), we weighted all captured birds, using an electronic balance OHAUS (Parsippany, New Jersey, USA) accurate to 0.1 g. To further adjust the body mass to the body size, we measured head-bill length with a calliper (with a 0.1 mm accuracy). We released all birds unharmed after ca 10 min of handling. In total, we sampled 92 breeders (23 males and 23 females during the incubation, 13 males and 11 females at the middle of chick rearing period, 11 males and 11 females at the end of chick rearing period). We kept the collected blood samples cool (2–5°C) for few hours, until making blood smears and centrifuging to separate the plasma (for OS parameters) and red blood cells (for molecular sexing). We kept separated plasma frozen (−20°C), and red blood cells suspended in 90% ethanol and stored in room temperature until laboratory analyses (3-4 months after collection).

2.2. Laboratory work

Measurement of reactive oxygen metabolites

We measured the serum concentration of reactive oxygen metabolites (ROMs), primarily hydroperoxides (ROOH), using the d-ROMs test (Diacron, Grosseto, Italy) following the procedure described by Costantini and Dell'Omo (2006). Firstly, we diluted the blood plasma (10 μL) with 200 μL of a solution containing 0.01 M acetic acid/sodium acetate buffer (pH 4.8) and N,N-diethyl-p-phenylenediamine as chromogen. Then, we incubated it for 75 min at 37°C. After incubation, we read the absorbance with a spectrophotometer (VISIBLE 722) at 490 nm. We expressed the measurements in mM of H_2O_2 equivalents in reference to a standard curve obtained by measuring the absorbance of a standard solution.

Measurement of antioxidant barrier

We measured the total antioxidant barrier (OXY) by the OXY-Adsorbent test (Diacron, Grosseto, Italy) following the procedure described by Costantini and Dell'Omo (2006). We diluted the blood plasma (10 μL) in proportion 1:100 with distilled water. We incubated a 200 μL aliquot of a tittered HOCl solution with 5 μL of the diluted plasma for 10 min at 37°C. Then, we added 5 μL of the same chromogen solution used for the ROM determination. We measured the intensity of the coloured complex, which is inversely related to the anti-oxidant power, with the same spectrophotometer at 490 nm. We expressed measurements as mM of HOCl neutralised in reference to a standard curve.

Leucocyte profiles

From the collected 5 μl of blood we made the blood smear, air-dried it and then stained using the May–Grünewald–Giemsa method (Lillie 1977) with a Wescor ‘‘Aerospray Hematology’’ stainer (Elitech Group, Puteaux, France). We examined blood smears under a microscope at a magnification of 1 000 × under oil immersion. We counted leukocytes until the cumulative total of 100 cells (Gross and Siegel 1983). We counted all types of leukocytes but we included in statistical analyses only heterophils and lymphocytes due to the small number of other leukocytes (basophils, eosinophils and monocytes). We also presented the proportion of heterophils and lymphocytes (i.e. H/L ratio). Moreover, we counted the number of heterophils (hereafter Hetero), lymphocytes (Lympho) and all types of leukocytes combined (Leuco) per 10 000 erythrocytes to estimate the overall allocation in the leukocyte production (Lobato et al. 2005). All smears were screened by the same person (IK), oblivious of the phase of breeding and sex of birds.

Molecular sexing

Because of the lack of considerable morphological dimorphism in the little auk (Jakubas and Wojczulanis 2007) we determined sex molecularly. We extracted DNA using the Blood Mini kit (A&A Biotechnology, Gdynia, Poland), after ethanol evaporation. We performed PCR using the primer pair F2550 and R2718, with 50^{o}C annealing temperature (Griffiths et al. 1998) . These primers amplify a 430-bp fragment on the W chromosome (in females only), and a 600-bp fragment on the Z chromosome (in both sexes) (Griffiths et

al. 1998). This size difference was clearly visible in UV-light when dyeing the fragments with Midori Green (Genetics, Germany) and separating on a 2% agarose gel.

Data analysis

We calculated an index of oxidative stress (OS) as the ratio of ROMs to OXY (x 1 000) following Costantini et al. (2006). In our study we focused on OS as an universal index of oxidative stress (Costantini et al. 2006), frequently reported in the literature. However, since OS may change due to various changes of ROMs and OXY (Costantini et al. 2006), we also separately analysed the two latter.

