ALESSANDRA TARODA

HEMOSPORÍDEOS E *EIMERIA* EM POMBOS *Zenaida auriculata* DO MUNICÍPIO DE LONDRINA, PARANÁ, BRASIL

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Tese apresentada ao Programa de Pós-graduação em Ciência Animal do Centro de Ciências Agrárias (área de concentração: Sanidade Animal) da Universidade Estadual de Londrina, como requisito para a obtenção do título de Doutora.

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RESUMO

A pomba-de-bando (*Zenaida auriculata*) é encontrada na maioria dos países da América Latina, tanto em áreas urbanas quanto rurais. Hemosporídeos (Apicomplexa: Haemosporida) são parasitas cosmopolitas encontrados em mamíferos, aves, répteis e anfíbios, e necessitam de vetores dípteros (Insecta: Diptera) no ciclo biológico. Parasitas dos gêneros *Plasmodium* e *Haemoproteus* ocorrem no mundo todo em diferentes aves e são agentes etiológicos da malária aviária. As co-infecções são comumente observadas em aves silvestres. *Eimeria* é um parasita intracelular obrigatório e hospedeiro específico, que infecta todos os vertebrados. Em Columbiformes, podem causar lesões intestinais graves. Este estudo objetivou determinar a presença de hemosporídeos e *Eimeria* de *Z. auriculata* capturados em Londrina, Paraná. Os pombos foram capturados em armadilhas e foram colhidas 211 amostras de sangue em tubos contendo EDTA e 214 amostras de fezes. Foi realizada a nPCR do gene do citocromo b mitocondrial (cit b) para a detecção dos hemosporídeos. Utilizou-se o Polimorfismo no Comprimento do Fragmento de Restrição para identificar os gêneros *Haemoproteus* ou *Plasmodium*. Além disso, realizou-se a detecção específica de *Plasmodium* sp. pela nPCR da subunidade ribossomal menor do RNA (18S SSU rRNA). O sequenciamento do produto amplificado na segunda reação do cit b foi utilizado para identificação dos gêneros. Árvores filogenéticas foram reconstruídas para inferir a relação entre espécies. A detecção de *Eimeria* foi realizada através da nPCR da subunidade I do gene mitocondrial do citocromo c oxidase (COI). Todos os 211 pombos foram positivos para presença de *Haemoproteus* e/ou *Plasmodium* na nPCR cit b e nPCR 18S SSU rRNA. Na RFLP, 155/211 (73,46%) foram positivos somente para *Haemoproteus* sp., 13/211 (6,16%) somente para *Plasmodium* sp., 41/211 (19,43%) para ambos os gêneros. A positividade para ambos os gêneros de parasitas demonstrou a ocorrência de infecções mistas. No sequenciamento genético foi identificado quatro pombos com *H. multipigmentatus* e um com *Plasmodium* sp. O DNA de *Eimeria* sp. foi identificado em 171 (79,91%) dos pombos pela nPCR. Houve significância estatística entre os locais de captura, na universidade e na indústria de grãos I (p=0,0008). Até o momento, este é o primeiro estudo molecular com a descrição de hemoparasitas e *Eimeria* em *Z. auriculata* no Brasil. Estudos adicionais devem ser realizados para identificar as infecções mistas por hemoparasitas e novas análises por sequenciamento devem ser utilizadas para identificar as espécies de *Eimeria* obtidas neste trabalho.

Palavras-chave: Eimeriose. Haemoproteidae. Gene citocromo b mitocondrial. COI locus. Columbidae.

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ABSTRACT

The eared dove (*Zenaida auriculata*) is found in most of Latin America countries in both urban and rural areas. Haemosporidians (Apicomplexa: Haemosporida) are worldwide hemoparasites found in mammals, birds, reptiles and amphibians, that need blood-sucking dipterans (Insecta: Diptera) as vectors in the life cycle. Both *Plasmodium* and *Haemoproteus* occur worldwide in several different birds and *Plasmodium* causes the avian malaria parasite. Coinfections are often observed in wild birds. *Eimeria* is a host specific and obligate intracellular parasite that infects all vertebrates. In Columbiformes, they can cause severe intestinal lesions. This study aimed to determine the presence of Haemosporidia and *Eimeria* from *Z. auriculata* captured in Londrina, Paraná State. *Z. auriculata* were trap-captured and 211 whole blood were collected in EDTA tubes and 214 fecal samples. nPCR mitochondrial cytochrome b gene (cyt b) of *Haemoproteus* sp./*Plasmodium* sp. Restriction Fragments Length Polymorfism (RFLP) was performed to identify the genus *Haemoproteus* or *Plasmodium*. Also, a nPCR based on small subunit ribosomal ribonucleic acid (18S SSU rRNA) specific for *Plasmodium* detection was done. The sequencing from the second reaction of nPCR of cyt b was carried out to identify the species. Phylogenetic trees were built to determine the closely related species. nPCR based on detecting sequences in the subunit I of the cytochrome c oxidase gene (COI) of the parasite mitochondrial genome was performed. All 211 doves were positive at nPCR cyt b, and nPCR 18S SSU rRNA, showing 100% of doves positive for the presence of *Haemoproteus* and/or *Plasmodium*. RFLP showed 155/211 (73.46%) positives only for *Haemoproteus* sp., 13/211 (6.16%) positives only for *Plasmodium* sp., 41/211 (19.43%) for both genera and 2/211 (0.95%) negatives. The positivity for both genera by the usage of the restriction enzyme suggests a mixed infection with both genera. The sequencing resulted in four doves with *Haemoproteus multipigmentatus* and one with *Plasmodium* sp. In 171 (79.91%) doves in was found *Eimeria* sp. DNA by nPCR. There were significantly differences between the places of capture (p=0.0008), between the University and the Crop Cooperative I. To this moment, this is the first molecular study and the first description of hemoparasites and *Eimeria* from *Z. auriculata* in Brazil. More studies need to be done to identify the mixed infections and the presence of other blood parasites and also, further analysis need to be done by sequencing to provide the species of *Eimeria* found in *Z. auriculata* in this work.

Key-words: Coccidiosis. Haemoproteidae. Mitochondrial cytochrome b gene. COI locus. Columbidae.

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“Daria tudo que sei pela
metade do que ignoro.”
René Descartes

REVIEW: HAEMOSPORIDIAN PARASITES AND *EIMERIA* IN PIGEONS

1 *ZENAIDA AURICULATA*

Zenaida auriculata (Des Murs, 1847) *(*Aves: Columbiformes) is a native bird in Brazil and can also be found from the Antilles to Southern Argentina (Tierra del Fuego), not only in the forests or rural areas, but it inhabits the urban centers (Goulart et al., 2011; Shibatta et al., 2009; Cândido Jr. et al., 2008).

The eared dove, as it is popularly known, is a medium-sized grey bird (about 23cm), the top of the head is blue-grey, shows dark spots on the wings and two horizontal black lines on the sides of the head, tanned neck and red foot and legs (*Figure* 1) (Sick, 1997).

Urbanization has changed ecosystems worldwide and the cities have replaced the vegetation and native forests. This process induces effects on the population of animals, and birds can be negatively affected because it can reduce the birds' richness or even extinct some species. However, many birds have adapted to urbanization becoming synanthropic, such as the pigeons and doves, as they benefit from human resources (food, garbage, places to live), and keep being able to reproduce rapidly (Villegas and Garitano-Zavala, 2010; Amâncio et al., 2008). *Z. auriculata* are found in Brazil in large flocks, and they have become pests in crops and even in the urban areas (Goulart et al., 2011, Cândido Jr. et al, 2008, González, et al., 2004, Adriano et al., 2003).

In Northeastern and Central Brazil, the eared dove is an important food resource for humans (Souza et al., 2007; Adriano et al., 2003). In many cities in Brazil including Londrina, the great number of doves has been a huge concern due to the transmission of diseases, losses in agriculture and the problem for their feces in the urban areas (Shibatta et al., 2009). It can be the largest population of birds in rural and urban areas in Londrina (Lopes and Anjos, 2006).

Many health problems can affect pigeons and parasitic diseases play a very important role, but little is known about parasites in *Z. auriculata* (Marques et al., 2007; González et al., 2004). Adriano (1999) found *Haemoproteus columbae*, *i*n *Z. auriculata* in São Paulo State, Brazil. *Ornithostrongylus volcani n. sp.* was described for the first time in this dove in Venezuela (Durette-Desset et al., 2000); Adriano et al. (2001) reported the first description of *Brachylaima mazzantii* in *Z. auriculata* in

Brazil; Adriano et al. (2003) described a new species of *Eimeria* from the eared dove in Brazil, named as *Eimeria zenaidae* sp. n. González et al. (2004) reported the presence of *Killigrewia delafondi*, *Raillietina* sp., *Heterakis gallinarum*, *Echinostomum* sp., *Eimeria labbeana* and the ectoparasites *Falculifer isodontus*, *Diplaegidia columbae*, *Amblyomma* sp., *Columbicola baculoides*, *Bonomiella* sp., *Hohorstiella* sp. and a mite from family Trombiculidae in *Z. auriculata* in Chile; Goulart et al. (2011) described the presence of mites in *Z. auriculata* from São Paulo State, and they found *D. columbae*, *D. coumbigallinae*, *Byersalges talpacoti*, *Pterophagus spilosikyus*, *Hypodectes propus*, *Ornithocheyletia columbigallinae*, *Ornithonyssus bursa* and *Tinaminyssus zenaidurae*.

In Londrina, Taroda et al. (2013) described for the first time in Brazil, the presence of *Ascaridia columbae*, *Tetrameres fissispina*, *Synhimantus nasuta*, *Raillietina allomyodes*, *Paratanaisia bragai* and *P. confusa* in *Z. auriculata* in Londrina, and they also found the already described species *Ornithostrongylus quadriradiatus*, and *Brachylaima mazzantii*. Barros et al. (2014) reported the presence of antibodies against *Toxoplasma gondii* in this dove and also isolated four known genotypes and a new one (ToxoDB#182), and also described the existence of clonal type II in *Z. auriculata* in Londrina. Seixas et al. (2016) studied the presence of Cryptosporidium in eared doves in Londrina by nPCR and they reported the detection of *C. galli* and *C. meleagridis* for the first time in this dove.

2 HAEMOSPORIDIA IN PIGEONS

Haemosporidians (Apicomplexa: Haemosporida) are worldwide hemoparasites found in mammals, birds, reptiles and amphibians, that uses blood-sucking dipterans (Insecta: Diptera) as vectors in the life cycle. The Family Plasmodiidae is one of the most studied and well known in blood parasites, which includes the parasite that causes malaria in humans. In birds, *Plasmodium* causes the avian malaria. Both *Plasmodium* and *Haemoproteus* occur worldwide in several different birds (Valkiunas 2005, Atkinson et al., 2008). However, there are several haemosporidians that needs to be more studied, such as families Haemoproteidae, Leucocytozoidae and Garniidae. Parasites from genera *Haemoproteus*, *Plasmodium*, *Leucocytozoon* and *Fallisia* were identified in Columbiformes (Valkiuñas, 2005). In the Neotropics, *Haemoproteus columbae* and *H. sacharovi* can be found, so as *Plasmodium columbae* (Valkiuñas, 2005). The effects of parasites infection provide reduction on the fitness of their hosts (Lefévre et al., 2008), may cause death (Donovan et al., 2008, Cannell et al., 2013). Consequently, these effects can be potentially the cause of population decline and extinction (Atkinson et al., 2000).

Many authors refer as avian malaria parasites for both *Haemoproteus* and *Plasmodium*, although several morphological and genetic differences have been described (Atkinson et al., 2008), despite they are closely related (Martinsen et al., 2008).

2.1 General Morphology

2.1.1 *Haemoproteus* sp. Morphology

The genus *Haemoproteus* belongs to the Family Haemoproteidae. Two subgenera are described: *ParaHaemoproteus* (parasites of birds from orders other than Columbiformes) and *Haemoproteus* (parasites from Columbiformes) (Valkiuñas, 2005, Krizanauskiene et al., 2013). Subgenus *ParaHaemoproteus* are transmitted by dipterans from family Ceratopogonidae and subgenus *Haemoproteus* by Hippoboscidae flies.

Most of *Haemoproteus* species are considered apathogenic although there are studies showing mortality in captive birds and birds from zoos, depending of the parasite species (Levin et al., 2011; Atkinson et al., 2008).

Mainly, the gametocytes of *Haemoproteus* can be found inside erythrocites. The microgametocyte and macrogametocyte show the nucleus and the cytoplasm with: discret parasitophorous vacuole, micropyles, electron-dense organelles, mitochondria, atypical centriole, osmiophilic bodies and ribosomes (Valkiuñas, 2005). Intraerythrocitic gametocytes are recognized by the presence of black or golden-brown pigment granules that are called hemozoin. These granules are products of digestion of host hemoglobin. *Haemoproteus* species have been morphologically described through the gametocytes, based on descriptions e. g. of their shape (round or elongated), how far they stay around the erythrocyte nucleus and the number and size of hemozoins (Atkinson et al., 2008).

The macrogametocytes have their nuclei with nucleolus, and the cytoplasm shows endoplasmic reticulum, Golgi apparatus and spherical bodies. Additionally, the cytoplasm of macrogametocytes is basophilic due to Giemsa's staining and it is eosinophilic in microgametocytes (Valkiuñas, 2005). The gametocytes inside the red blood cells are shown on *Figure* 2.

The micropyles are responsible for developing the food vacuoles, where the digestion of hemoglobin and other nutrients occur. These vacuoles are also responsible for the storage of pigment granules. A residual pigment called hemozoin is accumulated in these vacuoles that are present in *Haemoproteus* and *Plasmodium* gametocytes (Valkiuñas, 2005).

2.1.2 *Plasmodium* sp. Morphology

The morphology of gametocytes of *Plasmodium* sp. (subgenera *Giovannolaia* and *Huffia*) are very similar to the gametocytes of some species of *Haemoproteus* sp but it is different from *Haemoproteus* because of the presence of intraerythrocytic meronts. An example of gametocytes of *Plasmodium elongatum* can be seen at *Figure* 3. The acute stage, in which more gametocytes can be observed in blood, is very short, what makes the heavy findings more difficult in wild birds. (Valkiunas 2005). The hemozoin is also golden-brown or black, due to digestion of hemoglobin host (Atkinson et al., 2008).

The Genus *Plasmodium* is divided into five subgenera: *Haemomoeba, Giovannolaia, Novyella, Bennettinia* and *Huffia*, based at the morphology of gametocytes and meronts within erythrocytes (Valkiunas 2005; Atkinson et al., 2008). Some examples of each subgenera, according to Martinsen et al. (2006) are shown at Table 1.

Table 1 – Examples of *Plasmodium* species according to each subgenera.

Subgenus	Example species
Haemomoeba	*P. (H.) relictum, P. (H.) cathemerium, P. (H.) gallinaceum, P. (H.) tejerai*
Huffia	*P. (H.) elongatum, P. (H.) huffi, P. (H.) hermani*
Giovannolaia	*P. (G.) circumflexum, P. (G.) polare, P. (G.) lophurae*
Novyella	*P. (N.) columbae, P. (N.) nucleophilum*

Adapted from Martinsen et al. (2006)

2.2 Life Cycle

The general life cycle of haemosporidians in birds develop in vertebrate hosts (birds) and vectors (dipterans). Birds are the intermediate hosts and the dipterans are the definitive hosts, as the gametogony of the hemoparasites occurs in the midgut of the insects. The disease in birds can become latent and the parasites can persist for life, resulting in vectors been infected constantly (Valkiuñas, 2005, Atkinson et al., 2008).

2.2.1 *Haemoproteus* sp. Life cycle

The life cycles in the Family Haemoproteidae are obligate heteroxenous, as an intermediate host is needed to infect the definitive host. Dipterans, usually the biting midges (Diptera: Ceratopogonidae) and hippoboscid flies (Hippoboscidae) are the vectors. The insects inoculate sporozoites in birds while feeding. The sporozoites start the schizogony in the endothelial cells and fixed macrophages (exoerythrocytic meronts). Most of meronts can be located in the lungs, and less often in the liver, spleen, kidneys, heart and skeletal musculature (Valkiuñas, 2005, Atkinson et al., 2008).

