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ARTICLE

Leukocyte profiles and body condition of free-living Burrowing owls (*Athene cunicularia*) from rural and urban areas in the Argentinean Pampas

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ABSTRACT: Animals living in urban areas are exposed to novel and potentially stressful human disturbances. In response to the increased number of stressors in these habitats, they may manifest changes in their immune system, body condition and physiology. Many bird species are negatively impacted by urbanization, whilst other species thrive in urban areas. The capacity to adjust the physiological responses to the stressors associated with urban areas may play a key role in explaining the success of some bird species in these environments. In this study, we compared indicators of physiological stress response and body condition in free-living Burrowing Owls (*Athene cunicularia*) inhabiting urban and rural areas. We calculated a scaled index of body condition, and assessed leukocyte profiles and the heterophil/lymphocyte ratios of chicks and adults. We found no significant differences in these parameters between individuals from rural and urban areas. Chicks showed higher relative leukocyte counts than adults, which may be associated with ontogenetic development and immune system activation processes.

KEY-WORDS: heterophil, leukocytes, lymphocyte, stress, urbanization.

INTRODUCTION

With natural areas rapidly decreasing as human-altered habitats increase and human populations grow, bird species are finding themselves living ever closer to human habitats (Marzluff 2001). Some species have managed to co-exist with humans in urban areas while others failed to thrive in these new environments. Considering the rapid environmental change that occurs during urbanization, it may be predicted that only individuals capable of coping with challenges of urban life would succeed (Shanahan *et al*. 2014). Species living in urban habitats are exposed to many novel and potentially stressful anthropogenic disturbances, such as permanent presence of humans, higher densities of non-native predators (*e.g.*, cats and dogs), noise and light pollution as well as traffic (Sol *et al*. 2013). Such urban challenges are predicted to trigger physiological and behavioural reactions that ultimately increase the levels of stress responses (Partecke *et al.* 2006, Bonier *et al*. 2007, Bonier 2012, Johnstone *et al*. 2012). However, it is to be expected that if individuals are able to cope with different levels of urban disturbance, then living in urban contexts should not be perceived as more stressful than living in rural ones since a process involved in maintaining stability may occur (Wada 2015).

Two classes of hormones are involved in the modulation of physiological responses to stressors in vertebrates: catecholamines and glucocorticoids. The first involve the release of noradrenaline and adrenaline from the sympathetic nervous system and the adrenal medulla within seconds after detection of stressors, resulting in rapid physiological responses (Wada 2015). On the other hand, glucocorticoids are secreted through the hypothalamic-pituitary-adrenal axis and are mainly involved in recovery from stressors or preparing from future stressors. Glucocorticoids are secreted from adrenal cortex within minutes after detecting a stressor and acts redirecting energy and behavior to essential adjustments (Wada 2015). The release of glucocorticoid hormones after a stressor is one of the mechanisms involved in physiological coping mechanisms to long-term stress and, in vertebrates, is one of the mechanisms that ensure survival under adverse environmental conditions (Bonier 2012). However, prolonged or frequent stress responses can result in an individual entering a disease-like state of chronic stress leading to reduced fecundity and reduced chances of survival (Siegel 1980, Wingfield & Sapolsky 2003).

Measuring chronic physiological stress in free-living vertebrates can be problematic since reliable measurements of baseline levels of glucocorticoids are often difficult to obtain in the field because their levels rise immediately after capture (Davis 2005, Romero & Reed 2005). The use of haematological parameters such as relative leukocyte (or white blood cell) counts made from blood smears represents an alternative method for measuring stress in birds since their values are related to levels of glucocorticoids hormones such as corticosterone, the avian stress hormone (Davis *et al*. 2008). A prolonged increase of corticosterone levels in birds causes changes in the relative numbers of various specific leukocyte types present within the immune system. For example, in response to corticosterone, circulating lymphocytes adhere to the endothelial cells and subsequently a significant reduction in their circulating numbers is evidenced (Davis *et al.* 2008). Also, corticosterone stimulates an influx of heterophils (the avian equivalent of the mammalian neutrophils) into the blood from the bone marrow and attenuates the egress of heterophils from the blood to other compartments (see Davis *et al*. 2008).

