ORIGINAL ARTICLE

Factors affecting leucocyte profiles in the little auk, a small Arctic seabird

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Abstract Leucocyte profiles, especially the heterophil/lymphocyte ratio (H/L), are widely used to assess immune function and stress in birds. In this study, we investigated whether leucocyte profiles in a small colonial seabird, the little auk (*Alle alle*), are related to age (chicks, adults), sex or biometric traits, including body mass. We blood-sampled chicks at 4–8 and 14–18 days of life and chick-feeding adults with 14–20- and 24–30-day-old chicks. Relative numbers of heterophils and H/L generally differed among chicks and adults, with the highest values in adults with 14–20-day-old chicks and the lowest ones in 4–8-day-old chicks. Females had a higher proportion of lymphocytes than males. The relative numbers of eosinophils were similar in all the groups. The percentage of monocytes tended to differ among the age categories. Analyses of chicks revealed that the percentage of heterophils and the H/L ratios were significantly higher in older than in younger chicks. The opposite pattern was recorded for lymphocytes. In 14–18-day-old chicks, H/L (stress level) was negatively related to body mass, head-bill length and bill width. This time period is energetically stressful as nestlings start to exercise their wings outside the nest chamber. This suggests that the origin of stress in nestlings was mainly nutritional. We found a significant positive correlation between body mass at fledging and the relative number of eosinophils at 14–18 days of life, indicating a higher level of stress in lighter nestlings. The significant relationship between fledging age and the percentage of monocytes at 14–18 days of life suggested a poorer state of health in chicks that fledged at an older age.

Zusammenfassung

Einflussfaktoren auf Leukozyten Profile bei Krabbentauchern, einem kleinen arktischen Seevogel

Leukozyten Profile, insbesondere das Heterophilen/Lymphozyten-Verhältnis (H/L), wird häufig für die Beurteilung von Immunfunktion und Stress bei Vögeln genutzt. In dieser Studie wurde untersucht, inwieweit Leukozyten Profile beim Krabbentaucher (Alle alle), ein kleiner in Kolonien lebender Seevogel, in Verbindung stehen mit Alter (Küken, Adulte), Geschlecht oder biometrischen Eigenschaften inklusive Körpermasse. Dazu wurden Blutproben von 4–8 und 14–18 Tage alten Küken genommen. Außerdem wurden fütternde Altvögel mit 14–20 und 24–30 Tage alten Küken beprobt. Die relativen Anzahlen von Heterophilen sowie das H/L-Verhältnis im Allgemeinen unterschied sich zwischen Küken und Adulten, wobei die höchsten Werte bei Adulten mit 14–20 Tage alten Küken gemessen wurden. Die niedrigsten Werte fanden sich bei 4–8 Tage alten Küken. Weibchen hatten einen größeren Anteil an Lymphozyten als Männchen. Die relative Anzahl eosinophiler Granulozyten war gleich in allen Gruppen. Der Anteil von Monozyten unterschied sich leicht zwischen den Alterskategorien. Die Analyse der Küken zeigte, dass der Prozentsatz an Heterophilen und das H/L-Verhältnis signifikant höher war in älteren Küken. Ein gegensätzliches Muster wurde für die Lymphozyten ermittelt. Bei 14–18 Tage alten Küken war das H/L-Verhältnis

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(Stresslevel) negativ korreliert mit Körpermasse, Kopf-Schnabel-Länge und Schnabelbreite. Diese Phase ist energetisch gesehen anstrengend, da die Küken mit ersten Flugübungen außerhalb des Nestes beginnen. Das weist darauf hin, dass der Ursprung von Stress bei Jungvögeln vor allem mit der Ernährung zusammenhängt. Wir fanden eine signifikant positive Korrelation zwischen Ausflugsgewicht und der relativen Anzahl Eosinophiler bei einem Alter von 14–18 Tagen. Dies ist ein Zeichen für einen höheren Stresslevel bei leichteren Küken. Der signifikante Zusammenhang zwischen Ausflugsalter und dem Anteil an Monozyten bei einem Alter von 14–18 Tagen zeigt einen schlechteren Gesundheitszustand von Küken an, die später flügge werden.

Introduction

The immune system plays a pivotal role in defending an animal from attack of pathogens and parasites (Roitt et al. 1993). However, maintaining or activating immune function in vertebrates can be energetically costly and thus resource dependent (Saino et al. 1997, 2003; Norris and Evans 2000; Lochmiller and Deerenberg 2000; Alonso-Alvarez and Tella 2001; Martin et al. 2002). Therefore, it may compete for nutrient or energy resources with important life-history stages such as reproduction or growth and development (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Norris and Evans 2000; Zuk and Stoehr 2002; Lee 2006).