Some *Haemoproteus* species can develop megalomeronts in the endothelial cells in the lungs, myofibroblasts of the skeletal and in the myocardial muscle. After several generations at schizogony, the exoerythrocytic merozoites from these megalomeronts infect the erythrocytes and start to develop the sexual stages (gametocytes): the macrogametocytes that can produce macrogametes and the microgametocytes that may develop flagellated microgametes. These gametocytes infect the vectors that fed on infected birds, become rounded and go off the erythrocytes. The gamonts begin the gametogenesis in the midgut, resulting in oogamy (Valkiuñas, 2005, Atkinson et al., 2008).

The zygote is developed after a microgamete unites with a macrogamete extracellularly, and turns into an elongated motile ookinete. The ookinete crosses the epithelial tissue of the midgut, transform to a rounded shape at the basal lamina and develops into an oocyst (sporogony), with a capsule-like made from the hosts' cells. During the sporogony, a large number of elongated bodies named sporozoites are formed inside the oocyst (Valkiuñas, 2005, Atkinson et al., 2008).

Once the oocyst is mature, the sporozoites go to the haemocoele and penetrate the salivary glands of the dipteran. Then, these sporozoites are the infectious form to birds and the transmission occurs during bitting, when the insect injects the salivary secretion containing the sporozoites (Valkiuñas, 2005, Atkinson et al., 2008). The life cycle can be seen on *Figure* 4.

2.2.2 *Plasmodium* sp. life cycle

Family Plasmodiidae has a life cycle that presents the blood-sucking mosquitoes (Diptera: Culicidae) as their vector and definitive hosts. Insects from the genus Culex are usually pointed as the main vectors, but Anopheles can also infect some birds. The exoerythrocytic merogony occurs in the endothelial cells, in the hemopoietic cells (erythrocytic series) and lymphoid macrophage systems. Sporozoites presented in the salivary glands of the vectors are injected in the birds during the blood meal (Valkiuñas, 2005, Atkinson et al., 2008).

A first generation of the exoerythrocytic meronts results in cryptozoites containing the sporozoites that develop in the reticular cells of many tissues, including skin. These first sporozoites induce the generation of the second exoerythrocytic meronts that results in metacryptozoites full of merozoites, which

development occurs in macrophages. These second generation of merozoites can already infect cells from the erythrocytic series in hemopoietic system. A great number of these merozoites invade the erythrocytes, starting both agamic stages and gametocytes in blood (Valkiuñas, 2005, Atkinson et al., 2008).

Inside the erythrocytes, some of the parasites become rounded and give rise to trophozoites that can have a ring-like shape in some species of malaria parasites. It can start an erythrocytic meront after its first nucleus division, in which the merozoites are developed. Other sporozoites start to rise the gametocytes while the second exoerythrocytic merogony starts (phanerozoites) in the endothelial cells of capillaries of many organs (e. g. the brain). The phanerozoites keep the production of merozoites to maintain the parasitemia during the chronic infections (Valkiuñas, 2005, Atkinson et al., 2008).

The macrogametocytes and microgametocytes develop into the mature erythrocytes in the blood. During the acute stage of parasitemia, a large number of gametocytes can be found in the erythrocytes. However, in the chronic stage, the parasitemia decreases and a few number of parasites can be seen, and in the latent phase, the haemosporidians can disappear from the peripheral blood, persisting only within the organs. The number of *Plasmodium* in the blood can have some short-term recrudescences after relapses, creating a second parasitemia. The recrudescences can happen during the breeding and the offsprings may be infected, maintaining the parasites in the birds. These gametocytes infect the vectors that fed on infected birds and become rounded and escape from the erythrocytes. The gamonts start the gametogenesis in the midgut, resulting in the ookinetes (Valkiuñas, 2005, Atkinson et al., 2008).

The ookinete crosses the epithelial tissue of the midgut, becomes rounded at the basal lamina and develops into an oocyst (sporogony), with a capsule-like made from the hosts' cells. During the sporogony, a large number of sporozoites are formed inside the oocyst (Valkiuñas, 2005, Atkinson et al., 2008).

Once the oocyst is mature, the sporozoites go to the haemocoele and penetrate the salivary glands of the dipteran. Then, the sporozoites are transmitted to the birds during the bitting, when the insect injects the salivary secretion containing the sporozoites (Valkiuñas, 2005, Atkinson et al., 2008, Dimitrov et al., 2015). The *Figure* 5 shows the life cycle of *Plasmodium relictum*.

2.3 PATHOGENESIS, PATHOLOGY AND CLINICAL SIGNS

2.3.1 *Haemoproteus* sp.

Severe infection of *H. columbae* in *Columba livia* may cause weakness, anemia and anorexia, and can also be lethal, although most pigeons do not show any clinical signs (Valkiuñas, 2005, Atkinson et al., 2008).

Haemoproteus has been considered as apathogenic but a few cases of mortality were reported, while *H. meleagridis* can cause severe impact on turkey flocks as they might show weight loss and low growth rates (Atkinson et al., 2008, Earle et al., 1993). Earle et al. (1993) reported pathological findings in captive G*allicolumba luzonica* in Prettoria, Africa. These doves showed a high infection with *H. columbae* detected in blood films and histopathology. They also reported a high number of *P. canariensis* where these birds lived.

Usually the first generation of exoerythrocytic meronts of haemosporidians do not cause a severe damage to birds. Although in heavy infections, the capillaries of lungs of birds can be obstructed due to the presence of a large number of meronts in this organ, that can cause a severe inflammatory reaction. It can lead to pneumonia-like symptoms and may cause death in young birds (Valkiuñas, 2005, Atkinson et al., 2008).

The development of megalomeronts in the endothelial cells of capillaries, especially in skeletal and myocardial muscles cause a severe inflammatory reaction with a local concentration of erythrocytes and lymphocytes. The rupture of megalomeronts lead to necrosis and myopathy. Calcifications adjacent to meronts have been described (Valkiuñas, 2005, Atkinson et al., 2008).

Regardless of these exoerythrocytic alterations, the main lesions occur intravascularly, with the destruction of blood cells, causing anaemia. The lytic hemopoietic system can remove the erythrocytes from the circulation and an acute anemia can be diagnosed if the hematopoiesis does not compensate this loss of blood cells. Usually infections with *Haemoproteus* sp. cause less severe anemia (Valkiuñas, 2005, Atkinson et al., 2008). Earle et al. (1993) affirmed that only the birds that survive to acute myopathy can demonstrate gametocytes in the peripheral blood.

1 2.3.2 *Plasmodium* sp.
2
3 Avian malaria parasite *Plasmodium* cause severe disease of the blood and
4 reticuloendothelial system. During *Plasmodium* invasion, the phanerozoites and
5 metacryptozoites can cause the most severe lesions compared with *Haemoproteus*,
6 due to an obstruction of capillaries of the brain and principal organs, which can result
7 in anoxia with necrosis of the damaged tissues, resulting in cerebral paralysis. There
8 can be hemolysis associated with the development of erythrocytic meronts. Some
9 changes in the chemical composition of the blood plasma in birds cause a less
10 effective circulation of oxygen, leading to hypoxia (Valkiuñas, 2005, Atkinson et al.,
11 2008).
12 Biliverdin is the product of hemoglobin catabolism, that can be found in feces.
13 The feces color turns to green color four days after infections, and can produce thin
14 and mucoid diarrhea due to avian malaria (Atkinson et al., 2008).
15 Malaria parasites in birds can cause severe diseases. Weakness, reduced
16 motion coordination, anorexia, ruffled feathers, neurological symptoms, paralysis and
17 death have been described (Valkiuñas, 2005, Atkinson et al., 2008). *Plasmodium*
18 *relictum*, *P. elongatum*, *P. gallinaceum* and *P. durae* can cause death in zoo birds,
19 domestic fowls and turkey flocks, due to avian malaria (Earle et al. 1993). The reports
20 about avian malaria usually occur in captive birds (Atkinson et al., 2008).
21 Due to anemia, anoxia and erythrocytes agglutinations may occur, resulting in
22 endothelial damage, development of microemboli, especially in spleen. Edema,
23 hemorrhage can also be evident in the heart, lungs, kidneys and brain. Myopathy
24 with degeneration of capillaries in skeletal muscles and the presence of
25 macrophages is common (Atkinson et al., 2008).
26
27 2.4 DIAGNOSIS
28
29 2.4.1 Giemsa staining of blood films
30
31 Traditional techniques are based on morphologic descriptions of the
32 erythrocytic stages by optic microscopy, using blood smears (Waldestrom et al.,
33 2004, Dimitrov et al., 2015). The Giemsa staining is the gold stardard and is used
34 worldwide for the detection of hemoparasites (Waldestrom et al., 2004, Atkinson et

al., 2008). Usually young and mature intraerythrocytic gametocytes are identified and characterized morphologically according to Valkiuñas (2005) and Atkinson et al. (2008). Erythrocytic meronts can also be found in *Plasmodium* infections (Atkinson et al., 2008). However, if the meronts are not circulating, it is hardly possible to distinguish between *Haemoproteus* and *Plasmodium* (Atkinson et al., 2008).

The prevalence and intensity of parasitemia are also calculated, based on Bush et al. (1997) and Godfrey et al. (1987), respectively. The prevalence is described by Bush et al. (1997) as the number of hosts infected by a parasite from a total number of hosts. It is used in statistical analyse in percentage, to describe the presence-absence of parasites in a population of hosts. Godfrey et al. (1987) proposed a method to quantify the intensity of parasitemia of *Haemoproteus* in stained thin blood smears, examined at 1000x through microscopy. The parasitemia was quantified by counting the intraerythrocytic parasites in 20 replicates of 100 erythrocytes per field (approximately 2,000 erythrocytes).

2.4.2 Molecular characterization by PCR

Molecular diagnostics based on Polymerase Chain Reaction have been largely used for the identification and differentiation of genera (Atkinson et al., 2008, Krizanauskiene et al., 2013, Dimitrov et al., 2015). These methods have improved the studies of haemosporidian parasites, revealing a huge genetic diversity and the possibility to identify undescribed species (including cryptic species) (Beadell and Fleischer, 2005, Bensch et al, 2009, Valkiuñas et al, 2010, Levin et al., 2011), neverthless it is hard to distinguish individual species (Atkinson et al., 2008). However, many primers designed can distinguish one genus from the other (Hellgren et al., 2004, Atkinson et al., 2008). Still, the sequencing is necessary to identify the lineages and phylogenetic relationships (Vallkiunas, 2005, Atkinson et al., 2008).

Several investigations worldwide have described the occurrence of haemosporidians in birds in the last few years using primers that can be observed in Figure 6 (Hellgren et al., 2007, Martinsen et al., 2008, Valkiuñas et al., 2010; Krizanauskiene et al., 2013, Valkiunas et al., 2013). Most of these are not only based on morphology, but also genetic and phylogenetic analyse (Palinauskas et al., 2015, Tostes et al., 2015). In pigeons, molecular studies have revealed new species and

new hosts (Valkiuñas et al., 2010; Santiago-Alarcon, et al., 2010, Valkiunas et al., 2013).

In fact, four species of *Haemoproteus* were described in pigeons and doves (*H. columbae*, *H. sacharovi*, *H. maccallumi* and *H. palumbis*), and during a survey that found *H. columbae* and *H. saccharovi* in blood smears, *H. maccallumi* was identified in *Zenaida macroura*, but it is thought to be one of those species (Valkiunas 2005, Atkinson et al., 2008). Valkiuñas et al. (2010) described a new species named *Haemoproteus multipigmentatus* in *Zenaida galapagoensis*. All of these species are transmitted by hippoboscid flies (Valkiuñas, 2005, Valkiuñas et al., 2010). Santiago-Alarcon et al. (2010) reported several *Haemoproteus* lineages closely related to *H. multipigmentatus* in *Z. aurita* (Caribbean Islands), *Z auriculata* (Ecuador and Venezuela), *Z. meloda* (Peru), *Columbina buckleyi* (Ecuador), *C. cruziana* (Ecuador), *C. talpacoti* (Guatemala) and *Geotrygon montana* (Ecuador).

Despite the high sensitivity of PCR, the many developed assays for avian haemosporidian parasites detection underestimate mixed infections, which is why traditional microscopy is still needed to describe the species (Valkiunas et al., 2006, Krizanauskiene et al., 2013, Carlson et al., 2013).

2.4.3 Sequencing and Phylogenetic trees

Despite of the usage of different PCR assays to detect haemoporidian parasites, sequencing is still necessary to identify parasite lineages and to determine phylogenetic relationships (Atkinson et al., 2008).

Most of phylogenetic studies of avian haemosporidian parasites were based on mitochondrial DNA (mtDNA) cytochrome b (cyt b), revealing many parasite lineages of the genera *Haemoproteus* and *Plasmodium*. It has been used successfully to improve the hemosporidians taxonomy (Atkinson et al., 2008; Iezhova et al., 2011). Although most of studies were done in passerine birds, some lineages from Columbidae have been reported in the last years (Iezhova et al., 2011; Atkinson et al., 2008).

A public database called MalAvi has been organized to improve the studies on hemosporids diversity in birds based on mitochondrial cytochrome b gene (Bensch et al., 2009). According to MalAvi databases, there are five descriptions of *Haemoproteus* species in *Z. auriculata* (Bensch et al., 2009). No data is reported

1 relative to *Plasmodium* in this dove. The number of described hemoparasites in
2 doves from the *genus Zenaida* and other pigeons worldwide can be seen by
3 accessing http://mbio-serv2.mbioekol.lu.se/Malavi/.

4 Valkiuñas et al. (2010) described the presence of *Haemoproteus*
5 *multipigmentatus* in *Zenaida galapagoensis* for the first time, in Galapagos Islands
6 using blood smears and PCR.

7 In Socorro Island, Carlson et al. (2013) analyzed 25 *Z. macroura* and 23 *Z.*
8 *graysoni*, to identify haemosporidian in blood smears and PCR. In *Z macroura*, they
9 found 23/25 (92%) positives for haemosporidians and in *Z. graysoni*, 17/23 (74%).
10 They found 18 different lineages of *Haemoproteus* in doves, most of them were *H.*
11 *columbae* and *H. multipigmentatus* and one undescribed lineage. No *Plasmodium* or
12 Leucocytozoon were detected by PCR, although there was one blood film with
13 *Plasmodium* sp. from *Z. macroura*. They also found six *Z. macroura* co-infected with
14 more than one *Haemoproteus* species by PCR.

15 Krizanauskiene et al. (2013) studied 38 *Z. macroura* from Northern California
16 and 11 *Columbina passerina socorroensis* from Socorrro Island and identified *H.*
17 *saccharovi*.

18 *Haemoproteus (Haemoproteus) multivolutinus* n. sp. from dove *Turtur*
19 *timpanistria* of Uganda and *Haemoproteus (Haemoproteus) paramultipigmentatus* n.
20 sp. from *Columbina passerina socorroensis* of Socorro Island, Mexico, were first
21 described by Valkiunas et al. (2013), increasing the number of *Haemoproteus*
22 species in doves and pigeons.

23 Scaglione et al. (2015) examined 51 *Columba livia* from Italy by PCR and
24 found 15/51 (29.4%) birds positive for *Haemoproteus*/*Plasmodium* spp DNA. No
25 significant association was identified between age or sex and infection status.
26 However, the coinfection was very significant ($p<0.01$), showing that the presence of
27 a hemoparasite could predispose to another haemosporidian infection. DNA
28 sequencing showed P-SGS 1 (*P. relictum*) and H-HAECOL1 lineage 13 (*H.*
29 *columbae*). González et al. (2015) found one *Columba livia* parasitized by *H.*
30 *columbae* in Colombia. Martínez et al. (2016) found 100% *Z. auriculata* parasitized
31 by *H. multipigmentatus* in Chile (unpublished data).