To compare the OS, ROMs, and OXY (response variables) between breeding phases (incubation, mid and late chick-rearing) and sex we performed analysis of covariance (ANCOVA), with phase and sex as fixed factors. To control the effect of possible relationships between response variables and body mass (Totzke et al. 1999) , we added to all the analyses the size-adjusted body mass (scaled mass index, SMI) as a covariant. We also included in the analyses interaction of phase and sex, due to expected sex-specific response to reproductive effort in particular breeding phases (Weimerskirch et al. 2000). As a post-hoc tests, we used HSD test for unequal N.

To adjust body mass to body size we used scaled mass index (SMI) proposed by Peig and Green (2009), previously used for the little auk (Wojczulanis-Jakubas et al. 2015, Kulaszewicz et al. 2017). We used mean value of head-to-bill length for the target population. We chose the head-to-bill length for the linear body size measurement as this measurement was significantly related to the body mass in adults (both sexes combined; Pearson correlation coefficient, $r_{91} = 0.41$, $p = 0.002$). We did not analyse differences between the periods and sexes in body mass per se, as these have already been a subject of another study (e.g. Wojczulanis-Jakubas et al. 2015), and here we only wanted to control for a possible effect of body mass on the oxidative stress parameters (Totzke et al. 1999).

To examine relationships between oxidative stress parameters (OS, ROMs, and OXY) and immunological variables (H/L, and number of leucocytes, heterophils and lymphocytes per 10 000 erythrocytes), we applied a modelling approach (general linear model, LM). To avoid multicollinearity of predictors representing various haematological parameters, we run two separate models: 1) with H/L being treated as a continuous predictor, and 2) with the number of leucocytes, heterophils and lymphocytes per 10 000 erythrocytes, all treated as continuous predictors (the variables not correlated; all pairwise $r < 0.19$). We included sex as a categorical predictor in both models. We performed the models for two breeding phases separately (incubation and chick rearing period). We

combined mid and late chick rearing period due to lack of significant differences in OS, ROMs and OXY in previous ANCOVA (all $p > 0.12$).

To select the best model for the regression analyses including sex, phase of breeding and the number of leucocytes, heterophils and lymphocytes per 10 000 erythrocytes, we used Akaike's information criterion for small sample size (AIC_c) (Burnham and Anderson 2002, Mazerolle 2006). To compare the relative performance of the models, we calculated the difference (ΔAIC_c) between the AIC value of the best model and AIC value for each statistical significant model. We also calculated Akaike's weights, i.e. probability that a model is the best model for observed data given the candidate set of models (Burnham and Anderson 2002). We considered and presented results only for the models with $\Delta AIC_c \leq 2$ (Burnham and Anderson 2002). We checked significance of the candidate models using Wald statistics.

Before the analyses, we checked whether the data sufficiently satisfied the assumptions of the linear model using Q-Q plots (the quantile expected from normally distributed vs. the quantile from the observed residuals plot). We normalized the H/L ratio using logit transformation (Zar 1999), and the other haematological parameters using Box-Cox-transformation (Box and Cox 1964).

We performed the statistical analyses in STATISTICA 12 (StatSoft Inc., Tulsa, OK, USA), and R software (R Core Team 2015), using MuMIn package for the model selection (Bartoń 2013). We considered results significant at level of $p < 0.05$.

2. Results

Factors affecting oxidative stress components and leucocyte profiles

We found that oxidative stress level (OS) was affected significantly by the phase of breeding season, with lower values during the incubation compared to two phases of the chick-rearing period (ANCOVA; post-hoc test, $p < 0.01$, Table 1, Fig. 1A). OS was not affected significantly by a covariant, scaled mass index (SMI). Neither sex nor the interaction of sex and breeding phase affected OS significantly (Table 1).

Also, the ROMs were affected significantly by the phases of breeding season with higher values during incubation compared to two phases of the chick-rearing (ANCOVA;

post-hoc test, $p < 0.01$, Table 1, Fig. 1B). ROMs were not affected significantly by a covariant, scaled mass index (SMI). Neither sex nor the interaction of sex and breeding phase affected ROMs significantly (Table 1).