Leucocytes comprise the main part of the innate and acquired immune systems (Roitt et al. 1993). The innate immune system functions by means of phagocytosis, providing initial protection against various pathogens (Roitt et al. 1993). It is based on heterophils (primary phagocytic leucocytes that that are released from storage pools in response to infection, inflammation and stress), eosinophils (playing a role in the inflammation process and associated with defence against parasites), monocytes (long-lived phagocytic cells associated with defence against infections and bacteria) and basophils (involved in the inflammation process) (reviewed in Davis et al. 2008). Acquired immunity is more pathogen specific and works by means of cell-mediated and humoral responses by the lymphocytes (Roitt et al. 1993; Davis et al. 2008; Campbell and Ellis 2013). Leucocyte profiles, i.e. the relative numbers of particular leucocytes, have been used to gain insight into the immune system (e.g. Quillfeldt et al. 2008; Dehnhard et al. 2011a, b). Heterophils and lymphocytes are the two most frequent (together ca. 80 %) types of leucocytes in birds. Their ratio (hereafter the H/L ratio) is often used as a stress indicator in birds. It increases in response to various stressors,

including infectious diseases, parasite infestation, food or water deprivation, temperature extremes and psychological disturbance (e.g. Gross and Siegel 1983; Maxwell 1993; Totzke et al. 1999; Vleck et al. 2000; Lobato et al. 2005; Davis et al. 2008). This reaction is caused by the redistribution of lymphocytes and heterophils between the blood and other body compartments induced by the secretion of corticosterone (Davis et al. 2008). An increase in the H/L ratio takes place within 30–60 min after the initial exposure to a stressor and is detectable for several hours afterwards (Davis 2005; Cīrule et al. 2012). Such a time lag in reaction to handling or blood sampling stress makes this variable a useful indicator of avian stress (Maxwell 1993; Davis et al. 2008; Müller et al. 2011).

In chicks, the composition of leucocytes including the H/L ratio may predominantly reflect the ontogeny of the immune system rather than stress (e.g. Quillfeldt et al. 2008; Masello et al. 2009; Dehnhard et al. 2011b). Therefore, those haematological variables have been less frequently studied in chicks than in adults. However, some studies have reported that the H/L ratio in chicks is related to nestling survival and hence may be an indicator of offspring quality (e.g. Moreno et al. 2002; Suorsa et al. 2004).

To obtain insight into the factors affecting the development of the avian immune system, we focused on the little auk (or dovekie) (Alle alle). This is a small, zooplanktivorous seabird breeding colonially in the high Arctic. It is considered to be the most abundant alcid in the Palaearctic (Stempniewicz 2001). There are no apparent differences in male and female plumage, but males are slightly larger than females (Jakubas and Wojczulanis 2007). The species is socially monogamous with long-term pair bonds. The female lays a single egg annually, which is incubated by both sexes. The nest is situated mainly under boulders in mountain scree. Both parents incubate the single egg for 4 weeks, but males spend more time guarding the nest area during the mating and incubation periods. Both partners brood (for the few first days) and feed the semi-altricial chick with similar frequency for 21–31 days. Only at the end of the chick-rearing period, just before fledging (chicks start to fledge from the 21st day of life), does the female tend to leave the feeding duties to the male. The nestling spends the first 2 weeks of its life inside the nest chamber. Then, it starts to appear in front of the nest entrance, where it exercises its wings. After another 2 weeks, the fledgling leaves the colony, accompanied by the male parent (Stempniewicz 1995, 2001; Wojczulanis-Jakubas et al. 2009a, b; Wojczulanis-Jakubas and Jakubas 2012). Being long-lived seabirds, little auks are expected to invest strongly in their immune system to ensure survival. This species has relatively low rates of infection and prevalence of otherwise common pathogens, such as haemoparasites or fungi (Wojczulanis-Jakubas et al. 2010, 2011; Dynowska

et al. 2013). So far, leucocyte profiles in the little auk have been studied mainly in adults (Jakubas et al. 2008, 2011; Wojczulanis-Jakubas et al. 2012). Leucocyte profiles in chicks have been investigated only in one study (Jakubas et al. 2011) in the context of differences in body condition and stress level between two colonies situated in contrasting oceanographic conditions. We still know little about the ontogeny of leucocyte profiles in little auk chicks. Considering the small brood size (one chick only) and therefore no confounding effect of within-brood variation in the chicks' immunity, the little auk is an ideal model to investigate the investment in leucocyte profiles in relation to biometric variables and age.

The principal aim of the study was to investigate whether leucocyte profiles in the little auk chicks are related to age or biometric traits, including body mass. As development of the immune function can be associated with sex (Grossman 1985), we included sex in all the analyses. To investigate whether leucocyte profiles at the pre-fledging stage reached adult levels, suggesting a completion of the ontogeny of the immune system, we compared leucocyte profiles between chicks and adults. Specifically, we expected to find:

1. a negative correlation between chick body condition and the H/L ratios for both sexes as the H/L ratio increases in response to food deprivation (e.g. Davis et al. 2008; Gladbach et al. 2010; Müller et al. 2011);
2. few or no sex-related differences in leucocyte profiles and body condition in chicks as there are no differences in size or growth rate between the little auk males and females (Wojczulanis-Jakubas and Jakubas, unpublished data);
3. changes in leucocyte counts with nestling age reflecting the ontogeny of the immune system, as previously found in nestlings of other seabirds (Quillfeldt et al. 2008; Dehnhard et al. 2011a);
4. differences in leucocyte profiles between chicks and adults (especially an elevated H/L ratio in adults) as the blood profiles of nestlings develop with age (e.g. Quillfeldt et al. 2008).

Materials and methods

Study site and period

We collected the data in the large little auk breeding colony on the Høystakken and Alkekongen slopes in Magdalenefjorden (NW Spitsbergen; 79°35′N, 11°05′E) in July–August 2008. This is one of the main breeding areas of little auks on Svalbard. The estimated number of pairs breeding there is roughly 54,000 (Isaksen 1995).

Field procedures

To examine leucocyte profiles and identify the sex, a small blood sample (10–20 µl) was taken from the brachial vein (26-gauge needle) within 3 min of capture, following the standard procedure used in birds (Owen 2011). Part of each blood sample was used for leukocyte analysis; the remainder of each sample was stored in 1 ml 96 % ethanol until used for DNA-based sex identification. Two age categories of chicks were blood-sampled: 4–8 and 14–18 days old. These categories correspond to the early and middle phases of chick growth, differing in terms of energy demand (per chick per day). At the 4–8-day stage, chicks are brooded by their parents and stay inside the nest, and so their energy expenditures are relatively low. The 14–18-day stage covers the period of maximum energy demands, since the chicks reach peak body mass and intensively exercise their wings outside the nest chamber (Stempniewicz 1980, 1995; Konarzewski and Taylor 1989; Konarzewski et al. 1993). The ages of the chicks were known, as nests with sampled chicks were checked regularly to record the day of hatching. We sampled and measured two groups of chicks: (1) individuals that were sampled twice (i.e. at 4–8 and 14–18 days of life)—8 females and 12 males—and (2) individuals that were sampled once only (to avoid the possible effect of the first sampling on leucocyte profiles during the second blood collection)—8 females and 7 males at age 4–8 days and 13 females and 15 males at age 14–18 days. However, we were unable to control for the effect of previous handling, as the chicks in both groups were measured and weighed following the same schedule.

We weighed (with an electronic scale with an accuracy of 0.1 g) and measured all chicks every 3 days from hatching until fledging (at the age of 21–30 days). The following measurements were taken from chicks with dial calipers (0.1 mm accuracy; length of head-bill, culmen, tarsus, carpometacarpus and I primary, and bill width) and a ruler (1 mm accuracy; flattened wing length) by the same person (KWJ), experienced in measuring.

We assumed that a chick had fledged if it was no longer in the nest after 20 days (Harding et al. 2009; Wojczulanis-Jakubas and Jakubas 2012). Among the chicks sampled at 14–18 days of life, 26 individuals [14 sampled twice (4 females and 10 males) and 12 sampled once (3 females and 9 males)] had hatched early enough to leave the nest before the end of our field season. All those chicks survived up to 20 days. The other group of chicks had hatched too late to leave the nest before the end of our field season. Thus, we did not know their survival status.

To compare leucocyte profiles between chicks and adults, adult birds were captured in the colony during the chick feeding period when the chicks were 14–20 days old

(1–3 August; 11 females and 9 males) and 24–30 days old (11–13 August; 7 females and 20 males) according to the hatching median for the subcolony where they were captured (median: 16 July, 25–75 % quartiles: 14 July–18 July; $N = 36$ nests). The birds were caught randomly in the colony with mist-nets or noose-carpets. Only confirmed breeders (with gular pouch full of food for chicks) were blood-sampled.

Leucocyte profiles

Each blood sample was smeared on a microscope slide immediately after collection, air-dried and stored until staining using the May-Grünewald-Giemsa method (Lillie 1977) with a Wescor "Aerospray Hematology" stainer. We assessed the relative proportion of different types of leucocytes (lymphocytes, heterophils, monocytes, eosinophils and basophils) based on the examination of 100 leucocytes at ×1,000 magnification under oil immersion. All smears were screened by the same person (AK), who was unacquainted with the birds' age or sex.

Sex identification

We extracted DNA from the blood following evaporation of the alcohol using the Blood Mini kit (A&A Biotechnology, Gdynia, Poland). Molecular sexing was done with the primer pair F2550 and R2718 according to the protocol described by Griffiths et al. (1998), using a 50 °C annealing temperature for the PCR reaction. The sex differences in the PCR products were clearly visible when the fragments were separated on 2 % agarose gel and stained with ethidium bromide.

Statistics

Data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test). We normalised percentages of heterophils, lymphocytes and H/L ratios using an arcsin square-root transformation (Zar 1999). We used non-parametric tests for the percentages of eosinophils and monocytes in chicks, which were not normally distributed even after transformation.

To analyse the factors affecting leucocyte profiles in chicks (especially the sampling mode), we performed ANOVA with the relative numbers of heterophils, lymphocytes and the H/L ratio as the dependent variables, and fixed factors [sex, age of chicks (4–8-day-old chicks, 14–18-day-old chicks), sampling mode (once, twice)] and their interactions as explanatory variables. For the percentages of eosinophils and monocytes, we conducted two-way PERMANOVA (non-parametric MANOVA based on the Bray-Curtis measure; Anderson 2001) with fixed

factors [sex and sampling mode (once, twice)] and their interaction as explanatory variables. They were performed for both chick age categories separately.

Then, to analyse the factors affecting the leucocyte profiles in all age groups (including adults), we performed ANOVA (for the relative number of heterophils, lymphocytes and H/L ratio) or PERMANOVA (for the percentages of eosinophils and monocytes) with leucocyte profiles as the dependent variable and fixed factors [sex, age (4–8-day-old chicks, 14–18-day-old chicks, adults with 14–20-day-old chicks and adults with 24–30-day-old chicks)] and their interaction as explanatory variables.

