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Sex differences in body condition and hematological parameters in Little Auk *Alle alle* during the incubation period

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Incubation may be an important component of reproductive costs, and birds can spend considerable amount of energy during this period. We investigated how the stress associated with incubation affects hematological parameters (proportion of leukocytes) and size-adjusted body mass of males and females of Little Auk (*Alle alle*), a monogamous species. Body mass of both sexes increased significantly between the early and late phases of incubation; both sexes gained 3–4% of their initial mass on average. Stress level (heterophil/lymphocyte ratio) was higher in females than in males during the early incubation phase, which could result from physiological stress associated with egg formation and production, and/or allocation of resources from the immune system to reproduction. Female stress level decreased with the advance of incubation, whereas in males both stress level and body mass increased. The latter finding could be associated with the higher contribution of males in activities other than incubation. Our results show that body mass and hematological parameters respond independently to stressors. Thus, the assessment of the condition of an individual should be based on various parameters.

1. Introduction

Avian reproduction requires a high energetic expenditure (Drent & Daan 1980). Egg incubation may be considered less costly than chick rearing but may actually be an important component of reproductive costs. Birds can indeed spend about the same amount of energy in incubating as in feeding nestlings (Reid *et al.* 2002, Tinbergen & Williams 2002). Energetic constraints imposed during incubation may be related to parent condition (Moreno & Sanz 1994, Heaney & Monaghan 1996, Minguez 1998) and may influence the number of fledglings, reproductive decisions in subsequent breeding attempts, and parental survival (Minguez 1998, Reid *et al.* 2000). These costs can be different between males and females, as parental investments of females include a high initial investment in egg production (Monaghan *et al*. 1998). Even in monogamous seabirds with biparental care, males and females often differ in their specific parental roles, foraging behaviour, or the timing of certain forms of parental care across the breeding season (Creelman & Storey 1991, Weimerskirch *et al.* 1997, Gray & Hamer 2001, Ainley *et al.* 2002, Bradley *et al.* 2002, Paredes *et al.* 2006).

Costs of incubation can be measured by studying body condition and/or stress level. To assess changes in these parameters we applied size-adjusted body mass and hematological parameters, here leukocyte proportions. Measurement errors for these indices are relatively small as compared to their total variation, suggesting that these are adequate variables for ecological research purposes (Ots *et al.* 1998). Body mass is affected by a multitude of components of individual state. However, this easy measurement is widely used in studies dealing with captured animals (Ots *et al.* 1998). Size-adjusted body mass, on the other hand, allows a researcher to separate the effects of body condition from those of body size (Reist 1985, Krebs & Singleton 1993, Jakob *et al.* 1996). Hematological parameters, such as ratios of white blood cells, reliably indicate stress (Ots & Hõrak 1996; Ruiz *et al.* 2002) and provide a useful indicator of body condition (Zuk 1996).

Proportion of leukocytes, especially the heterophil/lymphocyte (H/L) ratio, are widely used stress estimators in poultry and are known to increase as a response to various stressors, including infectious diseases, starvation and psychological disturbance (Gross & Siegel 1983, Dein 1986, Maxwell 1993). Gulls and penguins also respond to stress conditions by increasing their H/L ratio (Averbeck 1992, Totzke *et al.* 1999, Vleck *et al.* 2000). This reaction is detectable within hours following an exposure to a stressor (Gross & Siegel 1983, Gross 1990, Ots & Hõrak 1996, Vleck *et al.* 2000, Ruiz *et al.* 2002). Thus, blood parameters reflect the current physiological status of an individual and are not affected by stress associated with animal handling and blood sampling. The H/L ratio is less variable than individual cell numbers and is generally more reliable than plasma corticosteroid level as an indicator of avian stress (Maxwell 1993).

Little Auk (*Alle alle*) breeds in the high Arctic and is the most numerous seabird in the Palaearctic. It is a colonial seabird with long-term pair bonds and socially monogamous breeding system. Both sexes incubate a single egg and feed the chick. However, females cease chick feeding a few days before fledgling, whereas males continue parental care, including escorting the chick in its first flight to sea (Stempniewicz 2001, Harding *et al.* 2004). Until now, the only study on changes in Little Auk body mass during the breeding season has been performed by Taylor (1994), and to our knowledge hematological studies focused on leukocytes have never been conducted in this species. Thus, our study is the first to report leukocyte reference values for Little Auk.

