



Variability in leucocyte profiles in thin-billed prions *Pachyptila belcheri*

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ABSTRACT

Because immune function competes for the resources that can be allocated to other activities, studies of immunological ecology may offer a powerful tool for explaining how reproductive effort links to reproductive costs and how conditions experienced early in their development affect growing chicks in later life. We studied the distribution of leucocyte types and the development of *H/L* ratio, which is indicative of heightened energetic stress, throughout the season 2004–2005 in chicks and adults of thin-billed prions *Pachyptila belcheri*. Adults decreased body condition throughout the season and increased *H/L* ratios. Likewise, chicks increased *H/L* ratios during the season, but this was age-related rather than condition-dependent. Chicks from earlier hatched eggs had lower *H/L* ratios initially, but this relationship became weaker with increasing age and had disappeared by fledging. The results suggest that the stress index may be a useful measure of condition in adult thin-billed prions, at least on a population level, although a larger sample size or repeated samples from the same individuals may be required to confirm the relationship on an individual level and to distinguish between seasonal and body condition effects. The data on chicks highlight our lack of knowledge of the ontogeny of immune function in wild birds. Studies of adults and chicks over several seasons may reveal how resources are allocated between immune and other functions under contrasting environmental conditions.

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1. Introduction

The condition of free-living animals is determined by a number of factors that influence health and vigour (e.g. Hōrak et al., 1998), including their nutritional state, the prevalence of parasites and the presence of stressors, such as extreme climatic conditions or reproductive effort. These factors are interrelated as, for example, birds in poorer nutritional condition may be more likely to be parasitized (e.g. Shutler et al., 1999). Because immune function interacts with the general health state of an organism and competes for the resources that can be allocated to other activities, studies of immunological ecology may offer a powerful tool for explaining how reproductive effort links to reproductive costs.

Thus, the need to study tradeoffs—between the immune system and general condition in free-living populations has been emphasized (e.g. Ewenson et al., 2001; Møller and Petrie, 2002), but so far, we still know little about sex, age and seasonal effects on immunological parameters in free-living birds. Knowledge of the ontogeny of immune function is also needed to establish how stressful conditions experienced early in their development can affect growing chicks in later life.

Acute and chronic stressors such as starvation lead to elevation of glucocorticoids (mainly corticosterone in birds) within minutes as well as chronically, but chronic stress may also elicit a response of the immune system (e.g. Ruiz et al., 2002). This response takes hours to several days in demonstrating itself, in particular by a higher proportion of heterophils (H) and low proportion of lymphocytes (L).

Leucocyte profiles (relative numbers of white blood cell types) have been used by a growing number of ecological studies to assess immune function and stress in wild birds (e.g. Figuerola et al., 1999; Ewenson et al., 2001; Hauptmanová et al., 2002; Ruiz et al., 2002; Mazerolle and Hobson, 2002; Davis et al., 2004). From the phenomenon of increase in the number of heterophils and diminution in the number of lymphocytes a quantitative indicator of stress calculated from proportions of circulating heterophils/lymphocytes (*H/L*) has been described as estimator of stress levels, following Gross and Siegel (1983). Increased *H/L* ratios were observed in clinically abnormal birds (e.g. Averbek, 1992; Davis et al., 2004; Vleck et al., 2000), in chicks during times of low food availability (Hoi-Leitner et al., 2001; Lobato et al., 2005; Hylton et al., 2006), in parasite-infested birds (e.g. Lobato et al., 2005), in birds attending experimentally increased broods (Illmonen et al., 2003; Suorsa et al., 2004) and in breeding adults that subsequently did not survive the winter (Kilgas et al., 2006a). The *H/L* ratio has also been found to differ between adults and chicks (e.g. Alonso et al., 1991), between breeding males and females (e.g. Hōrak et al., 1998; Ots et al., 1998), and between birds in different stages of

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the annual cycle, such as the breeding season versus migration (Owen and Moore, 2006).