Unequal N HSD or Dunn tests were used as post hoc tests after ANOVA and PERMANOVA, respectively.

To test whether the relative abundances of heterophils and lymphocytes and H/L ratio in chicks were affected by sex (coded as a dummy variable 0/1), age (in days) or biometric variables (body mass, length of head-bill, culmen, tarsus, carpometacarpus and I primary, and bill width), we performed multiple linear regression analysis (separate analyses for particular leucocyte and biometrical variables). We tested the correlation of eosinophil and monocyte frequencies with the biometric variables using the Spearman correlation (Zar 1999). We performed those analyses separately for 4–8- and 14–18-day-old chicks. The biometric variables included body mass, length of head-bill, culmen, tarsus, carpometacarpus and I primary, and bill width. To increase the sample size, and given the lack of a significant effect of sampling mode, we performed these analyses on the whole data set, comprising chicks sampled once and twice.

We investigated the relationship between various chick variables at fledging (body mass, fledging age, length of culmen, head-bill, tarsus and wing, and bill width) and leucocyte profiles in 14–18-day-old chicks (both sexes combined because of the small sample size and lack of sex differences; 7 females and 19 males) using the Pearson correlation coefficient (relative numbers of heterophils, lymphocytes and H/L ratio) or Spearman rank correlation coefficient (relative numbers of eosinophils and monocytes). We analysed two chick body mass variables at fledging: fledging mass, i.e. the last mass measured before the chick's departure from the colony, and mass recession, i.e. the difference between peak mass (the highest mass noted per chick) and fledging mass (after Stempniewicz 1980; Konarzewski and Taylor 1989). Peak and fledging masses were found to be effective growth indicators, even more so than the widely used growth-curve analysis (Zach 1988). We also investigated the age of fledging (date of the last presence in the nest).

We performed PERMANOVA using PAST 3.0 software (Hammer et al. 2001) and all other analyses using STATISTICA 8.0 (StatSoft, Inc., Tulsa, OK, USA).

Results

Factors affecting leucocyte profiles in chicks and adults

Heterophils and lymphocytes were the dominant types of leucocytes, each accounting for 43–56 % of all leucocytes in both chick age groups and 32–65 % in adults (Table 1). Eosinophils and monocytes were found in only 71 and 40 blood samples, respectively. Basophils were not found in any of the samples.

Analyses of chicks revealed a significant effect of age on the H/L ratio (ANOVA, $F_{1,75} = 5.51$, $P = 0.02$), relative number of heterophils ($F_{1,75} = 5.56$, $P = 0.02$) and lymphocytes ($F_{1,75} = 5.90$, $P = 0.02$). Percentage of heterophils and the H/L ratios were significantly higher in older than in younger chicks (Fig. 1). The opposite pattern was recorded for lymphocytes. ANOVA revealed no significant influence of sampling mode, sex or interactions (sampling mode × age, sampling mode × sex, age × sex, sampling mode × age × sex) on the relative numbers of heterophils ($P = 0.99$, $P = 0.39$, $P = 0.18$, $P = 0.62$, $P = 0.86$, $P = 0.83$, respectively) and lymphocytes ($P = 0.96$, $P = 0.37$, $P = 0.19$, $P = 0.68$, $P = 0.80$, $P = 0.83$, respectively) or the H/L ratio ($P = 0.77$, $P = 0.50$, $P = 0.27$, $P = 0.69$, $P = 0.75$, $P = 0.87$, respectively).

We found no significant influence of sampling mode, sex or their interaction on the percentages of eosinophils in 4–8 days old (PERMANOVA: $P = 0.66$, $P = 0.67$, $P = 0.85$, respectively) and 14–18 days old chicks ($P = 0.67$, $P = 1.00$, $P = 0.37$, respectively). The relative abundance of monocytes in 4–8 days old chicks was significantly affected by sex (PERMANOVA, $F_{1,31} = 3.81$, $P = 0.04$), with males having higher values (median 0, min–max 0–2) than females (median 0, min–max 0–1). We

found no significant influence of sampling mode ($P = 0.61$) or sampling mode × sex interaction ($P = 0.86$) on the relative number of monocytes in 4–8-day-old chicks. In 14–18-day-old chicks, there was no significant influence of sampling mode ($P = 0.68$), sex ($P = 0.73$) or their interaction ($P = 0.44$) on the relative numbers of monocytes (PERMANOVA).

In the light of these results, we combined the once- and twice-sampled chicks in all further analyses.