Similarly, we found that plasma anti-oxidant capacity (OXY) was affected significantly only by the phase of breeding season. In this case, however, lower values were revealed for the incubation than the two phases of the chick-rearing period (ANCOVA; post-hoc test, $p < 0.01$, Table 1, Fig. 1C). OXY was not affected significantly by a covariant, scaled mass index (SMI). Neither sex nor interaction of sex with breeding phase affected OXY significantly (Table 1).

Relationships among the studied variables

We found a positive and significant relationship of both OS and ROMs with H/L, both increased with an increase of H/L. For OXY and H/L, we recorded a negative significant relationship with OXY decreased with increasing H/L ratio (Table 2; Fig. 2).

According to the model selection in the analysis of the relationship between oxidative status variables and various haematological parameters, sex and phase of breeding, the best model contained only number of lymphocytes and total number of leucocytes per 10 000 erythrocytes (Supplementary Material 1, Table A1). OS level increased with increasing numbers of lymphocytes per 10 000 erythrocytes and total number of leucocytes per 10 000 erythrocytes. Similarly, ROMs increased with increasing numbers of lymphocytes per 10 000 RBC and total number of leucocytes per 10 000 erythrocytes. OXY increased with decreasing numbers of lymphocytes per 10 000 erythrocytes and total number of all leucocytes per 10 000 erythrocytes (Table 2, Fig. 3, 4).

3. Discussion

Due to increasing reproductive efforts with the progress of the breeding season (incubation < mid chick rearing < late chick rearing period), we expected the highest oxidative stress (OS) level in the little auk parents at the late chick rearing period. Also, due to assumed female-biased reproductive effort, we expected females to have higher indices of OS than males, especially at the end of the breeding season. Contrary to these expectations, however, we found that regardless of sex overall OS level and blood oxidative damage (ROMs) was lower during the incubation than at the later stages of

breeding in the parents. Consistently, the plasma anti-oxidant capacity (OXY) was higher during the incubation than at the later stages of breeding in both sexes.

Lower OS and lower ROMs with a simultaneous higher level of OXY during the chick rearing period compared to the incubation suggest a buffering the negative effects of high reproductive costs. Although surprising, given the initial expectations supported by relevant literature (e.g. Fletcher et al. 2013), similar changes in ROS level throughout the breeding season has been reported in some other species, e.g. in the brown booby (Sula leucogaster; Montoya et al. 2016), and the Magellanic penguin (Spheniscus magellanicus; Colominas-Ciuró et al. 2017). Apparently, costs of an increased reproductive effort are not always detected in OS level (Metcalfe and Monaghan 2013, Speakman and Garratt 2014, Costantini 2014). Although speculative, this may be the question of food resources availability at the time of the study. If the resources are not limited the reproductive effort does not extent a threshold, i.e. OS level is lower and/or ROMs is mitigated by the anti-oxidant barrier (van Noordwijk and de Jong 1986). In penguins, OS level in birds returning from the feeding areas is lower than before foraging trip (Shull et al. 2016), suggesting a body resources restoration of the breeding adults during the foraging trips. Little auks also seem to restore their body reserves during the foraging trips during the chick rearing period (Welcker et al 2012). According to match-mismatch concept (e.g. Visser et al. 1998, Durant et al. 2003), food availability during the chick-rearing period of seabirds should be the highest. It is therefore possible, that these birds (including the little auk) have enough resources to maintain the oxidative balance during the energetically demanding chick rearing period.