The main objective of our study was to compare the body condition and stress levels of Little Auk males and females during the incubation period. Because females invest more energy on egg production, we expected that, at the beginning of the incubation period, body condition would be poorer and stress level higher in females than in males. As both sexes similarly contribute to incubation (Stempniewicz & Jezierski 1987, Wojczulanis-Jakubas 2007), females may not have time to replenish their energetic reserves during egg laying and incubation. Hence, we hypothesized that females would be in poorer condition than males also in the late phase of incubation.

2. Methods

We conducted the study in 2006 in a Little Auk breeding colony at Ariekammen slopes in Hornsund, South Spitsbergen (77°00’ N, 15°33’ E). We caught adults in their nest chambers during early (3rd–11th day) and late (20th–25th day) phases of the incubation period. We estimated the advance of incubation in the sampled pairs by a backward calculation from the hatching date. After the first catching, we ringed and measured all individuals. We collected data on the flattened wing length (from the bend in the wrist to the tip of the longest primary), head–bill length (distance from the back of the head to the tip of the bill), bill width (from the left to the right edge of the bill at the base) and tarsus length (the diagonal distance from the middle of the joint between tibiotarsus and tarsometatarsus to the junction of the tarsometatarsus with the base of the middle toe). We took all measurements to the nearest 0.1 mm with dial callipers except for wing length, which was measured to the nearest 1.0 mm using a stopped wing ruler. All birds were measured by the same person. We

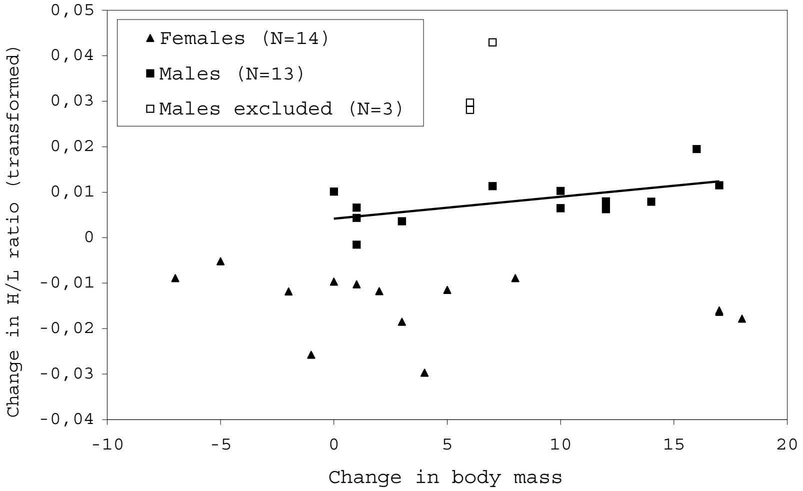

Fig. 1. Changes in body mass and heterophil/leukocyte (H/L) ratio (arcsin square-root transformed) between the early and late phases of incubation in males and females of Little Auk in Hornsund in 2006; regression line equation for males, $y = 0.0005\,x + 0.0042$.

weighed all birds to the nearest 1.0 g using 300 g PESOLA spring balance.

During both periods, we collected a small blood sample (ca. 10–20 µl) from the brachial vein of each individual for hematological analyses. The sample was smeared on a microscopic slide immediately after collection and stored until staining. One blood smear was prepared from each blood sample. Blood smears were stained using the May–Grünewald–Giemsa method. The proportion of different types of leucocytes (lymphocytes, heterophils, monocytes, eosinophils and basophils) was assessed on the basis of an examination of 100 leucocytes under oil immersion (1,000 × magnification).

To analyze the relative abundance of lymphocytes and heterophils, we used the heterophil/lymphocyte (H/L) ratio. As this proportion may not be normally distributed, we applied the non-parametric Mann-Whitney *U* test to compare between sexes and incubation phases.

During the first period we took an additional blood sample from each individual for a DNA-based sex identification. The sample was immediately suspended in 1 ml lysis buffer and stored until molecular analysis in the laboratory at the University of Gdańsk (Poland). DNA was extracted from 100 µl blood and buffer solution, using the Blood Mini kit (A & A Biotechnology, Gdynia, Poland). The protocol described by Griffiths *et al.* (1998) was optimized to amplify the CDH-W and CDH-Z genes located on the avian sex chromosomes using two sets of primers 2550 F and 2718 R. A gel electrophoresis revealed one band in the male and two in the female.