32 Some authors agree that the use of both PCR and blood microcopy should be
33 more sensitive than using just one technique (Carlson et al., 2013, Valkiuñas et

al.,2010) mainly to find co-infected birds. However, Valkiunas et a. (2008) showed no differences between both methods.

2.5 Co-infections

Co-infections are often observed in wild birds. PCR usually cannot identify mixed hemosporidian infections (Valkiuñas et al., 2006), although sometimes double peaks on sequence can be seen in electropherograms (Martínez et al., 2009, Dimitrov et al., 2015). The morphological studies could help to identify these mixed infections in blood films (Valkiuñas et al., 2010). Restriction enzyme-based assays have been studied to distinguish the haemosporidians (Martínez et al., 2009, Beadell and Fletcher, 2005, Kistler et al., 2013), but some enzyme restriction sites can show errors as sequences incorrectly assigned at the genus level, probably due to different lineages of the parasites (Martínez et al., 2009). PCR products not well digested can appear as multigenus infection (Beadell and Fleicher, 2005).

2.6 Vectors

Subgenus H. (*Haemoproteus*) is transmitted by hippoboscidae flies to pigeons. *Pseudolynchia canariensis* was described to be the vector of *H. columbae*, *H. saccharovi*, *H. maccallumi* and *H. turtur*, and *Ornithomyia avicularia* as the vector of *H. palumbis* (Atkinson et al., 2008). *Plasmodium* spp. has a life cycle that usually presents insects from genus Culex as their vector and definitive host, but Anopheles can also infect some birds (Valkiunas 2005, Atkinson et al., 2008).

Adriano (1999) found *H. columbae* and reported the presence of *P. canariensis* and *Stilbometopa* sp. in *Z. auriculata* in São Paulo State, Brazil. Valkiuñas et al. (2010) reported the presence of *H. multipigmentatus* in *Microlynchia galagoensis*. Levin et al. (2011) suspected that *Olfersia* sp., a hippoboscid fly found in Galapagos Islands, could transmit *Haemoproteus iwa* to frigatebirds.

3 *EIMERIA* IN PIGEONS

Eimeria is a host specific and obligate intracellular parasite that infects all vertebrates. It is a coccidian, belonging to the phylum Apicomplexa, order Eucoccidiorida, family Eimeriidae (Cole 1999, Atkinson et al., 2008, Berto et al., 2014). It is found in many animals, being the genus of the largest biodiversity in order Eucoccidiorida (Berto et al., 2014). The parasites are found primarily in the intestines, but there are some species that can also cause damages to the liver, spleen, lungs, kidneys and uterus, for its tissue development (Berto et al., 2014).

In wild birds, they can infect the intestines, kidneys and liver. In Columbiformes, they can cause severe intestinal lesions. However, most of free-living birds present asymptomatic infections, even if they shed oocysts. Usually young birds and unhealthy adults can develop clinical signs of coccidiosis, which also depend of inoculation dose, stress, previous infection, coinfection with other diseases and immunocompetence (Cole, 1999; Atkinson et al., 2008).

Eimeriosis is known worldwide for causing intestinal diseases, which produce important economic losses in poultry (chickens, geese and turkeys). Many species have already been described (around 200 species from different avian orders), but there are *Eimeria* species not well characterized, especially wild avian coccidia (Atkinson et al., 2008).

Columbiformes have already been known to host at least 12 *Eimeria* species (Atkinson et al., 2008). Despite the fact that *Eimeria* is highly host specific, closely related host can show the same *Eimeria* oocysts. Anseriformes, Passeriformes and Galliformes are the most reported orders with *Eimeria*, but they are usually infected by Isospora. Columbiformes are more likely to shed *Eimeria* oocysts rather than Isospora ones (Atkinson et al., 2008).

3.1 MORPHOLOGY

Eimeria sporulated oocysts contain four sporocysts and two sporozoites inside each sporocyst. In *Figure* 7 a drawing of the sporulated oocyst described in Berto et al. (2014) is shown.

The oocyst wall shows two layers surrounded by an outer membrane. Usually, the oocyst wall can show some color and it is used for species descriptions, but the

1 different colors could be due to the preservatives, light intensity or the filter used at
2 the microscope. However, the color (lighter or darker) between the two layers can be
3 important as in *Eimeria leuckarti*. The texture of the outer membrane can be smooth
4 or rough and some protruding structures such as spines and conic projections can be
5 found in this surface. The micropyle is a discontinuity either in the inner or outer
6 layer. A micropyle cap can be seen in some species, covering the micropyle. An
7 oocyst residuum can be seen between the sporocysts as a regular and compact
8 mass or an irregular mass of granules. A polar granule can also be located between
9 the sporocysts and its density and shapes can help to determine the species (Berto
10 et al., 2014).

11 The complex of Stieda and substieda bodies can show different sizes and
12 shapes. These differences can also be determinant to describe the species. Some
13 *Eimeria* can also show a parastieda body at the opposite end of the oocyst. The
14 sporocyyst residuum is described to be diffuse among sporozoites or as a compact
15 mass of granules. The sporozoites show refractile bodies, centralized nucleus and
16 striations (Berto et al., 2014).

17 Despite the morphologic characteristics, all these structures are also
18 measured, especially the length and width of the oocyst, sporocyst, Stieda and
19 substieda bodies. Depending on the length and width ratio, the oocysts are described
20 as (e. g.) ovoid, ellipsoidal and botttle-shaped (Berto et al., 2014).

21 On Table 2 it can be observed the 16 species described to parasitize birds
22 from order Columbiformes, adapted from Ball et al. (2012).
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1 **Table 2** – *Eimeria* species described in Columbiformes birds.

Columbiformes species	*Eimeria* species	References
Columba livia	*E. labbeana*	Labbé, 1896
	E. columbarum	Nieshoulz, 1935
	E. columbae	Mitra & Das Gupta, 1937
	E. tropicalis	Malhotra & Ray, 1961
	E. kapotei	Chatterjee & Ray, 1969
	E. janovyi	Bandyopadhyay, Bhakta & Shukla, 2006
	E. livialis	Alyousif, Al-Shawa & Al-Asiri, 2009
	E. columbadomestica n. sp.	Yang et al., 2016
Sphenocercus sphenurus	*E. sphenocerae*	Ray, 1952
Streptopelia decaocto	*E choudari*	Bhatia, Chauhan, Arora & Agrawal, 1972
Streptopelia turtur	*E. turturi*	Golemansky, 1976
Chalcophaps indica	*E. waiganiensis*	Varghese, 1978
Ducula spilorrhoa	*E. duculai*	Varghese, 1980
Goura victoria	*E. gourai*	Varghese, 1980
Zenaida galapagoensis	*E. palumbi*	*McQuistion, 1991*
Columbina talpacoti	*E. curvata*	Adriano, Thyssen & Cordeiro, 2000
Zenaida auriculata	*E. zenaidae*	Adriano, Thyssen & Carneiro, 2003
Nesoenas mayeri	*E. mauritiensis*	Ball et al., 2012

2 Adapted from Ball et al. (2012).

3

3.2 Life Cycle

Eimeria have direct life cycle and the asexual and sexual reproductions occur in intestinal epithelial cells. The unsporulated and not infective oocyst is found in feces, and after the sporulation in the environment, it becomes infective (sporulated). Sporocysts and sporozoites are produced during the sporogony, which occurs in the environment depending on the oxygen level, light and temperature. The sporulated oocysts are very resistant tolerating dehydration and freezing temperatures, although very low and very high temperatures may kill them (Cole 1999; Atkinson et al., 2008).

Inside the host by ingestion, the digestion ruptures the oocyst wall, releasing the sporocysts and after, the sporozoites. The sporozoites invade intestinal epithelial cells and become trophozoites. The trophozoites replicate to schizonts, which develop thousands of merozoites inside by merogony. The merozoites break the cells' wall and invade other cells to develop more merogony. After some generations, some merozoites start the gametogony. They become to gametocytes (macrogametocytes and microgametocytes) which develops the gametes. The macrogametocyte develops one macrogamete and the microgametocyte develops lots of flagellated microgametes. The microgametes exit the cell and go into the cell containing the macrogametes and their fusion develop an oocyst. The oocyst can exit the host cell and get the environment with the feces (Cole 1999; Atkinson et al., 2008).

3.3 Pathogenesis and Clinical Signs

The disease has not been described in free-living pigeons and doves, although *Eimeria labbeana* and *E. columbarum* have already caused losses in captive Columbiformes (Atkinson et al., 2008). Asymptomatic birds have low-intensity infections, which destroy a few epithelial cells that can be replaced rapidly. However, in moderate and high intensity enteritis, the intestinal absorption is damaged, causing less absortion of food and water, causing dehydration, mucoid diarrhea or intestinal hemorrhage, anorexia, loss of coordination, lethargy, ruffled feathers, decreased weight gain, loss of egg production and death (Cole 1999, Atkinson et al., 2008).

3.4 PATHOLOGY

Lesions associated with eimeriosis depends on the host, intensity of infection and the *Eimeria* species (Atkinson et al., 2008). The intestines can become discolored to hemorrhagic, with mucoid feces and ballooned shape. Loss of mucosa can also be seen. These lesions can predispose the gut to other infections, such as clostridiosis and salmonellosis. Intraepithelial forms (meronts, gamonts and oocysts) are easily observed (Atkinson et al., 2008).

3.5 DIAGNOSIS

The detection and identification of oocysts in feces by microscopy can be done in birds that show clinical signs or after necropsy. Flotation techniques are used to observe the oocysts, such as zinc sulfate and Sheather's sugar. To identify the species, the oocysts have to be sporulated. A potassium dichromate 5% is added to the feces, this solution is placed in a Petri dish at room temperature to be in contact with oxygen to sporulation. After sporulation, the solution must be stored at 4 °C to maintain the morphologic characteristics (Atkinson et al., 2008).

Morphologic characterization of oocysts is used for routine diagnosis and also for systematic studies with the species descriptions. Other studies can complement this characterization for species identification, including PCR and sequencing (Berto et al., 2014). The main DNA sequences used for *Eimeria* diagnostics are: nuclear 18S rDNA locus, ITS regions or mitochondrial cytochrome c oxidase subunit I (cox-1, COI). Dolnik et al. (2009) reported the molecular study of single isolated oocysts to provide more confident data for *Eimeria* speciation.

Baliska-Ramisz et al. (2014) studied 180 *Columba livia* from two different lofts in a husbandry in West Pomerania Province. They had respectively in loft I and loft II: 17/19 (89%) and 62/67 (93%) of young pigeons, 27/43 (63%) and 28/51 (55%) of adult pigeons with *Eimeria* sp. Morphologically, the oocysts found were described as *E. labbeana*, *E. columbarum* and *E. columbae*.

Ball et al. (2012) described for the first time the *Eimeria mauritiensis* in 5 *Nesoenas mayeri* in Mauritius Island; also in 2012, Jamriska and Modrý described

1 *Eimeria columbapalumbi* n. sp. for the first time, from *Columba palumbus* in Czech
2 and Slovak Republics.
3 Radfar et al. (2011) identified 41/102 (40.19%) of *Columba livia* with *Eimeria*
4 sp. by Sheather's method in Iran. Marques et al. (2007) studying *Columba livia* in
5 Santa Catarina, Brazil, found 37/43 (86.05%) of pigeons with *Eimeria* sp. oocysts by
6 Sheather's method.
7 Natala et al. (2009) verified 250 *Columba livia* and found 49.2% positive for
8 *Eimeria* sp. Sari et al. (2008) studied 136 domestic pigeons and 115 wild pigeons in
9 Turkey. In domestic pigeons, they found 81/136 (59.6%) positive domestic pigeons
10 and 35/115 wild pigeons with coccidian oocysts. They identified *E. labbeana*, *E.*
11 *columbarum*, *E. columbae* and *Isospora* sp. through morphology.
12 González et al. (2004), which studied *Z. auriculata* in Chile and found 13/235
13 (5.5%) of then shedding oocysts of *E. labbeana*, revealing a low prevalence too.
14 Adriano et al. (2003) first described a new species of *Eimeria* in *Z. auriculata*
15 in Brazil that was called *Eimeria zenaidae* n. sp. They studied 142 doves by flotation
16 with Sheather's sugar solution and obtained 34 (23.9%) animals shedding *Eimeria*
17 oocysts. Adriano et al. (2000) described *Eimeria curvata* n. sp. in 8/46 *Columbina*
18 *talpacoti* and 5/39 *Scardafella squamatta* in Brazil.
19 In 1991, McQuistion found a new coccidian in *Zenaida galapagoensis*, named
20 *Eimeria palumbi*, at the Galapagos Island. Conti and Forrester (1981) studied two
21 populations of *Zenaida macroura* and one population of *Zenaida asiatica* in Florida,
22 USA and they found *Eimeria* sp. in 33.3%, 49.1% and 6% pigeons, respectively.
23 As 18S rDNA has shown a lack of reliability on speciation, researchers
24 employed the mitochondrial COI sequence as an alternative marker for molecular
25 identification. Although the usage of COI gene has been increasing in the last few
26 years, there is not many information in public databases about coccidian COI
27 sequences (Berto et al., 2014).
28 The sequencing may be used to get definitive identifications, as it allows to
29 build phylogenetic studies between different species that can be or not closely-
30 related. Morphologic characterization of the oocyst and molecular identification
31 together would improve the knowledge of coccidian parasites and the course of
32 coccidiosis in animals (Berto et al., 2014).
33

4 FINAL CONSIDERATIONS

The haemosporidian parasites and *Eimeria* have been studied worldwide in several different hosts. However, there is a lack of information of these parasites in doves. The studies on parasitism in Columbiformes have increased recently, but more investigation is needed in the Neotropics. More descriptions are important to understand the diversity, distribution and prevalence of haemosporidian parasites and *Eimeria* in Brazil. This work was done because these informations can help to understand the impact of these parasitic diseases in pigeons.

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1 ***Figure* 1** – Specimen of *Zenaida auriculata* (Des Murs, 1847).

