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Experimental characterization of the complete life cycle of *Haemoproteus columbae*, with a description of a natural host-parasite system used to study this infection

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ABSTRACT

Characterization of complete life cycles of haemoparasites requires the maintenance of suitable susceptible vertebrate hosts and vectors for long periods in captivity, in order to follow the complete parasitic cycle in definitive and intermediate hosts. Currently, there are few host-parasite models established in avian haemosporidian research, and those have been developed mainly for species of Passeriformes and their parasites. This study aimed to develop an experimental methodology to access the complete life cycle of *Haemoproteus columbae* (*cytb* lineage HAECOL1), which parasitizes the Rock Pigeon (*Columba livia*) and louse fly (*Pseudolynchia canariensis*). A colony of louse flies, which are the natural vectors of this parasite, was established. Thirty newly emerged insects were exposed to *H. columbae* infection and used to infect naïve Rock Pigeons. The peak of parasitaemia (acute stage) was seen between 27 and 32 days p.i. when up to 70.8% of red blood cells were infected. The crisis occurred approximately 1 week after the peak, and the long-lasting chronic parasitaemia stage followed. Exo-erythrocytic meronts were seen mainly in the lungs where extensive tissue damage was reported, but also in the kidneys and spleen. In the vector, the sporogonic cycle of *H. columbae* was completed between 13 and 16 days p.i., at an average temperature ranging between 12 and 15 °C. This host-parasite model is tractable for maintenance in captivity. It is recommended for use in studies aiming for detailed characterization of host-parasite relationships in areas such as physiology, pathology, immunobiology, genetics, as well as for evaluative treatments and to follow the infection in any stage of parasite development both in the vertebrate or invertebrate host.

1. Introduction

Haemoproteus spp. (Haemosporida, Haemoproteidae) are vector-borne parasites widely distributed in birds worldwide (Valkiūnas, 2005; Thomas et al., 2007). There are approximately 170 described species in the genus *Haemoproteus*, which are classified into two subgenera based not only on their genetic differences but also the vectors where the sexual development takes place (Valkiūnas, 2005; Martinsen et al., 2008; Toscani et al., 2018). The parasites that belong to the subgenus *Parahaemoproteus* are transmitted by biting midges (*Culicoides*), while species of the family Hippoboscidae transmit parasites of the subgenus *Haemoproteus* (Adie, 1915; Baker, 1957; Valkiūnas, 2005; Valkiūnas et al., 2010).

The sexual process and sporogonic cycle of *Haemoproteus columbae* occur in *Pseudolynchia canariensis* (louse fly; Adie, 1915; Valkiūnas, 2005). In these louse flies, the eggs hatch into the mother's uterus, and three stages of larval development take place. The larvae feed on nutrient fluids secreted by paired milk glands in the louse fly uterine wall until they become pupae (Bishopp, 1929; Baker, 1967; Harwood and James, 1979). Female flies deposit pupae on the substrate in or around pigeon nests (Waite et al., 2012b).

The sporogonic cycle of *Haemoproteus* spp. in flies takes between 6.5 and 10 days until parasites reach the salivary glands (Adie, 1915; Baker, 1966). Coral et al. (2015) demonstrated that exflagel-

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lation in vitro occurs in as little as 3 min at 40 °C after exposure of infected blood to air (AEA), when micro- and macrogametes appear; zygotes appear 5 min AEA, developing ookinetes 45 min AEA and mature ookinetes were observed 20 h AEA. Gallucci (1974), indicates that there is no way to distinguish the ookinetes of *H. columbae* developing in vitro from those differentiating in vivo, but different sizes of these mature structures have been reported in other studies. In vitro, parasite development has been followed only to the ookinete stage to date. In vivo, the oocysts have been seen protruding outside of the midgut wall when mature (Baker, 1957); they are usually larger than 30 μm in diameter and contain numerous germinative centres; this may partly explain why the sporogonic cycle of the *Haemoproteus* parasite is longer than that of the *Parahaemoproteus* spp., in which tiny oocysts (<15 μm in diameter) with one germinative centre occur (Adie, 1915; Valkiūnas, 2005). In louse flies, the salivary glands are tightly packed and wrapped by the digestive tract (Adie, 1915). Sporozoites (infective stages for birds) are elongate bodies that appear 10–12 days p.i. (Adie, 1924). Histological changes (disruption of basal membranes and inflammation) have been reported in heavily infected salivary glands of louse flies (Klei and De Giusti, 1973).

The merogony and development of gametocytes of *H. columbae* occur in its natural host, the Rock Pigeon (*Columba livia*) (Valkiūnas, 2005). This bird species is of cosmopolitan distribution and is widely raised as a domesticated ornamental bird. In Colombia, these pigeons are considered an invasive species (Baptiste et al., 2010) and/or a pest that may cause health problems in humans due to the high number of associated pathogens (Haag-Wackernagel and Moch, 2004; Villalba-Sánchez et al., 2014).

Haemoproteus parasites do not multiply in peripheral blood, and for that reason, it is unlikely that they will infect a new avian host by direct inoculation of blood, as readily occurs with *Plasmodium* spp. (Valkiūnas, 2005). To access the infective stage (sporozoites) of haemoproteids, the sporogonic development must be followed in the louse fly vector. Sporozoites of the parasite reach the salivary glands of the vector, which inoculates them to birds during feeding. Another non-natural alternative to achieve an infection by *Haemoproteus* parasites is the inoculation of either the sporozoites from crushed infectious flies (Ahmed and Mohammed, 1978), or the mature tissue stages of the parasites (Atkinson et al., 1986) in the susceptible recipient avian host, but this mode of infection is difficult to achieve in practise.

Currently, there are few animal models available to study and characterise the host-parasite-vector interactions in avian haemosporidia. For example, the interactions between avian malaria parasites (*Plasmodium* spp.) and their hosts have been studied using two standardised models which involves *Culex* (*Cx.*) *quinquefasciatus* and *Cx. pipiens* mosquitoes to transmit infections to *Serinus canaria* or *Agelaius phoeniceus*. However, in the past, other models mainly involved chickens and ducklings (LaPointe et al., 2005; Valkiūnas et al., 2015). Additionally, various wild birds and biting midges of the genus *Culicoides* (mainly *C. impunctatus* and *C. nubeculosus*) were used to study sporogony of some species of the subgenus *Parahaemoproteus* (Bukauskaitė et al., 2015, 2016).

These animal models are tractable for use in experimental research due to the availability of both avian host and vectors that can be maintained under controlled laboratory conditions. This provides opportunities to sample parasitological material during experimental exposure of hosts. Model organisms to access development of haemosporidian parasites of the subgenus *Haemoproteus* remain unaccessed in experimental research. The main aims of this study were: i) to develop a useable methodology to carry out experimental research with *H. columbae* using its natural avian host (the Rock Pigeon) and its vector (louse fly) and ii) to follow the complete life cycle of *H. columbae* (*cytb* lineage HAECOL1) in experimentally infected insects and birds.

2. Materials and methods

2.1. Ethical considerations

The Bioethics Committee (Facultad de Ciencias of the Universidad Nacional de Colombia Act number: 04 of 2017 and 03 of 2018) approved the methodology used in this study. Fieldwork was done under permit No. 0255 granted by Autoridad Nacional de Licencias Ambientales (ANLA), Colombia.

2.2. Parasite lineage isolation and characterization

The cytochrome b lineage of *H. columbae* HAECOL1 was found in a naturally infected *C. livia* (pigeon No. 20) captured in Bogotá city, Colombia. In order to maintain the same clone of the parasite lineage, three naïve pigeons were infected experimentally by louse fly bites infected with the HAECOL1 lineage (see Fig. 1 for a detailed explanation of procedures used and their order; Table 1).

2.3. Cloning the infection of Haemoproteus columbae (lineage HAECOL1)

The exposure procedures of Rock Pigeons and louse flies to HAECOL1 lineage were as follows. Experimental pigeons were purchased from a pet shop (Table 1). Once in the laboratory, pigeons were screened for haemoparasites by microscopic examination and PCR-based testing.

Eighteen naïve adult louse flies (six per pigeon) were used to transmit *H. columbae* to three uninfected pigeons No. 68, 70 and 71; in these birds we characterised the infection cycle on the vertebrate host. Pigeons No. 70 and 75 were used to follow the infection cycle in the vector. Insects used in the experimental infection, were starved from 12 to 14 h in the incubator at 28 °C and 30–40% relative humidity. Then, the louse flies were allowed to feed for 24 h on bird N° 820, the pigeon harbouring mature gametocytes of the HAECOL1 lineage (2.4% parasitaemia with mature gametocytes). At this parasitaemia, one in six individuals were infected (Supplementary Table S1). Then the infected louse flies were collected manually and placed on uninfected pigeons to clone the infection with the same lineage of parasite. The entire sporogonic cycle occurred in the insects during their normal process of feeding, and the insects infected Rock Pigeons by natural bites when sporogony was completed. This procedure mimics the natural mode of infection and allows to clone the same lineage of the parasite (HAECOL1) in new individual pigeons. The last procedure guarantees maintenance of the same parasite lineage (HAECOL1) in its natural host. Preening was avoided by using restraint collars (Elizabethan bird collars) which were maintained on pigeons until the end of the experiment. It is important to mention that birds were able to feed and move freely despite the use of such collars.

2.4. Rock-pigeon sample and maintenance

The common Rock Pigeons were maintained in an outdoor aviary of the Biology Department, Universidad Nacional de Colombia, where the average year-round temperature ranged between 12 and 15 °C (Bernal et al., 2007). Each bird was kept separately in a cage (50 × 50 × 50 cm) under a natural photoperiod (approximately 12:12 h of dark:light). A silk net covered each cage to avoid contamination with external insects.

Eleven Rock Pigeons were purchased from a pet store and used in the present study as described in Table 1. Once the individuals arrived at the laboratory, and before any experiment, pigeons were tested for possible natural infection with *Haemoproteus* or another blood parasite using microscopic and molecular methods every

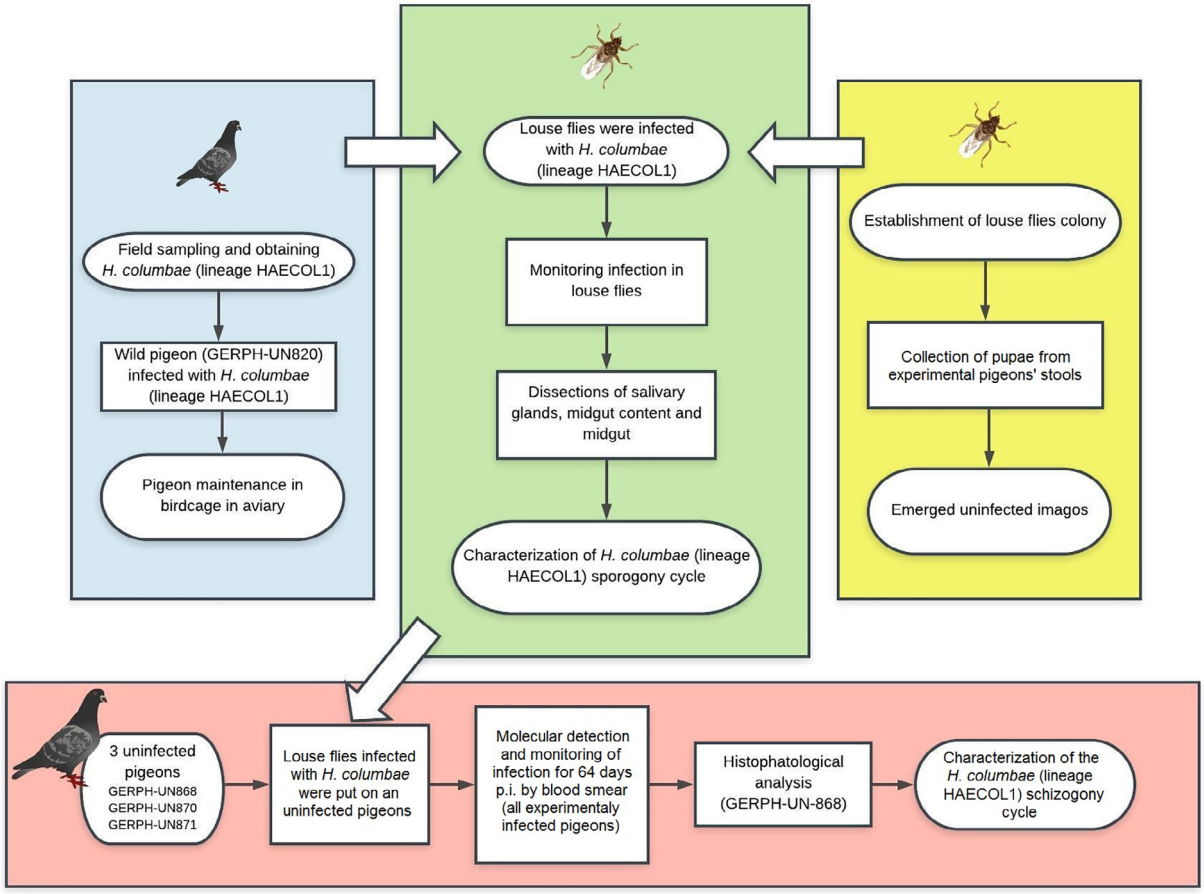

Fig. 1. Diagrammatic representation of the experimental approaches which were used for study of the *Haemoproteus columbae* life cycle in *Columba livia* and *Pseudolynchia canariensis*. dpi, days p.i.

Table 1
Identification of *Columba livia* used in the present study. All Rock Pigeons, except GERPH-UN820, were purchased from a certified pet store.

Rock Pigeon identification (number of individual)	Use in the experiment
GERPH-UN820 (20)	Original pigeon infected with *Haemoproteus columbae* (lineage HAECOL1)
GERPH-UN868[a] (68); GERPH UN870 (70) GERPH-UN871[a] (71)	To follow the infection in the pigeons
GERPH-UN870 (70); GERPH-UN875 (75)	To follow the infection in the louse flies
GERPH-UN873 (73); GERPH-UN869 (69)	Negative controls
And four pigeons without number	Naïve pigeons to maintain the colony of louse flies

[a] GERPH-UN871 died at 64 days p.i., GERPH-UN868 was euthanized 33 days p.i.

week during a 1 month period. These pigeons were ringed and fed three times daily, and water was provided ad libitum according to the requirements of this species. The main procedures of the experimental assays are shown in Fig. 1.

2.5. Microscopic examination and PCR protocols for detection of *Haemoproteus columbae*

Pigeons were bled from the brachial vein. Approximately 80 μl of whole blood were taken in heparinized micro haematocrit tubes (vitrex medical A/S Ref 161315, NRIS, Herlev, Denmark) to prepare three smears and the remainder was stored in EDTA for molecular analysis. Additionally, another 80 μL of blood were collected in capillary tubes and centrifuged for 5 min at 2096*g* (Scientific, Model HC-12A-Zenith Lab Inc., USA) and a Haematocrit Reader Card was used for haematocrit estimation.

Three blood smears were fixed in methanol and stained using 4% Giemsa solution (Valkiūnas et al., 2008). Parasitaemia and gametocytemia were estimated by counting the number of parasites per 10,000 erythrocytes. Blood films for microscopic examination were prepared daily starting from the first day until 64 days p.i. to follow the course of parasitaemia. Uninfected pigeons (controls) were tested once per week.

Haemoproteus columbae was identified according to Valkiūnas (2005). DNA from the blood was extracted using a DNeasy Blood & Tissue kit (Qiagen, GmbH, Hiden, Germany) and tested for *H. columbae* by amplifying 480 bp of parasite mitochondrial cytochrome *b* gene (*cytb*) according to Hellgren et al. (2004). The amplifications were evaluated by running 1.5 μl of the final PCR product on a 1.5% agarose gel.

2.6. Histopathological analysis

Pigeon No. 68 was euthanized at 33 days p.i. with the aims of evaluating the impact of the infection in selected tissues and identifying sites of exo-erythrocytic merogony. Brain, heart, kidney, liver, lungs, and spleen were fixed in 10% buffered formalin and embedded in paraffin for H&E routine staining. A veterinary pathologist (P.B.) evaluated the case with an Olympus BX43 light microscope. Tissue structures compatible with meronts and lesions

in organs were reported. Digital images of parasites were taken using an Olympus DP27 digital camera coupled to the microscope and processed with the cellSens™ Microscope Imaging Standard software (Olympus, Tokyo, Japan).

2.7. Collection and maintenance of louse flies infected with Haemoproteus columbae (HAECOL1)

Louse flies were captured by hand from pigeons in Bogotá, Colombia 4°35′53″ N, 74°4′33″W), and they were removed manually from more than 100 feral Rock Pigeons, placed in rearing silk mesh boxes (15 × 15 × 15 cm) and transported to the laboratory. The collected insects were placed on a non-infected caged pigeon for maintenance and reproduction. Since a female louse fly can produce one pupa every 2 days after they lay their first pupa (Herath, P.R., 1966. Colonising *Pseudolynchia canariensis* on hosts other than the pigeon: *Columba livia*. PhD, Wayne State University, Detroit, Michigan, USA), birdcages were examined once per week, looking for pupae that were mainly found in the cage litter. Pupae were placed in containers and maintained in an incubator at 28 °C and 30–40% relative humidity (Memmert model INB400, Germany) until the emergence of imagos. To increase the number of louse flies in the colony, all emerged adults were maintained on uninfected pigeons and used as parental lines for further reproduction, as described above.

The louse flies were identified to species level using morphological characters and the key by Hutson (1984). The species identity was confirmed by amplification of a 658 bp barcode fragment of Cytochrome Oxidase I (*cox1*) using the primers LCO1490/HCO2198 (Folmer et al., 1994) according to protocol of Colorado-Garzón et al. (2016).

2.8. The course of infection in louse flies

Twenty-three newly emerged non-infected louse flies (reared in the laboratory from pupae) were grouped in four cohorts and infected over a period of four consecutive days, one cohort per day. Louse flies belonging to each cohort were marked on the wings with a distinctive colour (Waite et al., 2012a,b), and left to starve for 12–14 h in an incubator at 28 °C and 30–40% of relative humidity. Then, they were allowed to take blood meals on an infected pigeon N° 870 (Table 1), whose parasitemia of mature gametocytes ranged between 1.3 and 2.0% during experiments. Detailed information about the number of individuals tested in each cohort and the corresponding parasitemia of the blood donor is provided in Supplementary Table S2. After 24 h, insects were collected from the infected pigeon No. 70 and transferred to a cage with one naïve pigeon No. 75. As a maximum, three individual louse flies were dissected every 2 or 3 days until 16 days p.i. The louse flies were dissected in order to follow the sporogonic development of the parasite according to the protocols suggested by Adie (1915). Dissections were carried out using a Carl Zeiss™ Stemi™ DV4 binocular stereo microscope (Oberkochen, Alemania). Salivary glands are tightly packed together with the intestine in the abdomen area. Using a blade, an incision was made in the posterior abdomen segments, following the middle line. Then, abdomen contents were gently pulled out using an entomological needle. Furthermore, to extract the goblet-shaped organ, which may contain sporozoites, a thin cross-section of the ventral chitin plate of the thorax was performed using a sterile razor blade. Thin films of midgut contents and salivary glands were prepared separately, fixed in absolute methanol and stained with Giemsa as blood films. The midguts were stained with Mercurochrome 2% for 10–15 min and then examined under the microscope. If oocysts were found, permanent preparations were prepared; entire midguts were fixed in formalin, stained with Ehrlich's hematoxylin and mounted in diluted Canada balsam following the protocols suggested by Valkiūnas (2005), Kazlauskienė et al. (2013) and Bukauskaitė et al. (2015). Images were prepared from both haematoxylin and Mercurochrome stained preparations (Supplementary Fig. S1).

2.9. Microscopic examination of vector preparations and parasite morphology

An Olympus BX43 light microscope was used to examine preparations, prepare illustrations, and to take measurements. All preparations were first examined at low magnification (400×) and then at high magnification (1000×). Digital images of parasites were taken using an Olympus DP27 digital camera coupled to the microscope and processed with the cellSens™ Microscope Imaging Standard software (Olympus, Tokyo, Japan). The morphometric features studied were those defined by Valkiūnas (2005). Voucher specimens of ookinetes (GERPH-UNI002:HAE; GERPH-UNI001:HAE), oocysts (GERPH-UNI006:HAE; GERPH-UNI0067:HAE) and sporozoites (GERPH-UNI022) of *H. columbae* lineage HAECOL1, and histological preparations of tissue stages (biological record ID: UNAL:GERPH:UN868:HAE), were deposited in the Biological collection GERPH, Biology Department, Universidad Nacional de Colombia, Bogotá, Colombia.

3. Results

3.1. The establishment of a natural model system for maintenance of Haemoproteus columbae infection

We developed a methodology to establish a natural avian host-parasite-vector model to maintain haemoproteid parasites belonging to the subgenus *Haemoproteus*. The described procedures were tested several times and allowed us i) to obtain *H. columbae*-infected avian hosts, ii) to sample sufficient numbers of infected and non-infected louse flies, iii) to infect louse flies and to follow the complete sporogonic development of *H. columbae*, and iv) to infect naïve birds and to follow the entire life cycle in the natural vertebrate host. This methodology and host-parasite model open opportunities for precise investigations of various aspects of the biology of haemosporidian infections and access to more details about the development of *H. columbae* (HAECOL1) infection.

Application of molecular and morphological tools in parallel indicated that the louse flies are cosmopolitan ectoparasites of Columbiformes birds. Also, these flies are competent vectors of the lineage HAECOL1 and are tractable to use in experimental vector research.

3.2. The course infection and dynamics of parasitaemia of Haemoproteus columbae (HAECOL1) in pigeons

The prepatent period was 19–20 days p.i. The acute phase, determined as the continuous active production and increasing number of immature gametocytes in the blood lower than 15% (Ahmed and Mohammed, 1978), started between 22 and 25 days p.i. in different individual birds (Fig. 2). After that, the crisis occurs; and it is characterised by an accelerated decrease of parasitemia (Ahmed and Mohammed, 1978), which dropped sharply from approximately 29–30 days p.i. and turned to the chronic stage within the next 7–8 days (Fig. 2).

Fig. 3 shows the development of gametocytes of *H. columbae* (HAECOL1) in pigeon No. 68. The immature gametocytes (Fig. 3A–D, G–H) predominated in the beginning of the acute stage. The first fully grown mature gametocytes (as determined by displacement of host cell nuclei and the presence of pigment granules

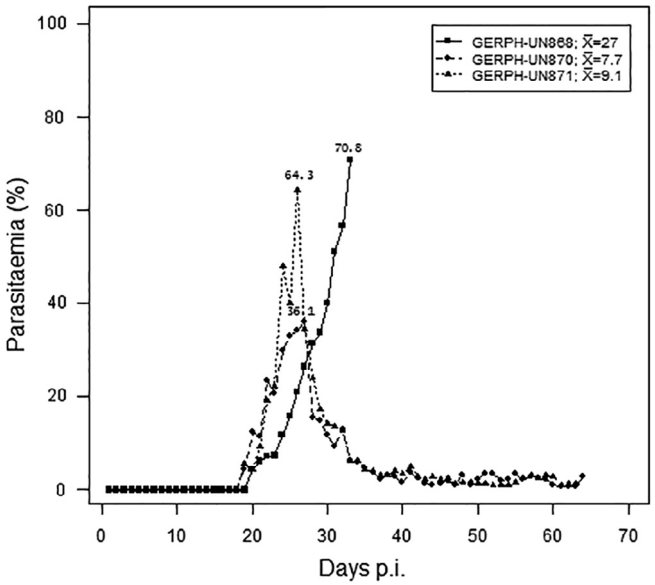

Fig. 2. Dynamics of *Haemoproteus columbae* parasitemia in three experimentally infected Rock Pigeons (*Columba livia*) (GERPH-UN868, GERPH-UN870, and GERPH-UN871).

on the ends of the parasite; Fig. 3E–F, I) were seen 3–5 days after the microscopical detection of parasites in the blood. The proportion of macro- to microgametocytes was 1:3 during the course of infection. Multiple infections of the same red blood cell with several growing gametocytes were common during high parasitaemia (Fig. 3G–I), but not during the chronic infection stage.

The intensity of parasitaemia varied among pigeons and days p.i., e.g. the peak of parasitaemia for pigeon No. 68 was 70.8% (33 days p.i.), but in pigeon No. 870 it was 36.1% (28 days p.i.) and for No. 871 was 64.3% (27 days p.i.; Fig. 2). Pigeon No. 871 died 64 days p.i. with a parasitaemia of 1.4% and an haematocrit value of 36% (Supplementary Table S2). The average haematocrit value for the two negative controls was 51%.

3.3. The establishment of a colony of louse flies and the sporogonic development of Haemoproteus columbae

This study shows that louse flies can be maintained under semi-natural conditions, and that several generations of these insects were successfully reared. A decline in the number of louse flies placed on the pigeons was also reported; the main reason for insect mortality was the killing of insects by grooming pigeons. Therefore, in order to maintain a sufficient number of louse flies in the colony and to increase the genetic variability of the flies, new wild-caught insects were introduced to the colony every month. Pupae were mainly found in the cage litter, and we were able to differentiate a scale of at least four colours grades of pupae varying from beige to black, and it was associated with the level of their maturation (Supplementary Movie S1). The development of pupae until the emergence of the imago took between 10 and 15 days under laboratory conditions, as described above.

Serial dissections of 23 louse flies allowed us to find ookinetes from 24 h post exposure (Fig. 4A, B), while the few oocysts observed appear in the midgut 4 days p.i., and mature oocysts can be seen 13 days p.i. (Fig. 4C, D and Supplementary Fig. S1). Sporozoites were detected from 13 to 16 days p.i. (Fig. 4E–F). The overall prevalence of sporogonic stages was 39.1% (9/23 flies infected). Both low prevalence and intensities of infection were observed particularly for oocysts (no more than of six oocysts were reported per infected insect, Supplementary Fig. S1A), and sporozoites (two to nine sporozoites were seen in each preparation; Supplementary Table S1). All parasite stages in the vector were measured and compared with values from previous studies (Table 2).

3.4. Histopathological findings

Examination of histological organ sections of the euthanized infected pigeon reveals that lungs presented the most critical damage generated by *H. columbae* infection, with moderate multifocal perivascular and mild lymphoplasmacytic pneumonia with abundant and multifocal exo-erythrocytic meronts located in capillary vessels (Fig. 5A). In the liver, moderate and multifocal lymphoplasmacytic hepatitis was observed, accompanied by moderate intracytoplasmic presence of meronts of *H. columbae* in mononuclear cells located in sinusoids (Fig. 5B and C). Also, multifocal and moderate hemosiderosis were associated with the presence of meronts (Fig. 5D). Meronts were characterised by an irregular, often lobular-like shape and variable size (Table 3).

4. Discussion

The key result of this study is the establishment of a usable avian host-parasite-vector model, which provides opportunities to access all stages of the life cycle in the haemosporidian parasite *H. columbae* under controlled laboratory conditions. This model system is recommended for the investigation of the biology of avian haemosporidian parasites of the subgenus *Haemoproteus*.

Several species of parasites belonging to this subgenus have been described parasitizing Columbiforme and marine birds (Valkiūnas, 2005; Valkiūnas et al., 2010; Levin et al., 2012), and the system described here will allow detailed studies of the life cycle of parasites of this subgenus. That is important because these haemoproteids cause severe diseases in pigeons and doves (Earlé et al., 1993).

Few *cytb* lineages of *H. columbae* have been reported around the world, and the genetic distances between them can be as large as 1% (e.g., hCOQUI05 and hCOLIV03; Chagas et al., 2016). Because different parasite lineages of same parasite species could produce different immune responses, or pathology in their hosts and vectors (Kazlauskienė et al., 2013; Žiegytė et al., 2014), in the present research we mimic the natural system of infection and transmission of *H. columbae*, and generate a population of pigeons infected with the same lineage of parasite HAECOL1 (clones). The lineage HAECOL1 is of wide distribution; there are reports of this parasite in Africa (Waldenström et al., 2002), Colombia (Coral et al., 2015; González et al., 2015), Italy (Scaglione et al., 2015) and Brazil (Chagas et al., 2016). This provides opportunities to develop comparative research using the same lineage in different sites and laboratories in the future.

It is important to note that a louse fly colony which is free of parasites was established. That provides opportunities to maintain the *H. columbae* infection in their natural hosts, the Rock Pigeon and louse fly. This study provided, to our knowledge, the first information about the development of the lineage HAECOL1 both in the insect vector and in the natural avian host *C. livia*.

The host-parasite model "*Haemoproteus columbae-Columba livia*" has been used in studies of avian haemosporida (Aragão, 1908; Valkiūnas, 2005). Some observations on the parasite life cycle, development and the course of infection have been published in France, Egypt, India and other countries (Aragão 1908, Adie, 1924; Ahmed and Mohammed, 1978; Coatney and Hickman, 1952; Mohammed, 1958; Rendtorff et al., 1949; Sergent and Sergent, 1906; Waite et al., 2014). Prevalence of this infection is high, and an active transmission occurs year-round in tropical countries, with a predominance of chronic infections in

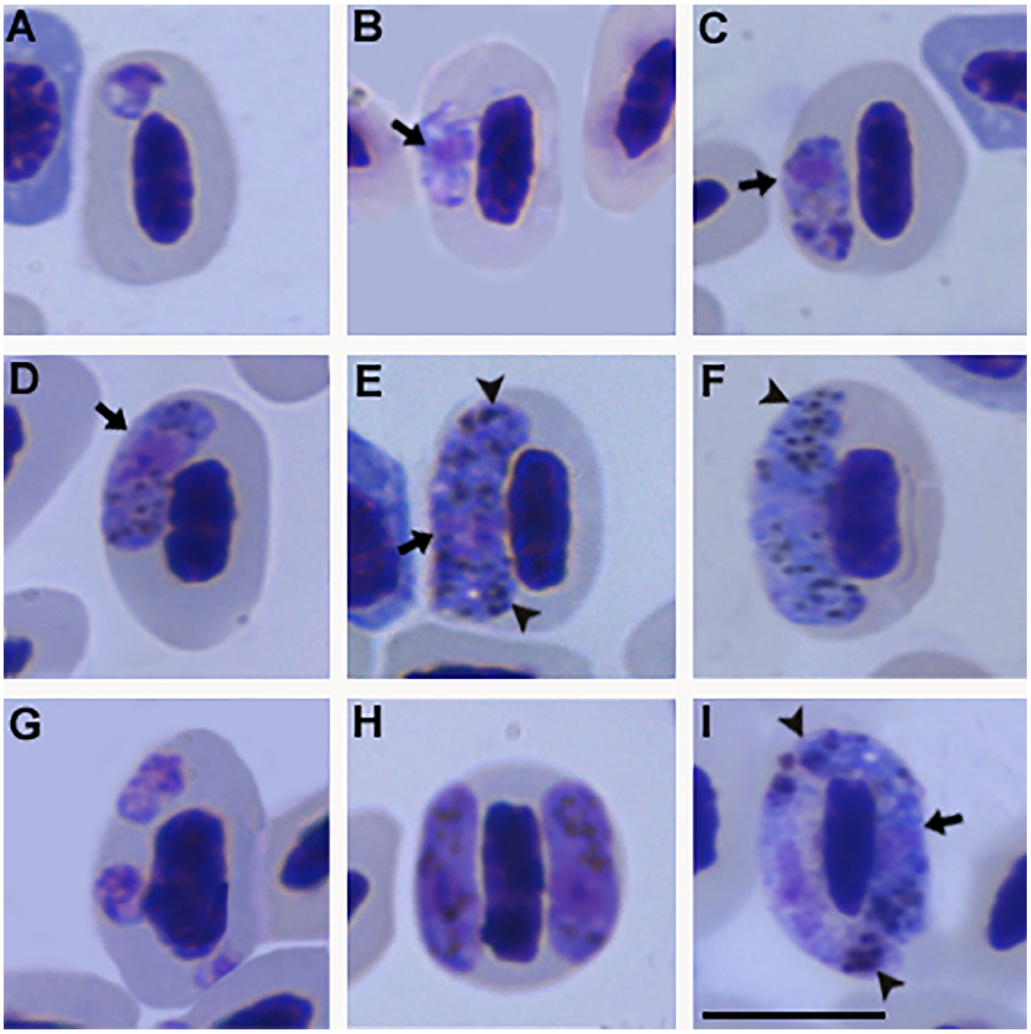

Fig. 3. Development of gametocytes of *Haemoproteus columbae* (lineage HAECOL1) in experimentally infected the Rock Pigeon No. 68. Immature gametocytes (A-B, G) 20 days p.i., 21 days p.i. (C-D), and 22 days p.i. (H). Mature macrogametocytes (E, F, I) and microgametocytes (H, I) 22 days p.i. (E), 23 days p.i. (F) and 24 days p.i. (I). Arrows show parasite nuclei and arrowheads indicate hemozoin granules. Scale bar = 10 μm.

pigeons (Adriano and Cordeiro, 2001; Villalba-Sánchez et al., 2014; Coral et al., 2015; González et al., 2015). This study provides, to our knowledge, the first experimental data on behaviour of the HAECOL1 lineage, both in the vector and vertebrate hosts.

The course of *H. columbae* infection reported in this study, particularly the prepatent period, was similar to that reported by Rendtorff et al. (1949) and Waite et al. (2014) despite the infection method used (louse fly bite or injection of infected macerated flies). Nevertheless, it is shorter in comparison to the prepatent period reported by other authors (Sergent and Sergent, 1906; Adie, 1924; Coatney and Hickman, 1952; Mohammed, 1958; Ahmed and Mohammed, 1978; Waite et al., 2014; see Supplementary Table S3). Indeed, the most prolonged period (15 days) was obtained by Ahmed and Mohammed (1978), who also used various modes of infection (louse fly bites, intramuscular, intravenous, and intraperitoneal inoculation of sporozoites). Porter et al. (1952) refer to these differences in time of the prepatent period, which may be due to how the infections were carried out. However, more in-depth studies should be designed for better understanding of this issue.

In this study, the acute stage was slightly shorter than reported by Ahmed and Mohammed (1978) and similar to Waite et al. (2014; Supplementary Table S3). Such differences can be due to numerous factors such as the climatic conditions of the aviary, the geographical origin of the sample, the immune response and physical condition of the hosts, sex, age, and the parasite lineage (Valkiūnas, 2005; