Do leucocytes reflect condition in nestling burrowing parrots *Cyanoliseus patagonus* in the wild?

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**Abstract**

The different leucocyte types are an important part of the immune system. Thus, they have been used in ecological studies to assess immune function and physiological stress in wild birds. It is generally assumed that increased stress and decreased condition are associated with an increase in the ratio of heterophils to lymphocytes, the H/L ratio. We studied leucocyte profiles in relation to body condition in nestling Burrowing Parrots (*Cyanoliseus patagonus*) in North-eastern Patagonia, Argentina. As in other wild parrots, heterophils were the most numerous leucocyte type, suggesting strong investment into innate immunity. Leucocyte profiles did not change with the age, while nestlings in better body condition increased the number of heterophils. Because the number of lymphocytes was independent of body condition, as a result we observed a positive correlation between body condition and the H/L ratio. The total number of leucocytes relative to erythrocytes increased in nestlings in better body condition, indicating a larger overall investment into immune function in well-nourished nestlings. The observed heterophilic profiles of nestling Burrowing Parrots together with the positive relationship between H/L ratio and body condition may indicate a favoured investment in a robust innate immunity that reduces the risk of infection taking hold in these long-lived birds.

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1. Introduction

Haematology has long provided some valuable indicators of individual health and condition. As a consequence of the close contact of the blood with all organs and the rapid replacement rate of the blood cells, the examination of peripheral blood can be used to study changes resulting from different physiological demands (e.g. Dein, 1986). More specifically, the relative proportion of the different types of circulating white blood cells may provide information about the health and condition of wild birds (e.g. Ots et al., 1988; Hörak et al., 1988; Moreno et al., 2002; Pap and Márkus, 2003; Lobato et al., 2005). It has been shown that immunological processes interact with an individual's nutritional condition (e.g. Lochmiller et al., 1993; Saino et al., 1997), among other life-history traits.

Leucocyte profiles (relative numbers of the different leucocytes) have been used in ecological studies to assess immune function and physiological stress in wild birds (e.g. Ewenson et al., 2001; Hauptmanová et al., 2002; Ruiz et al., 2002, Mazerolle and Hobson, 2002; Davis et al., 2004, 2008; Quillfeldt et al., 2008). Leucocytes form the basis of the immune system of vertebrates, their main function being the protection against pathogens. Leucocytes associated with the innate immune system act as an initial non-specific protection mechanism of wide range during the early stages of infestation. Monocytes and granulocytes, such as heterophils and eosinophils in birds, can thus offer an important measure of the non-specific host immune function and health status (e.g. Maxwell, 1993; Davis et al., 2004). Heterophils, usually the most numerous of the granulocytes found in birds (Hawkey and Dennett, 1989), are known to increase in response to abnormalities related to diet, stress syndrome, trauma, inflammatory processes and chronic bacterial infections (e.g. Gross and Siegel, 1983; Dein, 1986; Maxwell and Robertson, 1998). In contrast, lymphocytes, part of the acquired immune system, are highly specific and become abundant as a result of parasite infestation (e.g. Figuerola et al., 1999), viral infection (e.g. Hawkey and Dennett, 1989) and in response to immunization (e.g. Eeva et al., 2005). The acquired immune system acts more effectively against various pathogens, including viruses, bacteria and ectoparasites, but implies a high metabolic cost due to rapid cell proliferation (see e.g. Apanius, 1998; Lochmiller and Deerenberg, 2000; Blount et al., 2003; Pap and Márkus, 2003; Lee, 2006).
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 a lower proportion of lymphocytes (L) (see review by Davis et al., 2008). Hence, a quantitative indicator of physiological stress levels can be calculated from the proportions of circulating heterophils/lymphocytes (H/L), following Gross and Siegel (1983). As changes in circulating levels of both types of blood cells are much slower in comparison to hormonal changes (Dein, 1986; Maxwell, 1993), H/L ratios do not increase significantly within 1 h of capture and blood sampling (Davis 2005), thus making the H/L ratio independent of observer-induced biases and convenient for assessing environmental stress (see Gross and Siegel, 1983; Davis et al., 2008). Increased H/L ratios were observed in clinically abnormal birds (e.g. Averbeck, 1992; Veck et al., 2000; Davis et al., 2004), in parasite-infested birds (e.g. Lobato et al., 2005), and in nestlings during times of low food availability (e.g. Hoi-Leitner et al., 2001; Lobato et al., 2005). It has also been observed that the H/L ratio was negatively correlated with parameters of nesting growth and mass in Pied Flycatchers Ficedula hypoleuca (Moreno et al., 2002; Lobato et al., 2005). In a previous study, we found that an increase of H/L ratios in nestlings Thin-billed Prions Pachyptila belcheri was age-dependent and independent of condition (Quillfeldt et al., 2008).