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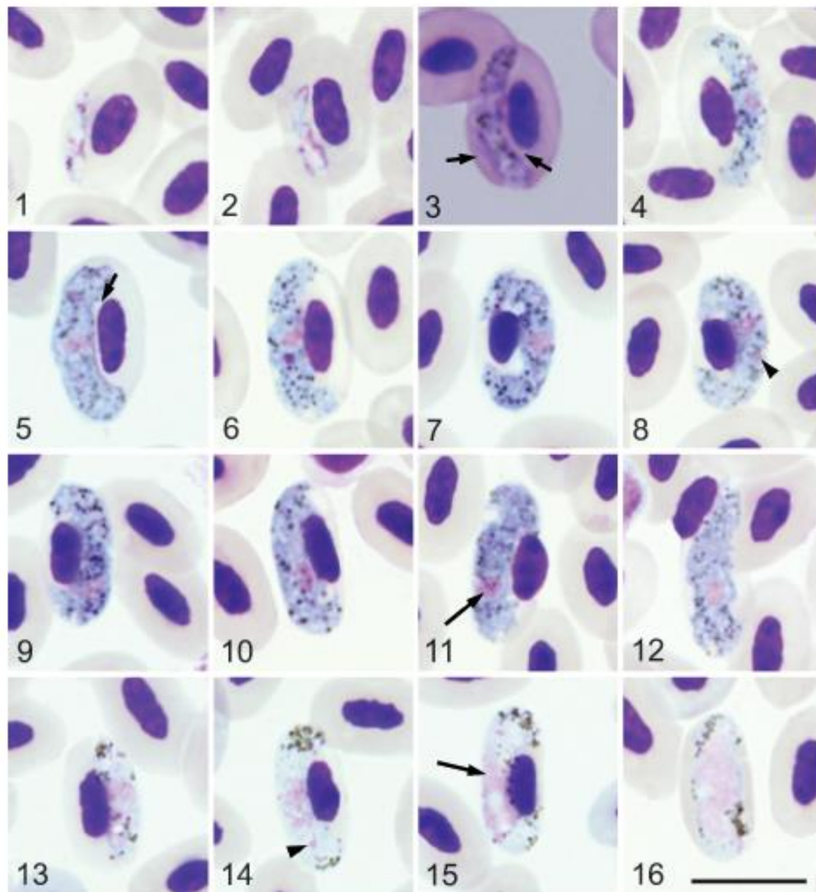
1 ***Figure 2*** – *Haemoproteus* (*Haemoproteus*) *multipigmentatus* from *Zenaida*
2 *galapagoensis*



3
4 (1, 2) Young gametocytes. (3–12) Macrogametocytes. (13–16) Microgametocytes. Long arrows, nuclei
5 of parasites. Short arrows, unfilled spaces among gametocytes and envelope and nuclei of infected
6 erythrocytes. Arrow heads, azurophilic granules. (1, 2, 4–16) Giemsa-stained thin blood films. (3)
7 Field-stained thin blood films. Bar = 10 μm.
8 From Valkiunas et al. (2010)
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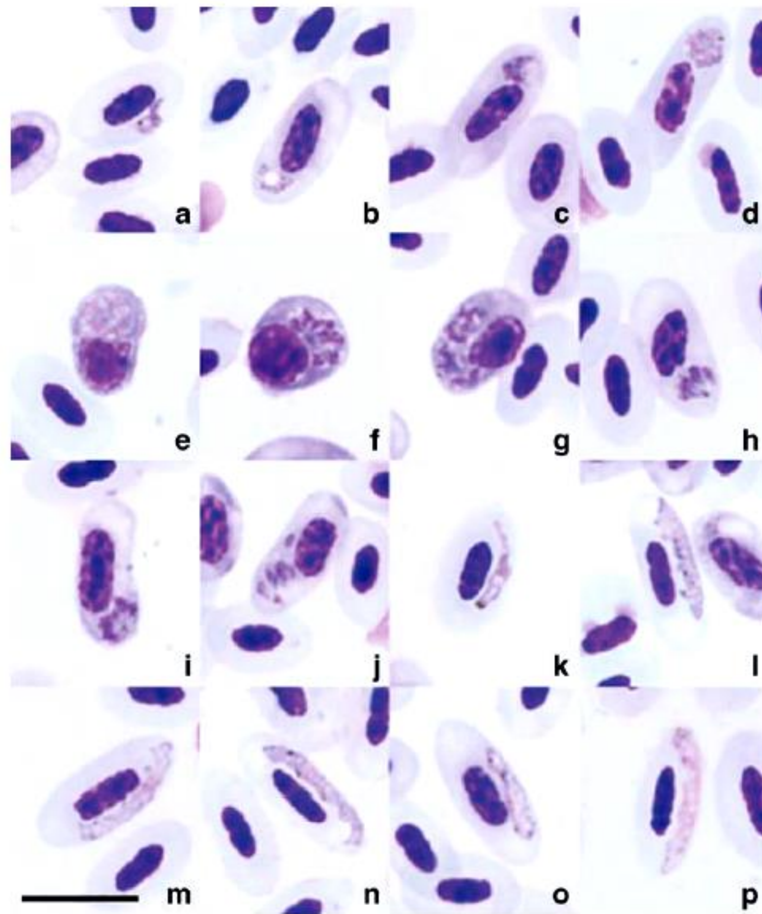
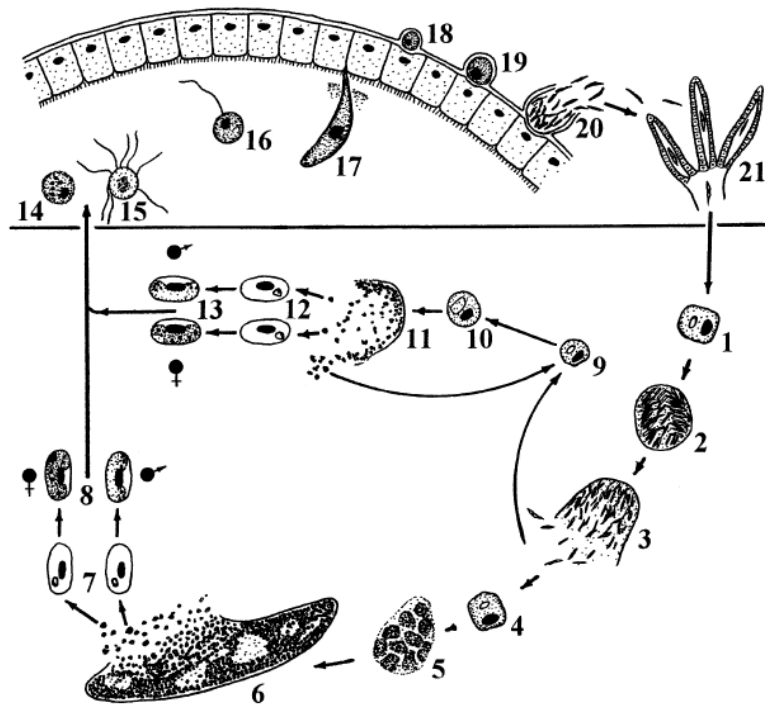
***Figure* 3** – *Plasmodium elongatum* from the blood of *Acrocephalus arundinaceus*



a,b = trophozoites, c-j = erythrocytic meronts, k-n macrogametocytes, o, p = microgametocytes.
From Valkiunas et al. (2008).

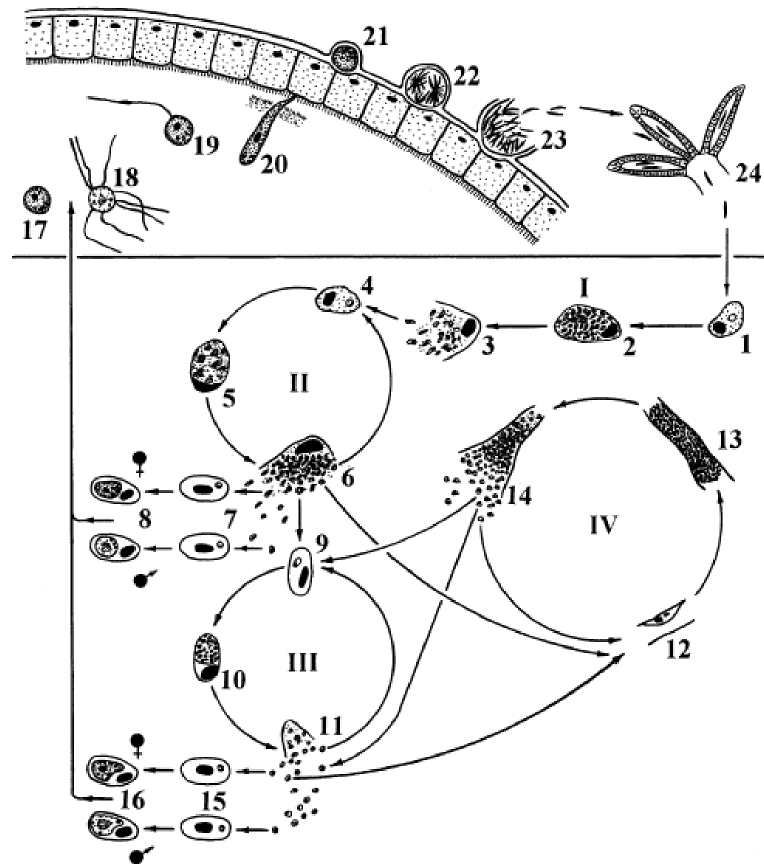
Figure 4 Diagrammatic representation of the life cycle of bird haemoproteids (*Haemoproteus mansoni* as an example).



Upper part, in vector; lower part, in bird: *1* – sporozoite in endothelial cell; *2*, *3* – exoerythrocytic meronts of the first generation with elongated merozoites; *4* – merozoite in endothelial cell; *5*, *6* – growing and mature megalomeronts in skeletal muscles, respectively; *7* – merozoites in erythrocytes; *8* – mature gametocytes; *9* – merozoite in reticuloendothelial cell in spleen; *10*, *11* – growing and mature meronts in spleen, respectively; *12* – merozoites in erythrocytes; *13* – mature gametocytes; *14* – macrogamete; *15* – exflagellation of microgametes; *16* – fertilization of macrogamete; *17* – ookinete penetrating the peritrophic membrane; *18* – young oocyst; *19*, *20* – sporogony; *21* – sporozoites in the salivary glands of vector.

From Valkiuñas (2005)

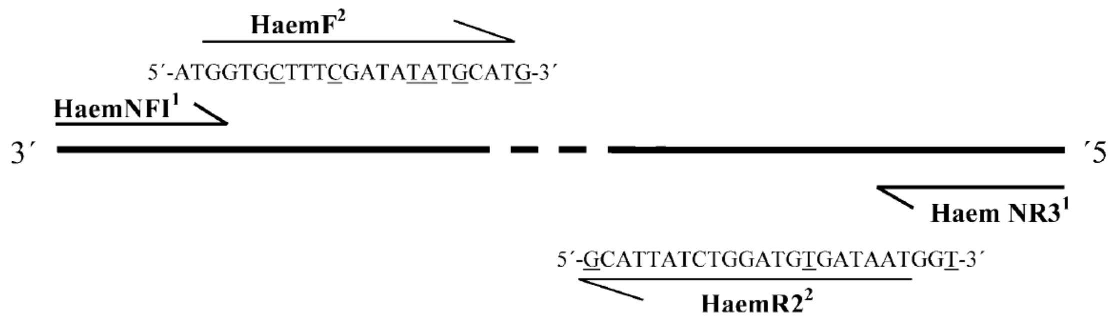
Figure 5 - Diagrammatic representation of the life cycle of bird malaria parasites (*Plasmodium relictum* as an example):



Upper part, in vector; lower part, in bird: I, II – primary exoerythrocytic merogony; III – erythrocytic merogony; IV – secondary exoerythrocytic merogony; *1* – sporozoite in reticuloendothelial cell; *2*, *3* – cryptozoites; *4* – merozoite in macrophage; *5*, *6* – metacryptozoites; *7* – merozoites in erythrocytes; *8* – gametocytes; *9* – merozoite in erythrocyte; *10*, *11* – erythrocytic meronts; *12* – merozoite in endothelial cell of capillaries; *13*, *14* – phanerozoites; *15* – merozoites in erythrocytes; *16* – gametocytes; *17* – macrogamete; *18* – exflagellation of microgametes; *19* – fertilization of macrogamete; *20* – ookinete penetrating the peritrophic membrane; *21* – young oocyst; *22*, *23* – sporogony; *24* – sporozoites in the salivary glands of vector.

From Valkiuñas (2005)

1 Figure 6 – Diagram of the directions and combinations of the primers HaemNFI,
2 HaemNR3, HaemF and HaemR2 according to Hellgren et al. (2004).



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4 Figure showing: **1.** Outer primers for DNA amplification at the first step of nPCR **2.** Inner primers for
5 amplification at the second step of nPCR of both *Haemoproteus* sp. and *Plasmodium* sp.

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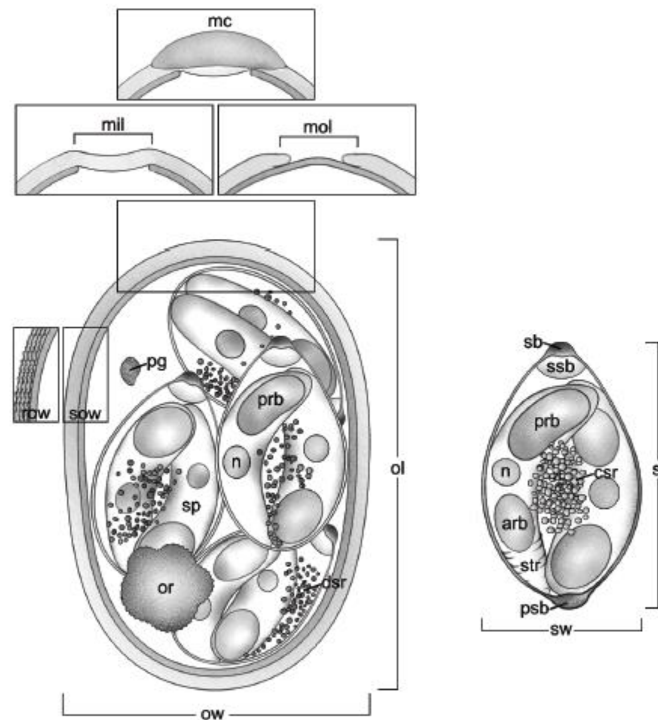
1 ***Figure* 7** – Diagramm of a sporulated oocyst of *Eimeria* with detailed intrastructures
2 to morphologic and morphometric characterization.



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4 (ow) oocyst width; (ol) oocyst length; (pg) polar granule; (or) oocyst residuum; (row) rough or (sow)
5 smooth outer wall, micropyle in (mil) inner layer or (mol) outer layer; (mc) micropyle cap; (sw)
6 sporocyst width; (sl) sporocyst length; (sb) Stieda body; (ssb) substieda body; (psb) parastieda body;
7 (csr) compact of (dsr) difuse sporocyst residuum; (sp) sporozoite; (prb) posterior and (arb) anterior
8 refractile body of the sporozoite; (n) sporozoite nucleus; and (str) sporozoite striations.
9 From Berto et al. (2014)
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5 OBJETIVOS

5.1 Objetivo Geral

- Determinar a presença de hemosporídeos e *Eimeria* sp. em *Zenaida auriculata* capturadas no município de Londrina, Paraná.

5.2 Objetivos específicos

- Verificar a ocorrência de hemosporídeos e *Eimeria* em *Z. auriculata* por meio de PCR;
- Identificar e caracterizar as espécies de hemosporídeos encontradas e verificar suas relações filogenéticas;
- Comparar as técnicas de Willis & Mollay e PCR para detecção de *Eimeria* sp. em fezes de pombos.

1 **6 ARTICLE I**

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16 # Occurrence of *Haemoproteus* sp. and *Plasmodium* sp. in *Zenaida*
17 # *auriculata* from Londrina-PR, Paraná State

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Occurrence of *Haemoproteus* sp. and *Plasmodium* sp. in doves *Zenaida auriculata* from Londrina, Paraná State

ABSTRACT

The aim of this study was to verify the presence and identify the species of haemosporidian parasites in trap-captured *Zenaida auriculata* from Londrina, Paraná, Brazil. Two hundred eleven male and female birds were trap-captured. Whole blood was collected in EDTA tubes through heart punction after euthanasia with CO_2. The samples were maintained at -20 ºC until they were used for PCR. A nPCR was performed to identify the mitochondrial cytochrome b gene (cyt b) of *Haemoproteus* sp./ *Plasmodium* sp. showing amplicons with 525bp lengh bands. A RFLP-PCR was performed to identify the genus *Haemoproteus* or *Plasmodium*. Moreover, a nPCR based on small subunit ribosomal ribonucleic acid (18S SSU rRNA) specific for *Plasmodium* was performed, searching for a fragment of DNA with 240bp. The sequencing from the second reaction of nPCR based on cyt b was carried out to identify the species. Phylogenetic trees were built to determine the closely related species. All 211 doves were positive at the nPCR based on cyt b, showing 100% of doves positive for the presence of *Haemoproteus* and/or *Plasmodium*, and nPCR and 18S SSU rRNA (*Plasmodium*). RFLP showed 155/211 (73.46%) positives only for *Haemoproteus* sp., 13/211 (6.16%) positives only for *Plasmodium* sp., 41/211 (19.43%) for both genera. The positivity for both genera by RFLP suggests a mixed infection with both genera. The phylogenetic trees showed four doves homologous with *Haemoproteus multipigmentatus* and one with *Plasmodium* sp. *Z. auriculata* had been previously described as host for *H. multipigmentatus* in Ecuador and Venezuela, showing that this parasite is present in South America. More accurate results in sequencing could be provided through amplification of the genes in specific enzyme restriction sites of *Haemoproteus* and *Plasmodium* after RFLP, showing more information about the species found in mixed infections. This is the first molecular study of hemoparasites from *Z. auriculata* in Brazil and it is also the first description of *H. multipigmentatus* and *Plasmodium* sp. infection detected by nPCR, RFLP-nPCR and sequencing in *Z. auriculata* in Brazil. Further studies are need to identify mixed infections and other blood parasites.