Analyses of all age categories (chicks and adults) revealed a significant effect of age on the H/L ratio (ANOVA, $F_{3,122} = 9.78$, $P < 0.0001$) and the relative number of heterophils ($F_{3,122} = 9.07$, $P < 0.0001$). The lowest values were recorded in 4–8-day-old chicks and the highest ones in adults with 14–20-day-old chicks (Fig. 1). The percentage of heterophils was significantly higher in adults with 14–20-day-old chicks compared to 4–8-day-old (unequal N HSD test, $P < 0.0001$) and 14–18-day-old chicks ($P = 0.03$). The H/L ratios in adults with 14–20-day-old chicks were significantly higher than in 4–8-day-old (unequal N HSD test, $P < 0.0001$) and 14–18-day-old chicks ($P = 0.01$). Values in 4–8-day-old chicks were significantly lower than in adults with 24–30-day-old chicks ($P = 0.049$). The percentage of heterophils and the H/L ratios tended to be lower in adults with 14–20-day-old chicks compared to adults with older chicks ($P = 0.054$ and $P = 0.056$, respectively) (Table 1; Fig. 1). ANOVA revealed no significant influence of sex or age × sex interaction on the relative number of heterophils ($P = 0.07$, $P = 0.35$, respectively) and H/L ratios ($P = 0.051$, $P = 0.35$, respectively). The percentage of lymphocytes was significantly affected by age (ANOVA, $F_{3,122} = 10.81$, $P < 0.0001$) and sex ($F_{1,128} = 4.52$, $P = 0.04$). The lowest values were recorded in adults with 14–20-day-old

Table 1 Leucocyte profiles in little auk chicks and adults

Variable	Chicks				Chick-feeding adults			
	Chick age in days of life (N chicks)				Chick age in days of life (N adults)			
	4–8 days ($N = 35$)		14–18 days ($N = 48$)		14–20 days ($N = 20$)		24–30 days ($N = 27$)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
H/L	0.92[a,d]	0.62	1.39[c]	1.07	2.19[a,c]	0.80	1.52[d]	1.05
Het (%)	43.2[a]	14.6	51.7[d]	16.3	64.8[a,d]	9.3	52.8	15.9
Lym (%)	55.6[a,c]	14.4	47.1[b]	15.9	32.5[a,b,d]	8.9	44.5[c,d]	16.0
Variable	Med	Min–max	Med	Min–max	Med	Min–max	Med	Min–max
Eos (%)	0.0	0–4	0.0	0–7	1.0	0–9	1.0	0–15
Mon (%)	0.0	0–2	0.0	0–4	1.0	0–4	0.0	0–2

Variables include the relative numbers of leucocytes (*Het* heterophils, *Lym* lymphocytes, *Eos* eosinophils, *Mon* monocytes) and the heterophil-to-lymphocyte ratio (H/L). *Med* median. The same letters indicate significant differences among age categories in particular variables

P values of post hoc unequal N HSD test for particular variables: [a] $P < 0.001$, [b] $P < 0.01$, [c] $P < 0.03$, [d] $P < 0.05$
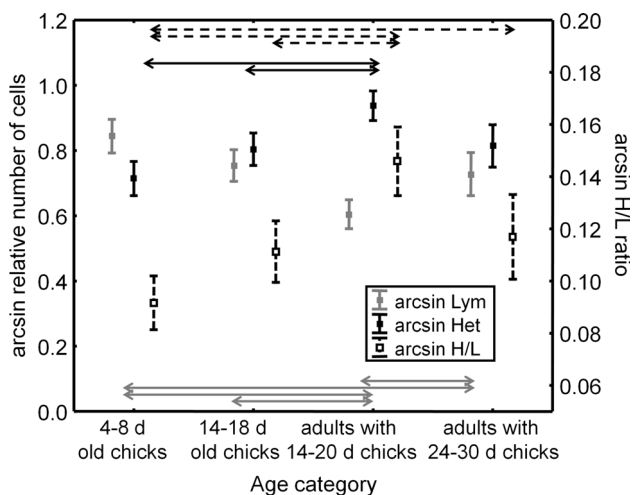
Fig. 1 Mean ± 95 % confidence intervals of relative numbers of heterophils (Het), lymphocytes (Lym) and H/L ratio (arcsin square-root transformed data) in little auk chicks and adults. The *arrows* indicate significant differences (ANOVA, post hoc unequal N HSD test, $P < 0.05$)
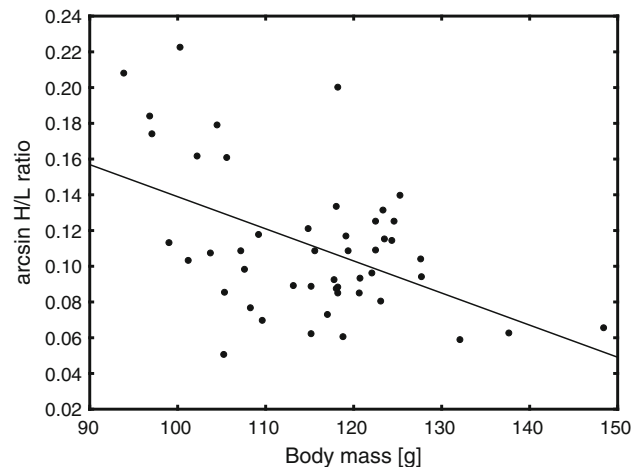

Fig. 2 Relationship between body mass and the arcsin square-root transformed H/L ratio in 14–18-day-old little auk chicks ($N = 48$). The least squares regression line is shown for a significant relationship

chicks and the highest ones in 4–8-day-old chicks (Fig. 1). The relative number of lymphocytes generally differed among all the groups (unequal N HSD test, $P < 0.05$) except for similar values in 4–8-day-old and 14–18-day-old chicks ($P = 0.06$) and in 14–18-day-old chicks and adults with 24–30-day-old chicks ($P = 0.92$) (Table 1). Regarding the sex effect, the frequency of lymphocytes in females (mean 49.1 ± 16.3, $N = 55$) was significantly higher than in males (mean 44.8 ± 16.4, $N = 75$). We found no significant influence of age × sex interaction on the relative number of lymphocytes ($P = 0.25$).

