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LEUKOCYTE PROFILE, MICRONUCLEI, AND NUCLEAR BUDS IN SPARROWS (*Centronyx bairdii* AND *Ammodramus savannarum*) OF THE CHIHUAHUAN DESERT DURING THE WINTER

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ABSTRACT

The populations of Baird's (*Centronyx bairdii*) and grasshopper (*Ammodramus savannarum*) sparrows are decreasing as a consequence of the loss of their natural habitat and the indiscriminate use of agrochemicals in farming areas bordering their breeding habitat. Although these two species have been studied from an ecological perspective, there is no research on the presence of abnormalities in their leukocyte and erythrocytes counts, both of them health biomarkers. The objective of this work was to determine the spontaneous rate of polychromatic erythrocytes (PCE), micronucleated erythrocytes (MNE), and nuclear buds (NBs), as well as the leucocyte differential and the heterophil/lymphocyte ratio (H:L), in order to establish the health status of these sparrow species that live in Cuchillas de la Zarca, Durango, Mexico. With this aim, we captured 20 organisms of each species with mist nets. We carried two blood smears per individual which we analysed under the microscope (100x). There were no statistically significant differences among species regarding the frequencies of the various biomarkers, which had similar values to those established for other healthy bird species. Therefore, there is no evidence of genomic instability, nor of genotoxic or cytotoxic effects. Likewise, we detected no alterations in the immune system, based on the leukocyte differential and the H:L ratio evaluation. This is the first study that establishes reference values for these biomarkers among these particular species of sparrows.

Keywords: erythrocytes, micronuclei, nuclear buds, leukocytes, grassland birds, *Centronyx bairdii*, *Ammodramus savannarum*.

INTRODUCTION

Some of the wild bird populations in North America are in severe decline; the most affected species are those that live in grasslands, since 74 % of them are in critical condition. This is the result of the loss of 700 million individuals of different bird species, as a consequence of the increasingly accelerated loss of their natural habitats

and of the intensive use of agrochemicals, among other factors (Quero *et al.,* 2016; Rosemberg *et al.,* 2019). Among the sparrow group, Baird's (*Centronyx bairdii*) and grasshopper (*Ammodramus savannarum*) sparrows have annual growth rates of -2.1 % and -2.5 %, respectively (Sauer *et al.,* 2017).

Baird's sparrow (*C. bairdii*) reproduces in southern Canada and the northern United States and migrates south in winter to Arizona, New Mexico, and Texas (USA) Chihuahua, Sonora, Durango, and Coahuila (Mexico). The reproduction area of the grasshopper sparrow (*A. savannarum)* covers from southern Canada to northern Mexico. This sparrow migrates south in winter to Mexico and western Central America. These two species belong to the family Emberizidae. They are seed-eating birds and have distinctively shaped beaks (Sierra Franco *et al.,* 2015).

Bird health is monitored through cell, tissue, and bodily fluid biomarkers, as well as physiological or biochemical changes (Ceyca *et al.,* 2014). The presence of erythrocyte abnormalities, such as micronucleated erythrocytes (MNE) and nuclear buds (NBs), are widely-used biomarkers in the assessment of genomic instability, as well as genotoxic and cytotoxic damage in different wild species, including birds (Martínez-Quintanilla *et al.,* 2017). Genotoxic damage is usually silent and goes unnoticed; however, it acts directly or indirectly on DNA and has various mutagenic, teratogenic, or carcinogenic manifestations. The result can be catastrophic, since genotoxic damage endangers the life of the organisms and can even cause the extinction of the affected species, causing irreversible alterations to ecosystems (Torres-Bugarín *et al*., 2014).

The immune system of birds comprises the nonspecific innate response system (mediated by phagocytic cells, granulocytes, and monocytes) and the acquired response system (mediated by lymphocytes, specifically for pathogens). Granulocytes (heterophils, eosinophils, and basophils) recognize, phagocytose, and degrade foreign agents. Heterophils are nonspecific cells that proliferate in tissues during bodily responses to inflammatory processes and kill pathogens. They play an important role in the innate immune response and their number increases in response to long-term bacterial and fungal infections, as well as to irregularities related to diet and stress. Heterophils represent 40 - 75 % of total leucocytes. Lymphocytes are leucocytes that participate in acquired, humoral (B cells that develop in the bursa of Fabricius) and cell-mediated (T cells that develop in the thymus) immune responses. Lymphocytes are the largest group of cells in the immune system of birds and represent 20 - 50 % of all white blood cells (Skwarska, 2018).

The heterophil/lymphocyte ratio (H:L) is commonly used as a haematological index; it is a relatively simple way to assess the immune system efficiency and individual health of birds. Furthermore, this parameter allows to assess the short- and long-term bodily response to environment-induced stress. Stress and immunosuppression levels not only manifest in a heterophil count increase (heterophilia) mediated by corticosterone (the "stress hormone"), but also in a lymphocyte count decrease (lymphopenia) (Genovese *et al.,* 2013; Skwarska, 2018; Minias, 2019).

As a result of their biological characteristics, *C. bairdii* and *A. savannarum* sparrows are frequently used in various ecological studies. This is, to our knowledge, the first study

that describes haematological parameters, which are fundamental for the diagnosis and monitoring of health in these sparrows. Therefore, the objective of this work was to describe the frequency of micronucleated erythrocytes and NBs, the normal leucocyte differential, and the H:L ratio among those mentioned sparrow species.

MATERIAL, METHODS, AND SUBJECTS OF STUDY

Study area

The study was conducted during the winter of 2016-2017, in three Grasslands (central coordinate 26° 16′ 37.902″ N, 105° 8′ 53.653″ W) situated in the Rancho Santa Teresa, that belongs to the municipality of Hidalgo, Durango, within the Cuchillas de la Zarca grassland priority conservation area (GPCA). This area comprises northern Durango and the southern end of Chihuahua, Mexico, and is part of the Chihuahuan Desert (Figure 1).

The mean altitude of the area is 1850 m and the temperature during the coldest month fluctuates between -3 and 18 °C (Sierra-Franco *et al*., 2015). The shrub stratum comprises species of the genera *Larrea*, *Prosopis*, *Acacia*, *Ephedra*, *Nolina*, *Opuntia*, *Juniperus*, and *Quercus*. The herb stratum is formed by the genera *Bouteloa*, *Aristida*, *Buchloe*, *Andropogon*, *Melinis*, *Muhlenbergia*, *Sporobolus*, *Heteropogon*, and *Pleuraphis* (COTECOCA, 1976).

The Baird's sparrow (*C. bairdii*) is a medium-sized grassland bird that measures between 10.5 and 13.0 cm length; the male is slightly larger than the female. It has a collar with fine dark brown lines in the upper part of the chest and sides, a buff-coloured eyebrow stripe, and a central ochre-coloured line in the crown (Figure 2a). This cautious bird prefers to walk over flying. It nests in central and southern Canada and in the northern United States. They build their nests on the floor; spend the winter in an area that spans from Arizona to central and

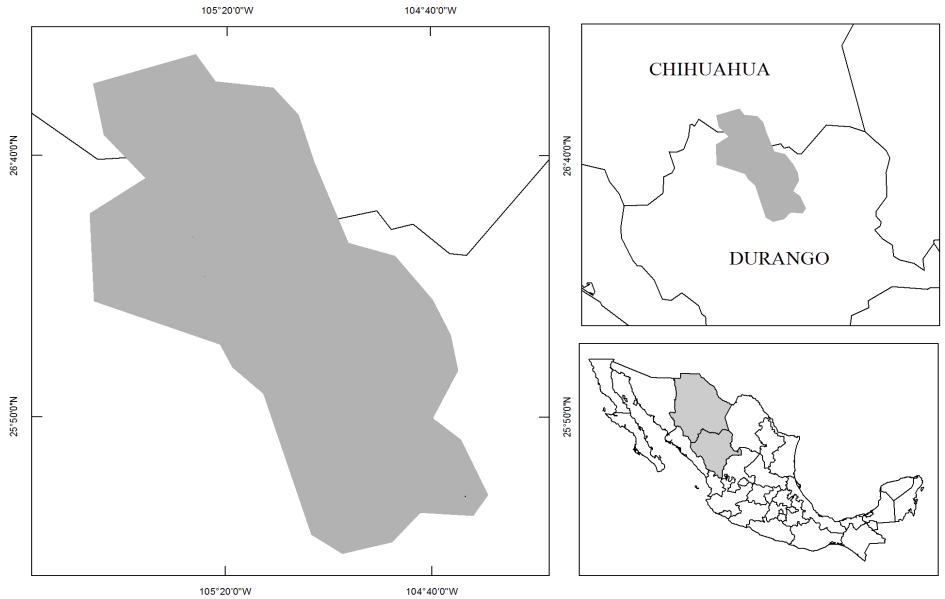

Figure. 1. Location of study area, Cuchillas de la Zarca region, between Durango and Chihuahua, Mexico.

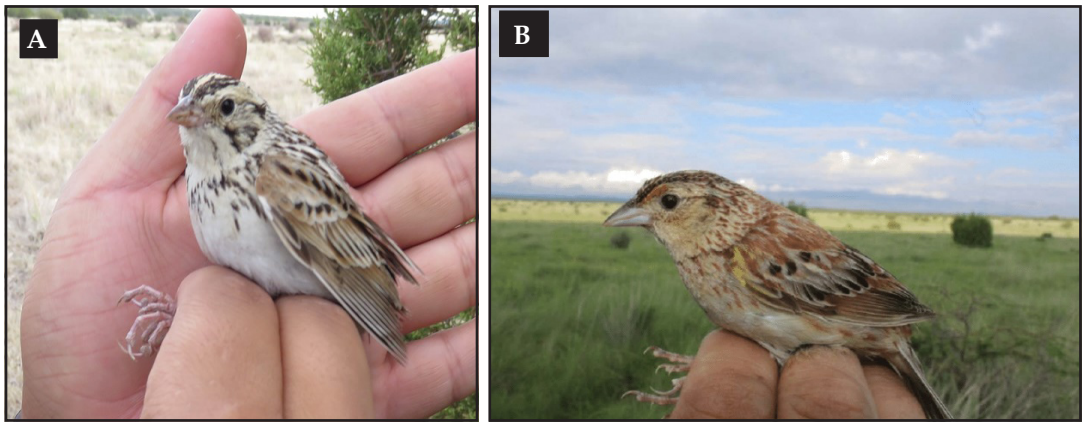

Figure 2. Species that were evaluated in this study. A: Baird's sparrow *(Centronyx bairdii)*. B: Grasshopper sparrow (*Ammodramus savannarum*). [Photos: Karina Ramírez Carreño (BSc in Animal Medicine and Animal Husbandry) and Samuel Arroyo Arroyo (ScD)].

northern Mexico (Panjabi *et al.*, 2007). This bird can migrate alone or in small flocks, although once they arrive to their wintering areas, they do not flock.

Baird's sparrow usually lives in open, slightly herded grasslands in which medium-sized and tall grasses are mixed with weeds and low shrubs. It mainly eats insects in summer and seeds in winter (Panjabi *et al*., 2007). According to the International Union for Conservation of Nature - IUCN red list (2021), it has been evaluated as a Least-Concern (LC) species. In spite of the downward trend of their populations, the decline is not believed to be quick enough to reach the vulnerability threshold. The decline in the population of Baird's sparrows is related to the loss of habitat as a consequence of agriculture and is in direct proportion to the reduction of grasslands in Canada (Rosemberg *et al.*, 2019).

The grasshopper sparrow (*A. savannarum*) is medium- to large-sized and short-tailed. It has a brown, black, grey, and white pattern on its back and wings, a brown head with a thin central line, and ochre chest and sides, frequently with a yellow-orange spot in front of the eyes. Its head is quite flat (Figure 2b). The song of this bird sounds like the buzzing of an insect. They migrate alone or in small flocks and spends the winter apart from others of their kind (Panjabi *et al*., 2007). The grasshopper sparrow nests in southern Canada and the western United States. It builds its nests on the floor, at the base of grass clusters. It spends the winter in the southern United States, Mexico, and part of Central America. Small populations of this species reside in the southern United States, as well as in Sonora and other parts of Mexico.

These birds live in wet or dry medium-height herbaceous grasses that often have vegetation clusters and shrubs or scarce underbrush, interspersed with patches of bare land (Arguedas-Negrini, 2001). They collect their food from the floor. They eat insects (preferably grasshoppers) and seeds, in summer and winter, respectively (Panjabi *et al*., 2007). According to the IUCN red list (2016), it is evaluated as a Low-Concern (LC) species. The 1966-1994 period recorded an annual 3.9 % decrease among grasshopper sparrow populations throughout North America. This decline is a result of the loss of habitat, the conversion of grasslands into intensive farming lands, and fire inhibition (Askins *et al*., 2007).

Capture and processing of birds

Birds were captured (with SEMARNAT collection permit SPGA/DGVS/13360/15) by moving and installing four 36-mm mesh polyester mist nets (2.6 m high x 12 m long) (model KTX, Avian Research Supplies, Association of Field Ornithologists, AFO) at the study site and by herding the birds towards the net (Sierra-Franco *et al*., 2019). Captures were carried out during the first morning hours (7:30 - 12:00 h). Recaptured birds were immediately freed to avoid taking second samples.

Drawing and processing samples

The blood sample was drawn by puncturing the brachial vein with a 1-mL insulin syringe with a 13-mm 29GX needle. Two blood smears per organism were prepared, left to dry at room temperature, and fixed in ethanol (80 %) during 10 min. They were then stained with acridine orange 8 (Sigma-Aldrich, USA), a nucleic-acid-specific staining. For this process, the smears were placed in two staining boxes, each one containing 0.5-L of phosphate buffer; 0.05 g of acridine orange were dissolved in one of the boxes. The samples were subsequently submerged in the colorant for 2 min and then washed with the phosphate buffer for 5 to 8 min. Finally, the samples were stored in a smear box awaiting analysis (Torres-Bugarín *et al*., 2014).

Analyses of micro-nucleated erythrocytes and nuclear buds

The analyses of MNE and NBs were conducted under a Zeiss® Axiostar Plus fluorescence microscope (100x). In order to identify the total MNE and NBs frequency, 10 000 total erythrocytes were counted per organism. In addition, the frequency of PCE was determined in 1000 total erythrocytes, while the frequency of MNPCE and NBsPCE were determined in 1000 PCE (Franco-Ramos *et al*., 2020).

Staining and leukocyte analysis

Smear slides were stained with the HYCEL® Diff-Quick staining kit —a quick blood stain— and analysed with a Leica DM-500 optical microscope. The zig-zag counting technique was used to record 100 leucocytes with the help of the WBC® mobile application; subsequently, their morphological characteristics were differentiated. The heterophil/lymphocyte ratio (H:L) was estimated as a relation between the amount of heterophils and lymphocytes. The individual parts of leucocytes (heterophils, eosinophils, basophils, lymphocytes, and monocytes) or only heterophils and lymphocytes were counted to add a total of 100 cells, using a microscope with a 1000x magnification and oil immersion.

Statistical analysis

Every variable of the study was subject to a descriptive analysis. Additionally, the Kruskal-Wallis nonparametric test was applied to compare the variable medians among species, using the NCSS 2000 statistical software.

RESULTS AND DISCUSSION

Population description

Forty blood samples from two sparrow species were collected: 20 from *C. bairdii* and the other 20 from *A. savannarum*. Not all samples were analysed due to various reasons, such as insufficient blood, quick clotting of the blood, and size of the organism. Determining the sex of the organisms was not possible, because they are a monomorphic species and, during the collection, they were not in their reproductive stage.

Polychromatic erythrocytes

Most erythrocytes in the blood of apparently healthy birds are "mature"; however, it is also possible to find immature erythrocytes (known also as PCE), mainly because its RNA content makes their cytoplasm thicker. Even their nucleus has less chromatin than a mature cell and consequently they develop a darker hue. These erythrocytes remain in circulation around 24 h and are used as biomarkers for erythrocyte regeneration (Jones, 2015).

When PCE frequency is analysed, the average values for both species fluctuate between 4.19 and 4.72 % (Table 1). These results match those described for healthy birds, where PCE frequency is 1-5 % of the erythrocyte total (Campbell, 2015; Jones, 2015). PCE frequency can increase when there is blood loss as a consequence of haemorrhage or anaemia and can decrease as a result of bone marrow suppression, some pathology, or cytotoxicity (Clark, 2015).

Polychromatic erythrocytes with micronuclei and nuclear buds

Polychromatic erythrocytes reach maturity in circulation within 24 h (MNPCE and NBsPCE); therefore, when they present micronuclei or lobulations, they are used as bioindicators of short-term effects resulting from exposure to both endogenous and exogenous genotoxic agents (Sommer *et al.*, 2020; Martínez-Quintanilla *et al.*, 2017). This study showed that MNPCE and NBsPCE in *C. bairdii* and *A. savannarum* sparrows

Table 1. Frequency of micronuclei and erythrocyte buds in Centronyx bairdii and Ammodramus savannarum sparrows.

Species	N	PCE/1000 TE	MNPCE/ 1000 PCE	NBsPCE/ 1000 PCE	MNE/ 10 000 TE	PNE/ 10 000 TE
		Min. - Max.	Min. - Max.	Min. - Max.	Min. - Max.	Min. - Max.
C. bairdii	20	41.9 ± 16.41a 20.2 - 83.2	0.01 ± 0.03 a 0 - 1	0.04 ± 0.07 a 0 - 2	2.55 ± 3.3 a 0 - 14	10.50 ± 14.58 a 0 - 57
A. savannarum	16	47.28 ± 24.14 a 23.6 - 97.7	0.01 ± 0.05 a 0 - 2	0.07 ± 0.09 a 0 - 3	2.18 ± 2.48 a 0 - 9	9.56 ± 11.48 a 0 - 37

PCE: Polychromatic erythrocytes, MNPCE: Polychromatic erythrocytes with micronuclei, NBsPCE: Polychromatic erythrocytes with nuclear buds, MNE: Micronuclei erythrocytes, NBs: Erythrocytes with nuclear protrusions. TE: Total erythrocytes. Mean values ± standard deviation were statistically similar ($z < 1.96$ Kruskal-Wallis, $p > 0.05$).

have < 1 % frequency, in relation to mature erythrocytes. This situation is considered normal, based on the frequencies described for other species (Martínez-Quintanilla *et al.*, 2017).

Micronucleated erythrocytes

Micronucleated erythrocytes frequencies observed in *C. bairdii* and *A. savannarum* are consistent with those described in 15 species of apparently healthy passerine species captured in a biosphere reserve in Argentina (Quero *et al*., 2016) or with those described in ten birds in captivity (Zúñiga-González *et al.,* 2000), including two passerines: the Mexican grackle (*Quiscalus mexicanus*) and the oriole (*Icterus* sp.), whose MNE frequencies were 0.5 MNE/10,000 TE (n=2) and 0 MNE/10,000 TE (n=1). Widely varying factors complicate establishing reference values for spontaneous micronuclei. For example, in most species, the spleen works as the natural filter for aged erythrocytes or erythrocytes with inclusions; given its physiology, it could quickly eliminate any abnormal erythrocytes from circulation, as the highly efficient human spleen does; although it could be less efficient, as occurs in mice. Either way, only through a histopathological analysis can the type of spleen that each organism possesses be determined. If MNE are detected in circulation, this will indicate the type of spleen. Other aspects that determine MNE frequency are the influence of anthropogenic activity, habitat, altitude, season, and kinds of available food, as well as age, sex, diet, and migratory behaviour, among others. Therefore, the sample size can help to discern the role that these factors play in this biomarker (Quero *et al.,* 2016; Zúñiga-González *et al*., 2000).

Erythrocytes with nuclear buds

Just like MNE, NBs are considered useful biomarkers of genetic damage. On the one hand, these cells were identified in different bird species with an 80.9 % incidence, within a range of 1 to 9.5/10 000 TE. On the other hand, Quero *et al.* (2016) determined that the minimum average was found in the scarlet flycatcher (*Pyrocephalus rubinus*, n=2), while the maximum was found in the buff-throated saltator (n=4). The frequency was similar to that observed in the species examined in this study.

Other authors agree that the formation of NBs is associated with the same causes that generate MNE. Both frequencies also seem to be related to the age and sex of individuals. In a study on the snow goose (*Anser caerulescens*), Martínez-Quintanilla *et al*. (2017) also observed great variability in NBS frequency; authors suggested that variability was caused by the heterogeneity of the collected birds. The difference in sex, age, and weight is related to the maturity of the reticuloendothelial system of birds and its ability to remove damaged cells from circulation.

Leukocyte differential and H:L ratio

Leukocyte description. The following leukocyte cells were observed: heterophils, eosinophils, basophils, monocytes, and lymphocytes. Observed under the microscope, heterophils were round, had a segmented nucleus with two or three elongated lobes,

and sometimes contained small cytoplasmic granules. Eosinophilic cells had a round shape with a two- or three-lobed nucleus. These cells are smaller than heterophils. Lymphocytes were spherical in shape, showed different sizes, and had a large and condensed nucleus. The nucleus takes up much of the cytoplasm in these cells. Basophils present a round shape and a lobed nucleus; they have a darker appearance as a consequence of the cytoplasmic granules that cover them. The cytoplasm contains granules that cover both the nucleus and the cytoplasm itself. Monocytes were characterized by large, irregular nuclei with granulations and have a compact and homogeneous cytoplasm with no granules.

Leukocyte differential. Lymphocytes were found to be the most abundant cells (69-77 %) in the leukocyte count of both species, probably because of the migratory nature of the studied birds. Campbell (2015) concludes that Anseriformes migratory birds are a lymphocytic species whose immune system is subject to multiple factors, such as climate change and a higher energy expenditure; therefore, they must generate an adaptive response in order to produce more lymphocytes.

H:L ratio. Evidence shows that cross-species variations of the H:L ratio are limited by development and respond to physiological factors such as age, sex, environmental stress, as well as phylogenetic aspects (Table 2). Non-passerine birds (more ancestral birds) are known to have a higher H:L ratio than passerines (Minias, 2019). In addition, the number of heterophils and lymphocytes has a positive correlation with the incubation period duration and the body mass; but has a negative correlation with the rearing period duration. This indicates that the constitutive immune function develops mainly during the embryonic phase (Pap *et al.*, 2015).

The second most important leukocyte group were the heterophils, which constitute the first natural protection against bacterial infections and other agents. This parameter is therefore related to immunosuppression, low growth rates, survival, high levels of glucocorticoids, and stress (Minias, 2019).

Eosinophils are one of the leukocyte groups observed in a smaller proportion (2 - 5.5 %). Eosinophilia in birds is difficult to interpret, since the exact function of avian eosinophils is unknown. Nevertheless, the eosinophil count may be useful for the

Table 2. Leukocyte count (%) and heterophil/lymphocyte ratio (H:L) in Centronyx bairdii and Ammodramus savannarum sparrows.

Species	N	Heterophils	Eosinophils	Lymphocytes	Basophils	Monocytes	H:L Ratio
C. bairdii	16	23.0 ± 18.0 a 4 - 72	5.5 ± 5.9 a 0 - 17	69.5 ± 19.5 a 16 - 84	0 ± 1.5 a 0 - 6	4 ± 4.2 a 0 - 13	0.65
A. savannarum	20	14.5 ± 7.6 a 2 - 33	2 ± 5.9 a 0 - 22	77.0 ± 14.0 a 40 - 90	0 ± 0.4 a 0 - 2	9 ± 5.9 a 1 - 24	0.23

Values were statistically similar ($z < 1.96$ Kruskal-Wallis, $p > 0.05$).

diagnosis of infections caused by extracellular parasites (such as giardiasis, ascariasis, and cestodiasis), allergic conditions, allergic reactions, and marked tissue damage. It is also an indicator of the stress status in birds (Campbell, 2015; Jones, 2015).