Key-words: haemosporidian, avian malaria, blood parasites, Columbidae, PCR

6.1 INTRODUCTION

Zenaida auriculata (Des Murs, 1847) *(*Aves: Columbiformes) is a native bird from Brazil that can also be found both rural and urban areas until Southern Argentina (Goulart et al., 2011; Shibatta et al., 2009; Cândido Jr. et al., 2008). These birds are pests of crops and even in the urban areas (Goulart et al., 2011, Cândido Jr. et al, 2008, González, et al., 2004, Adriano et al., 2003), as they have adapted easily to urbanization becoming synanthropic, housing and getting food from human resources rapidly (Villegas and Garitano-Zavala, 2010; Amâncio et al., 2008). In many cities in Brazil including Londrina, the great number of doves has been a big concern due to the transmission of diseases, the losses in agriculture and the problem for their feces in the urban areas (Shibatta et al., 2009). However, the documented information relative to hemoparasites in pigeons is scarce.

Haemosporidian parasites occur worldwide in several birds, however little is known about hemoparasites in pigeons (Valkiunas 2005, Atkinson et al., 2008). Despite they are genetically closely related (Martinsen et al., 2008), *Haemoproteus* and *Plasmodium* present different morphology, life cycle and pathogenesis, even though they are both known as avian malaria parasites, although the World Health Organization defines only *Plasmodium* as malaria parasites (Pérez-Tris et al., 2005).

Molecular diagnostic based on PCR have been largely used for the identification and differentiation of genera (Pérez-Tris et al., 2005, Atkinson et al., 2008) and the mitochondrial cytochrome b gene has been used to identify avian malaria parasites, although this method does not differentiate between *Haemoproteus* and *Plasmodium* species (Valkiuñas et al., 2006, Valkiunas et al., 2008). Studies of haemosporidian parasites showed a huge genetic diversity and the possibility to identify undescribed species (Beadell and Fleischer, 2005, Bensch et al, 2009, Valkiuñas et al, 2010, Levin et al., 2011). However, sequencing is necessary to identify the lineages and phylogenetic relationships (Vallkiunas 2005, Atkinson et al., 2008).

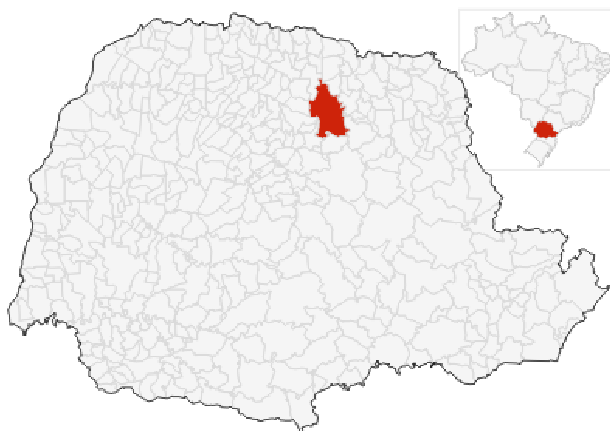
The aim of this study was to verify the presence and identify the species of haemosporidian in total blood from *Zenaida auriculata* trap-captured in Londrina, Paraná, Brazil.

6.2 MATERIAL AND METHODS

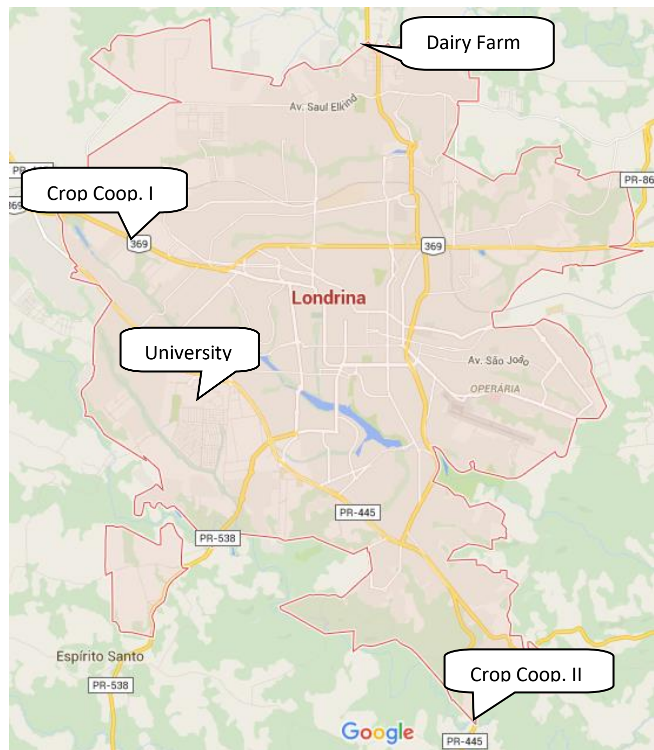
6.2.1 Study location and sampling

From September, 2010 to August, 2016, 211 *Z. auriculata* were trap-captured in Londrina, Paraná. Londrina is located in Southern Brazil, Northern Parana state (23°08’47” and 23°55’46” S, 50°52’23” and 51°19’11” W) (IBGE, 2010). The pigeons were trap-captured in four different locations in Londrina: Universidade Estadual de Londrina Campus (University) (n=53), Crop Cooperative I (Crop Coop. I) (n=133), Dairy Farm (n=16) and Crop Cooperative II (Crop. Coop. II) (n=9). The map of Londrina with the locations where the doves were captured can be seen on Figure 1.

Figure 1 - Map of Londrina and the locals where *Z. auriculata* were captured, 2016.



www.commons.wikimedia.org



Google Maps, 2016.

Total blood was collected in EDTA tubes through heart puncture after euthanasia with CO_2. The samples were maintained at -20 ºC until they were used for PCR. All the procedures were done at the Veterinary Protozoology Laboratory, Preventive Veterinary Medicine Department, State University of Londrina. This study was approved by the National Institute for the Environment and Renewable Natural

Resources (IBAMA) SISBIO N. 16428-1 and by the Ethics Committee of Animal Experiments of the Universidade Estadual de Londrina, no. 70/2008.

6.2.2 DNA Extraction

DNA was extracted from blood using BioPur Extraction Kit Mini Spin Plus® (Mobius Life Science Indústria e Comércio de Produtos para Laboratórios Ltda., Parana, Brazil), following the manufacturer's protocol. The extractions were maintained at -20 ºC until PCR assay.

In a microtube it was added 175 µL of ultrapure water, 200 µL of nuclei lysis buffer and 25 µL of Proteinase K to 25 µL of total blood. This was homogeneized and incubated for 25 minutes at 56 °C. After that, 400 µL of protein precipitation buffer was added, transferred to a column and incubated one minute at room temperature. The samples were centrifuged for two minutes at 13000g and the filtrated was discarded. 500 µL of washing buffer I was added, centrifuged for one minute at 13000g. The filtrate was discarded and 800 µL of washing buffer II was added, centrifuged for one minute at 13000g. The samples were centrifuged again for 4 minutes at maximum speed to dry. 50 µL of elution buffer was added and it was incubated for one minute at room temperature. Then finally it was centrifuged for one minute 8000g and DNA was collected in a microtube.

6.2.3 nPCR assay to amplify the mitochondrial gene cytochrome b

The first set of oligonucleotides used in this investigation was designed from the cytochrome b mitochondrial gene (cyt b), amplifying a 525pb fragment. The outer primers HaemNFI (5'-ATATATTAAGAGAAITATGGAG-3') and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3') are general for species of *Haemoproteus*, *Plasmodium* and *Leucocytozoon*. The inner primers HaemF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3') were described by Bensch et al. (2000), which can identify *Haemoproteus* spp and *Plasmodium* spp.

A nPCR was adapted from Hellgren et al. (2004). It was used 0.6 µM of each primer, PCR mixture (0,2 mM of each dNTPs, 2,5 mM $MgCl_2$, 1X PCR Buffer

(Invitrogen®) and 1.25 U of Platinum Taq DNA polymerase (Invitrogen®)) were added to 5 µl of genomic DNA. The DNA amplification was done in an Applied System Termocycle® (Applied Biosystems, Foster City, CA, USA), under the following conditions: 95°C for 3 minutes, 40 cycles at 95°C for 45 seconds, 48°C for 45 seconds and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. 50 µL of ultrapure water were added to each DNA amplification of the first round and 2 µL were used to the NPCR, under the same conditions described, except for the primer concentration (0,4 µM of each primer).

Two positive controls, one *Haemoproteus* sp. from pigeons and a genomic DNA from *Plasmodium gallinaceum* obtained from experimentally infected chicken, and a negative control (ultrapure water) were included in both reactions. The amplicons obtained from the second round PCR step were visualized in 1.5% agarose gel stained with SYBR Safe (Invitrogen®). Samples showing 525 bp length bands were considered as positive.

6.2.4 Restriction Fragment Length Polymorfism

The positive samples from the second reaction at nPCR of cytochrome b mitochondrial gene were used to identify the genus (*Haemoproteus* or *Plasmodium*) with the restriction enzyme EcoRV, following the description in Kistler et al. (2013). For enzymatic digestion, 0.25 µL (5 units) of EcoRV enzyme (New England Biolabs Inc., Hitchin, UK), 2.0 µL of 10x NEBuffer, 0.2 µL of BSA and 15.55 µL of ultrapure water were added to 2 µL of amplicons. It was incubated at 37°C for 60 min and inactivated at 80°C for 20 min. Electrophoresis in 2.5% agarose stained gel with SYBR Safe (Invitrogen®) was used to visualize the digested PCR products. Bans were expected to be seen as shown in Figure 2.

6.2.5 nPCR based on Small subunit ribosomal ribonucleic acid (18S SSUrRNA)

A nPCR genus-specific for *Plasmodium* was also used. Sequences of the small subunit ribosomal ribonucleic acid (18S SSU rRNA) were amplified according to Singh et al. (1999), with some modifications. A 1670bp fragment was amplified at the first round, using the following primers: rPLU1 (5'-TCA AAG ATT AAG CCA TGC

1 AAG TGA-3') and rPLU5 (5'-CCT GTT GTT GCC TTA AAC TCC-3'). It was added
2 0,4 μM of each primer, PCR mixture (0,2 mM of each dNTPs, 2,5 mM $MgCl_2$, 1X
3 PCR Buffer (Invitrogen®) and 1.25 U of Platinum Taq DNA polymerase (Invitrogen®))
4 were added to 5 μl of isolated DNA. The DNA amplification was carried out in an
5 Applied System Thermocycler under the following conditions: 94°C for 4 min, 39
6 cycles at 94°C for 30 sec, 55°C for 1min and 72°C for 1 min, finishing after a final
7 extension at 72°C for 4 min. Before proceeding to the second round, 50 μl of
8 ultrapure water was added to the first reaction. Two μl of the first PCR product were
9 used in the second amplification. The following primers were used: rPLU3 (5'-TTT
10 TTA TAA GGA TAA CTA CGG AAA AGC TGT-3') and rPLU4 (5'-TAC CCG TCA
11 TAG CCA TGT TAG GCC AAT ACC-3'). It was used the same PCR conditions from
12 the first reaction, except the aneelling temperature (62°C), obtaining a fragment of
13 240bp.
14 A positive control (genomic DNA from *Plasmodium gallinaceum* obtained from
15 experimentally infected chicken) and negative control were included in both
16 reactions. The amplified fragments were visualized in 1.5% agarose gel stained with
17 SYBR safe (Invitrogen®).
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19 **6.2.6 Sequencing**
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21 Sequencing was done with five samples to determine if the pigeons were
22 infected by *Haemoproteus* sp. or *Plasmodium* sp. or mixed infections, the
23 sequencing was carried out. The positive samples from the second reaction of nPCR
24 of mitochondrial gene cytochrome b were used for further evaluation by sequencing.
25 The amplicons were purified with QIAquick gel extraction kit (Invitrogen®). After
26 purification, they were sequenced using BigDye Terminator v3.1 Cycle Sequencing
27 Kit (Applied Biosystems, Foster City, CA, USA), through ABI3500 Genetic Analyzer
28 (Applied Biosystems, Foster City, CA, USA). Sequences were edited using Bioedit
29 7.2.5 and identified using the nucleotide BLAST application from NCBI. A neighbor-
30 joining tree was built with MEGA6 software (Tamura et al., 2013), using Kimura 2-
31 parameter distance matrix (Kimura 1980). Statistical analysis were determined with
32 bootstrap method in 1000 repetitions.

Standard samples from GenBank were included: *Haemoproteus* sp. (FJ462666.2, FJ462665.2), *H. multipigmentatus* isolate SocH15 (JN788946.1), *H. multipigmentatus* isolate SocH16 (JN788947.1), *H. belopolskyi* (DQ630006.1), *H. pallidus* (DQ630005.1), *H. ptyodactylii* (AY099057.1), *Plasmodium* sp. (KU057967.1), *Plasmodium* sp. (FJ462669.2), *P. gallinaceum* (KT290902.1), *P. relictum* (AY099032.1), *P. falciparum* (KM527163.1), *P. ovale wallikeri* (KP050437.1) and *P. ovale curtisi* (KP050436.1). *Toxoplasma gondii* was used as the outgroup.

6.2.7 Statistical Analysis

The Chi square ($\chi 2$) test corrected by Yates was used to compare the locations of capture and sexes (Epi Info Program, version 6.04). A p-value of ≤ 0.05 was considered as significant.

6.3 Results

6.3.1 nPCR

All pigeons were positive in the nPCR for mitochondrial gene citochrome b (cyt b) (Figure 3). This indicates that all birds had *Haemoproteus* sp. and/or *Plasmodium* sp. DNA. As these primers identify only the genera, it was not possible to identify mixed infections. All 211 (100%) pigeons were also positive by nPCR 18S SSU rRNA of *Plasmodium* sp. In Figure 4, the 240pb fragments can be seen in agarose gel.

6.3.2 RFLP

The RFLP with EcoRV enzyme resulted in 41/211 (19.95%) samples positive for both genera, indicating the mixed infection. Most of the samples evaluated with EcoRV enzyme showed just the presence of *Haemoproteus* sp. 155/211 (73.46%) and just a few samples 13/211 (6.16%) had *Plasmodium* sp. Despite of all samples were positive on nPCR, two samples (0.95%) were negative, possibly due to low quantity of DNA. Figure 5 shows the results after the digestion with EcoRV enzyme.

In total, 101/211 (47.87%) male pigeons and 110/211 (52.13%) female ones were captured. There was no statistical significance ($p>0.05$) between sexes in each place (University, Crop Coop. I, Dairy Farm and Crop Coop II). No statistical significance ($p>0.05$) was observed between the parasitism with only *Haemoproteus* sp. or *Plasmodium* sp. or both genera in each place by RFLP (Table 1).

6.3.3 Sequencing

Four (Pb 151, Pb 152, Pb 153, Pb 155) *Haemoproteus* species showed 99% of similarity with *Haemoproteus multipigmentatus* (JN788946.1 and JN788947.1) and one (Pb 154) *Plasmodium* showed 99% of similarity with *Plasmodium* sp. (KU057967.1). The phylogenetic analise (Figure 6 and Figure 7) from the consensus sequences obtained in this study agreed with the results obtained in comparison with the Blast tool.

6.4 Discussion

To this moment, this is the first molecular investigation of hemoparasites from *Z. auriculata* in Brazil. All doves showed DNA amplification of cyt b, demonstrating they presented *Haemoproteus* sp. and/or *Plasmodium* sp. genes, which agrees with Martínez et al. (2016), that found 100% *Z. auriculata* parasitized by *H. multipigmentatus* in Chile, showing that this parasite is present in these pigeons in South America. González et al. (2015) investigated 1487 birds of 166 different species and found just one *C. livia*, that showed to be parasitized by *H. columbae* in the blood smear also after sequencing. *H. columbae* is known to parasitize other species of Columbiformes, showing that this parasite can adapt in different Columbidae birds. This Haemoporida appeared to be closely related to *H. multipigmentatus*. It has already been recorded in eared doves in South America, by Santiago-Alarcon et al. (2010) in Ecuador and Venezuela, showing a wide range of transmission, especially in countries with warm climate in the Neotropics. Santiago-Alarcon et al. (2010) reported *H. multipigmentatus* also in *Z. aurita* (Caribbean Islands), *Z. meloda* (Peru), *Columbina buckleyi* (Ecuador), *C. cruziana* (Ecuador), *C. talpacoti* (Guatemala) and *Geotrygon montana* (Ecuador).