PERMANOVA revealed no significant influence of sex ($P = 0.12$), age ($P = 0.76$) or age × sex interaction ($P = 0.49$) on the relative number of eosinophils. The relative abundance of monocytes was affected significantly by age (PERMANOVA, $F_{1,122} = 2.61$, $P = 0.03$). However, the post hoc Dunn test did not reveal any tendency or significant differences ($P > 0.16$). We found no significant influence of sex ($P = 0.17$) or age × sex interaction ($P = 0.13$) on the relative number of monocytes (PERMANOVA).

Relationships between leucocyte profiles and biometric variables in chicks

Multiple linear regressions for 4–8-day-old chicks showed that the relative numbers of heterophils and lymphocytes and the H/L ratio were not affected by any of the factors examined [age (in days), sex or biometric variable (body mass, length of head-bill, culmen, tarsus, carpometacarpus and I primary, and bill width)] (all $P > 0.43$).

Multiple linear regression for 14–18-day-old chicks indicated that the leucocyte variables were affected significantly by body mass (heterophils, $t_{44} = -3.14$, $P = 0.003$; lymphocytes, $t_{44} = 3.19$, $P = 0.003$; the H/L ratio, $t_{44} = -3.68$, $P = 0.0006$), head-bill length (the H/L ratio, $t_{44} = -2.08$, $P = 0.04$) and bill width (the H/L ratio, $t_{44} = -2.23$, $P = 0.03$). The age and sex of chicks were not related to the relative number of heterophils or lymphocytes (body mass), or to the H/L ratio (body mass, head-bill and bill width) (all $P > 0.17$). Regarding the biometric variable effect, the H/L ratio decreased with increasing body mass (Fig. 2), head-bill length and bill width. The relative number of heterophils decreased, whereas the percentage of lymphocytes increased with increasing body mass. Relative numbers of heterophils and lymphocytes were not affected by age (in days), sex or the mentioned variables (head-bill length and bill width; all $P > 0.17$).

There was no significant relationship between the percentage of heterophils or lymphocytes, or the H/L ratio, and any other biometric variable (i.e. length of culmen, tarsus, carpometacarpus, I primary) investigated in 14–18-day-old chicks (all $P > 0.40$).

In 4–8-day-old chicks (both sexes combined), the relative number of eosinophils was not significantly related to the biometric variables (Spearman rank correlation coefficients, all $P > 0.13$). Owing to sex differences in the percentage of monocytes in 4–8-day-old chicks, we performed separate analyses for males and females. But in neither sex was there any significant relationship between the relative number of monocytes and the biometric variables (Spearman rank correlation coefficients, all $P > 0.10$). In 14–18-day-old chicks (both sexes combined), the percentages of

eosinophils and monocytes were not significantly related to the biometric variables (Spearman rank correlation coefficients, all $P > 0.14$).

Relationship between the leucocyte profiles at 14–18 days of life and the biometric variables at fledging

We found that the relationships between the percentages of heterophils and lymphocytes and the H/L ratio at 14–18 days of life and chick traits at fledging (i.e. body mass, body mass recession, fledging age, length of culmen, head-bill and wing, and bill width) were insignificant (Pearson correlations, all $P > 0.35$, $N = 26$).

We found a significant relationship between the relative number of eosinophils in 14–18-day-old chicks and fledging body mass (Spearman rank correlation coefficient, $r_s = 0.44$, $P = 0.03$, $N = 26$), indicating an increase in the percentage of eosinophils with increasing fledging mass (Fig. 3). The relative number of eosinophils tended to increase with increasing head-bill ($r_s = 0.38$, $P = 0.057$, $N = 26$) and culmen length ($r_s = 0.38$, $P = 0.055$, $N = 26$) at fledging. Relationships with other chick traits were insignificant (all $P > 0.08$). We found the percentage of monocytes to be significantly correlated with fledging age ($r_s = 2.99$, $P = 0.006$, $N = 26$), indicating an increase in the percentage of monocytes with increasing age of colony departure (Fig. 4). The relationships between the relative abundance of monocytes in 14–18-day-old chicks and their traits at fledging (i.e. body mass, body mass recession, length of culmen, head-bill, tarsus and wing, and bill width) were insignificant (all $P > 0.09$).