An alternative explanation of the observed pattern of OS parameters during the three breeding phases, could be some other physiological changes related to reproduction. A decline of the H/L ratio with the progress of the breeding season reported in the little auk in earlier studies (Wojczulanis-Jakubas et al. 2012, 2015, Kulaszewicz et al. 2017) could be related with an internal mechanism preparing adults to bring breeding activities to the end (Wojczulanis-Jakubas et al. 2012). This mechanism might also affect components of antioxidant defense. This whole process is triggered and modulated by hormonal changes. For example, it was shown that prolactin modulates function of vertebrate immune system (Matera 1996, Olavarría et al. 2012) serving as a regulator of the macrophage response. It has also been reported for fish that production of ROS by phagocytic leukocytes is stimulated by prolactin (Yada et al. 2001, Olavarría et al. 2010). Given a five-fold higher

level of prolactin during incubation compared to chick-rearing reported in the little auk (Wojczulanis-Jakubas et al. 2015), this hormone may be responsible for stimulation of immune system during incubation, which in turns is reflected in higher OS and ROMs levels (Fig. 1A, 1B).

Stimulation of the immune system may be expressed by increased oxidative damage since ROS (released by phagocytes to kill pathogens) are not able to discriminate between pathogens and host cells (Sorci and Faivre 2009). Indeed, we found that level of ROMs increased with increasing H/L ratio (Table 2; Fig. 2C, 2D) and numbers of lymphocytes per 10 000 RBC and total number of leucocytes per 10 000 RBC during both incubation and chick-rearing periods (Table 2; Fig. 3C, 3D, 4C, 4D). On the other hand, wild birds can also mount immune responses without suffering from systemic OS (Cram et al. 2015b).

Some other studies have reported sex-difference in OS level. For example, brown booby males had higher ROS levels than females during the courtship, which is likely to be related with their colourful ornaments developing at that time, which in turn might had entailed the oxidative cost (Montoya et al. 2016). We expected the little auk females to have higher OS than males due to higher initial investment (Wojczulanis-Jakubas et al. 2014).Our results, however, did not reveal any sex differences in plasma oxidative status during the three phases of breeding period. Although contradictory in the context of the tested hypothesis, this results is concordant with lack of sex differences in the little auk in other immune response components (H/L, killing bacteria capacity) reported in other studies (Wojczulanis-Jakubas et al. 2012, 2014, 2015, Jakubas et al. 2015, Kulaszewicz et al. 2017). Thus, all that together severely undermines the hypothesis about worse female body condition (immunological capacity, body mass, etc.) being a trigger of her earlier brood desertion.

In conclusion, our study indicates that OS level in adult little auks decreases throughout the breeding season, basically being higher during the incubation than chick rearing period. We suggest that those differences may be attributed to breeding stage dependent changes in hormone levels, that in turn, affect various haematological parameters, including the oxidative status. Lack of sex differences in the oxidative status, along with some other studies (Wojczulanis-Jakubas et al. 2012, 2014, 2015, Jakubas et al. 2015, Kulaszewicz et al. 2017), further undermines hypothesis about the little auk female ceasing the chick provisioning earlier than male due to her worse body condition.

Nevertheless, since our study is correlational with a cross-sectional design, to fully comprehend seasonal changes in OS and hormones as well as causality of the observed patterns, further, preferably experimental studies are needed, possibly also including earlier breeding stages.

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Figure Legends

Figure 1. The oxidative stress (OS), reactive oxygen metabolites (ROMs), total antioxidant barrier (OXY) (points are means, boxes are SD,whiskers are 5%-95% percentiles; values corrected for covariant, scaled mass index) in adult little auks (both sexes combined) captured during late incubation (LINC), mid (MCHR) and late chick-rearing (LCHR) periods. Arrows indicate significant differences among phases of breeding (ANCOVA, *post-hoc* HSD test for unequal N, $p < 0.01$).