1 Adriano et al. (2001) reported the first description of haemosporidians in blood
2 smears of three different species of Columbiformes in Brazil and they found
3 *Haemoproteus columbae* in 331 (100%) *Z. auriculata*, 32/62 (51.6%) *Columbina*
4 *talpacoti* and 11/57 (19.3%) *Squardafella squammata*. Natala et al. (2009) worked
5 with a survey in 250 *Columba livia* and found *H. columbae* (15.6%) and *Plasmodium*
6 *relictum* (0.8%). Radfar et al. (2011) used Giemsa method and detected 48/102
7 (47.05%) of *H. columbae* in *C. livia*. No molecular characterization was done in this
8 studies.

9 Haemosporidian parasites have been found in different pigeons from different
10 regions worldwide. The researches can lead to find new host-parasite relationships,
11 increasing the taxonomic knowledge about these parasites. In Socorro Islands,
12 Carlson et al. (2013) studied 25 *Z. macroura* and 23 *Z. graysoni*, searching for
13 haemosporidian infections by blood smears and PCR assay. In *Z macroura*, they
14 found 23/25 (92%) positives and in *Z. graysoni*, 17/23 (74%). They found 18 different
15 lineages of *Haemoproteus* in doves, most of them were *H. columbae* and *H.*
16 *multipigmentatus* and one undescribed lineage. No *Plasmodium* or Leucocytozoon
17 were detected by PCR, although there was one blood film with *Plasmodium* sp. from
18 *Z. macroura*. They also found six *Z. macroura* co-infected with more than one
19 *Haemoproteus* species by PCR. According to them, new methods are needed to
20 explore co-infections better. Our study could also find mixed infections between
21 *Haemoproteus* and *Plasmodium* parasites (19.95%), but intragenus coinfections
22 remain unknown.

23 Krizanauskiene et al. (2013) studied 38 *Z. macroura* from Northern California
24 and 11 *Columbina passerina socorroensis* from Socorro Islands and identified *H.*
25 *saccharovi*, which has not been documented in *Z. auriculata* in Brazil until this
26 moment, possibly for the few studies in this dove.

27 The number of described lineages from avian Haemosporida has increased in
28 the last few years in the world, as researches with these parasites became more
29 popular. More studies brought descriptions of new species and new hosts, as in
30 Valkiunas et al. (2010), that recently first described *H. multipigmentatus* in *Z.*
31 *galapagoensis* from Galapagos Islands. Valkiunas et al. (2013) reported the new
32 species *Haemoproteus (Haemoproteus) multivolutinus* n. sp. from dove *Turtur*
33 *timpanistria* of Uganda and *Haemoproteus (Haemoproteus) paramultipigmentatus* n.

1 sp. from *Columbina passerina socorroensis* of Socorro Island. These investigations
2 show that several new species can be identified worldwide. In this study, *H.*
3 *multipigmentatus* and *Plasmodium* sp. were 99% similar to our sequenced
4 amplifications, showing that native doves are infected with both genera.

5 In Brazil, most of genetic studies of hemoparasites in birds are done in non-
6 Columbiformes. A study with 119 birds from 36 different species was conducted by
7 Tostes et al. (2015) in the Atlantic Forest of Southeartern Brazil, showing that 99/119
8 (83.19%) of birds were infected with *Haemoproteus* and *Plasmodium*. From the 80
9 blood samples tested by nPCR amplification of cyt b gene, 66 were positive for
10 haemosporidians, describing a high prevalence of these parasites. From this work,
11 only one *C. talpacoti* was tested, resulting negative, despite it was positive by
12 microscopy. However, the sequencing was not done, so no genetic characterization
13 was done. Also, Fecchio et al. (2012) studied 790 birds from 54 species and found
14 166/790 (21%) positive for haemosporidian parasites. Most of birds were
15 Passeriformes and 91 *Haemoproteus* and 38 *Plasmodium* lineages were described,
16 revealing a huge diversity in Brazil, and the importance of more investigations in
17 haemosporidian parasites in the country.

18 Another survey with Passerine birds in the Atlantic Forest in Minas Gerais
19 (Sebaio et al., 2012) analyzed 925 birds from 109 species and found 9.2% birds
20 positive for *Plasmodium* and 3.2% for *Haemoproteus*, which shows lower prevalence
21 compared to this study with *Z. auriculata*. They also described 31 new hosts,
22 although *Haemoproteus* was less found, possibly because it is host-specific.
23 *Plasmodium* species instead, show lower specificity, so, the same species can be
24 found in different groups of hosts, as it can be seen in Silveira et al. (2013) that
25 identified *P. tejerai* in penguins, even if this *Plasmodium* was first described in
26 turkeys. Other *Plasmodium* species as *P. relictum*, *P. cathemerium* an *P.*
27 *juxtanucleare* are found in penguins, but they are also described in Passeriformes
28 and Galliformes.

29 A survey with free-living birds from São Paulo Zoo showed 100% of *C. livia*
30 positive for haemosporidian parasites in stained blood smears, which comprises our
31 molecular findings. Four *Haemoproteus* lineages were obtained and they clustered
32 with *H. columbae*, *H. iwa* and *H. multipigmentatus*, showing that *H. multipigmentatus*
33 is present in other Columbiformes in Brazil (Chagas et al., 2015). This result

corroborates with our sequencing, that showed this haemosporidian parasite infect eared doves from Londrina.

Belo et al. (2011) did not study Columbidae birds, but described *Plasmodium* and *Haemoproteus* lineages in wild birds from Cerrado habitats in Tocantins, Brazil. They first described 15 new haemosporidian lineages, indicating that there are several unknown parasites and maybe unknown host species in our country.

Scaglione et al. (2015) studied 51 *Columba livia* in Italy by PCR and found 15/51 (29.4%) positive for *Haemoproteus*/*Plasmodium* spp. No significant association resulted between age or sex and infection status. However, the coinfection was very significant ($p<0.01$), showing that the presence of a hemoparasite could predispose to another haemosporidian infection. DNA sequencing showed P-SGS 1 (*P. relictum*) and H-HAECOL1 lineage 13 (*H. columbae*).

The 18S SSU rRNA from *Plasmodium* sp. was used in this study to try to find this parasite beyond the cyt b nPCR in *Z. auriculata*. However, 100% samples were positive, which suggests that these doves may be more parasitized by mixed infection that other birds. Ribeiro et al. (2005) captured 275 Passeriformes in Minas Gerais, 16% of birds showed parasitemia and 39.6% were positive in rPLU nPCR. These set of primers were tested to identify *Plasmodium* from humans, primates and rodents, and also showed to be useful for detecting avian malaria parasites (Ribeiro et al., 2005), as we can also see in our study. No association between the infection and sexes was observed in both works, but Ribeiro et al. (2005) observed that insectivore birds showed to be more infected, probably because these birds were more exposed to infected vectors, as they could be destroyed and the sporozoites would penetrate in mucosa. *Z. auriculata* usually feeds with crops, but eventually they can eat other sources of food (ADANG et al., 2008).

Another study of Plasmodium infection detected by PCR based on 18S SSU rRNA of *Plasmodium* sp. was performed in Brazil (Belo et al., 2009), in captive psittacine birds from zoos in Minas Gerais and Ceará, showing the presence of *Plasmodium* sp. in 36% of the birds. However, they could not identify if those birds where infected before of after confinement, showing the possibility of getting infected in native habitat. So, we can observe that the detection of *Plasmodium* infection in eared doves was much higher by using nPCR, probably for its behavior for living in

1 large flocks and sharing the habitat with other species of birds, even in the urban
2 area.

3 Because *Plasmodium* and *Haemoproteus* are very genetically closely related,
4 it is difficult to design primers to distinguish one from the other, that could be useful
5 just in some particular regions. So, some researches were done with restriction
6 enzyme-based assays to try to develop new methods to provide more tools to detect
7 each parasite separately. Beadell and Fleischer (2005) described a RFLP with
8 combined Xmn I and Xba I enzymes that were tested in previously sequenced DNA
9 from cyt band they obtained full recorgnition of *Plasmodium*, *Haemoproteus* and
10 *Leucocytozoon*. Still, mixed infection could be underestimated, meaning that more
11 test were needed to increase the sensibility of this test.

12 Martínez et al. (2009) reported a restriction site specific to *Haemoproteus*,
13 using Hpy CH4III enzyme, which showed an error rate of 3%. After these studies,
14 Kistler et al. (2013) developed a RFLP with a EcoRV restriction enzyme cut sites for
15 *Haemoproteus* or *Plasmodium* or both, within the cyt b gene, which resulted in 98.8%
16 of sequences detected as the correct genus. In our study, 0.95% of samples were
17 negative, which agrees with this error rate. The results of single detection of
18 *Haemoproteus* of *Plasmodium* or mixed infection also showed agreement. These
19 assays can make the species genes diagnostics easier, as most of available PCR do
20 not distinguish the genera separately. This lack may cause subdiagnostics of species
21 through sequencing. The use of RFLP before sequencing could provide more
22 accurate informations about coinfections in pigeons.

23 An ectoparasite was recovered from a dove (0.4%) from Crop Cooperative I.
24 The identification was done by morphologic characteristics under stereomicrocopy
25 according to Graciolli and Carvalho (2003) and Lutz et al. (1915) and it could be
26 described as *Pseudolynchia canariensis*, a known vector of hemoparasites in
27 pigeons (Lutz et al., 1915, Graciolli and Carvalho 2003, Valkiunas, 2005). Adriano et
28 al. (2001) also reported the presence of *P. canariensis* in 193/331 (58.4%) *Z.*
29 *auriculata*, 23/62 (36.5%) *C. talpacoti* and 19/57 (33.6%) *S. squammata*. They
30 described that the transmission of vectors among the birds would have more
31 occurrence as they live in large flocks, permmiting the exchange of vector constantly.
32
33

Conclusion

In conclusion, this study reported a high number of doves infected with haemosporidian parasites. Additionally, to the best of our knowledge, this was the first molecular characterization of *Haemoproteus* and *Plasmodium* in *Z. auriculata* from Brazil. Still, more studies need to be done to identify the mixed infections and the presence of other blood parasites.

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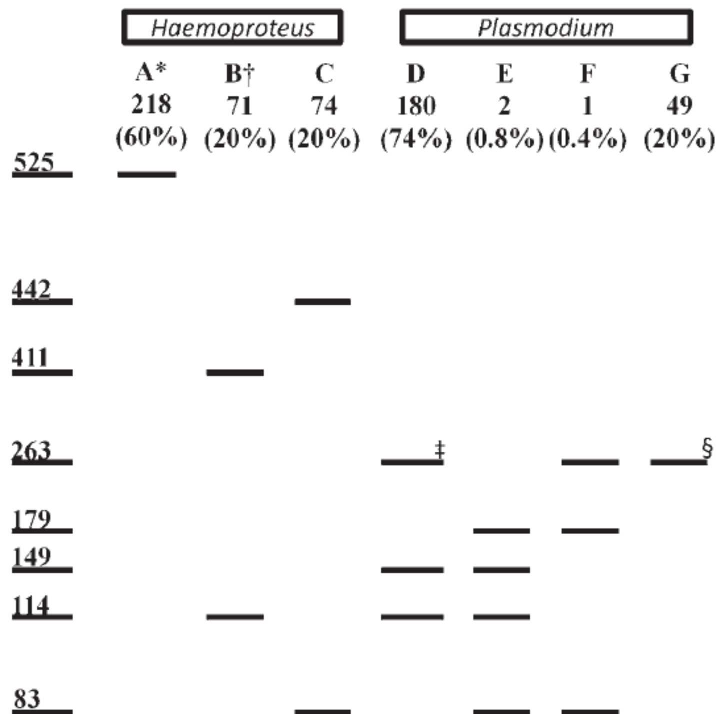
1 ***Figure 2*** – Diagram of band sizes based on EcoRV cut sites from sequences
2 presented in MalAvi database, according to Kistler et al. (2013).



3

4 Lanes A-C show *Haemoproteus* cut patterns and lanes D-G show *Plasmodium* cut patterns. The
5 numbers above each lane refer to the number of each cut-pattern expected and the numbers in
6 parentheses are the percentages of sequences in the MalAvi database in that particular genus that
7 would display that cut pattern. The 11 *Plasmodium* sequences that would have been misclassified
8 were represented by cut-patterns illustrated in lanes A and B. *Includes 5 misclassified *Plasmodium*
9 sequences. †Includes 6 misclassified *Plasmodium* sequences. ‡262 band size. §262 and 263 band
10 sizes.

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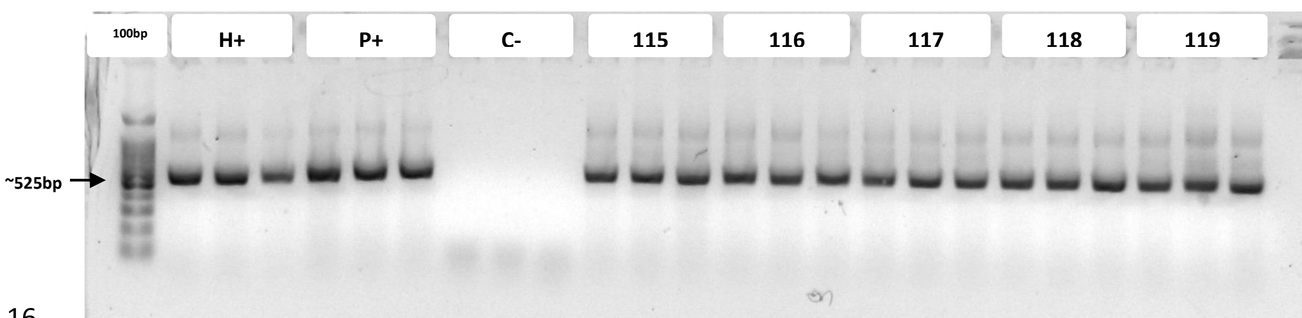
14 **Figure 3** – nPCR of cytochrome b of mitochondrial gene in 1.5% agarose gel, from
15 blood of *Z. auriculata*, Londrina, 2016.



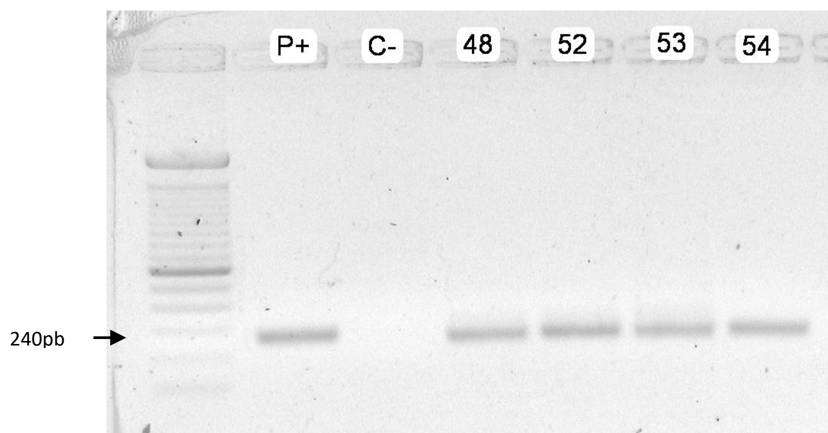
16

17 H+ = *Haemoproteus* sp. positive control; P+ = *Plasmodium* sp. positive control; C- = negative control;
18 115 – 119 = samples

19

1

2 **Figure 4** – nPCR of18S SSU rRNA of *Plasmodium* sp. in 1.5% agarose gel, from
3 blood of *Z. auriculata*, Londrina, 2016.