Discussion

Our analyses of chicks revealed significant differences in the relative numbers of heterophils and lymphocytes, and the H/L ratio between the two age classes, with higher H/L ratios in older chicks. A similar pattern of changes was reported in the thin-billed prion (*Pachyptila belcheri*) (Quillfeldt et al. 2008). However, the opposite pattern—with the highest H/L ratio in young chicks and the lowest in older ones—was found in another seabird species, the red-tailed tropicbird (*Phaethon rubricauda*) (Dehnhard et al. 2011a). In yet another seabird species, the southern rockhopper penguin (*Eudyptes chrysocome chrysocome*), the granulocyte/lymphocyte (G/L) ratio did not change with chick age [as heterophils are the most frequent type of granulocyte (G) compared to eosinophils and basophils, the G/L ratio can also be used instead of H/L; Dehnhard et al. 2011b]. Although all these species are classified as semi-altricial, they differ in the duration and mode of
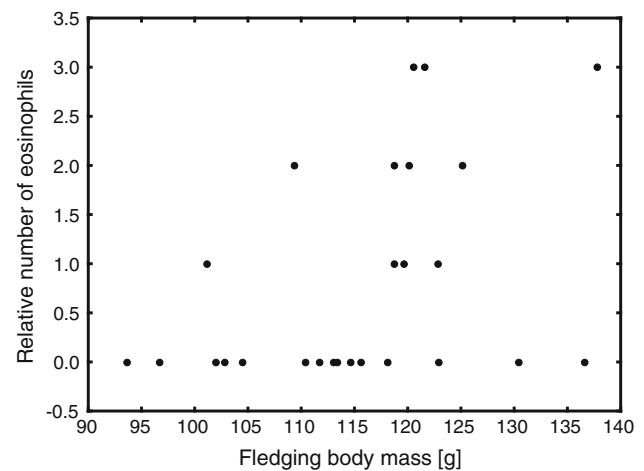

Fig. 3 Relationship between the body mass at fledging and the relative number of eosinophils in little auks on days 14–18 of their life ($N = 26$)
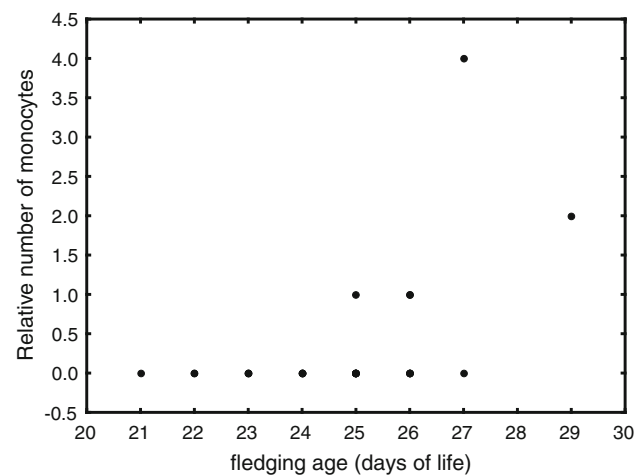

Fig. 4 Relationship between the fledging age (days of life) of little auk chicks and the relative number of monocytes on days 14–18 of their life ($N = 26$)

development. The little auk chicks leave their nests after 28 days (Stempniewicz 1980), whereas thin-billed prions and red-tailed tropicbirds spend 50 days and up to 91 days in the nest, respectively (Quillfeldt et al. 2008; Dehnhard et al. 2011a). The southern rockhopper penguin chicks leave their nests already around 20 days but then form crèches (Dehnhard et al. 2011b). These differences may affect the development of the immune system. Alternatively, such contradictory results may result from variations in immune system development because of spatio-temporal variability in food availability or climatic influences that lead to more or fewer resources for investment in the immune system or growth (Dehnhard et al. 2011a). Strong interannual variation in the H/L ratio has been demonstrated in adult birds, with higher values during seasons with adverse food conditions (Ochs and Dawson 2008;

Plischke et al. 2010). Thus, a similar pattern may be expected in nestlings. The southern rockhopper penguin chicks, with similar G/L ratios throughout their development, experienced favourable food conditions (Dehnhard et al. 2011b). In contrast, the thin-billed prion chicks, showing an increase in the relative number of heterophils and the H/L ratios with age, experienced poor feeding conditions (Quillfeldt et al. 2008). The study area (Magdalenefjorden) is generally considered a suboptimal feeding ground, with a prevalence of warmer water masses that affect the birds' foraging behaviour and chick growth (Kwasniewski et al. 2010; Jakubas et al. 2011, 2013). A study comparing body mass and the H/L ratio in little auk chicks between the colony investigated in the present study and in Hornsund (SW Spitsbergen) showed no differences in those variables in a year when the food supplied was of similar quantity and quality (Jakubas et al. 2011). However, a study from Hornsund investigating interseasonal differences in chick diet (Jakubas et al. 2007) indicated that at the end of chick-rearing, little auk parents under suboptimal food conditions (a warm year) supplied their chicks with poorer quality food than when conditions were optimal (a cold year). In this context, the recorded increase in the H/L ratio with chick age might reflect deteriorating food conditions at the foraging grounds in the late phase of chick-rearing. Further studies on the spatio-temporal variation of chick leucocyte profiles would be valuable.

The H/L ratio in the little auks studied here was lower in younger chicks than in adults, but in older chicks it reached levels close to those of adults at the end of the chick rearing period. The similar pattern observed in chicks and adults of the thin-billed prion and red-tailed tropicbirds was explained in the context of immune system development in chicks (Quillfeldt et al. 2008; Dehnhard et al. 2011a). Changes in leucocyte profiles throughout the breeding season in adult little auks, including a decrease of the H/L ratio at the end of this period, have already been reported (Wojczulanis-Jakubas et al. 2012). Low H/L ratios in adults at the end of the chick-rearing period could indicate the initiation of an internal mechanism preparing adults to bring breeding activities to an end (Wojczulanis-Jakubas et al. 2012).