We weighed 23 males and 23 females in the early phase and 18 males and 17 females in the late phase and prepared smears from blood samples for all of these; the latter numbers include 17 males and 15 females shared with the early phase. Therefore we were able to calculate changes in body mass and hematological parameters between both phases for the same 16 males and 14 females.

We adjusted the body mass of the studied individuals to head–bill length, a surrogate measure for body size, using analysis of covariance (ANCOVA; García-Berthou 2002) with the head–bill length as a covariate. We chose this measure to represent the individual size due to a significant correlation between this parameter and body mass during early ($r_{33} = 0.43$, $P = 0.009$) and late phase of incubation ($r_{33} = 0.42$, $P = 0.01$). The relationships of several other body-size parameters – namely wing and tarsus length, bill width, bill depth, and the first principal component of all mentioned measurements (PC1) – with body mass were not significant ($P > 0.05$). Because of the high degree of measurement overlap between the sexes (Jakubas & Wojczulanis 2007), we calculated all correlation coefficients for males and females combined.

We calculated changes in body mass between the first and second periods using raw data (i.e., not adjusted for head–bill length) because these changes were not correlated with body size.

We evaluated the relationship among hematological parameters, body mass and body size (i.e., head–bill length) separately for both sexes using multiple regression (Freckleton 2002). To approach data normality in regression, we arcsin square-root transformed all changes in hematological parameters (Zar 1999). In the case of the H/L ratio change in males, data were normally distributed after excluding three outliers representing individuals with peak values of H/L ratio increase (their values exceeded three inter-quartile ranges; Stanisz 2007; Fig. 1).

3. Results

3.1. Body mass

The ANCOVA revealed that body mass, corrected for body size by using head–bill length as a covariate, of males and females were similar during the early phase of incubation (respective mean values 166.1 ± SD 1.99 g and 165.4 ± SD 1.95 g; $F_{1,42} = 0.06$, $P = 0.80$). We observed a similar pattern also during the late phase (males: mean 171.5 ± SD 2.35 g; females: mean 171.7 ± SD 2.48 g; $F_{1,33} = 0.002$, $P = 0.96$). Body size (head–bill length), but not gender ($P > 0.05$), had a significant effect on body mass during the early ($F_{1,42} = 4.92$, $P = 0.03$) and late ($F_{1,33} = 6.44$, $P = 0.02$) phase of incubation.

The change in body mass between the early and late phases was independent of body size (i.e., the bill–head length) both in males and in females (Pearson correlation: $r_{16} = –0.12$, $P = 0.63$ and $r_{15} = 0.14$, $P = 0.60$, respectively). During the late phase, body mass was significantly higher as compared to the early phase (Student's *t* test for dependent variables; males: $t_{17} = –3.81$, $P = 0.001$, females: $t_{16} = –2.71$, $P = 0.01$). The mean increases of the male and female body mass were similar (6.2 and 4.9 g, respectively; Student's *t* test: $t_{33} = –0.52$, $P = 0.60$). The increase constituted on average 3.8% in males and 3.0% in females from the initial values. However, the direction of the body mass change varied among individuals. In 11% of males and 23% of females, body mass decreased between the early and late phase of incubation.

3.2. Proportion of leukocytes

Heterophils and lymphocytes were the most numerous leukocytes found in males (76% of all recorded leukocytes) and females (78%) during the early phase of the incubation. Eosinophils, basophils and monocytes were present less frequently (Table 1).

We found that the number of heterophils per 100 leukocytes was significantly higher in females than in males. A reverse was recorded for lymphocytes. The numbers of eosinophils, basophils and monocytes were similar in both sexes. The H/L ratio was higher in females than in males (Table 1).

During the late phase of the incubation, heterophils and lymphocytes were the most numerous leukocytes, together making up 78% of all recorded leukocytes in both male and female blood smears. Percentages of particular types of leuko-

Table 1. Baseline leukocyte reference intervals for Little Auks during the incubation period in Hornsund, SW Spitsbergen in 2006 (IQR = inter-quartile range).