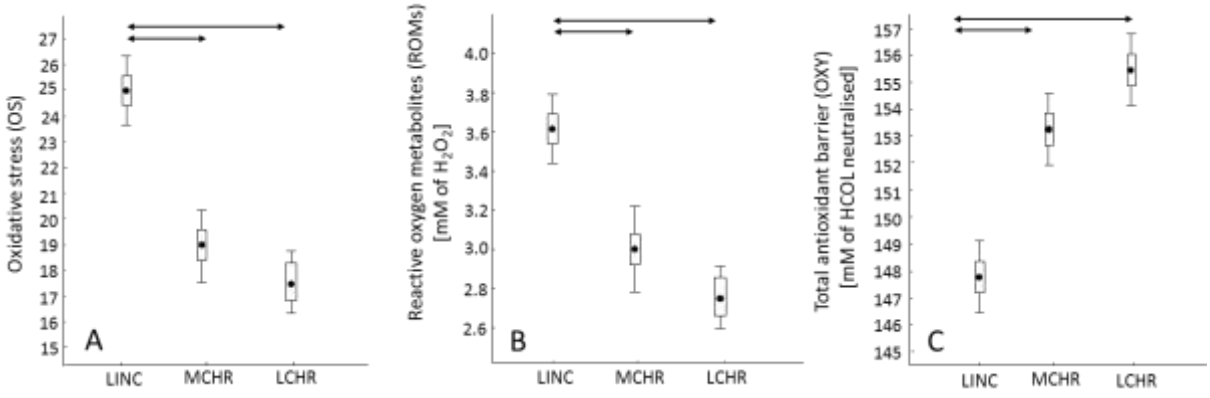

Figure 2. Relationship between the values of oxidative stress (OS), reactive oxygen metabolites (ROMs), total antioxidant barrier (OXY) and proportion of heterophils and lymphocytes (H/L ratio) in adult little auks during late incubation and chick rearing (mid and late phases combined) periods. Regression lines are shown for significant relationships. Analyses of regression performed on Box-Cox transformed data.