The function of avian basophils is poorly understood, but it is presumed to be similar to that of mammalian basophils, since their cytoplasmic granules contain histamine. It is common for no basophils to appear in normal hemograms, but basophilia seems to be linked to chronic and respiratory diseases, as well as to tissue damage. It may be common in active chlamydial infections (particularly in Amazon parakeets and parrots), as well as in the early stages of inflammatory processes (Campbell, 2015; Jones, 2015).

Monocytes are mobile cells that migrate using their movements to phagocytose (Campbell, 2015; Jones, 2015). In avian hemograms, these cells are observed in smaller proportions. In an exploratory study about various species of arctic birds, monocytes were observed in proportions of 5 to 11 % (Mallory *et al*., 2015). It is possible to encounter variations when comparing results, even between studies about the same or similar species. This is caused by factors such as sex, age, reproductive status, and environmental differences that may lead to adaptive physiological adjustments in birds. Monocytes have biologically active chemical substances used in inflammatory processes and to destroy invasive organisms. Monocytosis may be linked to infections with *Mycobacterium*, *Chlamydophila*, and *Aspergillus*, and even tuberculosis, mycosis and neoplastic diseases (Campbell, 2015; Jones, 2015).

The H:L ratio is useful to understand the immune response in birds. With this purpose, Martínez-Quintanilla *et al.* (2017) determined the values of this indicator in various bird species, including the Rufa red knot (*Calidris canutus rufa*) at two points in their migratory route: the first, an intermediate site where birds rest before reaching the wintering site; the second, the final destination of their migratory journey. The H:L ratio was different in both sites, because these birds are still energetically stressed in their stopover sites. The physical effort made during the flight leads to a compromised immune response, which in turn causes muscle cell damage and a redistribution of phagocytic cells, increasing the values of heterophils in order to start eliminating pathogens. However, when the birds arrive at their wintering site, their body mass starts to increase; then, they begin to store energy reserves, and therefore recover and maintain normal leukocyte levels. This is probably what we observed in our study, since, at the time of the sampling, the birds had already spent approximately 30 d at their wintering site.

The values of the H:L ratio for *A. savannarum* (0.23) and *C. bairdii* (0.65) were similar to those recorded by Martínez-Quintanilla *et al*. (2017) for 11 bird species. The immune response can be measured through leukocyte values and the H:L ratio, which change when defence mechanisms respond to external agents. Therefore, it is always possible that the reason why a particular leukocyte type increases or decreases is that the birds are undergoing or recovering from some undetermined infectious process (Demina *et al.,* 2019).

The H:L ratio is an index used to measure physiological avian stress, but it is also useful as an indirect indicator of animal welfare in birds, since it can be affected by different stress-inducing causes. In the initial phase of stress, birds exhibit heterophilia and lymphopenia, followed by heteropenia and lymphocytosis (Campbell, 2015). Many authors have considered this pattern as one of the variables with the highest predictive value for stress (Campbell, 2015), even more reliable than measuring cortisol. Finally, the group of leukocyte cells in which variations caused by (internal or external) parasites or in response to hypersensitivity reactions were observed matches eosinophils (Campbell, 2015). In the case of this study, the values of eosinophils for *A. savannarum* (2 %) and *C. bairdii* (5.5 %) may be considered similar, based on previous studies about different bird species (Mallory, 2015), where the range recorded for eosinophils was 1 to 6 %.

CONCLUSIONS

To our knowledge, prior to this study, the frequency of spontaneous micro-nucleated erythrocytes peripheral blood nuclear buds, leucocyte differential and heterophil/lymphocyte ratio had not been described for *C. bairdii* and *A. savannarum* sparrows. Therefore, this research constitutes the first effort to establish reference values for these species, which are facing severe population decline.

The cell values determined in this study for the target birds are consistent with those observed in other apparently healthy bird species; therefore, they do not constitute evidence of the presence of any disease in the analysed organisms.

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