We found that smaller (in terms of body mass, head-bill length and bill width) little auk nestlings at age 14–18 days had an higher H/L ratio (suggesting a higher stress level) than bigger ones. At that stage of a chick's growth, the estimated costs of its activity are very high because it is exercising its wings (Konarzewski et al. 1993). The experimental study of Øyan and Anker-Nilssen (1996) on the Atlantic puffin (*Fratercula arctica*) found that the growth rate of various biometric variables, including body mass and head-bill length, was depressed in food-stressed chicks. This suggests that the origin of stress in lighter little

auk nestlings in our study was mainly nutritional. A negative relationship between the H/L ratio and body condition has been recorded in other bird species (Hoi-Leitner et al. 2001; Moreno et al. 2002; Suorsa et al. 2004; Lobato et al. 2005; Ortego et al. 2009; Parejo and Silva 2009; Müller et al. 2011), suggesting that this haematological variable is sensitive to food shortage and may be a good indicator of nutritionally based physiological stress in nestlings.

There was no significant relationship between the H/L ratio at 14–18 days of life and fledging mass. Given the significant relationship with body mass at age 14–18 days, this may suggest that nutritional stress was only periodic and was experienced only by the lightest chicks. An experimental study by Azis (2012) showed that chickens subjected to food restriction at age 7–21 days had elevated H/L ratios and lower body masses compared to chickens fed ad libitum. However, after the realimentation period from 22 to 42 days of life, there were no significant differences in leucocyte profiles and body mass between food-restricted and ad libitum chickens. Little auk parents are capable of intensifying their parental efforts under suboptimal food conditions (Harding et al. 2009; Jakubas et al. 2007, 2011, 2013; Kwasniewski et al. 2010) and can increase their provisioning effort. It is hypothesised that such a reaction is triggered by the increased begging behaviour in chicks with an elevated corticosterone level (Kitaysky et al. 2003), which is related to the H/L ratio (Davis et al. 2008). Thus, it is possible that parents could have intensified feeding of lighter chicks after 14–18 days of life. In consequence, there was no relationship between the H/L ratio at 14–18 days of life and their body mass at fledging.

Two other variables appear to indicate the condition/state of health little auk chick at fledging. Individuals with a low body mass at fledging had a low relative number of eosinophils at 14–18 days of life. Eosinophils are involved in the defence against intestinal parasites (Beutler 2004). In amphibians and mammals, glucocorticoid-induced stress leads to a reduction in eosinophil numbers (Belden and Kiesecker 2005; Davis et al. 2008 and literature therein). This may suggest a higher stress level and/or parasite load in lighter fledglings. Fledglings departing the colony at an older age had an elevated percentage of monocytes at 14–18 days of life. In southern rockhopper penguin chicks, relative monocyte numbers were negatively correlated with body condition (Dehnhard et al. 2011b). A higher number of monocytes in birds is typically associated with infectious and inflammatory disease (Campbell and Ellis 2013). Thus, the high relative number of monocytes might suggest poor health. Our data, therefore, may suggest that a poorer state of health was the reason for the slower growth rate in chicks that fledge at an older age. However, considering the small number of chicks with elevated percentages of monocytes, this result should be interpreted with caution.

As we had predicted, given that there were no sex differences in chick growth rate or size (Wojczulanis-Jakubas and Jakubas, unpublished data), there were no differences in the H/L ratio between the male and female little auk chicks either. A similar result was reported for southern rockhopper penguin (G/L ratio; Dehnhard et al. 2011b) and Kestrel (*Falco tinnunculus*) nestlings (Parejo and Silva 2009). Another study on Kestrel nestlings (Fargallo et al. 2002) showed a sex difference in immune function, but only in food-restricted nests. The lack of sex differences in biometric traits in 4–8- and 14–18-day-old little auk chicks and fledglings suggests a similar provisioning level for nestlings of both sexes. The lack of sex differences in the H/L ratio in chick-feeding adults suggests similar male and female contributions to parental care, which was also reported in a previous study performed in Magdalenefjorden (Wojczulanis-Jakubas et al. 2012). The significant sex difference in the relative number of lymphocytes in adults was therefore unexpected, also considering the lack of sex differences in the percentage of lymphocytes in chicks. This result could be interpreted in the context of resource allocation from the more costly acquired immunity (Lochmiller and Deerenberg 2000; Norris and Evans 2000; Lee 2006) to innate immunity. However, as our work was based on percentages of leucocytes and not absolute numbers per blood volume (see below), it is not possible to confirm or refute this interpretation.

The present study revealed that leucocyte profiles in the little auk vary not only among age categories (chicks and adults), but also within them. We conclude that both the age and phase of season or development should be considered when investigating leucocyte profiles in seabirds. The observed variability in leucocyte profiles and its relationship with biometric variables are difficult to interpret in terms of investments in the immune system, as our work was based exclusively on percentages of leucocytes. In contrast to the ratios, absolute numbers of leucocytes (cells per ml) must be measured to infer changes in the investment in immunity (e.g. Masello et al. 2009). We therefore recommend that future studies interpreting investments in the immune system should include both total leucocyte numbers per ml of blood and percentages of particular leucocytes.

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