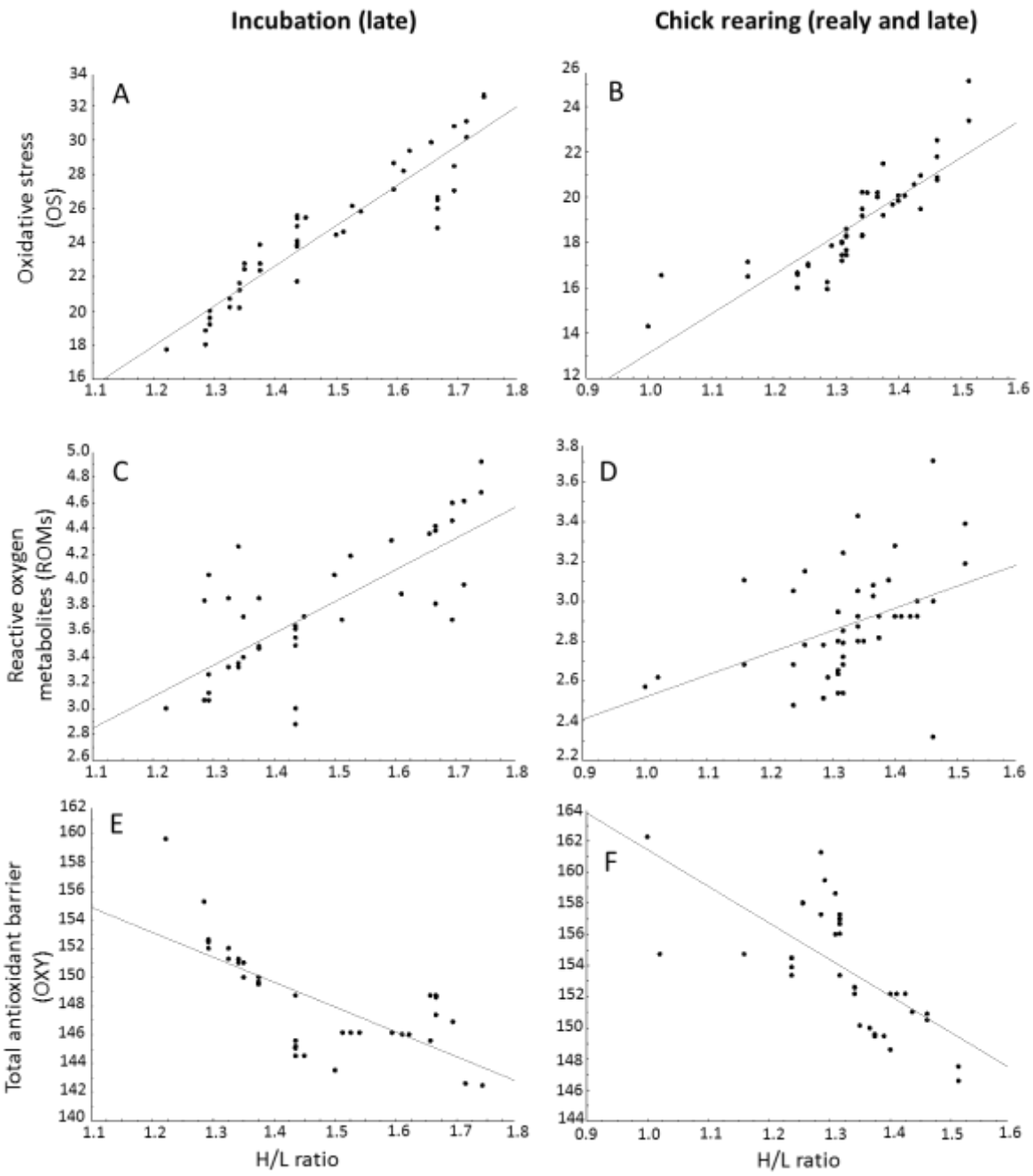

Figure 3. Relationship between the values of oxidative stress (OS), reactive oxygen metabolites (ROMs), total antioxidant barrier (OXY) and number of leucocytes per 10 000 erythrocytes in adult little auks during late incubation and chick rearing (mid and late phases
combined) periods. Regression lines are shown for significant relationships. Analyses performed on Box-Cox transformed data.