Pelagic seabirds of the Order Procellariiformes provision their chicks infrequently compared to other birds, and their chicks therefore show large day-to-day variations in body condition during their nestling period of nearly 2 months. Due to oceanographic conditions, nestling and adult seabirds often experience fluctuations in food availability. For example, thin-billed prions *Pachyptila belcheri* had large differences in provisioning both within and between seasons (Quillfeldt et al., 2007a) in connection with differences in the sea surface temperature. In previous studies, we have developed methods to monitor provisioning in this species (Quillfeldt et al., 2003, 2007a,b), which is now a good model to study the development of leucocyte profiles (having an extended nestling period), and to distinguish seasonal and condition effects on leucocyte profiles of nestlings and parents. Although no difference in chick provisioning rates between male and female parents has been observed in this species (Quillfeldt et al., 2007b), parental investment over the breeding season may differ between the sexes, for example due to the production of the very large egg by the females.

In the present study, we analyse leucocyte profiles in thin-billed prions during the course of the breeding season 2004–2005, in relation to chick and adult body condition. Specifically, we test the following hypotheses:

1. The H/L index is negatively correlated to body condition in adults and chicks,
2. The H/L index differs between the sexes, and with the stage of the reproductive cycle.
3. The H/L index varies with chick age.

2. Materials and methods

2.1. Study site and study species

The study was carried out at New Island, Falkland Islands, between October 2004 and March 2005. Thin-billed prions breed in the Falkland Islands, Crozet and Kerguelen, New Island being the most important known breeding site.

At New Island, recent studies explored variability in provisioning and parent–chick interactions (Quillfeldt et al., 2003, 2006, 2007a,b,c). Thin-billed prions show the typical procellariiform pattern of a single-egg clutch and slow chick development, with an average fledging period of 50 days (Strange, 1980). Under good conditions, the chicks accumulate large lipid reserves, attaining peak masses of up to 190% of adult mass. At the end of nestling development, chicks lose mass and fledge close to adult mass. Variation in water temperatures and upwelling, coupled with larger scale processes (e.g. El Niño/La Niña) may affect the availability of prey, and an analysis of provisioning in relation to ocean climate and prey availability (Quillfeldt et al., 2007a) suggested that periods of elevated sea surface temperatures were associated with periods of low food availability for thin-billed prions. The year 2005 was especially poor during the chick-rearing period, and most chicks were not able to obtain peak body masses above fledging mass (a phenomenon described in Procellariiformes as chick obesity, e.g. Hamer et al., 1997, 1999) during that season. Thin-billed prions are burrow nesters, and we reached chicks in their nest chambers via short access tunnels in the roof of each burrow, capped with removable stone lids. This system facilitated rapid access to chicks, reducing overall disturbance. Marked nests were monitored for eggs and hatching chicks.

2.2. Chick and adult measurements

We determined the hatching dates of chicks (to the nearest day) by calibrating wing length against wing growth in chicks of known age.

Chicks were weighed daily to the nearest 1 g using a digital balance (Kern CM320-1 N, Germany). Wing length was measured every three days to the nearest 1 mm with a stopped wing rule to determine the wing growth rate (the daily increase during the approximately linear phase between 20 and 45 days of age) and the wing length at fledging. Tarsus length was measured every three days to the nearest 0.1 mm using callipers to determine the tarsus growth rate (the daily increase during the approximately linear phase between 10 and 25 days of age) and the asymptotic tarsus length, reached around 40 days of age. An index of chick body condition was calculated relative to the mean mass for study chicks of each age (m_{mean}), using the following formula: $BC = m^*100/m_{\text{mean}}$ (e.g. Quillfeldt et al., 2006). In species with a pattern of peak mass and mass recession (e.g. Procellariiformes, Psittaciiformes), body condition indices relative to age are more adequate than mass controlled for measures of body size. In these chicks, growth of structural size and body mass does not occur in a parallel fashion (e.g. approx. quadratic growth of mass, but logistic tarsus growth, with maximum tarsus often reached well before the end of the nestling stage). Thus, mass vs. structural size in chicks of these groups is not independent of chick age, and body condition of older chicks would be overestimated compared with chicks in mass peak, as tarsus stays constant and body mass decreases during mass recession. A mean body condition was calculated for each chick from all body condition measurements over the nestling period.

