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Physiological stress on Rufous-collared Sparrow *Zonotrochia capensis* on an urban-rural matrix in the high tropical Andes

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Abstract

Urbanization has dramatically changed landscapes, representing a novel challenge to many species whose habitats have been affected by this process. Some species appear to adapt better than others to urban environments. However, in some cases, even when population data shows good levels of adaptation, physiological data shows oppose patterns and species are under greater level of stress than in natural habitats. Stress includes several factors that can provide information about how species are adapting to novel environments, providing very informative data that allows researchers to make predictions about evolution, population ecology, and long-term conservation. For this thesis, (i) we conducted a systematic literature review in order to determine challenges, gaps of knowledge and future research directions about the physiological and behavioural effects of urban life for birds; (ii) studied corticosterone levels and ectoparasites of three populations of Rufous-collared Sparrow *Zonotrichia capensis*, a common bird in the Neotropics, in urban-suburban-rural matrix on the valley of Quito (Ecuador), including small urban gardens, medium-sized green periurban spaces, and large suburban parks; (iii) provide the first report of avipox virus in northern Ecuador, including a discussion about preliminary genetic information of the virus; and (iv) describe a detailed protocol for future studies about bird adaptation to urban environments in order to provide a useful guide for other researchers. This project provides valuable information about the adaptation of *Zonotrichia capensis* to urban environments as well as we stablish a baseline for further studies.

Keywords: stress, corticosterone, bird, urban, poxvirus, ecophysiology

Resumen

La urbanización ha cambiado dramáticamente los paisajes, esto representa un nuevo desafío para varias especies, cuyos hábitats se han visto alterados por este proceso. Algunas especies parecen tener mejor adaptación que otras a los ambientes urbanos. Sin embargo, en algunos casos, incluso cuando los datos poblacionales muestran una Buena adaptación, los datos fisiológicos muestran patrones opuestos y las especies se encuentran en condiciones más altas de estrés que en sus ambientes naturales. El estrés incluye varios factores que pueden proveer información sobre como las especies se adaptan a nuevos ambientes, arrojando datos informativos que permiten a los investigadores hacer predicciones sobre evolución, ecología poblacional y conservación a largo plazo. Para esta tesis nosotros (i) condujimos una revisión sistemática de literatura para identificar desafíos, vacíos de información y futuras direcciones de investigación sobre el efecto fisiológico y comportamental de la vida urbana en aves. Luego nosotros (ii) estudiamos niveles de corticosterona de tres poblaciones del gorrión ruficollalero *Zonotrichia capensis* (un ave común en el neotrópico) en la matriz urbano-rural, incluyendo pequeños jardines de la matriz urbana, espacios verdes medianos en la matriz periurbana, y parques grandes en áreas suburbanas, en estos lugares también identificamos los macroparasitos externos encontrados en las aves. La tercera parte de este proyecto de investigación es (iii) el primer reporte de virus avipox en la región norte de Ecuador, incluyendo una discusión sobre la información genética preliminar del virus. Finalmente, (iv) describimos un protocolo detallado para futuros estudios sobre la adaptación de aves a ambientes urbanos para proveer una guía de utilidad a otros investigadores. Este proyecto provee valiosa información sobre la adaptación de *Zonotrichia capensis* a ambiente urbanos, así como también establece una línea base para futuros estudios.

Palabras clave: estrés, corticosterona, aves, urbano, poxvirus, ecofisiología

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Chapter 1

Birds physiological and behavioural stress in urban context: A systematic literature review

Birds physiological and behavioural stress in urban context: A systematic literature review

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Abstract

Birds that inhabit in urban landscapes face several challenges that requires physiological and behavioural adaptation in order that their populations could still be using urban niches. Several studies have evaluated how birds are adapting their stress response. Some birds have good levels of adaptation and maximize exploitation of urban resources, some other cannot adapt so well. Corticosterone and heterophil/lymphocyte ratio are the most common stress indicators; however parasitic relationships are still misunderstood. Research suggest that urban environment acts as selective pressure over genetic and social traits. In this systematic literature review, we present data from stress in urban birds.

Introduction

Urban environments represent novel challenges and opportunities for birdlife (Herrera-Dueñas et al., 2017). In urban environments there is a different availability of resources, compared to natural environments (Wingfield, 2013). Several aspects of urban landscapes constitute disturbations for animal life, those disturbations could interfere in the normal development of their behavioural and physiological processes (Partecke et al., 2006; Schlesinger et al., 2008). Different types of pollution that is present in the city is also a disturbance for animal life (Dominoni et al., 2013; Meillère et al., 2016). The interaction between different ecological factors makes urban landscapes environments difficult to colonize by wildlife.

Avian urban fauna is been used to study these phenomena in order to understand how physiological and behavioural processes are affected by the urbanizing environments (Meillère et al., 2016). Studies in birds can lead to know how the availability of resources and ecological niche affect in the colonization of urban landscapes as it is been reported in the Black Sparrowhawks, in which urban development is related with health status due to prey abundance (Suri et al., 2017). Other studies in birds can be used to understand the role of physiological processes in urban life (Bauerová et al., 2017). Birds could also serve to analyse how pollution is shaping communities' structure and health status of populations (Hargitai et al., 2016). Due to some characteristics of birdlife, this is a group of major interest to study the behavioural and physiological effects of life at urban environments.

Stress measurements are used as a proxy to determine adaptation to the nouvelle environments, these measurements constitute a health indicator for animals' populations (Heiss et al., 2009; Meillère et al., 2016). A common way in which researchers can quantify physiological changes in wild populations is using hormonal assessment, mainly corticoids (Wingfield, 2013). Corticosterone levels can be used for both measuring stress response and baseline stress at birds' populations (Partecke et al., 2006). The use of endocrine approaches to study birds´ urban life could be linked to body condition and behaviour as well (Møller, 2010; Powell et al., 2013). The use of different measurements of physiological and behavioural stress are useful indicators to understand how birds are adapting to urban life.

Anthropogenic pressures in urban landscapes can lead to changes in behaviour and physiology of urban fauna (Atwell et al., 2012; Meillère et al., 2015). Physiological responses to stress could be evidenced at metabolic processes that are associated with a reduction of immune response (Constantini et al., 2014). Anthropogenic pressures could be also altering breeding behaviour of birds, consequently their fitness (Brahmia et al., 2013). Physiological and behavioural effects of urban life over birds could also be reflected in infectious epidemiology

(Fokidis et al., 2011). By the fact that urban settlements constitute a significant disturbance to environment, it is highly important to understand its effect over birds' populations.

In this systematic literature review we present all published information related to behavioural and physiological effects of urban life over birds. In this work we identify challenges and gaps of knowledge in the understanding of how birds are adapting to urban environments in order to determine future research questions and priorities. This work is aimed to give a better understanding of how birds are adapting to urban settlements. Information compiled in this review can be used to make information about landscape management and urban planning. Other aim of this review is to facilitate comparison of the different studies related to the topic in order to identify new prospectus for future research and applicability.

Methods

This work aims to identify and report all the available information about evidence-based physiological and behavioural effects of urban landscapes. We conducted a Systematic Literature Review following the PRISMA statement patterns (Moher et al., 2009). We used Scopus database and the research equation was *("stress" OR "corticosterone") AND ("bird" OR "aves") AND ("urban" OR "city" OR "cities").* The terms *stress* and *corticosterone* were used due to its general usage as proxy of adaptation and facing environmental challenges (Meillère et al., 2016). Corticosterone measurements and other stress related analysis are used to provide information about the physiological responses of birds to disturbance (Partecke et al., 2006). The study was carried out including birds due to the availability of enough information to be compared. We used *urban*, *city* and *cities*, in order to cover every study in which those landscape were a variant. Our search strategy focused on titles and abstracts on the Scopus® research engine. We used the PICOS (Population, Intervention, Comparison,

Outcomes, and Study Types, Table 1) framework to define the study question (Higgins & Green, 2011).

Table 1 PICOS framework used to define the research question

Item	Definition
Population	Birds
Interventions	Living in urban landscapes, or under urbanization process
Comparators	Not applicable
Outcomes	Any impact
Study Type	Academic literature, primary and secondary bibliography, including observational and natural history studies, prospective ecological studies, interventional studies, and all types of reviews

The search was carried out on October the 28th, 2017. The screening included all the papers written in English, Spanish or French, without limitation on the date. All studies that included behavioural or physiological stress effects in birds were included. We discarded all studies that focused on stress measurements in any other taxa than birds, we also discarded studies in which researchers did not analyse urban populations or in which any proxy of stress was analysed, our eligibility criteria are presented in table 2. In the earliest stage of our research we evaluated 213 that turned into 46 after the application of our eligibility criteria, our PRISMA statement flowchart is showed at graphic 1. At the extraction table we identified the species in which the studies were carried out, as well as the family. We also identified the number of sampled individuals, the stress biomarker that was used, location of the study, main outcomes and in some cases, other relevant data presented in the paper.

Table 2 Eligibility criteria

Category	Exclusion criteria	Notes
0. Null entries, duplicates, not in the	01 - Null entries	No information is reported in title and abstract fields.

language of interest, abstract is reported elsewhere and not in the time period of interest	02 - Duplicates	Duplicate of an existing entry.
	03 - Not in the language of interest	Documents in other languages that English, Spanish or French
	04 - Abstract that is reported elsewhere	Abstracts and contents that have been reported in another publication will be excluded. It should be noted that this criterion should **only** be applied if the numerical values are the same in the full publication.
1 - Nature of study	10 - Not study type of interest	Conference proceedings, non-edited or peer-review documents; related to objective
	11 - Not study type of interest **(but useful for discussion)**	
	12 - Not study type of interest **(FLAG)**	
2 - Study population	20 - Not animal	Focussing on plants, fungi, unicellular or other groups of biodiversity but not on animals
	21 - Not vertebrate animal	Focussing on invertebrates, not on vertebrates
	22 - Not tetrapod	Focussing on fishes
	23 - Not bird	Not focussing on birds
	24 - Not Stress Related	Does not include effects of stress
	25 - Not Urban	Does not refer to urban landscapes
3 - Outcome	30 - Not including any outcome (impact) of interest	The endpoints of interest is stress effects in urban landscapes
4 - Potential	40 - Potential	Citation with selected population, study type and outcomes.
Cannot decide	xxx CANNOT DECIDE xxx	The title and abstract cannot provide enough information for a decision.

After the extraction we analyzed the obtained data in order to compare results, identify common conclusions and establish directions for future research.

Stressors

Research shows that habitat fragmentation and human activity are primary stressors in urbanizing environments (Schlesinger et al., 2008). In an experiment done at Lake Tahoe basin in California and Nevada, USA urbanization was confirmed as the main force structuring bird communities (Schlesinger et al., 2008). The same study identified human disturbance and landscape-scale factors as more important that habitat availability and local-scale factors for richness and abundance (Schlesinger et al., 2008). Researchers suggest that a complex diversity of responses can occur to bird communities that are confronting urbanization processes (Schlesinger et al., 2008). Stress response can be caused liable or permanent, in each case physiological or behavioural response is different and also depends on the frequency and intensity of the stressor (Wingfield, 2013).