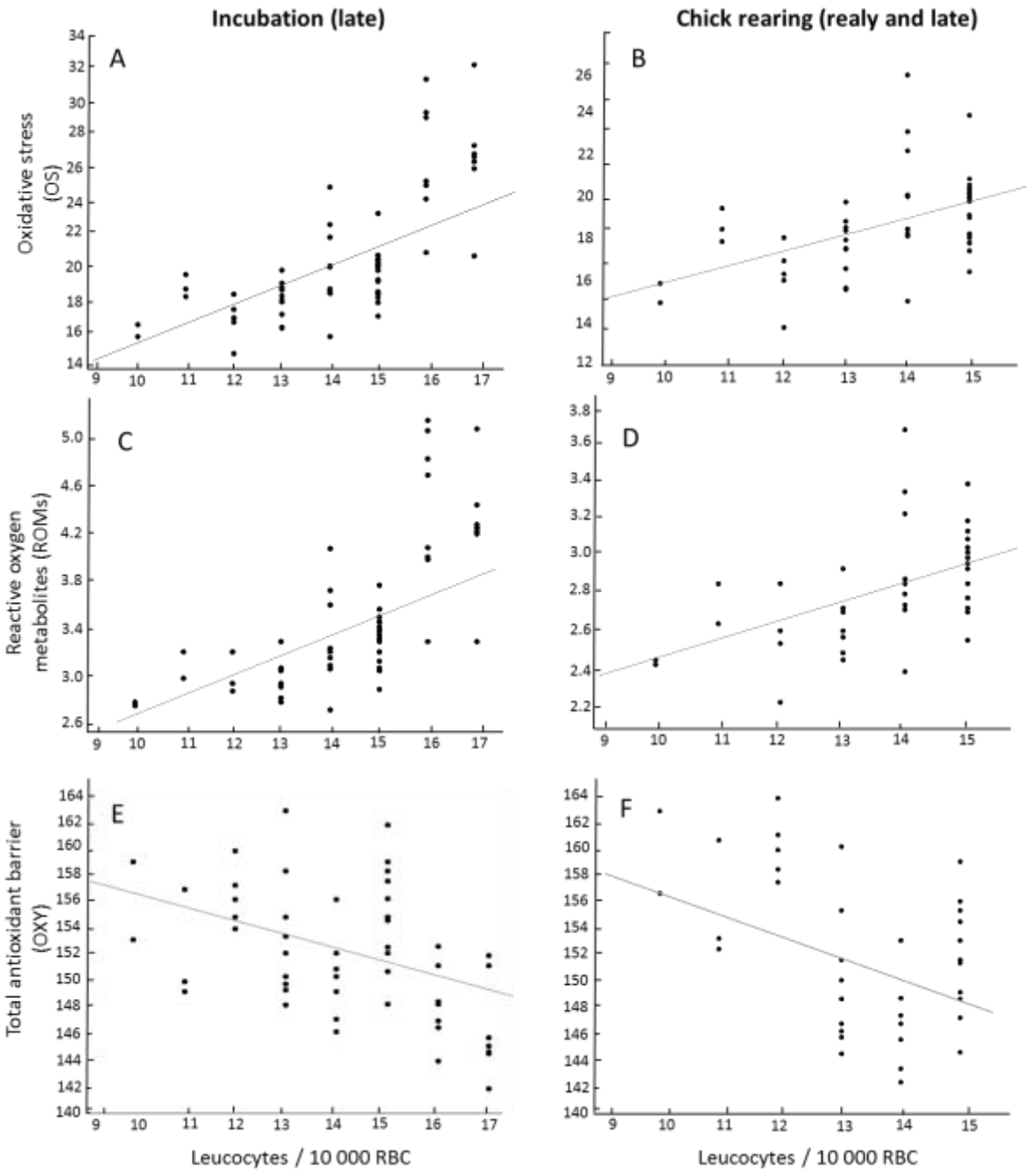

Figure 4. Relationship between the values of oxidative stress (OS), reactive oxygen metabolites (ROMs), total antioxidant barrier (OXY) and number of lymphocytes per 10 000 erythrocytes in adult little auks during late incubation and chick rearing (mid and late phases combined) periods. Regression lines are shown for significant relationships. Analyses performed on Box-Cox transformed data.