Given the effect of stress hormones on leukocytes, leukocyte profiles and the heterophils/lymphocytes ratio have been widely used as indicators of response of individuals to several stressors such as temperature, muscular exhaustion, food or water deprivation, captivity and contaminants among others (Siegel & Gross 2000, Davis *et al*. 2008). Even though relative leukocyte ratios have been widely used as an estimator of long-term stress or baseline hormones levels (Davis *et al.* 2008), some studies have shown that in some avian species the handling stress can significantly affect leukocyte counts within 30 and 60 min after capture (Cirule *et al.* 2012), showing that leukocyte response to stress may be quicker than traditionally assumed. For this reason, the interval between bird capture and sample collection should be minimized to obtain a more precise estimation of baseline stress level. Also, Müller *et al.* (2011) found in free-living kestrel nestlings that baseline corticosterone levels and the heterophils/lymphocytes ratio differ in the sensitivity to various stressors suggesting that both measures should be taken when possible.

The Burrowing Owl (*Athene cunicularia*) is a ground-nesting raptor widely distributed throughout the mid to low latitude regions of Americas. Burrowing Owls' habitat has experienced important changes in the last decades due to human activities, such as agriculture, tourism and urbanization. It has been reported that some Burrowing Owl populations have been strongly affected by habitat change in its northern range, to the point of being considered as threatened in some areas of North America (Poulin *et al.* 2011). In contrast, at its southernmost distribution this species has managed to survive and prosper in urban habitats, often reaching higher densities in such heavily disturbed habitats than in their natural habitats (Martínez *et al*. 2017). Living in close proximity to humans presupposes a frequent exposure to anthropogenic stress factors, which Burrowing Owls have overcome mainly through behavioral plasticity (Cavalli *et al.* 2016a, 2016b).

The aim of this study was to compare leukocyte profile, the heterophil/lymphocyte ratio and body condition of Burrowing Owls inhabiting urban and rural habitats of south-eastern Pampas region. Considering that Burrowing Owls showed no breeding and behavioural limitations to establish in urban habitats (Cavalli *et al*. 2016a, 2016b, Martínez *et al.* 2017) and have demonstrated a good ability to live in a wide variety of habitat types and with different levels of disturbance (Baladrón *et al.* 2016, Martínez *et al.* 2017), we hypothesize that this species presents good adaptability to live in urban areas and should not perceive urban habitats as more stressful than rural areas.

METHODS

The study was conducted in the southeast portion of the Pampas region of Argentina. The area was dominated in the past by sand dunes, wetlands and grasslands (Vervoost 1967), and is nowadays a mosaic of different land-uses where agroecosystems (grazing fields, croplands, and pasturelands) and thriving urban centers share the landscape (Pedrana *et al.* 2008). Here, Burrowing Owls inhabit rural habitats, vegetated sand dunes, and urban habitats (Baladrón *et al*. 2016). Urban and rural habitats were sampled in Mar Chiquita district (37°44.6'S; 57°25.7'W) and General Pueyrredón district (38°00.8'S; 57°33.1'W). In these regions, urban habitat is mostly represented by peri-urban areas (small touristic villages with < 800 inhabitants and scattered houses) and, to a lesser extent, suburban areas of larger cities (Pedrana *et al.* 2008, Zelaya *et al.* 2016). Rural habitats are mostly devoted to livestock production, thus grazing fields are the dominant landscape unit, whereas croplands are limited to best-quality upland soils. In this context, we defined urban habitats as built-up areas where owls will regularly encounter humans and considered as urban owls those whose nests were surrounded by more than 35 houses within a radius of 200 m. Rural habitat comprised open farmlands, grazing fields, and croplands, and distance from owl nests to houses in rural habitats was always greater than 1 km.

Adults and chicks (~10 days old) from different nests were sampled during January 2014 and 2015. Burrowing Owls were captured with noose carpets (Bloom *et al*. 2007). Two researchers remain constantly

watching traps with binoculars from a blind and run to handle owls immediately after they were trapped. Owls were weighed using a spring scale (d = 5 g, precision ± 0.3%). A preliminary inspection of each captured individual was performed in order to register health status. Health status of each individual was classified as healthy (*i.e.*, no external signs of illness based on the brightness of plumage, good flight capacity in adults owls, absence of feather damage from ectoparasites, and standard body mass: 198.5 ± 22.8 g for adults, Baladrón *et al.* 2015) or non-healthy (*i.e.*, missing feathers, presence of ectoparasites, below normal body mass), and only those of the former group were used for further analyses. A drop of blood was extracted from their brachial vein using 0.5 mm needles. Samples were collected within 5 min of capture to minimize capture and handling stress (Davis 2005). After collecting a blood sample and taking morphometric measurements, we released owls at the same place that they were captured. For all these procedures, we adhered to guidelines for the use of animals in research and to the legal requirements of Argentina (permit numbers: 2145-14331 and 22500-24871).