Researchers in Czech Republic studied the relationship between stress levels and high metal pollution in the european great tit *Parus major* at 13 cities across the country (Bauerová et al., 2017). They have found that lower heterophil/lymphocyte (H/L) ratio is linked with higher concentrations of trace metals in feathers (Bauerová et al., 2017). Even when their results showed that pollution is clearly affecting the immunological response of the birds, in this study they did not found decrease in the bacteriolytic function of the complement (Bauerová et al., 2017). However, researchers did find anaemia-like haemolytic conditions and increased haematopoiesis (Bauerová et al., 2017). For three years researchers in Algiers studied breeding performance of Blue tits *Cyanistes cæruleus ultramarinus* in relationship with lead pollution and different levels of urbanization (Brahmia et al., 2013). They found that lead concentrations

in tissues were higher in urban birds than in rural ones, that does not lead to a clear effect over nesting morphology (Brahmia et al., 2013). Researchers suggest that other factors related to urbanization can be negatively affecting breeding performance (Brahmia et al., 2013).

As animal research commonly requires handling animals during a period of time, researchers in Catalonia have studied the differential response to this stressor across urban and rural populations of *Parus major* (Torné-Noguera et al., 2014). By analyzing breath rate as a proxy of stress, researchers have found that urban birds tend to present a higher stress response than rural ones (Torné-Noguera et al., 2014). This difference remains during the whole year but increases in winter, possibly because the challenges that birds confront during this time of the year (Torné-Noguera et al., 2014).

In Argentina, researcher analysed the relationship between survival and stress of urban and rural populations of *Athene cunicularia,* they found that annual survival of this birds was twice higher in urban landscapes than in the rural ones (Rebolo-Ifrán et al., 2015). Closer interaction with human settlements was not associated with higher levels of corticosterone nor for males nor females' individuals (Rebolo-Ifrán et al., 2015). Owls living in urban landscapes seems to have lower fear to humans in relationship to their rural counterparts (Rebolo-Ifrán et al., 2015). The results of this research suggest that life in the city does not represent an extra stressor for these owls (Rebolo-Ifrán et al., 2015).

Corticosterone related studies

Researchers at Germany assessed hormone levels at European Blackbirds living in a urban-rural gradient and determined that there is an effect of night artificial lights on hormone production (Russ et al., 2015). Elevated artificial light appeared to increase corticosterone levels and decrease feminine estrone levels, while it appears not to be any effect over testosterone levels (Russ et al., 2015). Researchers suggest that stress response may be different

for both sexes and that female blackbirds can be more affected urban landscapes, which can lead to a reduction at the population fitness (Russ et al., 2015).

In another study carried out in European Blackbirds researchers found significative differences between corticosterone levels between urban and rural populations (Meillère et al., 2016). At this study levels of non-essential trace elements contamination were also assessed, and there was determined a positive correlation between those contaminants and corticosterone levels (Meillère et al., 2016). Researchers suggest that a possible cause to high levels of corticosterone can be associated with bioaccumulation of non-essential trace elements caused by urbanization more than stress-related urbanization factors by itself, there is a lack of evidence to prove that causation and more studies should be done to determine interaction between trace elements and corticosterone secretion (Meillère et al., 2016).

Stress-response adaptation is a way in which animals can avoid possible deleterious effects of chronic stress caused by changes in habitat (Partecke et al., 2006). A study carried out in Munich, Germany shows that European Blackbirds *Turdus merula* born in urban environments have fewer stress response that the ones born in natural environments (Partecke et al., 2006). In this study researches hand-raised the birds of both populations under the same conditions and assessed corticosterone levels at the ages of five, eight and eleven months and found significative differences between populations (Partecke et al., 2006). As researchers had control over every external variable, results show that the difference is genetically determined (Partecke et al, 2006). Due to the strength of their results researchers concluded that these differences are ubiquitous and could be necessary for all animals that live in urban areas or that are constantly exposed to human caused stress (Partecke et al., 2006).

One of the concerns of high corticosterone levels is that it can interact in several metabolic pathways in the body of the study animal (Davies et al., 2012). Researchers have artificially

administered corticosterone to a passerine bird in urban and rural localities in the Phoenix area and assessed how does this addition interacts with plasma metabolites (Davies et al., 2012). The addition of corticosterone did not affect fat scores nor body mass in none of the study populations (Davies et al., 2012). However liver glycogen, increased in urban birds after corticosterone administration and decreased in rural birds, this suggest that stress responses are more different between urban and rural birds than how we hypothesized before (Davies et al., 2012).

In Mexico, researchers have studied two species of birds living in different land uses, and determined that stress response is highly dependent of the species that is been studying (Chávez-Zichinelli et al., 2013). *Melozone fusca* and *Columbina inca*, both presented the highest corticosterone levels in croplands, with different concentrations in the city (Chávez-Zichinelli et al., 2013). Researchers also concluded that only 30% of birds collected in the city were under chronic stress, which represents that there is a good adaptation to urban environments (Chávez-Zichinelli et al., 2013).

Researchers in Australia have tested if corticosterone in feather is a confident measure of stress in populations or if it depends on the stage of formation and interindividual variation, as part of their study they measured corticosterone in feathers of two populations of house sparrow *Passer domesticus* in Australia (Aharon-Rotman et al., 2017). They found higher levels of corticosterone in birds sampled in a suburban area than in the ones founded in an urban area (Aharon-Rotman et al., 2017). Researches demonstrated that corticosterone measures in feathers did not have interindividual significative differences, so that this indicator does provide relevant information about the population (Aharon-Rotman et al., 2017).

In China, researchers have evaluated the levels of basal corticosterone of the tree sparrow *Passer montanus* that inhabits in a gradient of urbanization, they have found that as

urbanizations increases corticosterone increases too (Zhang et al., 2011). This correlation could be explained because of the poor adaptation to of the bird to urban landscapes (Zhang et al., 2011). A poor adaptation of the sparrow to an urban landscape can depend on the lack of proper habitat or the poor plasticity of the species (Zhang et al., 2011).

The only study carried out in South America about this topic evaluated physiological stress and body condition in the rufous collared sparrow *Zonotrichia capensis* in Chile (Ruiz et al., 2002). In this study, researchers concluded that the sparrow does not have a good adaptation to urban environments (Ruiz et al., 2002). By doing a relation of the heterophil/lymphocyte ratio with the body mass, researchers found that urban and rural birds form two clearly separated groups with a significant difference (Ruiz et al., 2002). Another study in the *Zonotrichia* genera evaluated if sex is determinant for corticosterone variation in a gradient of urbanization (Bonier et al., 2006). Researchers found that males do have a higher level of corticosterone in urban habitats than in rural ones, indicating poor adaptation to this landscape (Bonier et al., 2006). However, the level of corticosterone in females did not differ between urban and rural individuals (Bonier et al., 2006). Researchers are concerned about the fitness of populations and suggest further research in the topic (Bonier et al., 2006).

Corticosterone variation, as well as other biomarkers of stress can be dependent of the context in which the organism develops (Fokidis et al., 2011). Researchers in Arizona studied the variation in corticosterone in two species of passerines in urban and rural landscapes and determined that context is more influential than the level of urbanization (Fokidis et al., 2011). In another study, researchers evaluated how peptide administration interact with corticosterone production in urban and rural birds, they confirm that urban individuals could have a higher adrenal gland sensitivity than their counterpart (Fokidis, H. B., & Deviche, 2011).

In Phoenix, Arizona researchers assessed levels of free baseline and stress induced corticosterone and corticosterone binding globulin in five species of passerine, testing differences between birds living in different urban densities and the ones living at the native desert (Fokidis et al., 2009). Nor corticosterone levels nor corticosterone binding globulin had significative differences between urban and rural birds (Fokidis et al., 2009). Even when corticosterone binding globulin had no much variation between different assessed individuals, corticosterone had a very much bigger variation caused by the different life stages of individuals (Fokidis et al., 2009). Variation of corticosterone binding globulin was less in birds living in the city than in birds living in rural areas, a possible explanation is that at the city the availability of resources is constant and that can cause fewer stress to populations living in there (Fokidis et al., 2009). In another study, they evaluated the interaction between cortisol and the neuropeptide arginine vasotocin that is the avian homolog of vasopressin (Fokidis & Deviche, 2012). They confirmed that stress response is different among the brains of urban and rural birds (Fokidis & Deviche, 2012). However, researchers suggest that their results are linked to differences in territorial defence (Fokidis & Deviche, 2012). Further research is needed in the topic, especially because in this study the lack of water availability for rural birds could cause bias of results (Fokidis & Deviche, 2012).

Oxidative stress

Comparing oxidative stress in European Blackbirds *Turdus merula* was compared between hand-raised individuals from urban and rural environments, finding that city birds have lower levels of oxidative stress than their rural counterparts (Costantini et al., 2014). Birds from urban landscapes tend to have lower levels of enzymatic and non-enzymatic antioxidants than their counterparts (Costantini et al., 2014). Significative results such as the ones presented in this study can suggest that urban fauna is adapting to city life at the physiological level (Costantini et al., 2014). Several environmental factors that are characteristic of urban landscapes

facilitates invasion by species with high levels of antioxidants (Møller et al., 2010). Differences in concentrations of antioxidants seem to be caused by diet, and also to the time since the beginning of urbanization process (Møller et al., 2010). Researchers also suggest that there are selective pressures favoring individuals with higher levels of antioxidants over those with lower levels of antioxidants, this suggestion was done after finding that birds killed by cats had lower levels of antioxidants that birds collected by other methods (Møller et al., 2010).

Researchers in Spain have evaluated levels of oxidative stress in the house sparrow *Passer domesticus* and found that individuals living in cities showed higher levels of oxidative damage as well as a higher activity of antioxidants enzymes (Herrera-Dueñas et al., 2017). Urban sparrows seem to have lower levels of antioxidant capacity, in comparison of their rural counterparts (Herrera-Dueñas et al., 2017). Researchers also found that differences between rural and urban sparrows seems to increase during the breeding season (Herrera-Dueñas et al., 2017). The results of this research can be used to explain the decrease of urban populations of the common house sparrow (Herrera-Dueñas et al., 2017).

In Mexico, researchers evaluated the lipid peroxidation rates and liver histopathology of populations of *Fulica americana* in a rural and an urban area, they found that for the two reported pathologies the highest prevalence was in the rural population (López-Islas et al., 2016). Lipid peroxidation varied depending on the season of the year, but the highest rates were reported in winter, possibly because ecological challenges that birds have at that time of the year (López-Islas et al., 2016). This biomarker was reported to be higher at the rural population than in the urban one (López-Islas et al., 2016).

In a study carried out near a small city in Sweden researchers compared stress responses to urban landscapes in two species of tits and two of sparrows, demonstrating that responses

highly vary among species and also among families (Isaksson et al., 2017). For the four species the tendency seems to be that in urban environments birds have a higher level of inflammation and oxidative stress than their rural counterparts (Isaksson et al., 2017). The peroxidation index showed high difference between rural and urban sparrows of both species, so that this index was higher in urban birds than in rural ones, however it did not show significant differences for the tits populations of any of the species (Isaksson et al., 2017). Great tits *Parus major* shows that there seems no significative difference between lipid peroxidation, measured as TBARS, in populations of this bird living in urban and rural landscapes (Isaksson et al., 2009). At the same study researchers found that levels of catalase increased at urban landscapes, however there were no differences at the levels of this enzyme at the liver (Isaksson et al., 2009). There were significant differences between concentration of dietary carotenoids such as lutein and zeaxanthin and its ratio, this difference was found between sexes and environment (Isaksson et al., 2009). Results shows that there is no negative impact of urbanization at the lungs or liver of the birds, regarding to oxidative stress (Isaksson et al., 2009).

The only study that evaluated differential expression of genes between urban and rural birds was carried out in Sweden, in this study researchers used last generation research tools to perform a transcriptome analysis of urban and rural great tits (Watson et al., 2017). They found that there is a significant difference between populations at an epigenetic level (Watson et al., 2017). Genes related to stress regulation had a higher expression at the urban individuals that in their rural counterparts (Watson et al., 2017). Most of these genes were related to immunological and inflammatory responses (Watson et al., 2017). This study is the first that provides evidence of epigenetic difference between urban and rural dwelling animals, this kind of research opens the door for further research in a finer scale in the topic (Watson et al., 2017).

Physical condition

A study carried out over 1375 cape sugarbirds *Promerops cafer* used several stress indicators to evaluate how this bird is adapting to habitat changes (Mackay et al., 2017). Researchers found higher levels of fault bars in feathers and fluctuating asymmetry in birds that were closer to urban settlements (Mackay et al., 2017). In this study there was no clear relationship between stress indicators that were tarsal disease, fluctuating asymmetry, body condition and feather fault bars (Mackay et al., 2017). In Hungary researches have evaluated physiological levels of stress and body condition through an urbanization gradient (Bokony et al., 2012). They have found that the house sparrow does not have different body mass across the gradient of urbanization (Bokony et al., 2012). Urban and rural birds presented similar levels of heterophil/lymphocyte ratio, indicating that there is a good level of adaptation to human activity (Bokony et al., 2012).

After evaluating body size and condition on house sparrow *Passer domesticus* at four cities with different levels of urbanization in France researchers proved that urban birds have reduced body size and mass par opposition of their rural counterparts (Meillère et al., 2015). There is no evidence that sparrows are suffering from nutritional stress due to the fact that there are no differences between populations for any of the assessed indicators, that were scaled mass index, muscle score, haematocrit, baseline and stress-induced corticosterone levels (Meillère et al., 2015). At juvenile individuals there was a difference at fat scores between urban and rural ones, this could be because urban landscapes are inadequate at satisfying nutritional necessities of the birds (Meillère et al., 2015).

Several studies have found a relationship between body condition and the stress biomarker that is been used, however researchers in Australia have found that in *Manorina melanocephala*, a passerine bird, body condition is not related to the heterophil/lymphocyte ratio (Powell el al., 2013). According to this study, urban and rural populations did not present significative differences referring to body condition, even when urban individuals tended to be a little

heavier (Powell et al., 2013). However, the heterophil/lymphocyte ratio did present statistical difference between populations (Powell et al., 2013). As urban birds had a higher heterophil/lymphocyte ratio this could stand for elevated chronic stress or infections due to the anthropogenic activity (Powell et al., 2013).

Different species can have different levels of tolerance to urbanization, some can adapt thanks to human-provided resources (Suri et al., 2016). Researchers in Cape Town, South Africa assessed the physiological stress of Black Sparrowhawk *Accipiter melanoleucus* using heterophil/lymphocyte ratio as stress indicator and determined that they could not find negative impact of urbanization over nesting health (Suri et al., 2016). In this study researches neither found significant differences of body condition or parasitism across the urban gradient (Suri et al., 2016). Researchers proposed that a factor influencing their results is that diet composition of the Black Sparrowhawk in terms of quantity and quality has little or no variation across the urban gradient (Suri et al., 2016).

A study near Los Angeles, California, evaluated the physiological and behavioural differences between urban and rural populations of *Junco hyemalis*, a passerine bird with a large distribution (Abolins-Abols et al., 2016). Researchers concluded that according to reproductive and territorial behaviour, as well as corticosterone levels, rural populations had higher responses to stress than the urban population (Abolins-Abols et al., 2016). Differences between individuals' levels of corticosterone did not explain the dynamics of the territorial behaviour in these birds (Abolins-Abols et al., 2016).

Egg characteristics have been studied to understand stress and pollution effects over *Parus major* in Hungary (Hargitai et al., 2016). In urban habitats eggshells presented higher levels of metals and calcium than in the rural habitats (Hargitai et al., 2016). Egg volume and eggshell spotting intensity did not show significative difference between urban and woodland

populations (Hargitai et al., 2016). Eggs from the rural environments presented a lower level of antioxidants than the ones of the woodland (Hargitai et al., 2016). Researchers suggest that their results showed that egg characteristics is not a sensitive indicator of chronic stress across populations (Hargitai et al., 2016).

One of the constant challenges that urban fauna has is light pollution, this can affect endocrine functions and the circadian rhythm (Dominoni et al., 2013). Researchers in Munich, Germany have used testosterone measures and testicular development as a comparator between urban and rural blackbirds (Dominoni et al., 2013). Urban birds seem to have an early development of testicles in comparison to their rural counterparts (Dominoni et al., 2013). During the first year of exposure to artificial night light urban male blackbirds did not present any difference in their annual reproductive cycle, but at the second year there was no sexual development at all (Dominoni et al., 2013). Researchers suggest that artificial night light is a way of chronic stress that can be affecting the reproductive physiology of several species, there is still more research to be done to understand the mechanisms behind this process (Dominoni et al., 2013).

Carotenoid pigmentation is used to study urbanization processes due to its multifactorial expression, at the phenotypic level it is seen that urban birds display less colourful ornaments that rural birds (Giraudeau et al., 2015). At a controlled environment *Haemorhous mexicanus* of the city seems to be less colourful than its counterpart of rural areas but there was no difference in the way carotenoid provisioning or oxidative stress affect coloration (Giraudeau et al., 2015). However, researchers suggest that urban birds can be better assimilating carotenoid from food, because with the same diet after four months urban birds had higher levels of circulating carotenoids than their rural counterparts (Giraudeau et al., 2015).

Stress and infections

Researchers had evaluated prevalence of a viral and a protozoan infection in wild male house finches *Haemorhous mexicanus* across an urban gradient, they also quantified stress levels by using oxidative stress as indicator (Giraudeau et al., 2014). Prevalence of the viral infection increased as urbanization increased, but there is no correlation with prevalence of the protozoan infection nor the oxidative measures of stress (Giraudeau et al., 2014). Even when there is no a clear conclusion of what is happening with parasitism across an urban gradient, researchers suggest that at urban landscapes humans can act as facilitators to increase infections in birds, as an example by bird-feeders, but also by the increase of stressors (Giraudeau et al., 2014).

In a study in Mexico researchers assessed immunoglobulin and corticosterone levels as indicator of stress responses of House Sparrow *Passer domesticus* to different urban land uses (Chávez-Zichinelli et al., 2010). With a variety of results researchers conclude that House Sparrows that live in urban areas are both stressed and not stressed, levels of cortisone could represent differences in frequency and intensity of stressors more than the habitat per se (Chávez-Zichinelli et al., 2010). Based on the immunoglobulin levels the immune response of the House Sparrow could be not responding properly to pathogenic agents (Chávez-Zichinelli et al., 2010). At industrial areas researchers found negative relationship between immunoglobulin and cortisone (Chávez-Zichinelli et al., 2010).

A possible factor that allows birds' adaptation to urban environments is the reduced presence of parasite vectors and predators (Fokidis et al., 2008). The facilitated access to water and food can enhance immune system of birds and promote defences to parasitism (Fokidis et al., 2008). In a research done with five species of songbirds at a desert habitat, it was determined that birds in urban areas have less prevalence of blood parasitism than their counterparts at rural areas (Fokidis et al., 2008). At the same research they found that the Northern Mockingbird *Mimus polyglottos* had a higher level of heterophil to lymphocyte ratio at urban environments that at rural ones, this ratio is used as a chronic stress indicator, however body condition of this bird

was significantly better at urban environments (Fokidis et al., 2008). In the other four species analysed researches did not find habitat-related differences (Fokidis et al., 2008). Researchers suggest that vector abundance or body conditions are not determining factors to differences in parasitism across urban gradients, but resources availability and immune function are relevant to species capability to adapt to cities (Fokidis et al., 2008).

Behavioural studies

The great tit *Parus major* is being used as a model to study behavioural traits in urban birds compared to their rural counterparts in several studies (Charmantier et al., 2017). Researchers in the south of France found that the breath rate seems to be accelerated in urban individuals compared to the rural ones (Charmantier et al., 2017). Broods from urban landscapes were smaller than the ones of the forest, and the hatching success was lower, by the other side the fledging success did not show significant differences between populations (Charmantier et al., 2017). By using an experimental open-field cage for the novel environment test researchers determined urban individuals have higher handling aggression and a higher exploratory activity (Charmantier et al., 2017).

Tolerance to human approaches can be used as a proxy of chronic stress in birds (McGiffin et al., 2013). Researchers in Melbourne determined that for the common mynas *Acridotheres tristis* the variation of the flight-initiation distance is high between urban and rural populations (McGiffin et al., 2013). In the rural environments this distance can reach up three times larger than it is in the urban landscapes (McGiffin et al., 2013). The time that the birds spent in vigilance did not present difference across populations (McGiffin et al., 2013). Researchers in Taiwan studied 36 species in urban and rural context and determined that flight initiation distance is related with population size and dispersal, even more than with the degree of urbanization (Lin et al., 2012). Using flight initiation distance, researchers evaluated flight

initiation distance in birds among different sites in France and Denmark, showing that urban birds have a decreased flight initiation distance (Moller, 2010).

Successful urban-exploiters are those species that have managed to adapt to urban environments in order to persist on urban landscapes, behavioural traits are critical to ensure adaptation (Minias, 2015). There are several studies passerine adaptation to urban environments, but there is a lack of information about what is happening with other birds or other animals (Minias, 2015). Research with Eurasian Coot *Fulica atra* shows that this bird is welly adapted to anthropogenically transformed littoral zones with scarce or no emergent vegetation, those places seem to be appropriate for nesting and birds nesting there had lower levels of heterophil/lymphocyte ratio compared to birds nesting in rural areas (Minias, 2015). Birds nesting at urban areas tend to spend less time incubating and tend to be more aggressive than their counterpart of rural areas (Minias, 2015). Birds nesting in urban areas also approached more to human introduced in order to defend their nest (Minias, 2015). In New York, researchers have evaluated the differential growth and nutritional rate of crows in rural and urban landscapes, they have found that rural individuals have higher level of total protein in blood plasma and growth larger (Heiss et al., 2009). In the same study, researchers did not find significative differences between corticosterone levels across populations (Heiss et al., 2009).