4

5 P+ = *Plasmodium* sp. positive control; C- = negative control; 48 – 54 = samples

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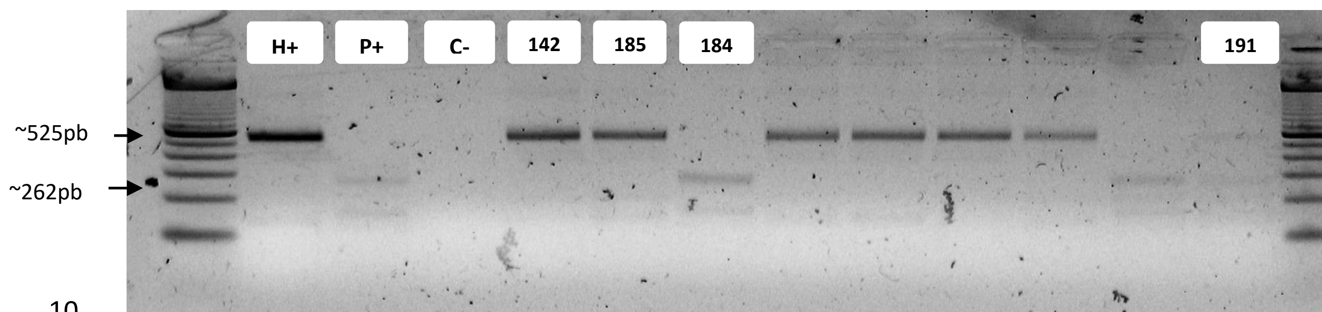
9 ***Figure* 5** –2.5% agarose gel from RFLP from blood of *Z. auriculata*, Londrina, 2016.



10

11 H+ = *Haemoproteus* sp. positive control; P+ = *Plasmodium* sp. positive control; C- =
12 negative control; 142 - 191 = samples

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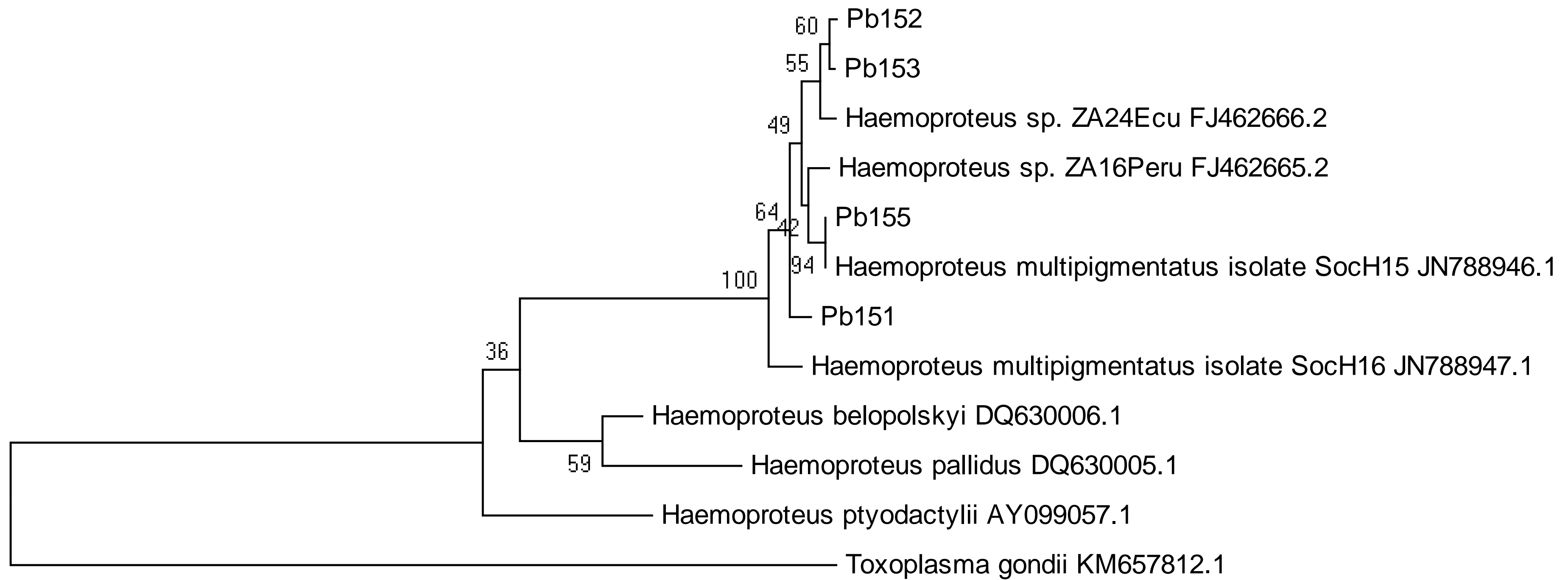
1 **Figure 6** – Phylogenetic tree based on the mitochondrial cytochrome b gene sequences of *Haemoproteus* species from *Zenaida*
2 *auriculata* of this study and other *Haemoproteus* species found in GenBank.



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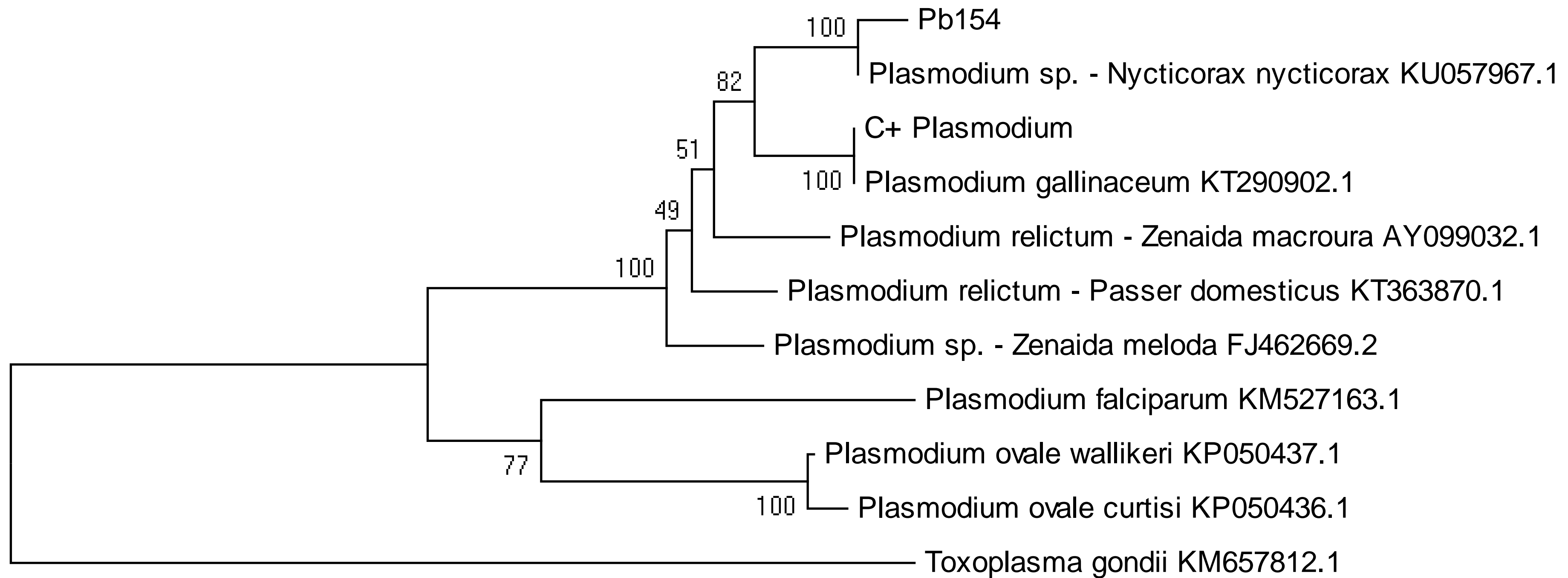
1 **Figure 7** – Phylogenetic tree based on the mitochondrial cytochrome b gene sequences of *Plasmodium* species from *Zenaida*
2 *auriculata* of this study and other *Plasmodium* species found in GenBank.



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1 **Table 1** – Results of RFLP of mitochondrial cytochrome b gene in blood of *Zenaida*
2 *auriculata* from Londrina, Parana State, 2016.

	N	POS	(%)	X^2	p-value
Haemoproteus sp.					
University Campus	53	19	(35.8)	3.64	0.30
Crop Coop. I	133	117	(88.0)		
Dairy Farm	16	10	(62.6)		
Crop Coop. II	9	6	(66.7)		
Plasmodium sp.					
University Campus	53	11	(20.8)	nc	nc
Crop Coop. I	134	1	(0.8)		
Dairy Farm	16	1	(6.2)		
Crop Coop. II	9	0	0		
Mixed infection					
University Campus	53	23	(43.4)	0.64	0.89
Crop Coop. I	133	14	(10.6)		
Dairy Farm	16	4	(25.0)		
Crop Coop. II	9	3	(33.4)		

3 N = total number of samples in each local of capture, POS = positive samples, χ^2 = qui-square, p-
4 value, nc = not calculated
5
6

7 ARTICLE II

Occurrence of *Eimeria* sp. in doves *Zenaida auriculata* from Londrina, Paraná State, Southern Brazil

Occurrence of *Eimeria* sp. in doves *Zenaida auriculata* from Londrina, Paraná State, Southern Brazil

ABSTRACT

The aim of this study was to verify the presence of *Eimeria* sp. in fecal samples of *Zenaida auriculata* trap-captured in Londrina, Paraná. The fecal samples from 214 *Z. auriculata* trap-captured in Londrina, Parana, were collected from the guts in plastic microtubes after euthanasia. The samples were maintained at -20ºC until they were used for PCR. After the DNA extraction, a nPCR based on the subunit I of the cytochrome c oxidase gene (COI) of the parasite mitochondrial gene was performed. From 214 animals, 171 (79.91%) were positive for *Eimeria* sp.. There were significantly differences between the places of capture (p=0.0008), at the University and from the Crop Cooperative I. From 214 pigeons, 103/214 (48.13%) were male and 111/214 (51.87%) were female. There were no differences between male and female doves, considering the locality of captures. To the authors' knowledge, this is the first study with molecular prevalence of *Eimeria* sp. in *Z. auriculata* in Brazil. Further analysis need to be done by sequencing to provide the species of *Eimeria* found in *Z. auriculata* in this work.

Key-words: Eimeriidae, coccidiosis, PCR, Columbiformes, cytochrome c oxidase I gene

7.1 INTRODUCTION

The eared dove (*Zenaida auriculata*) is found in most of Latin America countries in both urban and rural areas (Adriano et al., 2003). It belongs to family Columbidae, which comprises over than 300 avian species world-wide (Jamriska and Modry, 2012). In Londrina, it has been described 10 Columbidae species, including *Z. auriculata* (Shibatta et al., 2009).

Eimeria is the most diverse coccidia from Order Eucoccidiorida, causing damages in all classes of vertebrates (Berto et al., 2014). Eimeriosis has been causing losses especially in poultry industry for its high patogenicity (Reid et al., 2014; Cardozo e Yamamura, 2004). The species of *Eimeria* described infecting pigeons are: *E. labbeana* (Labbé, 1896), *E. columbarum* (Nieshoulz, 1935), *E. columbae* (Mitra & Das Gupta, 1937), *E. tropicalis* (Malhotra & Ray, 1961), *E. kapotei* (Chatterjee & Ray, 1969), *E. janovyi* (Bandyopadhyay, Bhakta & Shukla, 2006), *E. livialis* (Alyousif, Al-Shawa & Al-Asiri, 2009), *E. sphenocerae* (Ray, 1952), *E choudari* (Bhatia, Chauhan, Arora & Agrawal, 1972), *E. turturi* (Golemansky, 1976), *E. waiganiensis* (Varghese, 1978), *E. duculai* (Varghese, 1980), *E. gourai* (Varghese, 1980), *E. palumbi (McQuistion, 1991), E. curvata* (Adriano, Thyssen&Cordeiro, 2000), *E. zenaidae* (Adriano, Thyssen & Carneiro, 2003) and *E. mauritiensis* (Ball et al., 2012), according to Ball et al. (2012).

Along the years, the methodologies to diagnose *Eimeria* in birds have been developed, not only in the traditional morphologic characterization but in molecular biology (Carvalho et al., 2011). The molecular findings have been used for diagnostics and also to describe the genetic variability of *Eimeria* oocysts (Carvalho et al., 2011).

The sequences in the subunit I of the cytochrome c oxidase gene (COI) have been used to identify *Eimeria* and *Isospora* species in birds. However, there are still a few studies (Ogedengbe et al., 2011, Ogedengbe et al., 2015, Rathinam et al., 2015, Hafeez et al., 2015, Yang et al., 2016) of coccidia in birds worldwide, which is shown by the lack of descriptions (Berto et al., 2014).

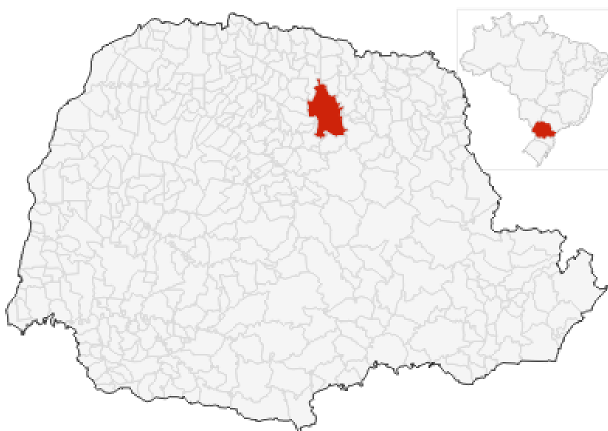
The aim of this study was to verify the presence of *Eimeria* sp. in fecal samples of eared doves (*Z. auriculata)* trap-captured in Londrina, Paraná.

6.2 Material and Methods

6.2.1 Study location and sampling

From September, 2010 to August, 2015, the fecal samples from 214 male and female *Z. auriculata* trap-captured in Londrina, Parana Southern Brazil (23°08'47" and 23°55'46" S, 50°52'23" and 51°19'11" W) (IBGE, 2010), were collected from the guts in plastic microtubes after euthanasia with CO_2. The samples were maintained at -20 ºC until they were used for nPCR. All the procedures were done at the Veterinary Protozoology Laboratory, Preventive Veterinary Medicine Department, State University of Londrina. The pigeons were trap-captured in four different locations in Londrina: State University of Londrina Campus (University) (n=54), Crop Cooperative I (Crop Coop. I) (n=136), Dairy Farm (n=16) and Crop Cooperative II (Crop. Coop. II) (n=9). The map of Londrina City with the locations of capture of doves can be seen on Figure 1.

Figure 1 - Map of Londrina and the locals where *Z. auriculata* were captured, 2016.



www.commons.wikimedia.org

Google Maps, 2016.

This study was approved by the National Institute for the Environment and Renewable Natural Resources (IBAMA) SISBIO N. 16428-1 and by the Ethics Committee of Animal Experiments of the State University of Londrina no. 70/2008.

6.2.2 DNA Extraction

DNA from fecal samples of all eared doves were extracted. Before the DNA extraction, feces were submmited to three cycles of freeze and thaw (-80 °C freezing and water bath 70 °C). One militre of Tris – EDTA (TE) was added for each 50μl of fecal sample in a 1.5ml microtube. After centrifugation (4000g, 15 min), the supernatant was discarded and the pellet was used for extraction with Nucleospin Tissue® (Macherey – Nagel, Germany). The pellet was resuspended in 200 μl of buffer T1 plus 25 μl of proteinase K, and kept overnight at 56 °C. Two hundred microlitres of lysis buffer B3 were added, and incubated at 70°C for 10 min. 210 μl of 100% ethanol was also added and the content was transferred to a column provided by the kit. After these steps, the manufacturer's protocol was followed. For elution, 25 μl of elution buffer was used. The column was centrifuged 11000g for 1 minute and liquid discarded. 500 μl of buffer BW was added and it was centrifuged again 11000g for 1 minute. 600 μl of buffer B5 was used and it was centrifuged again under the same conditions and the elution was discarded. The column was centrifuged to dry. DNA was eluted with 20 μl of elution buffer for further analysis.