	Early incubation		Late incubation	
Leukocytes (%)	Males, N=23 Median; IQR; Range	Females, N=23 Median; IQR; Range	Males, N=17 Median; IQR; Range	Females, N=15 Median; IQR; Range
Heterophils	37*; 7; 32–46	45*; 9; 35–54	42; 6; 34–57	42; 7; 38–56
Lymphocytes	38*; 4; 35–45	33*; 12; 21–41	37; 3; 27–39	35; 10; 27–42
Eosinophils	9; 4; 3–15	8; 4; 0–13	7; 2; 1–12	7; 4; 2–10
Basophils	5; 5; 2–14	7; 6; 1–23	5; 2; 2–12	6; 4; 1–10
Monocytes	8; 4; 4–17	7; 4; 0–11	9; 7; 4–15	11; 5; 4–17
H/L ratio	1.0*; 0.2; 0.7–1.1	1.3*; 0.6; 0.9–2.5	1.2; 0.1; 0.9–2.1	1.2; 0.2; 1.0–2.1

* – significant (*P* <0.001) intersexual difference (Mann-Whitney *U* test).

Table 2. Differences in percentage of particular leukocyte types between the early and late incubation phases in the Little Auk (Wilcoxon matched-pairs test).

	Males, N=17			Females, N=15		
Leukocytes	Change	*T*	*P*	Change	*T*	*P*
Heterophils	increase	15.0	0.004	decrease	16.5	0.02
Lymphocytes	decrease	24.0	0.01	increase	19.5	0.02
Eosinophils	decrease	22.0	0.03	no change	32.5	0.21
Basophils	no change	41.5	0.29	no change	32.0	0.348
Monocytes	no change	66.5	0.94	increase	9.5	0.004
H/L ratio	increase	1.0	<0.001	decrease	15.0	0.01

cytes and the H/L ratio were similar for both sexes (Table 1).

For males, the number of heterophils was significantly lower during the early than during the late phase of incubation. We found the opposite for females (Table 2). The number of lymphocytes, however, was significantly higher during the early than during the late phase for males, whereas we found the opposite for females. The numbers of basophils were similar during the whole incubation period for both sexes, as were the numbers of eosinophils for females. For males, on the other hand, values recorded were lower in the late than in the early phase. The numbers of monocytes in males remained relatively constant throughout the incubation period; however, in females, values recorded were lower in the early than in the late phase. The H/L ratio for males increased significantly between the early and late phase of incubation, whereas a decrease was recorded for females (Table 2). Changes in all these hematological parameters did not significantly correlate with body size (i.e., bill–head length; Pearson correlation; $P > 0.05$).

3.3. Relationship between the size-adjusted body mass and hematological parameters

Multiple regression revealed no significant relationships among changes in body mass, hematological parameters and body size (i.e., head–bill length) ($P > 0.05$ for both males and females). In males, however, an exclusion of three individuals with the highest values of H/L ratio increase led the analysis to reveal a significant relationship between changes in body mass and H/L ratio ($\beta = 0.72$, $P = 0.02$; $R^2 = 0.46$, $F_{2,10} = 4.25$, $P = 0.046$; see Fig. 1 and Material and methods). The influence of body size (i.e., head–bill length) remained non-significant ($\beta = -0.34$, $P = 0.20$). In males, the H/L ratio value increased the most in individuals with the highest increase in body mass (Pearson correlation; $r_{11} = 0.60$, $t = 2.49$, $P = 0.03$; Fig. 1).

4. Discussion

The increase in body mass of males and females during incubation that we found is consistent with Taylor's (1994) results obtained from the same colony. Taylor (1994) found that this increase was almost solely due to fat deposition and was associated with high lipid content in the diet (Taylor & Konarzewski 1992). This pattern is not common in birds (Moreno 1989) but has been recorded in some other alcids and gulls (Hario *et al.* 1991, Gaston & Perin 1993, Jones 1994). These findings seem to support the "Incubatory Reserves Constancy Hypothesis" which suggests that the incubation period may be a departure from breeding stress that allows for the maintenance or recovery of body reserves (Houston *et al.* 1983, Moreno 1989, Hario *et al.* 1991). Body reserves may be beneficial for incubating Little Auks which often have long incubation shifts (the longest recorded bout was 33 hours; Stempniewicz & Jezierski 1987). These reserves function as insurance against the parents being forced to abandon their egg (Taylor 1994).