The loss of habitat in urban areas can generate proliferation of some species over others, to adapt to those changes in landscape some species present novel behavioural traits that are useful for prevalence of the population at the altered environment (Galbreath et al., 2014). Researchers in Japan combined animal behaviour with geographic information systems to determine if land-use type is a major factor to predict aggressive tendencies in Black Kites *Milvus migrans* (Galbreath et al., 2014). In this study researchers found that aggressive

behaviour was significantly affected by the amount of forest area available (Galbreath et al., 2014).

As part of behavioural and hormonal response to urbanization bolder exploratory behaviours is included (Atwell et al., 2012). In a study carried on in populations of dark-eyed junco *Junco hyemalis* in San Diego, California, researchers found consistent correlations between reduced corticosterone levels and bolder exploratory behaviour in the colonist population (Atwell et al., 2012). Human caused disruption can lead to changes in physiology and behaviour of animals living in this environment (Atwell et al., 2012). Even when there are not clear established patrols about hormonal response to urbanization researchers agree that urbanization does have an effect over endocrine response to stress (Bonier, 2012).

Researchers suggest that certain behaviours are under selective pressure, when birds are living in the cities (Scales et al., 2011). In Pennsylvania, researchers evaluated differences in boldness and aggressive behaviour between urban and rural populations of song sparrow *Melospiza melodia,* they have found that these behaviours are positively selected in an urban landscape (Scales et al., 2011).

Studies' characteristics

Endocrine response to stress seems to be affecting health status of birds' populations living in the city, both immunological response and infectious rates present correlations with hormonal changes due to urban life (Bauerová et al., 2017; Fokidis et al., 2008; Giradeau et al., 2014). Over the 80% of studies that measured corticosterone found that there are significant differences between corticosterone concentrations among urban birds and their counterparts from natural habitats. Endocrine responses to stress seem to be different among males and females, in which females could be negatively more affected by living in urban habitats (Bonier et al., 2006; Russ et al., 2015). Differences in stress responses among birds living in urban

habitats and their counterparts could alter other physiological processes, including immunological response, as corticosterone is considered an immunosuppressant (Bonier, 2012). Another immunological-related proxy of stress are differences in heterophil/lymphocyte ratio, studies using this biomarker are showing poor adaptation of birds to urban settlements (Powell et al., 2013; Ruiz et al., 2002). Human driven activities could lead to a facilitation of parasites and pathogens transmission, this risk is higher for immunocompromised populations such as those with increased levels of corticosterone (Cascio et al., 2011; Giraudeau et al., 2014). Poxvirus and coccidians infections have been reported to be positively related to the degree of urban urbanization (Giraudeau et al., 2014). However, the decrease of vectors in cities could be associated with lower levels of parasitism in urban birds than in rural ones (Fokidis et al., 2008). Endocrine analysis serves as a proxy to understand immunological function and health status in wild populations, results generally show that birds in the city are more vulnerable than their rural counterparts (Costantini et al., 2014; Dominoni et al., 2013).

In urban environments, birds are showing higher levels of boldness behaviour and aggression (Atwell et al., 2012; Galbreath et al., 2014). Endocrine differences between urban and rural birds could be one factor behind the previous behaviours (Atwell et al., 2012). Flight initiation distance and tolerance to human approaches seems to have significant differences between populations in every study, however these differences are not the same across species (Lin et al., 2012; McGiffin et al., 2013; Møller, 2010).

As a result of the constant need of populations' adaptations it emerges the raise of selective pressures over those populations (Møller et al., 2010). Current evidence suggest that stress response is genetically determined, however it poorly understood in which at what stages of development this response is determined (Partecke et al., 2006). Latest research shows that differences in stress responses could be managed at the gene expression level (Watson et al., 2017).

As it is shown in table 1, the most common proxy of stress used was corticosterone levels, other common measurements for stress were related to immune function as well. The second and third most common proxy of stress were heterophil/lymphocyte ratio and oxidative stress. Other stress biomarkers are highly related to the results of corticosterone studies because of its relationship to immune function and endocrine releases. The most commonly studied birds are the Passeriformes, just three of the 46 reviewed studies were carried out in another bird group. The distribution of the studied birds by families is shown in graph number two.

Most of the studies reviewed were carried out in similar geographic locations, see graph number 2, the 19 out of 46 studies have been done in Europe. Another big proportion of the studies of this type was carried out in the United States, were most of the research done its been carried out by the same researchers. Arizona, with ten studies, is the state in which the majority of the studies have been developed. In Africa there have been carried out three studies of which two are from South Africa. We only found three studies about this topic in Asia. The three identified studies of Oceania were carried out in Australia. There are only two studies about the topic in South America. Geographic distribution of the reviwed studies is shown at the graphic 2.

Graphic 1: PRISMA diagram showing the selection of studies analysed

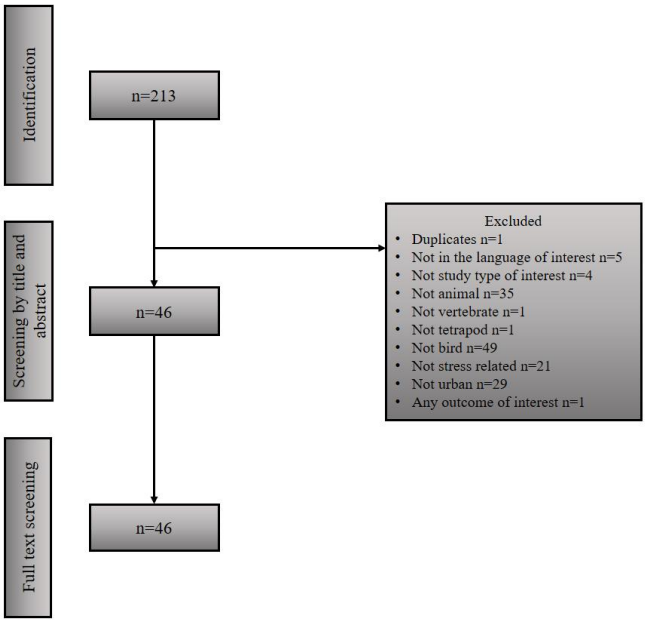
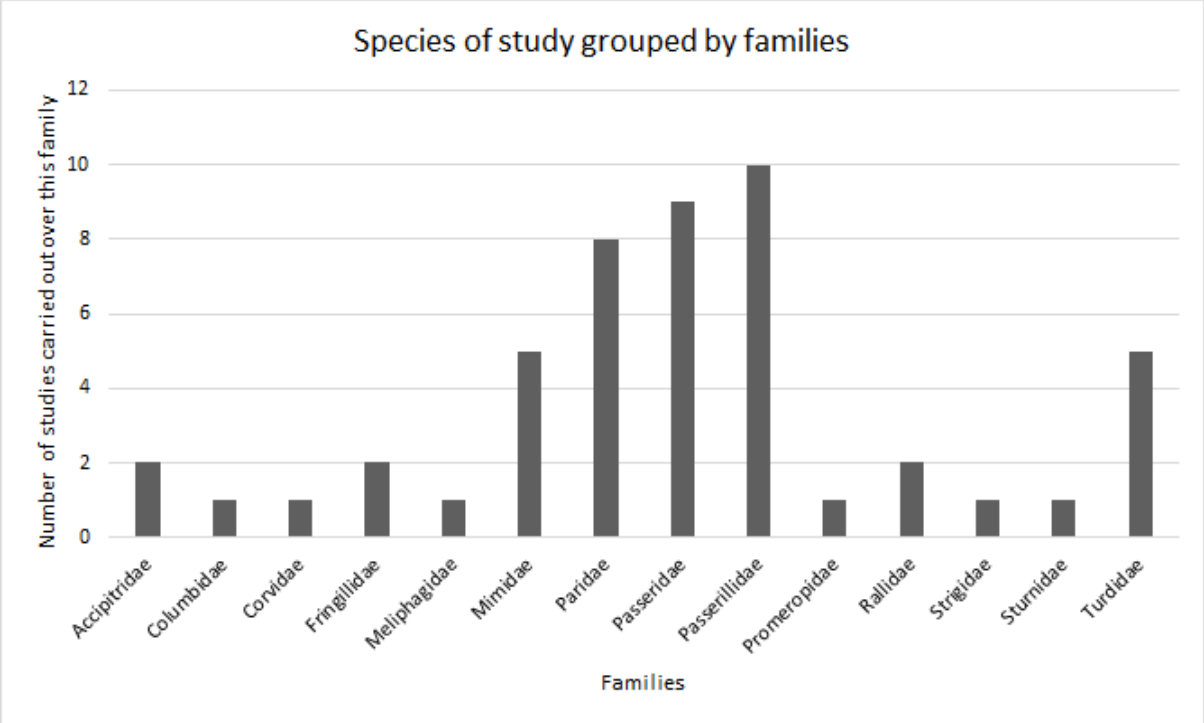


Table 1: Number of studies that uses each physiological stress biomarker

Stress biomarker	**Number of studies reporting the use of this biomarker**
Annual Survival	1
Antioxidants	2
Bioaccumulation	2
Body condition	4
Brain Arginine Vasotocin Immunoreactivity	1
Breath rate	3
Breeding performance	1
Corticosterone	17

Corticosterone Binding Protein	1
Egg characteristics	1
Estrone	1
Fatty acids	1
Glucose concentration	1
Growth and nutritional rate	1
Heterophil/Lymphocyte ratio	6
Infection	3
Lipid peroxidation	1
Liver histopathology	1
Metabolite assay	1
Testicular development	1
Testosterone	2
Transcriptome analysis	1
Plumage coloration	1
Oxidative stress	5

Graph 1: Distribution of studied species grouped by families



Graph 2: Geographic distribution of reviewed studies

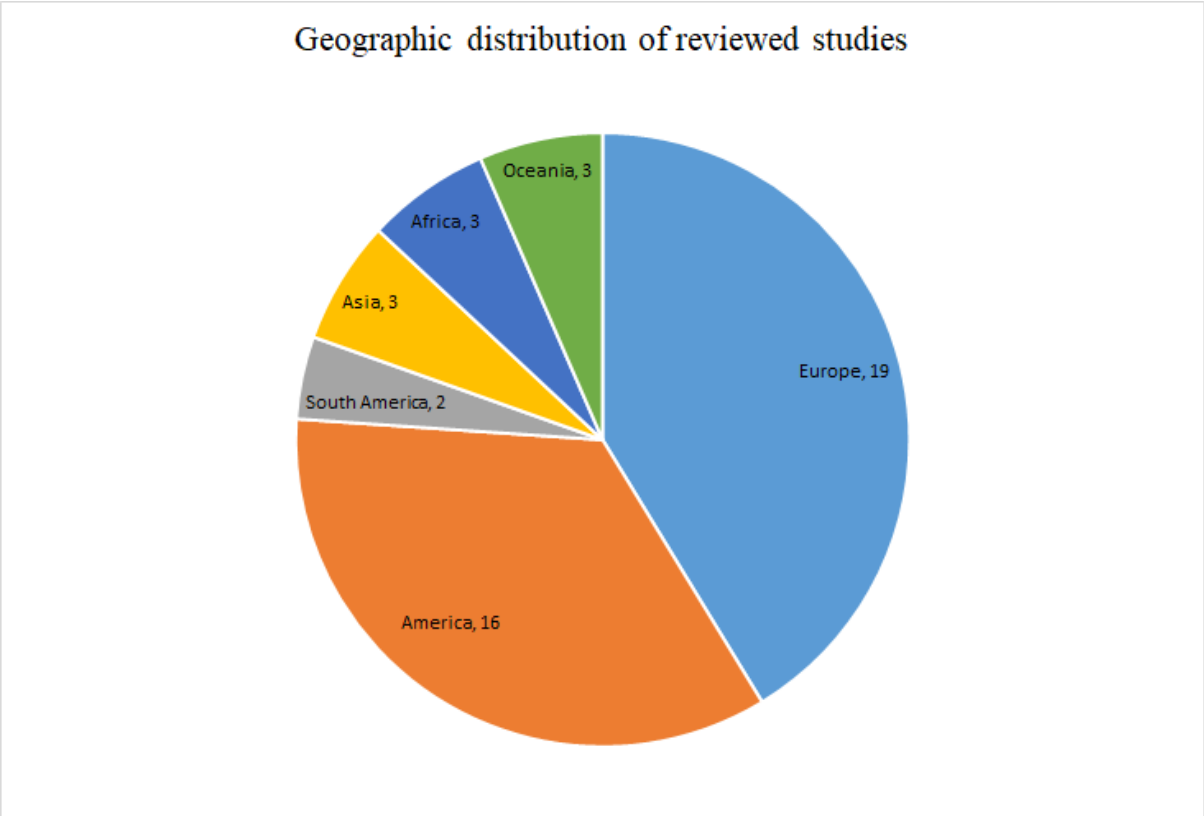


Table 2: Extraction table of the reviewed papers

Table 2: Extraction table of the reviewed papers

Authors	Title	Year	Birds´species	Birds´families	Number of birds used for this study	Location	Stress biomarker	Main conlussion	Other relevant outcome
Bauerová P., Vinklerová J., Hraníček J., Čorba V., Vojtek L., Svobodová J., Vinkler M.	Associations of urban environmental pollution with health-related physiological traits in a free-living bird species	2017	*Parus major*	Paridae	N = 57	13 cities across the Czech Republic	blood and plumage contamination with heavy metals; heterophil/lymphocyte (H/L) ratio	High heavy metal contamination in bird feathers was linked to a high heterophil/lymphocyte (H/L) ratio, indicating longterm stress in individuals inhabiting heavily polluted environments.	did not find any association of heavy metal contamination with the bacteriolytic activity of plasma complement, feather growth or ornamentation
Mackay B., Lee A.T.K., Barnard P., Møller A.P., Brown M.	Urbanization, climate and ecological stress indicators in an endemic nectarivore, the Cape Sugarbird	2017	*Promerops cafer*	Promeropidae	N = 1375	Cape Floristic Region, South Africa	tarsal disease; fluctuating asymmetry; body condition; feather fault bars	Birds closer to urban settlements had higher levels of fluctuating asymmetry and fault bars in feathers.	

Herrera-Dueñas A., Pineda-Pampliega J., Antonio-García M.T., Aguirre J.I.	The influence of urban environments on oxidative stress balance: A case study on the house sparrow in the Iberian Peninsula	2017	*Passer domesticus*	Passeridae	N = 210	six locations of central Iberian Peninsula	Oxidative Stress	The maintenance of oxidative balance increases in an urban environment in comparison to a rural one	urban sparrows showed higher levels of oxidative damage and higher activity of antioxidant enzymes, but lower antioxidant capacity in comparison with the rural birds; and these differences especially increase during the breeding season.
Charmantier A., Demeyrier V., Lambrechts M., Perret S., Grégoire A.	Urbanization is associated with divergence in pace-of-life in great tits	2017	*Parus major*	Paridae	N = 498	two main study areas in the south of France	personality traits; breath rate; four breeding traits	high urbanization in nest-box surroundings was associated overall with earlier breeding and smaller clutches, but also with faster breath rate	Urban birds laid smaller broods earlier in spring compared to their rural conspecifics, and city broods resulted in lower hatching success yet interestingly fledging success was similar.
Isaksson C., Andersson M.N., Nord A., Von Post M., Wang H.-L.	Species-dependent effects of the urban environment on fatty acid composition and oxidative stress in birds	2017	*Parus major; Cyanistes caeruleus; Passer domesticus; Passer montanus*	Paridae; Passeridae	N = 243	Scania, Sweden	plasma fatty acids; oxidative stress	the urban environment affect tits and sparrows primarily via two different pathways—inflammation and oxidative stress, respectively,—with potential consequences for the health of urban populations.	the discriminant analyses revealed species- and family-specific fatty acids profiles.

Aharon-Rotman Y., Buchanan K.L., Klaassen M., Buttemer W.A.	An experimental examination of interindividual variation in feather corticosterone content in the house sparrow, Passer domesticus in southeast Australia	2017	*Passer domesticus*	Passeridae	N = 45	Victoria, Australia	Corticosterone in feathers	higher levels of corticosterone in birds sampled in a suburban area than in the ones founded in an urban area	confirms the use of feathers as a non-invasive tool to estimate plasma CORT during moult in birds, but importantly demonstrates the potential for intrinsic differences in stress characteristics between populations and individuals to obscure the effects extrinsic stressors might have on CORT
Watson H., Videvall E., Andersson M.N., Isaksson C.	Transcriptome analysis of a wild bird reveals physiological responses to the urban environment	2017	*Parus major*	Paridae	N = 12	Malmö, Sweden	Transcriptome analysis	Many genes linked to stress responses were expressed at higher levels in the urban birds, in accordance with our prediction that urban animals are exposed to greater environmental stress.	Differentially expressed genes had functions related to immune and inflammatory responses, detoxification, protection against oxidative stress, lipid metabolism, and regulation of gene expression.

Suri J., Sumasgutner P., Hellard É., Koeslag A., Amar A.	Stability in prey abundance may buffer Black Sparrowhawks Accipiter melanoleucus from health impacts of urbanization	2017	*Accipiter melanoleucus*	Accipitridae	N = 343	Cape Town, South Africa	heterophil/lymphocyte ratio; body condition; risk of infection	no negative effects of urbanization on nestling health, with no significant relationships with heterophil/lymphocyte ratio, body condition, risk and intensity of infection by *Haemoproteus* or intensity of infection by *Leucocytozoon.*	
Meillère A., Brischoux F., Bustamante P., Michaud B., Parenteau C., Marciau C., Angelier F.	Corticosterone levels in relation to trace element contamination along an urbanization gradient in the common blackbird (Turdus merula)	2016	*Turdus merula*	Turdidae	N = 44	Poitou-Charentes region, France	Levels of trace elements; Corticosterone	the increased feather non-essential trace element concentrations were also associated with elevated feather corticosterone levels, suggesting that urbanization probably constrains birds and that this effect may be mediated by trace element contamination.	
Abolins-Abols M., Hope S.F., Ketterson E.D.	Effect of acute stressor on reproductive behavior differs between urban and rural birds	2016	*Junco hyemalis*	Passerillidae	N = 153	California, US	Flight initiation distance; breath rate; Corticosterone	The rural population had a higher physiological and behavioral stress response than the urban population, and acute capture stress had a lasting (24 h) negative effect on territorial behavior, but only in the rural habitat.	individual-level differences in measures of the stress response did not explain variation in the impact of stress on territorial behavior

López-Islas M.E., Ibarra-Meza I., Ortiz-Ordóñez E., Favari L., Elías Sedeño-Díaz J., López-López E.	Liver histopatology, lipidperoxidation and somatic indices of fulica Americana in xochimilco (Urban) and tecocomulco (Rural) wetlands in the Mexico Basin [Histopatología del hígado, lipoperoxidación e Índices somáticos de fulica Americana en los humedales de xochimilco (Urbano) y tecocomulco (Rural) de la Cuenca de México]	2016	*Fulica americana*	Rallidae	N = 59	Mexico city, Mexico	Liver Histopatology; Lipidperoxidation; Somatic Indices	maximum lipid peroxidation levels were detected during winter at rural populations	The highest prevalence of liver pathologies were observed in rural coots
Hargitai R., Nagy G., Nyiri Z., Bervoets L., Eke Z., Eens M., Török J.	Effects of breeding habitat (woodland versus urban) and metal pollution on the egg characteristics of great tits (Parus major)	2016	*Parus major*	Paridae	N = 43	Pilis Mountains, Hungary	egg characteristics	In the urban habitat, soil and eggshells contained higher concentrations of metals, and soil calcium level was also higher than that in the woodland.	
Minias P.	Successful Colonization of a Novel Urban Environment is Associated with an Urban Behavioural Syndrome in a Reed-Nesting Waterbird	2015	*Fulica atra*	Rallidae	N = 64	Łodz, Poland	heterophil/lymphocyte (H/L) ratio; Nesting behaviour	Urban individuals had a reduced level of physiological stress when compared with suburban individuals.	Urban individuals spent, on average, less time incubating due to more frequent bouts off the nest than suburban conspecifics.