Adults were captured in the nest only once, either during courtship (between 17 and 31 October 2004) or chick feeding (between 31 January and 6 February 2005, when chicks were in the second third of their development i.e. 20–40 days old). They were weighed with a digital 100 g balance to the nearest 0.1 g using a weighing cone. To calculate adult body condition, we corrected body mass for a measure of body size derived from a Principal Component Analysis (PCA) with varimax rotation of measures of structural size (wing length, tarsus length, bill length, bill width and bill height). This procedure was justified as indicated by the Kaiser–Meyer–Olkin Measure of Sampling Adequacy of 0.5 and Bartlett's test of sphericity revealing significance ($\chi^2=23$, $df=10$, $P=0.013$). The PCA extracted two factors: PC1 explained 32% of the total variation and described mainly bill height and width, while PC2 explained 27% of the total variation and was positively correlated to tarsus ($R=0.823$), bill length ($R=0.690$) and wing length ($R=0.449$), while bill height and width had very little influence on this factor. PC2 thus best described body size, and was used to the first power to calculate body condition.

Molecular sex determination of adults and chicks was carried out as described in Quillfeldt et al. (2007b).

2.3. Blood sampling and blood cell counts

Blood samples were collected within 2 min after capture by hand by puncture from the brachial vein in heparinised capillaries. Nestlings were sampled at 3 weeks (58 chicks) and 6 weeks of age (45 chicks) and pre-fledging (1–2 samples between 50–56 days of age from 22 chicks). The sample sizes became smaller in the older chicks when chicks either died or moved to inaccessible parts of the burrow. If 2 samples were taken from chicks between 50–56 days of age, then a mean was used for age-effect analyses, e.g. Fig. 1. Samples of chicks and adults during the courtship period were taken during daytime (0800 to 1800 h), and samples of chick-feeding adults at night (2300–0200 h).

The differential leucocyte count was determined as described previously (Ruiz et al., 2002) by examining whole blood air-dry smears. In the laboratory, smears were stained by the May Grünwald and Giemsa technique, modified by Robertson and Maxwell (1990). The differential count included relative percentages of lymphocytes (L), heterophils (H), monocytes, basophils and eosinophils, which were identified according to the criteria of Hawkey and Dennet (1989). A total of 100 leucocytes were counted per slide. Using the percentages

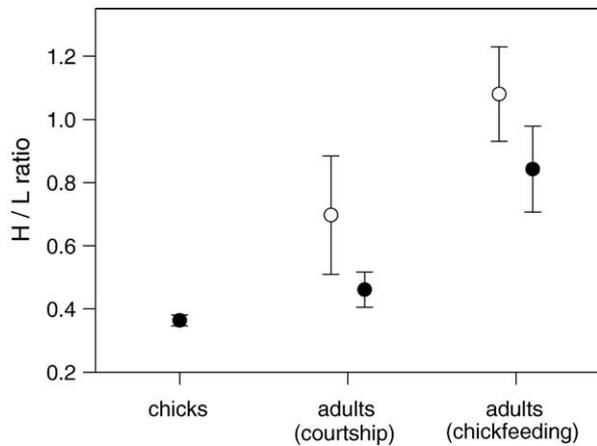


Fig. 1. *H/L* ratios (means±standard errors) of chicks and adults of thin-billed prions during the breeding season (for sample sizes see Table 1). Among adults, values for females are shown with open circles, while values for males are shown with filled circles.

of heterophils and lymphocytes, the *H/L* ratio was determined for each sample.

2.4. Data analysis

Statistical tests were performed in SPSS 11.0. Normality was tested with Kolmogorov–Smirnov tests. Means are given with standard errors. Results of General Linear Models (GLM), based on Type III Sum of Squares, carried out using SPSS 11.0 and were cited with effect sizes (η^2). To avoid unreliable assessments of the effects of covariates (see below), when testing for within-chick effects we included only those chicks into the analysis of which we had obtained at least three cell counts (sometimes four). In order to control for individual differences between chicks and in order to avoid pseudo-replication (e.g. Quillfeldt, 2002) we included chick as a categorical independent variable ('factor') into these analyses. Initially, we included the interaction between the factor chick and the covariate into the model, but removed it, as it did not reveal significance (all $P > 0.22$).

3. Results

3.1. Distribution of leucocyte types with age, sex and season

Chicks were similar to adults in the relative proportions of eosinophils (Mann–Whitney $U_{133,44} = 2869$, $P = 0.729$) and monocytes (Mann–Whitney $U_{133,44} = 2650$, $P = 0.228$), but differed in the other types. Chicks had more basophils (Mann–Whitney $U_{133,44} = 2361$, $P = 0.032$) and lymphocytes ($t = 5.81$, $df 172$, $P < 0.001$), but less heterophils ($t = 3.31$, $df 172$, $P = 0.001$) than adults (Table 1).

Table 1
Distribution of leucocyte types with age, sex and season

Group	N	Eosinophils	Basophils	Heterophils	Lymphocytes	Monocytes
Chicks	133	1.99±0.159	1.49±0.153	24.17±0.811	71.71±0.937	0.64±0.076
Females (courtship)	9	1.78±0.596	0.33±0.236	34.78±4.564	62.33±4.770	0.78±0.278
Females (chick feeding)	10	2.80±0.929	1.20±0.389	43.00±3.399	52.70±3.847	0.30±0.153
Males (courtship)	14	1.93±0.339	0.43±0.137	29.07±2.702	67.79±3.105	0.79±0.281
Males (chick feeding)	11	1.82±0.464	1.18±0.377	41.00±3.211	55.91±3.299	0.09±0.091

All age groups of chicks were combined.

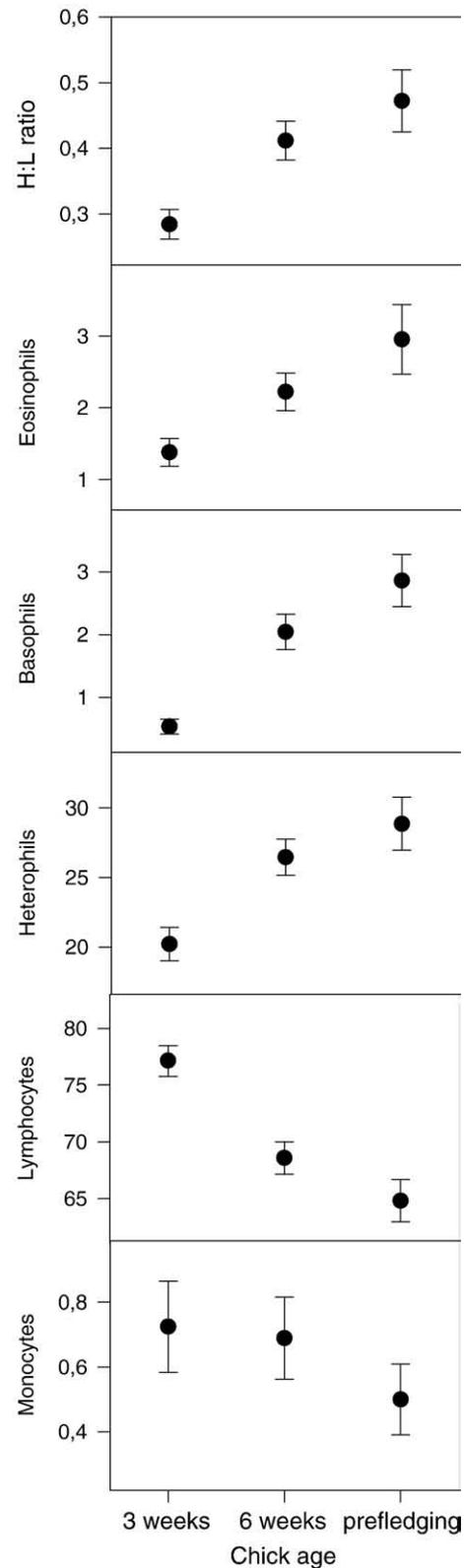


Fig. 2. *H/L* ratios and leucocyte types (mean in counts of 100 leucocytes) of chicks of thin-billed prions during the nestling season 2005 ($N = 58$, 45 and 22 chicks for ages 3 and 6 weeks and pre-fledging, respectively).

Among adults, sexes did not differ during either courtship (t -tests or Mann–Whitney U -tests for all cell types $P > 0.200$) or chick feeding (t -tests or Mann–Whitney U -tests for all cell types $P > 0.200$). The frequency of eosinophils and monocytes of adults did not change over the season (Spearman correlation with Julian date; eosinophils:

$R_s=0.008$, df 43, $P=0.959$; monocytes: $R_s=-0.225$, df 43, $P=0.136$), while the other types changed. Basophils increased ($R_s=0.313$, df 43, $P=0.036$), as did heterophils ($R_p=0.383$, df 43, $P=0.009$), while lymphocytes decreased ($R_p=-0.381$, df 43, $P=0.010$).

3.2. Variability in H/L ratio with age, sex and season

Adults had higher H/L values than chicks (Fig. 1; 0.72 ± 0.07 vs. 0.36 ± 0.07 ; t -test: $t=7.23$, df 172, $P<0.001$). Among adults, the H/L ratio increased over the breeding season, and females tended to have higher mean H/L ratios than males (Fig. 1, GLM with H/L ratio as dependent, and sex and period as factors; influence of sex: $F=3.6$, $P=0.064$, $\eta^2=0.092$; influence of season: $F=9.8$, $P=0.003$, $\eta^2=0.214$). Male and female chicks did not differ in their H/L ratios at any age (t -tests at 3 weeks, 6 weeks and pre-fledging, all $P>0.400$).

3.3. Variability of chick leucocytes with age, hatch date and body condition

Within chicks, the relative abundance of eosinophils, basophils and heterophils as well as the H/L ratio increased with age, independently of body condition (Fig. 2, Table 2). Monocytes did not vary, and lymphocytes decreased with age (Fig. 2, Table 2). We found no relationship between the H/L ratio at 3 weeks, 6 weeks or pre-fledging and either the mean body condition of chicks, their wing growth rate, fledging wing length, tarsus growth rate or asymptotic tarsus length (correlations, all $P>0.07$). Chicks from earlier hatching eggs had lower H/L ratios initially (age 3 weeks: $R_s=0.320$, df 57, $P=0.014$), but this relationship became weaker with increasing age (6 weeks: $R_s=0.302$, df 44, $P=0.041$) and had disappeared by fledging ($R_s=0.241$, df 21, $P=0.280$).

3.4. Adult body condition and H/L ratios

Adults were sexually monomorphic in body mass ($t=0.491$, df 37, $P=0.665$) and lost weight over the breeding season (137.8 ± 2.2 g during courtship vs. 129.2 ± 2.1 g during chick feeding, $t=2.8$, df 37, $P=0.008$). Among adults, there was a trend for a negative correlation between body condition and H/L ratio ($R=-0.275$, df 38, $P=0.091$). This was consistent with the pattern observed within the population, where H/L ratios increased (see above) and body conditions decreased over the course of the season (Table 1, t -test courtship vs. chick feeding period: $t=3.1$, df 37, $P=0.003$).

Table 2

Relationship between chick body condition, age and leucocyte distributions of thin-billed prion chicks

Parametric tests (GLM)	Body condition			Age		
	$t(bc)$	$p(bc)$	$\eta^2(bc)$	$t(age)$	$p(age)$	$\eta^2(age)$
Heterophils	-0.353	0.725	0.003	3.862	<0.001	0.241
Lymphocytes	0.076	0.940	<0.001	-5.004	<0.001	0.348
H/L ratio	0.181	0.857	0.001	3.672	0.001	0.209
Nonparametric tests*	R_s	P	N	χ^2	P	N, df
Eosinophils	-0.023	0.882	45	9.15	0.010	21, 2
Basophils	0.125	0.411	45	23.15	<0.001	21, 2
Monocytes	-0.121	0.430	45	0.75	0.687	21, 2

General linear models were carried out for each normally distributed parameter independently, consisting of "chick body condition" and "age" as independent variables, the test parameter as dependent variable and "chick number" as factors. t -values are included to indicate the direction of the relationship, and η^2 for effect sizes. The values $t(bc)$, $p(bc)$ and $\eta^2(bc)$ refer to the effect of body condition on leucocytes, while $t(age)$, $p(age)$ and $\eta^2(age)$ refer to the effect of age. (*) For not normally distributed variables, correlation of cell frequencies with body condition was tested using Spearman correlations, controlling for age by using only data of chicks of 6 weeks. Age effects were tested within chicks using Friedman tests. Significant correlations are marked bold.

4. Discussion

In the present analysis, we found that the stress index H/L increased over the season in both adults and chicks. In adults, this may be explained by the stress of parental care in the relatively poor conditions experienced during the breeding season 2005, but in chicks the increase was age-dependent and independent of condition.

4.1. Seasonal variation in leucocytes and stress index in adult birds

Variation in immune function in adult birds over the breeding season has been observed previously. For example, Adelie penguins *Pygoscelis adeliae* decreased H/L during the breeding season (Vleck et al., 2000). Likewise, H/L of great tits *Parus major* declined from the first to the second breeding attempt (Kilgas et al., 2006b). In contrast to those studies, Hōrak et al. (1998) found the opposite trend, i.e. increasing H/L ratios, in female great tits, and we observed the same in both sexes of thin-billed prions. Previous studies have shown that the H/L ratio in birds is positively related to measures of reproductive effort (Ots and Hōrak, 1996; Moreno et al., 2002; Sanz et al., 2004), and this may explain the trend observed in Adelie penguins, where the incubation stage is extremely demanding (e.g. Davis, 1982), and in the present study, where chick feeding is probably more demanding than the courtship period. A study of leucocyte profiles in great tits (Ots et al., 1998) has shown diurnal variation in leucocyte numbers relative to red blood cell numbers, but the H/L ratio did not differ significantly between daytime and nighttime samples. Therefore, we assume that the seasonal differences observed here are not due to differences in sampling time, but this should be confirmed in future studies.

We found only slight, statistically non-significant, sex differences in adults, and this is consistent with observations of similar investment in chick provisioning in male and female thin-billed prions (Duriez et al., 2000; Quillfeldt et al., 2007b).

Among individual adults, we found a trend for a negative correlation between body condition and H/L ratio, consistent with the temporal pattern observed within the population. When body conditions decreased over the course of the season, H/L ratios increased.

The reason why the correlation, using a single value of each adult, was not very strong, may be explained by large mass fluctuations of adults, which influence the measure of body mass. Adult thin-billed prions carry large food loads relative their body mass (up to 52 g vs. 130 g, Quillfeldt et al., 2003), which may strongly influence the measurements of body condition from body masses. Former studies have suggested that single measurements of adult body mass cannot be used as a reliable measure of body condition in highly pelagic seabirds with large food loads (e.g. Quillfeldt et al., 2004). Thus, more realistic measures of condition should be independent of the last meal and integrate a timescale of several days, and the H/L stress index would appear a useful measure of condition in adult thin-billed prions, at least on a population level.

However, our limited sample size did not allow us to distinguish between seasonal and body condition effects, a larger sample size or repeated samples from the same individuals may be required to confirm whether H/L ratios reliably reflect body condition on an individual level.