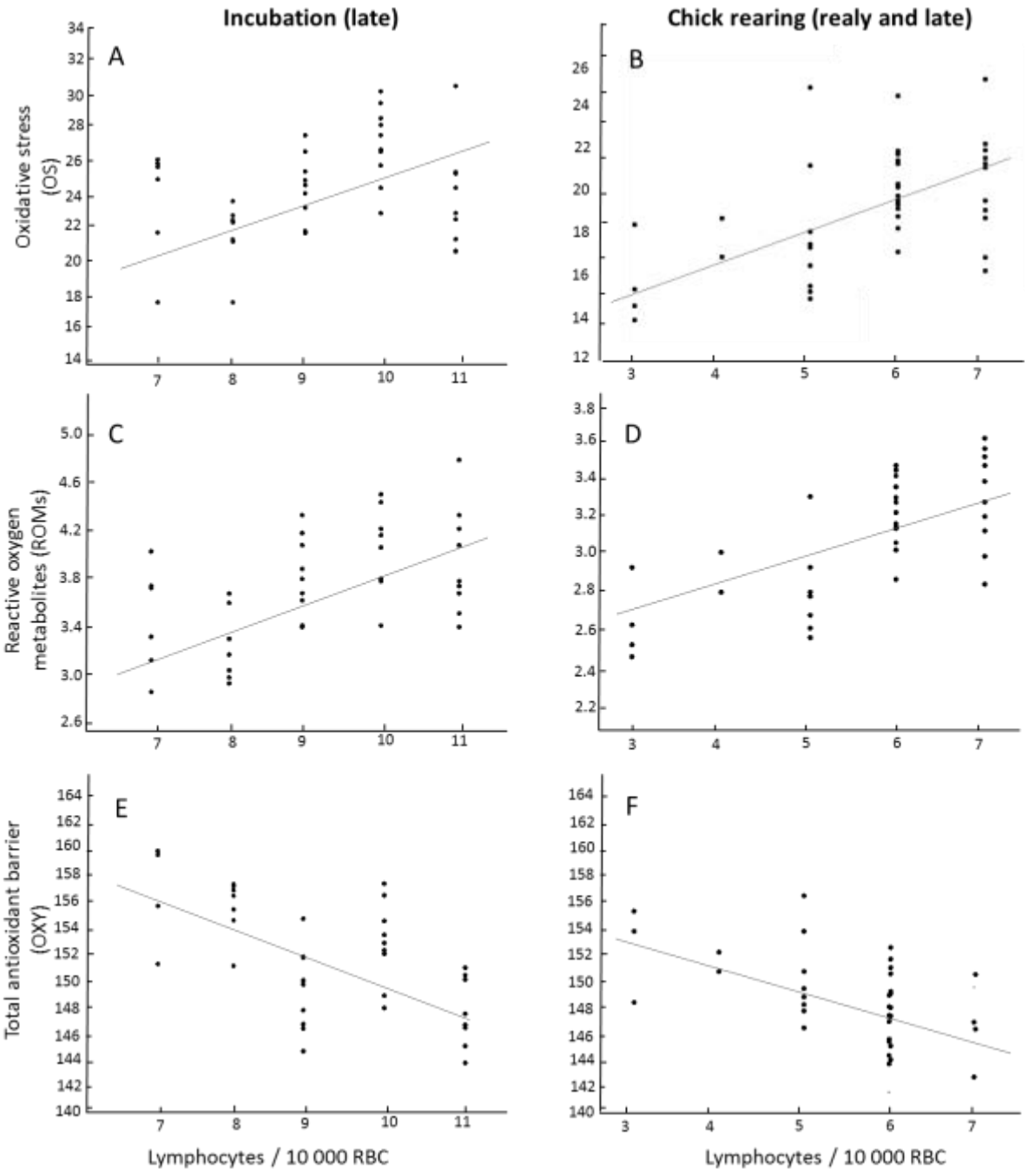

Table Legends

Table 1. The effects of breeding phase (Phase), sex and their interaction on reactive oxidative stress (OS), oxygen metabolites (ROMs) and total antioxidant barrier (OXY) in adult little auks, with scaled mass index (SMI) as a covariant (ANCOVA). Significant effects ($p <$ 0.05) are bolded.

	OS			ROMs			OXY		
Parameter	df	F	p	df	F	p	df	F	p
Intercept	1	34.86	< **0.001**	1	35.34	< **0.001**	1	906.83	< **0.001**
SMI	1	0.79	0.37	1	0.67	0.41	1	0.27	0.61
Phase	2	37.58	< **0.001**	2	29.34	< **0.001**	2	23.27	< **0.001**
Sex	1	0.15	0.69	1	0.07	0.78	1	0.99	0.32
Phase × Sex	1	0.62	0.53	1	0.55	0.57	1	0.31	0.77
Error	86			86			86		

Table 2. The relationship between oxidative stress (OS), reactive oxygen metabolites (ROMs), total antioxidant barrier (OXY) and H/L ratio, number of lymphocytes (Lymph), leucocytes (Leuco) per 10 000 erythrocytes during incubation and chick rearing (mid and late phases combined). adj. R^2 – adjusted determination coefficient.

Model parameters	**Incubation**			**Chick rearing**		
	β	p	Eta-Squared	β	p	Eta-Squared
OS ~ H/L, adj. R^2 = 0.48						
Intercept		**<0.001**			**<0.001**	
H/L	0.52	**0.038**	0.25	0.45	**0.027**	0.24
OS ~ Leuco + Lymph, adj. R^2 = 0.33						
Intercept		**0.004**			**0.001**	
Leuco	0.38	**0.011**	0.22	0.35	**0.029**	0.28
Lymph	0.41	**0.022**	0.27	0.38	**0.025**	0.31
ROM ~ H/L, adj. R^2 = 0.44						
Intercept		**<0.001**			**<0.001**	
H/L	0.47	**0.019**	0.38	0.33	**0.024**	0.32
ROM ~ Leuco + Lymph, adj. R^2 = 0.39						
Intercept		**<0.001**			**0.002**	
Leuco	0.36	**0.011**	0.41	0.31	**0.017**	0.34
Lymph	0.32	**0.004**	0.28	0.36	**0.014**	0.26
OXY ~ H/L, adj. R^2 = 0.52						
Intercept		**<0.001**			**<0.001**	
H/L	-0.43	**0.033**	0.21	-0.44	**0.017**	0.28
OXY ~ Leuco + Lymph, adj. R^2 = 0.38						
Intercept		**0.001**			**0.003**	
Leuco	-0.38	**0.018**	0.37	-0.32	**0.027**	0.36
Lymph	-0.31	**0.003**	0.23	-0.33	**0.018**	0.38