Russ A., Reitemeier S., Weissmann A., Gottschalk J., Einspanier A., Klenke R.	Seasonal and urban effects on the endocrinology of a wild passerine	2015	*Turdus merula*	Turdidae	N = 192	Leipzig, Germany	Estrone; testosterone; corticosterone	Elevated artificial night light intensities were significantly positively correlated with corticosterone and negatively with female estrone levels	
Rebolo-Ifran N., Carrete M., Sanz-Aguilar A., Rodriguez-Martinez S., Cabezas S., Marchant T.A., Bortolotti G.R., Tella J.L.	Links between fear of humans, stress and survival support a non-random distribution of birds among urban and rural habitats	2015	*Athene cunicularia*	Strigidae	N = 121	Buenos Aires, Argentina	corticosterone; annual survival	Survival was twice as high in urban as in rural birds and links with corticosterone varied between habitats	
Meillère A., Brischoux F., Parenteau C., Angelier F.	Influence of urbanization on body size, condition, and physiology in an urban exploiter: A multi-component approach	2015	*Passer domesticus*	Passeridae	N = 110	Western France	Morphological measurements; corticosterone; heterophil/lymphocyte (H/L) ratio	sparrows in more urbanized habitats have reduced body size and body mass compared to their rural conspecifics.	significant difference in juvenile fat scores, suggesting that food types provided to young sparrows differed highly between habitats.
Giraudeau M., Chavez A., Toomey M.B., McGraw K.J.	Effects of carotenoid supplementation and oxidative challenges on physiological parameters and carotenoid-based coloration in an urbanization context	2015	*Haemorhous mexicanus*	Fringillidae	N = 80	Phoenix, Arizona, US	Carotenoids; Oxidative stress; Plumage coloration	Urban finches were less colorful than desert birds at capture, but we found no differences between urban and desert finches in how carotenoid provisioning or oxidative stress affected plumage coloration.	urban birds circulated higher levels of carotenoids than desert birds after receiving the same diet for 4 months

Galbreath D.M., Ichinose T., Furutani T., Yan W., Higuchi H.	Urbanization and its implications for avian aggression: A case study of urban black kites (Milvus migrans) along Sagami Bay in Japan	2014	*Milvus migrans*	Accipitridae	Not reported	Sagami Bay, Japan	avian aggression	aggression was higher amongst populations with less forested or agricultural area within their foraging zones, and aggression increased during spring, which is the breeding season.	
Giraudeau M., Mousel M., Earl S., McGraw K.	Parasites in the city: Degree of urbanization predicts poxvirus and coccidian infections in house finches (Haemorhous mexicanus)	2014	*Haemorhous mexicanus*	Fringillidae	N = 174	Phoenix, Arizona, US	oxidative stress; poxvirus and coccidian infections	Prevalence of poxvirus infection and severity of coccidial infection were significantly associated with the degree of urbanization, with an increase of infection in more urban areas.	levels of oxidative damage in plasma were not associated with infection or with urbanization metrics
Costantini D., Greives T.J., Hau M., Partecke J.	Does urban life change blood oxidative status in birds?	2014	*Turdus merula*	Turdidae	N = 43	Munich, Germany	oxidative stress	city birds had lower oxidative damage during stressful conditions compared with rural birds	
Torné-Noguera A., Pagani-Núñez E., Senar J.C.	Great Tit (Parus major) breath rate in response to handling stress: Urban and forest birds differ	2014	*Parus major*	Paridae	N = 177	Catalonia	breath rate	Urban birds displayed higher breath rates than forest birds.	
Dominoni D.M., Quetting M., Partecke J.	Long-term effects of chronic light pollution on seasonal functions of European blackbirds (Turdus merula)	2013	*Turdus merula*	Turdidae	N = 40	Munich, Germany	Testicular development; testosterone	urban birds developed testes faster than their control rural conspecifics	

Powell C., Lill A., Johnstone C.P.	Body condition and chronic stress in urban and rural noisy miners	2013	*Manorina melanocephala*	Meliphagidae	N = 99	Melbourne, Australia	body condition; heterophil/lymphocyte (H/L) ratio	Urban individuals had a significantly higher HL (mean 0.995) than rural con-specifics (0.719)	There were no significant relationships between body condition indices and blood variables of the kind demonstrated in some bird species
McGiffin A., Lill A., Beckman J., Johnstone C.P.	Tolerance of human approaches by Common Mynas along an urban-rural gradient	2013	*Acridotheres tristis*	Sturnidae	N = 160	Melbourne, Australia	tolerance to human approacres	Urban and suburban Mynas had a 2.3–5.7 times shorter FID, were 1.6–2.5 times less likely to retreat by flying and were 1.9–3.1 times more likely to flee >5 m than their peri-urban and rural conspecifics.	
Chávez-Zichinelli C.A., Macgregor-Fors I., Quesada J., Rohana P.T., Romano M.C., Valdéz R., Schondube J.E.	How stressed are birds in an urbanizing landscape? Relationships between the physiology of birds and three levels of habitat alteration	2013	*Melozone fusca; Columbina inca*	Passerellidae; Columbidae	N = 223	Michoacán, Mexico	corticosterone	corticosterone concentration was significantly higher in croplands	

Brahmia Z., Scheifler R., Crini N., Maas S., Giraudoux P., Benyacoub S.	Breeding performance of blue tits (Cyanistes cæruleus ultramarinus) in relation to lead pollution and nest failure rates in rural, intermediate, and urban sites in Algeria	2013	*Cyanistes cæruleus ultramarinus*	Paridae	N = 25	Annaba, Algiers	Breeding performance	Although lead concentrations were higher in the tissues of urban birds than in intermediate and rural individuals, we did not detect a clear influence of this variable on nestling morphology	
Davies S., Rodriguez N.S., Sweazea K.L., Deviche P.	The effect of acute stress and long-term corticosteroid administration on plasma metabolites in an urban and desert songbird	2013	*Melozone aberti*	Passerellidae	N = 28	Phoenix, Arizona, US	corticosterone administration and metabolite assays	There was no difference between urban and desert localities in body mass, fat scores, and the response to acute stress. CORT administration decreased body mass but had no effect on glucose and uric acid, pectoral muscle glycogen, or kidney PEPCK-C.	
Atwell J.W., Cardoso G.C., Whittaker D.J., Campbell-Nelson S., Robertson K.W., Ketterson E.D.	Boldness behavior and stress physiology in a novel urban environment suggest rapid correlated evolutionary adaptation	2012	*Junco hyemalis*	Passerillidae	N = 80	San Diego County, CA, USA	Boldness behavior; corticosterone	apid adaptive shifts in both stress physiology and correlated boldness behaviors in a songbird, the dark-eyed junco, following its colonization of a novel urban environment.	

Bonier F.	Hormones in the city: Endocrine ecology of urban birds	2012					Review	Urban ecologists have documented numerous biotic and abiotic consequences of urbanization, such as altered climate, species interactions, and community composition, but we lack an understanding of the mechanisms underlying organisms' responses to urbanization	
Lin T., Coppack T., Lin Q.-X., Kulemeyer C., Schmidt A., Behm H., Luo T.	Does avian flight initiation distance indicate tolerance towards urban disturbance?	2012	*36 bird species from 3 orders, 7 families and 23 genera*	7 families	254 trials	Xiamen, Taiwan	Flight initiation distance	bird species with a high propensity to disperse and with large population sizes tend to decrease their FID more strongly along the urban–rural habitat gradient.	
Fokidis H.B., Deviche P.	Brain arginine vasotocin immunoreactivity differs between urban and desert curve-billed thrashers, toxostoma curvirostre: Relationships with territoriality and stress physiology	2012	*Toxostoma curvirostre*	Mimidae	N = 160	Phoenix, Arizona, US	Brain Arginine Vasotocin Immunoreactivity; corticosterone	divergence in the AVT system between urban and desert thrashers may help explain observed differences in both the adrenocortical stress response and territorial behavior between populations	

Bókony V., Seress G., Nagy S., Lendvai T.Z., Liker A.	Multiple indices of body condition reveal no negative effect of urbanization in adult house sparrows	2012	*Passer domesticus*	Passeridae	N = 89	Hungary	corticosterone; hematocrit; heterophil:lymphocyte ratio	urban and rural birds had similar levels of corticosterone, hematocrit, and heterophil:lymphocyte ratio	birds had similar scaled mass index (body mass corrected for body size) along the urbanization gradient at all times of the year
Heiss R.S., Clark A.B., McGowan K.J.	Growth and nutritional state of American crow nestlings vary between urban and rural habitats	2011	*Corvus brachyrhync*	Corvidae	N = 113	Ithaca, New York, US	Growth and nutritional state; corticosterone	Rural nestlings were significantly larger than suburban-residential crows and had higher total serum protein	Nestling corticosterone levels did not differ significantly among habitats, indicating that, although suburban nestlings may be food-limited
Scales J., Hyman J., Hughes M.	Behavioral syndromes break down in urban song sparrow populations	2011	*Melospiza melodia*	Passerillidae	N = 60	Pensylvania, US	boldness; aggression	Urban habitats may select for bold and aggressive birds, and yet the traits can vary independently	
Zhang S., Lei F., Liu S., Li D., Chen C., Wang P.	Variation in baseline corticosterone levels of Tree Sparrow (Passer montanus) populations along an urban gradient in Beijing, China	2011	*Passer montanus*	Passeridae	N = 298	Beijing, China	corticosterone	corticosterone levels of Tree Sparrow populations were strongly and positively correlated with the degree of urbanization of the habitat.	

Fokidis H.B., Deviche P.	Plasma corticosterone of city and desert Curve-billed Thrashers, Toxostoma curvirostre, in response to stress-related peptide administration	2011	*Toxostoma curvirostre*	Mimidae	N = 156	Phoenix, Arizona, US	corticosterone	Urban adult male thrashers showed greater responsiveness than desert birds to an injection of arginine vasotocin or adrenocorticotropin hormone, suggesting a population difference in pituitary and adrenal gland sensitivity.	
Fokidis H.B., Orchinik M., Deviche P.	Context-specific territorial behavior in urban birds: No evidence for involvement of testosterone or corticosterone	2011	*Melozone alberti; Toxostoma curvirostre*	Passerillidae; Mimidae	N = 69	Phoenix, Arizona, US	corticosterone; territorial behaviour	Urban Abert's Towhees displayed more territorial behaviors in areas where their population densities were high than in areas of low population densities. Urban Curve-billed Thrashers displayed more territorial behaviors in areas with a high proportion of desert-type vegetation.	
Chávez-Zichinelli C.A., MacGregor-Fors I., Rohana P.T., Valdéz R., Romano M.C., Schondube J.E.	Stress responses of the House Sparrow (Passer domesticus) to different urban land uses	2010	*Passer domesticus*	Passeridae	N = 38	Morelia, Mexico	corticosterone	birds in the three studied urban conditions are both stressed and non-stressed	the immune system of industrial House Sparrows could not be responding successfully to pathogens.

Møller A.P., Erritzøe J., Karadas F.	Levels of antioxidants in rural and urban birds and their consequences	2010	Oportunistic sample of different bird species		N = 44	Copenhagen, Denmark	Levels of antioxidants	Bird species that had successfully colonized urban areas had signicantly higher levels of vitamin E and total carotenoids than species that did not succeed	
Møller A.P.	Interspecific variation in fear responses predicts urbanization in birds	2010	Several, not reported		N = 48	France; Denmark	flight initiation distance	rural populations of birds characterized by short mean flight distances and large variances in flight distances differentially colonized urban areas.	
Fokidis H.B., Orchinik M., Deviche P.	Corticosterone and corticosteroid binding globulin in birds: Relation to urbanization in a desert city	2009	*Mimus polyglottos; Toxostoma curvirostre; Pipilo aberti; Pipilo fuscus; Passer domesticus*	Mimidae; Passerellidae; Passeridae	N = 312	Phoenix, Arizona, US	corticosterone; corticosterone binding protein	Baseline total CORT levels were generally similar in urban and rural birds.	
Isaksson C., Sturve J., Almroth B.C., Andersson S.	The impact of urban environment on oxidative damage (TBARS) and antioxidant systems in lungs and liver of great tits, Parus major,	2009	*Parus major*	Paridae	N = 35	Goteborg, Sweden	oxidative damage	There was no significant difference in lipid peroxidation in lungs between the environments. Among the antioxidant enzymes measured in lungs, only CAT showed a tendency towards increased activity in the urban environment.	

Bobby Fokidis H., Greiner E.C., Deviche P.	Interspecific variation in avian blood parasites and haematology associated with urbanization in a desert habitat	2008	*Passer domesticus; Pipilio alberti; Pipilo fuscus; Mimus polyglottos; Toxostoma curvirostre*	Passeridae; Passerellidae; Mimidae	N = 195	Phoenix, Arizona, US	blood parasites; haematology	Urban birds generally exhibited less blood parasitism than rural birds	
Bonier F., Martin P.R., Sheldon K.S., Jensen J.P., Foltz S.L., Wingfield J.C.	Sex-specific consequences of life in the city	2007	*Zonotrichia leucophrys*	Passerillidae	N = 60	Monterrey; San Francisco; Seattle	corticosterone	in urban habitat had significantly higher baseline corticosterone levels than males in rural habitat, females did not differ	Females with blood parasites had lower heterophil:lymphocyte ratios, lower body condition scores, fewer mates, and fledged fewer offspring than females without parasites.
Partecke J., Schwabl I., Gwinner E.	Stress and the city: Urbanization and its effects on the stress physiology in European Blackbirds	2006	*Turdus merula*	Turdidae	N = 20	Munich, Germany	corticosterone	their corticosterone stress response at an age of 5, 8, and 11 months. The results suggest that the difference is genetically determined, although early developmental effects cannot be excluded	the results support the idea that urbanization creates a shift in coping styles by changing the stress physiology of animals

Ruiz G., Rosenmann M., Novoa F.F., Sabat P.	Hematological parameters and stress index in Rufous-collared Sparrows dwelling in urban environments	2002	*Zonotrichia capensis*	Passerillidae	N = 169	Chile	heterophil/lymphocytes ratio; body condition; glucose concentration	urban Rufous-collared Sparrows have lower body weight, higher blood glucose concentration, higher proportion of heterophils (H), lower proportion of lymphocytes (L), and consequently, a larger H:L stress index, than rural ones.	

Discussion

We have found a variety of results regarding to both physiological and behavioural responses to stress lead by urbanization. Most studies found that there are significant differences between corticosterone measurements of urban birds and their rural counterparts. These differences could be reflected in other physiological processes, especially with immunological response due to the fact that corticosterone is an immunosuppressant (Bonier, 2012). Differences in heterophil/lymphocyte ratio, an immunological related proxy of stress, are also indicating that there could be a negative physiological impact of urbanization over birds (Bauerová et al., 2017). Several studies even suggest that birds could have a higher risk of infections living in the city than in natural environments, possibly because humanly driven habitat changes can cause facilitation for the transmission of zoonotic diseases in immunocompromised populations (Cascio et al., 2011; Giraudeau et al., 2014). However, in urban environments there is a lack of certain parasites, this reflects in urban birds having less infectious charge than rural ones (Fokidis et al., 2008). This perturbation of immunological function could be associated with other metabolic processes, including reproduction and development (Costantini et al., 2014; Dominoni et al., 2013).

Behavioural adaptations to urban environments seem to include higher aggression and boldness behaviour (Atwell et al., 2012; Galbreath et al., 2014). These behavioural traits could possibly be associated with endocrine differences between birds living in urban and natural environments (Atwell et al., 2012). Flight initiation distance and tolerance to human approaches seems to have significant differences between populations in every study, however these differences are not the same across species (Lin et al., 2012; McGiffin et al., 2013; Møller, 2010).

The necessity of permanent adaptations of populations lead to the existence of selective pressures (Møller et al., 2010). There is some evidence that stress response to urban landscapes is genetically determined, and not as we thought before that these responses depend on experiences on early stages of development of the individual (Partecke et al., 2006). Recent research shows that differences in stress response could be processed in a transcriptomic level (Watson et al., 2017).

Most of our results are based on corticosterone related studies, other commonly used stress biomarkers also have a close relation to immune function. As shown in graph number one, the most common stress indicators are corticosterone, heterophil/lymphocyte ratio and oxidative stress. The results of other stress biomarkers are highly related to the ones of corticosterone due to their clear relationship to immune function and endocrine releases. The higher availability of information is about passerines birds, specially to common ones, that serve as models for the study of urban adaptation. In graph number two there is the distribution of studied clades grouped by families, there we can notice a bias in the research about this topic due to the fact that most studies have been carried in the *Passer* & *Parus* genera.

Urban environments constitute a threat to wildlife populations not only in a physiological level but also in fitness (Isaksson et al., 2018). Stress responses differs across species and the stage of development of the individual, this emphasizes the need of studying this phenomenon in specific contexts (Salmón et al., 2018). Future research needs to emphasize in signalling which ecological factors are determine for a species adaptation to urban surroundings. Ecological aspects of studies species should deeply discuss in order to understand the reasons for some species better adaptation than others.

Most studies are not comparable between them, because of the differences in data collection and in the lack of details about environments. For future studies it should be preferably to use

just corticosterone or heterophyl/lymphocyte ratio, the most common proxies of stress, in order to be able to compare between studies. However, those data could not be compared in a quantitative way because of the different conditions in which studies have been carried out. Those differences do not mean that the studies are not replicable, but only that as a proxy of stress and physiological adaptation most of the biological markers depend on a series of factors that can influence over the system of study (Isaksson et al., 2018).

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Chapter 2

Physiological stress on Rufous-collared Sparrow *Zonotrochia capensis* on an urban-rural

Physiological stress on Rufous-collared Sparrow *Zonotrochia capensis* on an urban-rural matrix in the high tropical Andes

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Introduction

Urban environments constitute a major challenge to several animals' species that need to adapt in order to inhabit those landscapes. Several ecological factors are taking into account to determine the success of species colonizing urban environments (Suri *et al.,* 2017). Researchers have been studying how the response to stress could be a determinant to the success of a determined species at an urban settlement (Chávez-Zichinelli *et al.,* 2010). The processing of stress at the hypothalamic–pituitary–adrenal axis allows vertebrates to face external and internal factors in order to adapt, this processing involves the secretion of glucocorticoids (Sapolsky *et al.,* 2000).

Several biomarkers can be used to study how well an animal is adapting to its environment. Behavioural analysis allows us to make preliminary assessment about how species are interacting with disturbance (Scales *et al.,* 2011). However, behavioural traits are related to physiological quantitative data that can be determined at corticosterone measurements, glucocorticoid hormone that is involved in stress response (Fokidis & Deviche, 2011; Partecke et al., 2006). Corticosterone is also used to understand other physiological traits that could be affected by the life in urban environments (Bonier, 2012). Corticosterone measurements are generally used as a proxy of stress and physiological adaptation of birds to urban environments (Partecke et al., 2006).

At this study we measured corticosterone levels at three populations of the rufous-collared sparrow *Zonotrichia capensis*, a common bird in the Neotropics, in order to determine how are these birds adapting to urban environments. The study was performed in Quito, at the northern high tropical Andes, a region in where there is a lack of information about the current status of fauna adaptation to urbanisation.

Methods

The area of study was the Metropolitan District of Quito (DMQ, by its Spanish acronym), where the main human settlement is the City of Quito, founded at sixteenth century during Spaniards´ colonization to at the mentioned area. During the last decades the city and other human settlements at the DMQ have experienced rapid growth and landscapes' changes (Carrión & Erazo-Espinoza, 2012). We determined three sites for sampling. The first site, referred as urban, correspond to small gardens at the residential northern part of the city of Quito. This site is characterized by high population density and high human disturbance; it has been previously reported that the City of Quito has reduced levels of birds' diversity than the suburban and periurban parts of the DMQ (Cisneros-Heredia *et al.,* 2015). Our second population of study was located at the Guangüiltagua Metropolitan Park (PMG, by its Spanish acronym), a remnant of 500 hectares of forest located near the city border. This place constitutes habitat for a large variety of bird species due to the presence of high and medium complexity areas (Cisneros-Heredia *et al.,* 2015). The third population was located at medium-sized gardens at the periurban town of Cumbayá, which host a large variety of birds' species due to its reduced level of urbanization in comparison to the City of Quito and the presence of areas of medium structural complexity, including the water reservoir and the University campus (Cisneros-Heredia *et al.,* 2015). Coordinates of sampling sites are shown at table 1.

Site	Classification	Coordinates
Guangüiltagua Metropolitan Park	Natural, remnant of forest	-0.180284, -78.471047
Cumbayá	Periurban	-0.195623, -78.429890
City of Quito	Urban	-0.132070, -78.462323

Table 1: Sampling sites

Birds were captured using passive mist netting at all the sampling points. Captures were performed form March to November 2017 between 0600 and 1030 h. Blood sample (~100µl) was taken within 3 minutes since the capture. Samples were always taken from the brachial vein, following standard biosafety and bioethics protocols. Blood was saved into a 1.5ml Eppendorf® tube without anticoagulant, and the tube was placed in a cooler until its mobilization to the laboratory. At the laboratory samples were centrifuged at 5 thousand revolutions per minute for five minutes and serum was transferred into another tube in order to be stored at minus twenty degrees Celsius until performing the corticosterone measurements. The remnant of blood was discarded following standard protocols.

For corticosterone measurements, we performed an enzyme-linked immunosorbent assay ELISA, using a commercial kit from the German brand Labor Diagnostika Nord LDN®. This kit was selected because commercial kits from other brands may only work for measure mammals' cortisol. Each measure was done by duplicate and absorbance was measured at 450nm as recommended by the kit manufacturer. Absorbance measurements were transformed into corticosterone concentration using four parameters logistic. Data from populations was compared using an ANOVA test and post hoc proves.

Results

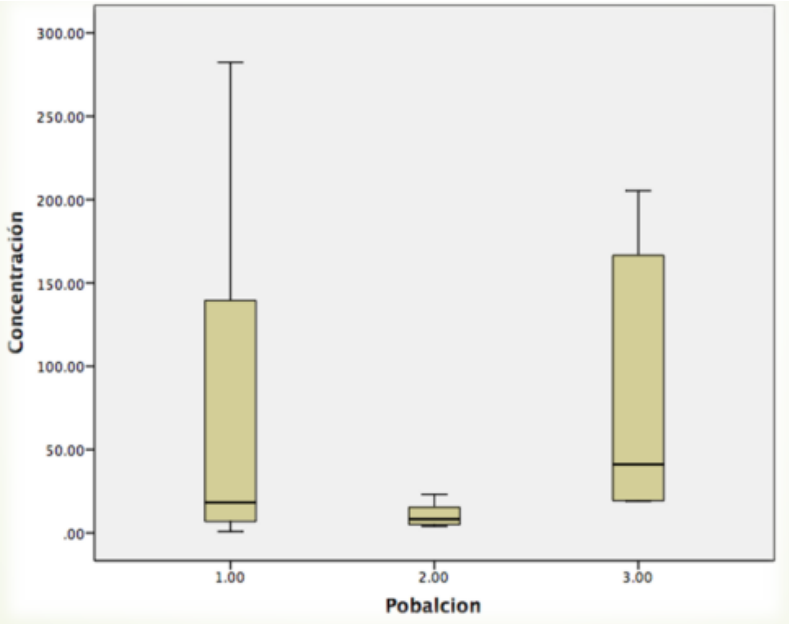

Figure 1: Corticosterone measurements of the three populations

ANOVA

Concentración

	Suma de cuadrados	gl	Media cuadrática	F	Sig.
Entre grupos	28370.283	2	14185.141	3.054	.062
Dentro de grupos	139323.350	30	4644.112		
Total	167693.632	32			

Table 2: ANOVA results for comparing the three populations measurements

We did not find significant differences across the populations, even when the periurban population shown lower levels of corticosterone in comparison to the other two groups. This population also shows the lest variance in comparison to the other two populations. The higher levels of corticosterone where at the remnant of forest.