4.2. Development of leucocytes and stress index in chicks

The nestling stage is critically demanding for the parents and offspring of altricial birds. During this period, a range of factors including environmental conditions, parental investment and genetic quality, determine whether nestlings have a better or worse chance to survive to independence. Measures of immunological condition, such as the leucocyte stress index, may reflect superior physiological condition, and thus better chances to cope with hazards like fluctuating food abundance, especially in the presence of disease or

parasitic infestation. Surprisingly little, however, is known about the ontogeny of immune function of birds in the wild.

Blood profiles of nestling birds develop with age and therefore, values often differ between nestlings and adults. While it is well established that altricial bird chicks hatch with low contents of red blood cells, compared to adults, and hematocrit of chicks steadily increases in the nestling period (e.g. Kostelecka-Myrcha and Myrcha, 1980; Merino and Barbosa, 1997; Villegas et al., 2002; Gayathri et al., 2004), leucocytes of neonatal and nestling birds have not been studied extensively. Procellariiform seabirds, with their single-egg clutches and slow development make excellent models for this type of study, because they exclude confounding effects of clutch size, and allow sufficiently long time of study in the nest to observe changes in body condition and immune parameters.

In the present study, we found that nestling thin-billed prions progressively increased the relative number of eosinophils, basophils and heterophils (Fig. 2), while lymphocytes and monocytes decreased in abundance (Fig. 2). The stress index was lower in chicks than adults, on average, but reached levels close to adults at pre-fledging age. The avian, as the vertebrate immune system in general, has two major components: the innate immunity and the acquired immunity (see e.g. Roitt et al., 1998; Norris and Evans, 2000). Heterophils are part of the innate immune response, which is carried out by phagocytosing cells that react nonspecifically to a variety of pathogens (e.g. bacteria and fungi). Lymphocytes are part of the acquired immune system. The acquired immunity is antigen-specific and includes cell-mediated and humoral (antibody) responses, as well as immunological memory (i.e. towards viruses). In the chicks, thus, as the lymphocyte numbers decrease and heterophile numbers increase with age, the investment of the immune system is shifted towards more investment into innate immunity.

The few studies, which relate the H/L stress index to chick survival or measures of chick growth, yield contrary results. Two studies reported effects: H/L at 12 days predicted nestling survival from hatching to fledging in great tits (Nadolski et al., 2006), and the stress index negatively correlated with food availability in nestling serins *Serinus serinus* (Hoi-Leitner et al., 2001). In contrast, H/L at 12 days was independent of feeding rates in experimentally manipulated broods of barn swallows *Hirundo rustica* (Pap and Markus, 2003), and there was no relation between H/L in wood storks *Mycteria americana* aged 4–6 weeks and their post-fledging survival (Hylton et al., 2006). However, in the latter study, chicks tended to have higher H/L in a poorer season.

In thin-billed prions, chicks that hatch and survive the first days have a very good chance of survival to fledging, even in poor conditions such as those experienced in 2005 (e.g. Quillfeldt et al., 2007a). Therefore, we could not test between surviving and non-surviving chicks. In the present study, H/L ratios were independent from body condition, which reflects feeding rate. However, the austral summer 2004–2005 was very poor in food abundance, and chicks were generally underfed. Thus, the condition of all chicks may have been depressed. It would be instructive to compare the data with H/L ratios determined in a season of higher feeding rates.

We found that chicks from earlier hatching eggs had lower H/L ratios up to 6 weeks of age. Because earlier egg-laying is often associated with parental quality, this may indicate an advantage of early hatching chicks in terms of immune function or general condition. The relationship between hatching date and H/L ratio became weaker with increasing age and had disappeared by fledging, probably reflecting the impoverished conditions experienced by all chicks.

The results suggest that the stress index is a useful measure of condition in adult thin-billed prions, while they highlight our lack of knowledge of the ontogeny of immune function. Studies of adults and chicks over several seasons may reveal how resources are allocated between immune and other functions under contrasting environmental conditions.

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