Lack of intersexual differences in body mass during early and late phases of incubation and in magnitude of body-mass increase suggest similar conditions for both sexes during the incubation, despite varying energy investments before this period. Our analysis of hematological parameters revealed a different pattern, however. In males, we observed an increasing H/L ratio (i.e., a rise in response to stressors; Ots *et al.* 1998) and a decreasing number of lymphocytes (i.e., a sign of immunodepression; Gross & Siegel 1983, Dohms & Metz 1991, Hõrak *et al.* 1998, Totzke *et al.* 1999, Lobato *et al.* 2005) with the advancement of the incubation period. We recorded the opposite for females: despite equal sharing of incubation duties by both sexes of Little Auk, males spend more time than females in the colony and outside the nest, and they also more frequently take part in aggressive interactions (Wojczulanis-Jakubas 2007). Thus, the increase in H/L ratio during the incubation period in males probably reflects this additional activity. The recorded higher H/L ratio in females during the first phase of incubation could be a consequence of physiological stress associated with egg formation and production (Latimer & Bienzle 2000). Moreover, although body and fat mass did not differ before and immediately after egg laying (Taylor 1994), other types of activity such as self-defense against forced extra-pair copulations (Wojczulanis-Jakubas *et al.*, in prep.) and pre-laying foraging at sea (Wojczulanis-Jakubas 2007) are certainly stressful and may cause immunodepression. Alternatively, the higher H/L ratio may result from buffering against increased reproductive demands by allocating resources from immunity to reproduction (Folstad & Karter 1992, Sheldon & Verhulst 1996, Norris & Evans 2000).

Body condition and stress level of females at the end of the incubation period were similar to males and better than during the early phase. This result contradicts the proposed reason of cessation of feeding young by a female shortly before fledging. Early termination of chick rearing by females has been hypothesized to balance the high initial investment in egg production (Harding *et al.* 2004). Our results suggest that after egg-laying and during incubation, females rebuild their body reserves. Thus, the reason for them leaving a chick earlier should be investigated during the chick-rearing period.

Contrary to our predictions, in the majority of the studied males the increase of stress level peaked in individuals with the highest body mass increase. Such a pattern could result from trade-offs within or between components of the immune system (Salvante 2006), suggesting a need for further studies that examine other components of the immune system. The mentioned relationship was obtained after excluding three analysis outliers. These excluded males might have been in a poorer state of health than the rest of the males or were more stressed by food resources (e.g., they may use less profitable foraging areas).

Our results showed that body mass and hematological parameters respond independently to stressors, as earlier reported for Zebra Finch (*Taeniopygia guttata*; Ewenson *et al.* 2001). Little Auk males were able to gain body reserves independently of stress levels. We conclude that the assessment of the actual body condition of a bird individual is a complex procedure and should therefore be based on various parameters.

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Ruumiinkunnon ja veren parametrien erot pikkuruokin *Alle alle* sukupuolten välillä haudonta-aikana

Haudonta voi muodostaa merkittävän osan jälkeläistuoton kustannuksista, ja lintuyksilöt voivat käyttää huomattavasti energiaa tämän jakson aikana. Tutkimme, miten haudonnanaikainen stressi vaikuttaa veren parametreihin (valkosolujen osuuteen) ja ruumiin kokoon suhteutettuun massaan pikkuruokkikoirailla ja –naarailla; laji on monogaminen. Molemmilla sukupuolilla massa kasvoi merkitsevästi haudonnan edetessä; molemmilla sukupuolilla nousu oli keskimäärin 3–4 % lähtötasosta. Stressitaso (heterofiilien ja lymfosyyttien suhde) oli naarailla koiraita korkeampi varhaisessa haudonnan vaiheessa, mikä voi liittyä munantuotannon elimistöön kohdistamaan stressiin, ja/tai resurssien jakoon immuunisysteemin ja lisääntymispanoksen välillä. Naaraiden stressitaso laski haudonnan edistyessä, mutta koirailla sekä stressitaso että massa nousivat. Jälkimmäinen havainto voi liittyä koiraiden korkeampaan aktiivisuuteen muussa kuin haudontaan liittyvässä toiminnassa. Tuloksemme osoittavat, että ruumiin massa ja veren parametrit muuttuvat toisistaan riippumatta stressitekijöiden vaihdellessa. Yksilön kunnon arvioinnin tulisi siten perustua useampaan parametriin.

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