Discussion

Our results showed that there are no significant differences between the analysed populations. However, tendencies should be attributed to ecological factors such as competence and infections rates. The population with the higher levels of corticosterone is also the population with the higher levels of apparent infections. Previous research suggests that infections rates in

birds' populations are related to the degree of urbanization of their environment (Giraudeau *et al.,* 2014). We suggest further research in order to determine if infections rates are a major ecological factor for corticosterone analysis.

Even when research suggests that *Z. capensis* endocrine physiology in does not have severe fluctuations during the year (González-Gómez *et al.,* 2018), we suggest that for future studies should be done at one delimitated season of sampling. Research in other birds, clearly suggest that seasonal variation is a strong determinant for hormonal studies (Russ *et al.,* 2015), that could let to a limitation for this type of studies, for instance it should be a controlled variable.

For further research, we suggest the use of less invasive samples. Corticosterone levels in blood have high probabilities to increase due to the manipulation of the bird during the extraction of the net and taking out the blood, corticosterone levels in feathers may be a more reliable measurement. Feathers are recently being used to this kind of measurements because data is not suggested to change with manipulation of the bird (Johns *et al.,* 2018). However, it is important to have controlled variables during the study and possible another marker of stress, as corticosterone in feathers may not be as informative as corticosterone levels measured in blood (Sepp *et al.,* 2018). We also suggest that other standard measurements of the birds should be done in the field before the liberation of the individual, research shows that physical condition may be affected by the degree of urbanization (Meillère *et al.,* 2015).

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Chapter 3

First report of avian poxvirus for northern Ecuador with discussion of preliminary genetic data

First report of avian poxvirus for northern Ecuador with discussion of preliminary genetic data

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Introduction

Pox virus infection is a serious concern for some birds' populations, this pathogen is a common cause of mortality in wild populations (Ruiz-Martínez *et al.,* 2016). Avian pox infections have been reported in 232 species in 23 orders but there still a lack of information about another possible host (Bolte *et al.,* 1999). Information about avian pox infections is limited in the Neotropic even when this information is critical to avoid the introduction of the virus to new environments (Niemeyer, 2015).

In this study we confirm the presence of the virus in a geographic region where it has not been reported previously and we also report a new host species. Genetic data about the virus suggests possible concerns for avian wildlife.

Methods

In November 2017, during fieldwork for a major project near the city of Quito, we captured an individual of the Rufous-Collared Sparrow *Zonotrichia capensis* that presented a pustule in its leg, the individual was humanely sacrificed and taken to the lab. Using a commercial kit, we extracted DNA and RNA from the pustule. Amplification of the 4b core region of the virus

was carried out using primers P1 and P2 first reported in Lee & Lee (1997). The polymerase chain reaction was carried out in a total volume of 40µL, consisting in 4µL of buffer PCR (1X) (Invitrogen), 0.68µL of bovine serum albumin (170µg/mL), 5.36µL of $MgCL_2$ (6.7 mM), 4µL of each primer (1µM), 6µL of dNTPs (6mM), 0.2µL of Taq polymerase platinum Invitrogen (1U/µL), and 2µL of DNA. Amplification was performed in a thermal cycler using the protocol reported in Lee & Lee (1997). Amplicons obtained were amplified again using the same protocol. We used DNA extracted from a commercial fowl pox vaccine as positive control and molecular grade water as negative control. Amplified fragments were separated by electrophoresis for 40 minutes at 100V using an agarose gel (1.5%). The amplicon was sent to Macrogen for secuenciation.

Results

With the obtained sequence we confirmed Canarypox as the infectious agent with 80% of similarity to a virus collected in Morocco in 2012 and reported in Le Loc'h *et al.* (2014). This is the first time that the virus is been reported in northern Ecuador, the only previous register of the virus in the country was in the southernmost part of the country (Moens *et al.,* 2017). This is also the first time that the virus is reported to infect an individual of the Rufous Collared-Sparrow *Zonotrichia capensis,* even when there are registers of the infection in other species of this genera (Bolte *et al.,* 1999).

Discussion

Even when there are no previous registers of the virus it does not mean that it is a recently introduced infection, it is possible that the lack of information of the virus in the region is due to the low quantity of avian research that has been done in this locality. As there are previous registers of the disease infecting other birds in the same genre as the Rufous-Collared Sparrow it was expected that this bird could constitute a potential host of the virus. This report

constitutes a concern about the health status of birds' populations in the northern Andes, mainly because of this pathogen as a potential cause of mortality for wild birds (Ruiz-Martínez *et al.*, 2016). The obtained sequence does not have very high similarity with previously reported ones, which is not very common for a mainly conservative region (Binns *et al*., 1989). We suggest further molecular research about this particular virus mainly because it could be an emergent virus or a new strain, complete sequencing of the virus is highly recommended.

Accession number

Still pending

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Chapter 4

Protocol for studying birds' adaptation to urban environments

Protocol for studying birds' adaptation to urban environments

Definition of the system of study

It is very important to choose an appropriate system of study, this included the birds that are going to be analysed, but also the places where you are going to sample. It is important to use a group of study in which you are going to be able to have a significant sample, therefore common birds are recommendable. The chosen species would preferably have relatively high abundances across the urban-rural matrix but also at each sampling point. It is preferable to use a species that would be relatively easy to capture, in order to be able to have a significant sample. Granivorous and omnivorous birds tend to have relatively high abundances across the urban-rural matrix and their ecological roles tend to be equivalent to relative species in other cities (Clergeau *et al.,* 1998).

For choosing the sampling sites it is preferable to review places that would be accessible during multiple visits. I recommend visiting sampling points in order to take in consideration logistic details before the sampling. Chosen sites should represent different degrees of urbanization, a standard study should include an urban site, a suburban one, a periurban one and a rural one. As urban site it is generally better to use a downtown location, suburban places refers to residential locations outside downtown, periurban sites refers to mixed landscapes between suburbs and rural landscapes. It is recommendable to use replicas for each environment; however this could be difficult depending of the city structure, at those cases it is preferable to analyse data assuming that each population does have a different degree of urbanization than the others.

Sampling birds by mist nets

Once that the system of study is defined now you have to define adequate mist nets for the species of study. Generally, nets with a size of 36mm could be used, but are not very efficient

for capturing birds bigger than a thrush or very small passerines (Jenni *et al.*, 1996). Mist nets are generally the most recommendable way to capture birds for research proposals, however depending on other ecological data (e.g. habitat use) that researchers would like to measure during the study you may use other sampling methods (Karr, 1981).

Before placing the nets, it is appropriate to determine a correct place, this place should be a path for the target bird and normally would stay aside with shrubbery. To determine the location of the nets, it would depend of the structure of the site but it is preferred to avoid direct sunlight and strong wind exposure (Jenni *et al.*, 1996). If you are using more than one net at the time you may be sure that you are able to manage all the birds that could fall at the net at the same time. Fieldwork would require two persons in order to coordinate work and be able to manipulate the bird in the less disturbing way.

Corticosterone and other metabolites levels tend to fluctuate across the day and the year (Touma & Palme, 2005). At non-seasonal environments the variation across the year decreases (González-Gómez *et al.*, 2018). We strongly recommend that the sampling should be done always at the same period of the day and in a delimitated season of the year. During summer it is a great opportunity for researchers to conduct fieldwork.

Samples management

For taking blood samples of the birds you should do it in less than three minutes since it has felt into the net until you take the blood sample, samples taken after longer periods of time may not be used for measuring basal corticosterone levels (Breuner *et al.*, 1999). Blood samples should be taken from the brachial vein of the bird, preferably using an insulin syringe, if the bird is too small, it is possible to use a lancet to make hurt the vein and wait for the blood to come out and be took by the syringe. The amount of blood taken from the bird would depend on the species, however for passerines is recommended to take less than one millilitre. Blood

samples should be stored at tubes with anticoagulant, it is important to remember that the anticoagulant may be well mixed with the blood. Samples could be stored at a regular cooler until being frozen at minus twenty degree Celsius in an appropriate space.

At fieldwork researchers may work together in order to take the bird out of the net as soon as it falls into it. One of the researchers should take the bird from the neck and the body in order to avoid it from being hurt. This researcher should extend one of the wings and identify the brachial vein, at the elbow joint between the humerus with the radius and ulna. The other researcher may clean the joint with a swab with grease, in order to make it visible the vein and take the blood sample. After researchers have taken the sample, they should put a piece of cotton with alcohol al the hurt joint, and wait until it stops bleeding before releasing the bird.

It is important to have all the materials ready before beginning fieldwork, so that it would not lack something when needed. You may also have some microcentrifuge tubes and an indelible marker in order to save parasites that could be found at feathers or any other biological material of interest such as faeces. For collecting feathers, you may take one or two from the tail and store them in a paper bag. If the bird presents evidence of poxvirus infection (i.e. pustules) you should use a swab with TBE to clean over it and then save the swab in a tube.

Measuring corticosterone

For measuring corticosterone, an ELISA assay should be performed, the initial sample could be feathers or blood samples. For blood samples, the blood with anticoagulant should have been centrifuged after the collection, and plasma must be stored alone. In an ELISA plate 20µl of the sample should be dispensed to a well. After this you should dispense 200µl of enzyme conjugate and mix. Then you should incubate the plate at room temperature for one hour and wash it three times with 400µl of diluted wash solution. Right after the wash, you should add 100µl of substrate solution and incubate for a quarter of an hour. To stop the enzymatic

reaction, you should add 100µl of stop solution. The absorbance is measured at 450nm at a microplate reader.

For each assay it is required to have a standard curve with several known concentrations of corticosterone. The absorbance value is directly related to a concentration value. Conversion of results from absorbance to concentration should be done by using a four parameters logistics. It is recommended that for performing an assay you have samples to complete the whole plate, because by the necessity of a standard curve, doing small groups of samples can lead to waste wells of the plate and reactants. For easier performance is helpful to use a multicarrier micropipette, however for people that does not have experience working with this instrument is preferably to use a regular micropipette. Even though it is not very common to have crossed contamination, it is preferred to take all the measurements to make sure that there are no possible contaminants nearby during the assay.

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