Thin smears from fresh blood were prepared on individual slides, air dried, fixed with methanol (Reagents, Inc.) for 10 min and then stained with May-Grünwald (BIOPUR®) and Giemsa (BIOPUR®). Smears were examined using a light microscope scanning monolayer fields with similar densities of erythrocytes for all individuals (Campbell 1994). The proportion of each leukocyte type was obtained from a sample of 100 leukocytes viewed in 1000× magnification (oil immersion) and expressed as percentage of basophils (B%), heterophils (H%), eosinophils (E%), lymphocytes (L%), and monocytes (M%) (Campbell 1994). The heterophil/lymphocyte ratio (H/L) was calculated from the leukocyte counts and was used as an indicator of stress (Davis *et al.* 2008). In general, higher H/L ratio values indicate higher levels of individual stress while lower values indicate the opposite. Relative leukocyte count (RLC) per 10,000 erythrocytes was estimated by counting the number of all erythrocytes in one microscopic visual field and multiplied by the number of the microscopic visual fields that were scanned until reaching 100 leukocytes, following Lobato *et al.* (2005).

Body mass (g) and tarsus length (mm, measured with a digital caliper) was measured to calculate a body condition index (Peig & Green 2009). Since both variables do not differ between Burrowing Owls sexes (see Baladrón *et al.* 2015) we pooled them indistinctly. To quantify body mass in relation to body size, we calculated a scaled mass index of body condition following the procedure described by Peig & Green (2009). The index was calculated as follows:

$$\text{Body mass of individual } i \times \left(\frac{\text{Average tarsus length of the study population}}{\text{Individual tarsus length of infividual } i} \right) \text{bSMA}$$

The scaling exponent bSMA was calculated indirectly by dividing the slope from an ordinary least squares regression on log transformed tarsus length and body mass variables by the Pearson's correlation coefficient (r). We chose tarsus length as the proxy for skeletal body size as this measure has been routinely taken during all captures.

Leukocyte values were compared between owls from rural and urban habitats (only for adult owls since no chicks were captured at rural habitats) and between age groups (chicks and adults) using Student's t-test and the nonparametric Mann-Whitney test when data did not show a normal distribution. Normality was assessed by performing Shapiro-Wilk's test (Zar 2010). RLC was compared between habitats (rural and urban adult owls) and between age groups (chicks and adults) using Mann-Whitney test. Scaled mass index of body condition were compared between rural and urban adult owls by Student's t-test (Zar 2010). A part of the data used to calculate body condition belonged to a previous data set that was partly published by Baladrón *et al.* (2015), but none of the blood smears examined in this study were obtained from owls examined in that study.

RESULTS

Relative leukocyte counts, H/L ratios and RLC showed no significant differences between rural and urban adult Burrowing Owls ($P > 0.05$, see Table 1). No significant difference was found between adult rural and urban Burrowing Owls in scaled mass index of body condition (mean urban owls = 205.5 g, SE = 3.8, $n = 42$; mean rural owls = 204.1 g, SE = 11.1, $n = 9$; $t = 0.15$, $df = 49$, $P = 0.88$). Only one adult owl from a rural habitat was excluded from our data set since it was classified as a non-healthy individual (missing feathers, body mass below the population mean: scaled mass index of body condition 149.7 g, had only one eye while the other showed infection signs evidenced by its color).

No significant differences were identified between age groups in heterophils (H), monocytes (M), basophiles (B) and relative leukocyte counts (RLC) ($P > 0.05$, see Table 2). Percentage of lymphocytes (L) was higher for chicks than for adult Burrowing Owls while percentage of eosinophils (E) was higher for adults (Table 2). The H/L ratio was higher for adult than for chick Burrowing Owls. Figure 1 shows the morphology of erythrocytes, heterophils, eosinophils, lymphocytes, monocytes and thrombocytes.

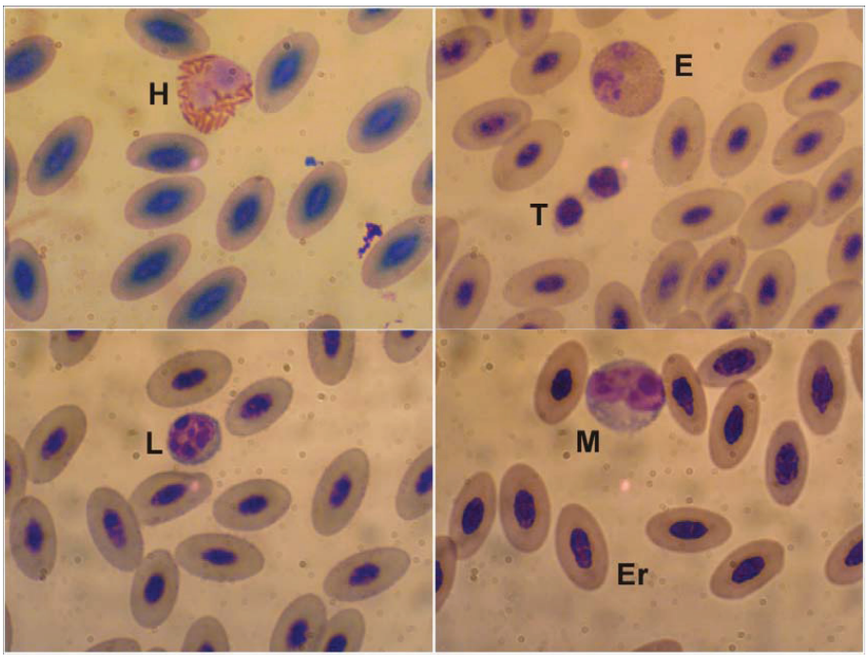

Figure 1. Burrowing Owl (*Athene cunicularia*) blood smears. Erythrocytes (Er), thrombocytes (T), heterophils (H), lymphocytes (L), eosinophils (E) and monocytes (M). × 100 objective.

Table 1. Mean ± standard error (SE), range (minimum – maximum) of relative leukocyte counts, relative leukocyte count (RLC) and heterophil/lymphocyte ratio (H/L) from rural and urban adult Burrowing Owls (*Athene cunicularia*) from southeast of Pampas region in Argentina. Sample size (n) is shown in parentheses. Leukocyte types are expressed as percentage of heterophils (H%), lymphocytes (L%), monocytes (M%), basophils (B%) and eosinophils (E%).

	Rural (*n* = 4)		**Urban (*n* = 11)**		**Statistical test**	***P*-values**
	Mean ± SE	**Range**	**Mean ± SE**	**Range**		
H%	36.1 ± 0.4	33.6 – 37.1	37.5 ± 3.6	19.0 – 61.4	t = - 0.2	0.81
L%	40.3 ± 0.7	38.9 – 42.0	35.7 ± 1.9	25.8 – 47.6	t = 1.3	0.13
M%	1.0 ± 0.7	0.88 – 3.1	0.9 ± 0.3	0.75 – 3.3	U = 21	0.94
B%	0.2 ± 0.2	0.0 – 0.7	0.1 ± 0.1	0.0 – 1.7	U = 19	0.58
E%	22.3 ± 1.9	18.1 – 27.2	25.5 ± 2.6	15.3 – 37.1	U = 27	0.55
H/L	0.9 ± 0.02	0.8 – 0.9	1.1 ± 0.1	0.4 – 1.5	U = 29	0.39
RLC	94.5 ± 25.2	121.0 – 160.0	91.0 ± 9.5	59.0 – 132.0	U = 23.5	1.00

Table 2. Mean ± standard error (SE), range (minimum – maximum) of relative leukocyte counts, relative leukocyte count (RLC) and heterophil/lymphocyte ratio (H/L) from chick and adult Burrowing Owls (*Athene cunicularia*) from southeast of Pampas region in Argentina. Sample size (*n*) is shown in parentheses. Leukocyte types are expressed as percentage of heterophils (H%), lymphocytes (L%), monocytes (M%), basophils (B%) and eosinophils (E%).

	Chicks (*n* = 10)		**Adults (*n* = 15)**		**Statistical test**	***P*-values**
	Mean ± SE	**Range**	**Mean ± SE**	**Range**		
H%	34.3 ± 2.2	27.3 – 46.4	37.2 ± 2.5	19.0 – 61.4	U = 91	0.36
L%	51.9 ± 2.3	39.5 – 63.0	36.9 ± 1.5	25.8 – 47.6	t = -5.6	<0.001
M%	0.5 ± 0.2	0.0 – 1.4	0.9 ± 0.3	0.0 – 3.3	U = 82	0.69
B%	0.0 ± 0.0	0.0 – 0.0	0.2 ± 0.1	0.0 – 1.6	U = 85	0.26
E%	13.3 ± 2.3	6.0 – 27.2	24.7 ± 1.9	12.9 – 37.1	t = 3.7	0.001
H/L	0.6 ± 0.1	0.5 – 1.0	1.1 ± 0.1	0.4 – 2.4	U = 120	0.01
RLC	76.6 ± 7.1	48.0 – 127.0	92.0 ± 9.3	39.0 – 160.0	U = 96.5	0.24

DISCUSSION

Animals often respond to the challenges found in urban areas through changes in their body condition and physiological stress responses (Davis *et al*. 2008). The magnitude and prevalence of stress levels may vary according to the species and its tolerance to the different stressor agents associated to urban life (*i.e.*, if it is an avoider, adaptable or exploiter of urban areas (Blair 1996, McKinney 2002). Our results show that leukocyte profiles and the body condition of wild Burrowing Owls did not differ significantly between urban and rural habitats, corroborating the notion that this is an urban-adaptable species. This finding is similar to that reported for other urban-exploiter such as House Sparrows (*Passer domesticus*). Urban and rural House Sparrows have been show to display similar levels of stress hormones (Bókony *et al*. 2012) and their immunological status does not vary among urbanization levels (Chávez-Zichinelli *et al.* 2010). However, the range of indicators of physiological stress response (immunoglobulin and corticosterone concentrations) is wider in more habitats with more stressful stimuli. In line with this idea, we found that urban areas housed both high- and low-stressed owls, as relative counts for all leukocyte types showed wider ranges in urban areas in comparison to rural areas.

In addition, we found that the heterophil-lymphocyte (H/L) ratio, a parameter widely used as indicators of stress response, does not differ between urban and rural Burrowing Owls. This is similar to the pattern reported by Fokidis *et al.* (2008) for the Northern Mockingbird (*Mimus polyglottos*), which is also an urban-adaptable species. However, these authors also reported the same for the Curve-billed Thrasher (*Toxostoma curvirostre*), an urban avoider species. This suggests that the ability to cope with human stressors in urban habitats might be associated with intrinsic characteristics of each species and with its capacity to adapt to new environmental conditions, or alternately it could be interpreted as indicating that the physiological responses to urban stressors might not necessarily involve substantial changes to the H/L ratio.

Even though leukocyte counts and the H/L ratio are considered reliable stress indicators in birds (Davis *et al*. 2008), some authors argue that these variables should be interpreted cautiously since they may vary in response to inflammatory or infectious processes. In this sense, it has been suggested that the H/L ratio should be considered a complementary measure to the corticosterone level in blood and that these parameters are not interchangeable (Müller *et al*. 2011). Regarding this, it would be interesting to incorporate corticosterone level information into future studies comparing physiological stress between urban and rural owls. Rebolo-Ifrán *et al*. (2015) recently reported that feathers from urban and rural Burrowing Owls showed similar values of corticosterone, suggesting that life in urban settings might not represent an additional source of stress for individuals living in this environment.

The percentage of eosinophils we observed in circulating blood was similar between owls from rural and urban habitats and, in both habitats, values were higher than the typical reported for birds in general (*e.g.*, E% = 2.5 – 5.6; Davis *et al*. 2008). This type of cell is strongly associated with helminth parasite load and activity (Johnstone *et al*. 2012). However, the high counts of eosinophils observed for Burrowing Owls in our study is consistent with numbers reported for most healthy raptor species studied to date (Copete-Sierra 2013). In addition, the low percentages of other leukocyte types, such as monocytes (associated with defense against infections and bacteria) and basophils (associated with inflammatory processes) (Campbell 1994), support the idea that the Burrowing Owls examined in this study were healthy.

We found that chicks and adult Burrowing Owls showed differences in relative leukocyte counts, which could be explained by different developmental stages of the immune system in young Burrowing Owls. The thymus and bursa are proportionally up to 10 times larger in chicks than in adults, and lymphocytes are naturally more abundant in earlier stages of development (Maxwell & Robertson 1998). As chicks grow, the number of lymphocyte cells decrease and the thymus and bursa decrease in size (Maxwell & Robertson 1998, Dunbar *et al*. 2005). For these reasons, even though the H/L ratio has been considered a reliable index to determine individual physiological condition and a stress indicator (Maxwell & Robertson 1998, Davis *et al.* 2008), the differences observed in Burrowing Owls' H/L ratio between ages is probably related to the ontogenetic development rather than to a difference in how they respond to stress situations.

In summary, our study indicates that urban Burrowing Owls show similar relative leukocyte counts, H/L ratios, and body condition than rural individuals. Even when such similarity may be influenced by the analytical tool employed (*e.g.*, another parameter different of H/L ratio and RLC should have been used) or the limited sample (*e.g.*, a relatively small number of rural individuals was sampled), our findings suggest that living in urban habitats might not significantly affect the haematological parameters of Burrowing Owls.

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