As shown, haematological parameters can provide useful tools for ecological research. Yet, data from wild birds are still scarce due to the difficulties obtaining enough good samples from wild birds, and the relatively time-consuming analysis. In the particular case of the Psittaciformes, one of the most endangered order of birds during the relatively time-consuming analysis. In the particular case of the Psittaciformes, one of the most endangered order of birds during the last few decades (Snyder et al., 2000), haematology values are scarce and, with a few exceptions (Joyner et al., 1992; Karesh et al., 1997; Sheridan et al., 2004; Deem et al., 2005; Low et al., 2006; Tompkins et al., 2006), correspond to captive birds (e.g. García del Campo et al., 1991; Harper and Lowe, 1998; Polo et al., 1998; Scope et al., 2000; Foldenauer et al., 2007).

Burrowing Parrots Cyanoliseus patagonus are colonial Psittaciformes that, in Argentina, mainly inhabit the phytogeographical province of ‘Monte’, a semi-desert scrubland, characterized by bushy steppes and xerophytes forests (see Cabrera, 1971; López et al., 2006). Burrowing Parrots need sandstone, limestone or earth cliffs where to excavate their nests (see e.g. Masello et al., 2001, 2006a). Recently, various aspects of the breeding biology of this species have extensively been studied (see e.g. Lubjuhn et al., 2002; Masello et al., 2001, 2002, 2004, 2006a,b, in press; Masello and Quillfeldt, 2002, 2003, 2004a,b; Mey et al., 2002). A typical brood of Burrowing Parrots is composed of two to five nestlings, which hatch asynchronously and remain in the nest for an average of 63 days (Masello and Quillfeldt, 2002, 2004a, b). Until now, information on haematological parameters of Burrowing Parrots was only available from adult captive birds (Polo et al., 1998).

Here, we report results of a study of Burrowing Parrots where we analysed leucocyte counts in relation to nesting body condition and age. Specifically, we test the following hypotheses:

1. Relative leucocyte abundance is related to nesting condition,
2. Particularly, the H/L index is negatively correlated to body condition in nestlings, and,
3. Leucocyte counts vary with nesting age, as we previously found in nestlings of another wild bird species (Quillfeldt et al., 2008).

2. Materials and methods

Fieldwork was carried out during November to January 2003–2004 at the largest colony of Burrowing Parrots, located in a sandstone cliff facing the Southern Atlantic Ocean in north-eastern Patagonia, Argentina. A detailed description of this colony has been previously published in Masello et al. (2006a,b). The habitat in the surroundings of the colony belongs the phytogeographical province of Monte, more specifically to its north-eastern Patagonian sector also known as Austral Monte (see e.g. Bran et al., 2007).

According to accessibility, 32 nests were monitored in the easternmost sector of the colony as part of an ongoing study of the breeding behaviour of the species (see e.g. Masello et al., 2006a and references therein, Masello et al., 2006b; Blank et al., 2007). Nests were inspected every five days by climbing the cliff face. Regular nest monitoring started after all nestlings of a brood were, at least, five days old. As previously described (e.g. Masello et al., 2002), this was due to the tendency of Burrowing Parrots to desert their broods if disturbed during the incubation or hatching periods.