7.2.3 nPCR COX tenella

A nPCR based on detecting sequences in the subunit I of the cytochrome c oxidase gene (COI) of the *Eimeria* mitochondrial genome was done, according to Dolnik et al. (2009).

The first PCR was performed using the outer primers COX tenella F4 5'–G(AT)TCATTAGTATGGGCACATCA–3' and COX tenella R 5'–CCAAGAGATAATAC(AG)AA(AG)TGGAA–3'. The second round was performed with the inner primers COX tenella F2 5'–GGGCACATCATATGATGAC–3' and COX tenella R2 5'–ATAGTATGTATCATGTA(AG)(AT)GCAA–3'.

For the DNA amplification, 0.8 µM of each primer, PCR mixture (0,2 mM of each dNTPs, 2.5 mM $MgCl_2$, 1X PCR Buffer (Invitrogen®) and 1.25 U of Platinum Taq DNA polymerase (Invitrogen®)) were added to 2 µl of isolated DNA. The first PCR was carried out in an Applied System Termocycle® (Applied Biosystems, Foster City, CA, USA) under the following conditions: 94°C for 3 min, 12 cycles at 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec, 15 cycles at 94°C for 30 sec, 48°C for 30 sec and 72°C for 30 sec, finishing after a final extension at 72°C for 10 min. Two µl of the first PCR product were used in the second amplification. This second PCR was carried out under the following conditions: 94°C for 3 min, 35 cycles at 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec, and a final extension at 72°C for 10 min. Samples with 250bp length bands were considered positive.

A positive control (genomic DNA from *Eimeria acervulina* obtained from experimentally infected chicken) and negative control (ultrapure water) were included in both reactions. The amplified fragments were visualized in 1.5% agarose gel stained with SYBR Safe (Invitrogen®).

7.2.4 Statistical Analysis

The Chi square ($\chi 2$) test corrected by Yates was used to compare the locations of capture and sexes (Epi Info Program, version 6.04). A p-value of ≤0.05 was considered as significant.

7.3 RESULTS

From 214 animals, 171 (79.91%) were positive for *Eimeria* sp. We can see on Figure 2 the positive and negative controls, and some of the samples tested by nPCR COX tenella, showing the amplicons with approximately 250 pair of basis. There were significantly differences of positivity between the places of capture (p=0.0008), especially when we compared the pigeons from the University and from the Crop Cooperative I (Table 1).

From 214 pigeons, 103/214 (48.13%) were male and 111/214 (51.87%) were female. There were no differences between male and female doves, considering the locality of captures, as it is shown on Table 2.

7.4 Discussion

To the best of the authors' knowledge, this is the first study with molecular prevalence of *Eimeria* sp. in *Z. auriculata* in Brazil. There is just one investigation with molecular characterization of *Eimeria* in pigeons, which sequence (KT305929) can be found in GenBank according to Yang et al. (2016). It was found in *Columba livia* and named *Eimeria columbadomestica* n. sp. Most of the molecular characterization of *Eimeria* oocysts were done in chickens and turkeys (Ogedengbe et al., 2011, Ogedengbe et al., 2015, Rathinam et al., 2015, Hafeez et al., 2015).

Molecular methods are needed to complement morphological data, as it may not be enough to give full information about species identity and host specificity. Besides, molecular characterization might show the genetic diversity, the phylogenetic relationships and also provide information relative to geographic distribution. However, PCR methods can underestimate mixed infections, that can sometimes be observed as double peaks on sequence electopherogram, as the sequencing selects the amplification of only one species of parasite (Valkiunas, Iezhova et al., 2008, Pérez-Tris and Bensch, 2005, Valkiunas et al., 2006).

A significantly difference was observed between the positivity on samples from the University and those from Crop Coop I (61.11%, 87,51%, p=0.0008). The reason for that might be because there were more doves captured from Crop Coop I, giving a higher prevalence of *Eimeria* in this place, or because the *Z. auriculata* population from there was much bigger than the one found at the University.

There was no statistically importance between the sexes and the locals of capture of pigeons, demonstrating that both male and female get infected equally.

Most of studies with *Eimeria* in pigeons around the world are done with the prevalence of oocyst shedding and morphometric descriptions:

In 1991, McQuistion found a new coccidian in *Zenaida galapagoensis*, named *Eimeria palumbi*, at the Galapagos Island. Conti and Forrester (1981) studied two populations of *Zenaida macroura* and one population of *Zenaida asiatica* in Florida,

USA and they found *Eimeria* sp. in 33.3%, 49.1% and 6% pigeons, respectively. In Brazil, Adriano et al. (2000) described *Eimeria curvata* n. sp. in 8/46 *Columbina talpacoti* and 5/39 *Scardafella squamatta*. A few years later, Adriano et al. (2003) first described a new species of *Eimeria* in *Z. auriculata* that was named *Eimeria zenaidae* n. sp. They studied 142 doves by flotation with Sheather's sugar solution and obtained 34 (23.9%) animals shedding *Eimeria* oocysts.

Some studies with other species of pigeons also found *Eimeria* oocysts in feces by flotation methods. Sari et al. (2008) studied 136 domestic pigeons (*Columba livia*) and 115 wild pigeons in Turkey, by Sheather's method. They found 81/136 (59.6%) positive domestic pigeons and 35/115 (30.4%) wild pigeons with coccidian oocysts. They identified E. *labbeana*, *E. columbarum*, *E. columbae* and *Isospora* sp. through morphology.

Also in *C. livia* by Sheather's method, Natala et al. (2009) verified 250 birds and found 49.2% positive for *Eimeria* sp and Radfar et al. (2011) identified 41/102 (40.19%) of pigeons with *Eimeria* sp. in Iran. In Santa Catarina, Brazil, Marques et al. (2007) found 37/43 (86.05%) of domestic pigeons with *Eimeria* sp. oocysts.

Ball et al. (2012) described for the first time the *Eimeria mauritiensis* in 5 *Nesoenas mayeri* in Mauritius Island. In the same year, Jamriska and Modrý described *Eimeria columbapalumbi* n. sp. for the first time, from *Columba palumbus* in Czech and Slovak Republics.

Baliska-Ramisz et al. (2014) studied 180 *Columba livia* from two different lofts in a husbandry in West Pomerania Province. They had respectively in loft I and loft II: 17/19 (89%) and 62/67 (93%) of young pigeons, 27/43 (63%) and 28/51 (55%) of adult pigeons with *Eimeria* sp. Morphologically, the oocysts found were described as *E. labbeana*, *E. columbarum* and *E. columbae*.

Most of these studies showed higher presence of oocysts in fecal samples, but it does not seem to be related to the total number of samples. As 171/214 (79.91%) of the doves were positive by PCR, we can say that *Z. auriculata* had a high prevalence of *Eimeria* in Brazil.

Molecular studies with *Eimeria* in pigeons are still limited, so little is known about genetic characterization of this parasites in Columbiformes. Dolnik et al. (2009) developed the nPCR based on the sequences in the subunit I of the cytochrome c oxidase gene from a single oocyst of *Isospora*. Moreover, this technique can be used

for *Eimeria* in birds, because the primers were designed from *E. tenella*, *E. mivati*, *E. maxima* and *E. acervulina*. Still, the sequencing is needed to describe the genetic characterizations of the species.

The evaluation of Eimeriidae coccidia species is very important not only to contribute to taxonomy and phylogeny, but to estimate the risk of infection of other birds species that could be living at the same environment of *Z. auriculata*.

7.5 CONCLUSION

Eimeria sp. in *Zenaida auriculata* was first described by nPCR in the present study. More than 79% of the samples were considered positive, but there was no difference between males and females from the four different locations of capture. Further analysis need to be done by morphometry and gene sequencing to provide the species of *Eimeria* found in *Z. auriculata* in this work.

Acknowledgements

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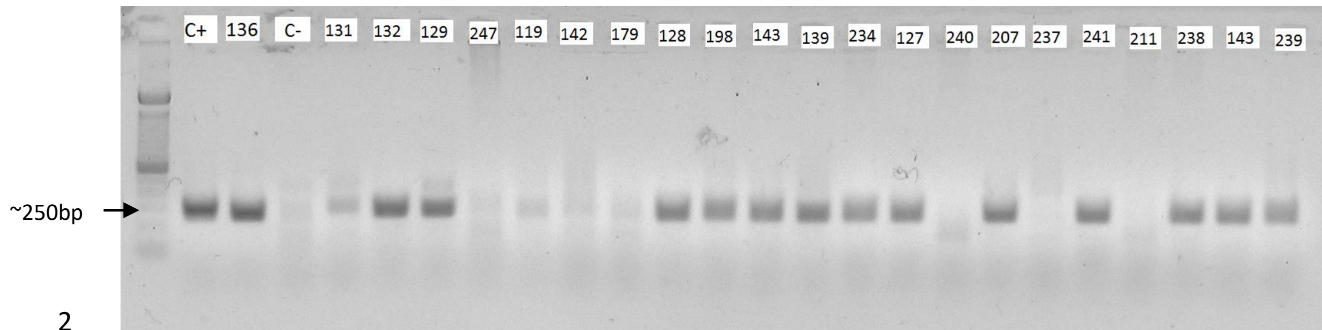
Figure 2 – nPCR of COX tenella of feces of *Z. auriculata*, Londrina, 2016.



C+ = positive control (*E. acervulina*), C- = negative control, numbers = samples

1 **Table 1** - Association between the number of positive *Zenaida auriculata* for *Eimeria*
2 detection by PCR and the local of capture, Londrina, 2016.

	N	Positive	%	Negative	%	X^2	p
University[a]	54	33	61.1	21	38.9	16.79	0.0008
Crop Coop. I[b]	136	119	87.5	17	12.5		
Dairy Farm[a,b,c]	15	12	80.0	3	20.0		
Crop Coop. II[a,b,c]	9	7	77.8	2	22.2		
Total	214	171	79.9	43	20.1		

3 N = total number of samples, χ^2 = qui-square, p-value, different letters, a and b, indicate significant
4 differences ($p < 0.05$) in prevalence for the different haemoparasites.

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1 **Table 2** - Association between the number of positive male and female eared doves
2 for *Eimeria* detection by nPCR and the local of capture, Londrina, 2016.

		Male		Female		X^2	p
	N	POS	%	POS	%		
University	54	11	20.37	22	40.74	4.17	0.2437
Crop Coop. I	136	63	46.32	56	41.18		
Dairy Farm	15	6	40.00	6	40.00		
Crop Coop. II	9	4	44.44	3	33.33		
Total	214	84		87			

3 N = number of doves, POS = number of positive samples, χ^2 = qui-square, p-value
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CONCLUSÃO

Este é o primeiro trabalho com a descrição da presença de DNA de hemosporídeos em *Zenaida auriculata* no Brasil. Todos os pombos se apresentaram positivos através da nPCR baseada no gene mitochondrial citocromo b e 18S SSU rRNA. Atráves de RFLP pode-se verificar que os pombos estavam parasitados somente por *Haemoproteus* ou *Plasmodium*, mas 19.43% das amostras apresentaram infecção mista entre estes dois gêneros. No sequenciamento genético, obteve-se quatro sequências com 99% de similaridade com *H. multipigmentatus* e uma sequência de similaridade de 99% com *Plasmodium* sp., demonstrando que este parasita pode se adaptar em diferentes espécies de pombos, provenientes de diferentes localidades no mundo.

A presença de DNA do gênero Eimeria foi primeiramente descrita em *Z. auriculata* no Brasil através da nPCR. Mais de 79% das amostras de fezes foram consideradas positivas e não houve diferença significativa entre machos e fêmeas ou entre os locais de captura, demonstrando que este parasita pode ser encontrado nos animais em diversas localidades.

Os estudos de parasitas em aves silvestres vêm aumentando no mundo todo. No entanto, deve-se dar uma maior importância na identificação destes parasitas para uma maior contribuição para a taxonomia clássica e molecular, e também para um maior entendimento das parasitoses nestes animais.

PERSPECTIVAS

Outros estudos nos hemosporídeos devem ser realizados neste trabalho, como o sequenciamento genético de mais amostras, assim como das amostras após a RFLP. Deve-se realizar o índice parasitêmico nos esfregaços sanguíneos para ralizar uma comparação entre microscopia e nPCR.

No estudo de *Eimeria*, a morfometria e morfologia devem ser realizadas com oocistos esporulados para determinação das espécies através da caracterização morfológica e fotodocumentação, assim como o sequenciamento genético para a determinação genética das espécies encontradas.

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ANEXOS

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ANEXO A

Comitê de Ética em Experimentação Animal da Universidade Estadual de Londrina

COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL

OF. CIRC. CEEA Nº 70/2008 — Londrina, 10 de setembro de 2008.

Prezado Pesquisador

O CEEA/UEL, reunido aos 09 de setembro do ano corrente, avaliou o projeto de pesquisa intitulado **"Caracterização genética de isolados de *Toxoplasma gondii* de pombas (*Zenaida auriculata*) do município de Londrina, Paraná"**, registrado no CEEA sob o nº 35/08, desenvolvido sob sua responsabilidade, julgando-o *aprovado* para execução por entender que os princípios éticos postulados pelo Colégio Brasileiro de Experimentação Animal estão respeitados.

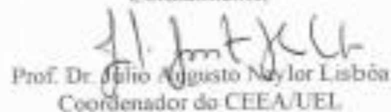
Serão utilizados 384 pombos capturados em praças públicas do município de Londrina e 768 camundongos adultos procedentes do Biotério central da UEL.

Tendo em vista o grande número de pombos capturados e o ineditismo e a complexidade desse procedimento, o CEEA/UEL sugere que outras investigações relevantes sejam realizadas aproveitando material biológico colhido desses animais.

Cumpre orientar que caso se pretendam quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação do CEEA/UEL anteriormente à execução das modificações.

Sem mais para o momento, subscrevo-me.

Cordialmente,



Prof. Dr. Júlio Augusto Naylor Lisbôa
Coordenador do CEEA/UEL

Ilmo. Sr.
Prof. Dr. João Luis Garcia
Coordenador do Projeto
Departamento de Medicina Veterinária Preventiva
Centro de Ciências Agrárias

LONDRINA - PARANÁ - BRASIL

ANEXO B

Autorização de captura e eutanásia de *Z. auriculata* - Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) SISBIO N. 16428-1

Ministério do Meio Ambiente - MMA
Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis - IBAMA
Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 16428-1	Data da Emissão: 14/07/2008 10:12	Data de Validade: 14/07/2009

Dados do titular

Registro no Ibama: 2509292	Nome: João [illegible]	CPF: 605.644.319-15
Título do Projeto: Caracterização genética de isolados de *Toxoplasma gondii* de Pombas (*Zenaida auriculata*) do município de Londrina, Paraná.		
Nome da Instituição : Universidade Estadual de Londrina		CNPJ: 78.640.489/0001-53

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1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
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6	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico.
7	Em caso de pesquisa em Unidade de Conservação Federal, o pesquisador titular deverá contactar a administração dessa unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1	LONDRINA	PR	Universidade Estadual de Londrina	Fora de UC

Atividades X Táxons

#	Atividade	Táxons
1	Captura de animais silvestres in situ	Zenaida auriculata
2	[illegible] in situ	Zenaida auriculata
3	[illegible] de espécimes da fauna silvestre in situ	Zenaida auriculata (*Qtde: 364)

* Qtde. de indivíduos por espécie/localidade/unidade de conservação, a serem coletados durante um ano.

Material e métodos

1	Amostras biológicas (Aves)	Fezes, Ectoparasita, Sangue, Fragmento de tecido/órgão
2	[illegible] (Aves)	Outros métodos de captura/coleta(Arapucas-Armadas [illegible])

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	Universidade Estadual de Londrina	Pesquisa

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