As described in previous studies (e.g. Masello and Quillfeldt, 2002, 2003, 2004a,b) body mass was recorded, using a digital balance to the nearest 1 g. Mass growth in nesting burrowing parrots follows a quadratic regression reaching a peak mass around a mean age of 38 days, which is followed by a large mass recession (Masello and Quillfeldt, 2002). An index of nesting body condition (BC) was calculated relative to the mean mass for nestlings of each age (m̄mea), using the following formula: BC = m / m̄mea, as previously described in Masello and Quillfeldt (2004a) and Quillfeldt et al. (2008). In species with a pattern of peak mass and mass recession (e.g. Procerculiformes, Psittaciformes), 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 (see e.g. Quillfeldt et al., 2008).

Blood samples of nesting Burrowing Parrots were taken by puncture of the cutaneous ulnar vein, using a hypodermic needle and heparinised capillary tubes, immediately after capture i.e., between 11.00 and 19.00 h. Birds were sexed using PCR amplification of a highly conserved W-linked gene as previously described (Lubjuhn and Sauer, 1999; Masello and Quillfeldt, 2004b). Blind duplicate and triplicate blood samples of burrowing parrots were analysed in order to ensure the accuracy of the gender determination. In all cases duplicates and triplicates confirmed the results. Blood sampling had no detectable adverse effects on the birds.

After measuring and sampling, the birds were released in their burrows. No desertion occurred. The number of fledglings and the fledgling size of handled nests were within previously reported ranges (Masello and Quillfeldt, 2002, 2003, 2004a).

Immediately after sampling, a drop of blood was smeared on a glass slide, air dried, and fixed with absolute methanol later in the lab. All smears were stained with Giemsa. Following previous studies (see e.g. Merino et al., 1999; Moreno et al., 2002; Lobato et al., 2005), differential leucocyte counts were carried out with a light microscope (1000×, magnification with oil immersion) in parts of the smear where erythrocytes had separated in a monolayer. As in Merino et al. (1999) we crossed the sample from down to up to minimize differences in the thickness of the blood smear. Heterophils, eosinophils, basophils, lymphocytes and monocytes were determined following Dein (1986) and Hawkey and Dennett (1989). Examination was ended when a total of 100 leucocytes were reached, thereby obtaining relative numbers of the different types of leucocytes. The number of leucocytes per 10,000 erythrocytes was calculated by counting the number of erythrocytes per field and multiplying by the number of fields viewed to count 100 leucocytes following e.g. Merino et al. (1999), Moreno et al. (2002), Lobato et al. (2005). Thrombocytes were excluded as they are usually present in small numbers and hard to distinguish from leucocytes.
irregularly distributed (see e.g. Lobato et al., 2005). Differential leucocyte counts were carried out in samples of 103 nestlings of wild Burrowing Parrots (57 males, 46 females).

H/L ratio was calculated as an index of individual stress. This haematological parameter has shown a low measurement error under field situations (see e.g. Gross and Siegel, 1983; Maxwell, 1993; Ots et al., 1998; Merino et al., 1999; Moreno et al., 2002; Hanssen et al., 2003; Eeva et al., 2005). All birds were sampled within 30 min of capture ensuring that the H/L ratios did not changed due to routine handling (see Davis, 2005).

The H/L ratios, the total number of leucocytes per 10,000 erythrocytes, the number of heterophils per 10,000 erythrocytes, and the number of lymphocytes per 10,000 erythrocytes were normally distributed (Kolmogorov–Smirnov Test: p < 0.001). We normalized these parameters using a natural logarithm transformation. We decided to pool the data of both male and female nestlings together as t-tests revealed no significant differences for any of the studied haematological parameters (all P > 0.2).

Data were analysed using Sigma Stat 2.03 and SPSS 11.0. We tested for correlations between age, body condition (BC) and haematological parameters of nestling Burrowing Parrots using General Linear Models (GLMs), with differential leucocyte counts as dependent variables and ‘age’ and ‘body condition’ as covariates. We initially included interaction terms between factors and covariates in the models but removed them, when no significance was revealed (all P > 0.2). In all cases where general linear models (GLM) were performed, covariates were always entered in separate models. In those cases where homogeneity of variance was not achieved according to Levene’s test, we followed the suggestion of Zöfel (2002) and considered the homogeneity of variance was not achieved according to Levene’s test. Note that sample sizes for different analyses varied as not all measurements could be taken on all birds.

Results correspond to General Linear Models (GLM), with ln H/L ratio, ln(leucocytes/10,000 erythrocytes), heterophils/100 leucocytes, and lymphocytes/100 leucocytes as dependent variables, and both nestling age and body condition as covariates. t-values are included to indicate the direction of the relationship, and η² for effect sizes.

### 3. Results

Mean values of the haematological parameters and body condition of nestling Burrowing Parrots in the wild are shown in Table 1.

We found heterophils the most abundant leucocytes of nestling Burrowing Parrots (Table 1), as only in 5% of the nestlings lymphocytes were more abundant (i.e. H/L ratios lower than 1 in Fig. 1). Although in most cases H/L ratios were below 6, some nestlings reached values as high as 15.8 (Table 1, Fig. 1). Eosinophils and monocytes were rare or even totally absent (Table 1). In the studied samples, basophils were not detected.

General Linear Models (GLM) revealed that the leucocyte numbers, the relative proportions of the different white blood cells and the H/L ratio of nestling Burrowing Parrots did not change with age (Table 2A to F).

The proportion of heterophils and lymphocytes within leucocytes was related to body condition (Table 2A, B); with a rising proportion of heterophils (Table 2A) and a decreasing proportion of lymphocytes (Table 2B). This led to a positive correlation between H/L ratio and body condition (Fig. 2, Table 2C). Nestlings in better body conditions have lower H/L ratios.
also increased the total number of leucocytes relative to erythrocytes (Table 2D). This was caused by an increase in the number of heterophils (Table 2E), while the number of lymphocytes was not related to body condition (Table 2F).

4. Discussion

A number of haematological tests, among them differential leucocyte counts, can be used to study nestling condition. We demonstrate here that differential leucocyte counts are related to nestling body condition in wild Burrowing Parrots of North-eastern Patagonia, Argentina. Contrary to our prediction, but in line with some previous studies (Dufva and Allander, 1995; Figuerola et al., 1999; Møller and Petrie, 2002; Maney et al., 2008), the observed relationship was positive, i.e. Burrowing Parrot nestlings in better body condition showed the highest H/L ratios. This was caused by an increase in the number of heterophils in nestlings in better body condition. Because the number of lymphocytes was independent of body condition, as a result we observed an increase in the total number of leucocytes relative to erythrocytes and the aforementioned increase in H/L. In nestling Thin-billed prions (Quillfeldt et al., 2008), we found that H/L ratio increased with age. This was not the case in nestlings Burrowing Parrots: the leucocyte numbers, the relative proportions of the different white blood cells or H/L ratio showed no relationships with nestling age.

The nestling stage is a critically demanding period in altricial birds, when 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 (Quillfeldt et al., 2008). The quality and the amount of food available to nestlings in connection with the environmental quality might affect the development of the immune system (Hoi-Leitner et al., 2001). Nestlings growing under better conditions will attain higher body conditions. This, in turn, may allow the maintenance of a higher level of metabolism, as here revealed in nestling Burrowing Parrots, where the total number of leucocytes relative to erythrocytes increased as a consequence of increased numbers of heterophils in nestlings in better body condition. As a consequence, these nestlings might develop a more vigorous immune system (see e.g. Norris and Evans, 2000; Quillfeldt et al., 2008).

Heterophilic profiles, as observed in nestling Burrowing Parrots in the wild have also been described in most of the few studies conducted in wild Psittaciformes (mainly in adults: Joyner et al., 1992; Sheridan et al., 2004; Deem et al., 2005; Low et al., 2006). Heterophilic profiles and increased H/L ratios have been interpreted as a symptom frequent in environmental and physiological stress (e.g. Maxwell, 1993) and infection (e.g. Hawkey et al., 1985). However, it has also been suggested that increased H/L ratios could be indicative or enhanced resistance to parasites (Dufva and Allander, 1995; Figuerola et al., 1999). The observed heterophilic profiles of nestling Burrowing Parrots together with the positive relationship between H/L ratio and body condition could be interpreted as a favoured investment in a robust innate immunity that reduces the risk of infection taking hold. Nestling Burrowing Parrots with higher body condition could afford to produce larger numbers of heterophils as an insurance against possible parasite or pathogen infections. The costs of constitutive innate immunity are usually considered to be comparatively low, because of the lack of a diversification process such as that required for lymphocyte development and low rates of cell turnover when an immune response is not necessary (see e.g. Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Lee, 2006). Moreover, long-lived species (like many Psittaciformes) are thought to have evolve higher levels of immune response, mainly innate immunity, related to an increased investment in self-maintenance (see Tieleman et al., 2005). A previous study (Masello et al., 2006a) supports our interpretation of a larger investment of Burrowing Parrots in innate immunity: a subset of the same samples studied here revealed the absence of blood parasite (Plasmodium spp. and Haemoproteus spp.) in nestlings, either in blood smears or with the use of a PCR detection method (the primers 621 and 983 that amplify a fragment of the parasites’ cyt b gene; see Richard et al., 2002). Additional support is provided by a literature review of hemoparasites in wild Psittaciformes (Masello et al., 2006a) that showed absence of blood parasites in all cases where the wild origin of the birds, together with no contact with poultry, pets, rehabilitation centres or zoos, was certain. Further support is brought by the same study that showed an absence of intestinal parasites in Burrowing Parrots as well as a generally low incidence of this kind of parasites in wild Psittaciformes (Masello et al., 2006a). These results have been confirmed by other recent studies that further report absence of hemoparasites and/or intestinal parasites in wild Psittaciformes (Deem et al., 2005; Gordon Stone et al., 2005; Chahota et al., 2006; Tompkins et al., 2006). However, alternative interpretations cannot be excluded without direct manipulation of both the innate and acquired immune system.

As suggested in a recent review by Davis et al. (2008), a positive correlation between condition-dependent parameters of the organism (e.g. body condition, brighter plumage colouration, absence of parasites or infectious diseases, higher cell-mediated immunity) and H/L ratios can be interpreted in a different way. The positive correlation may occur if individuals showing better condition-dependent parameters are subject to greater stress perhaps due to e.g. greater energy expenditure on mate seeking and/or territory defence (see Mazerolle and Hobson, 2002; Davis et al., 2008, Maney et al., 2008). However, it is difficult to conceive how the latter would work in altricial nestlings. In line with these authors (Davis et al., 2008; Maney et al., 2008), a possible alternative explanation to our results might be related to two aspects of the life history of Burrowing Parrot: a) the species is a burrow-nester subject to ectoparasitic pressure (see Mey et al., 2002; Masello et al., 2006a), b) hatching asynchrony can lead, under certain unfavourable environmental conditions, to brood reduction (see Masello and Quillfeldt, 2002, 2004b). This could generate a situation of stress among the nestlings that could explain the observed very high heterophil counts. But in this scenario, as Davis et al. (2008) review shows, corticosterone increases associated to a stressful situation should lead to an increase in heterophils with a simultaneous decrease in lymphocytes, which was not the case in our study. In nestling Burrowing Parrots the number of lymphocytes was independent of body condition. In the observed absence of blood and intestinal parasites (Masello et al., 2006a), it still remains possible that
Burrowing Parrot nestlings show inflammation response to some undiagnosed fungi, virus of bacterial infection. But all these three sources of stress (ectoparasites, brood reduction, inflammation) should stronger affect last-hatched nestlings, with lower body conditions (see Masello and Quillfeldt, 2002, 2004b), rather than the first-hatched nestlings with higher body conditions, which showed the highest heterophilic profiles in our study.

Total leucocytes, heterophils and lymphocytes numbers per 10,000 erythrocytes have been shown to produce highly repeatable differential leucocyte counts (e.g. Merino et al., 1999; Moreno et al., 2002; Lobato et al., 2005). Nevertheless, in many behavioural studies, and in almost all studies on Psittaciformes, these parameters have largely been ignored. Without these parameters, however, the observed variability in H/L ratios and its relationship with body condition would have been difficult to interpret in the present study. We therefore recommend that future studies calculate total leucocytes, heterophils and lymphocytes numbers per 10,000 erythrocytes and interpret investment in immune system using these parameters in addition to H/L ratios.

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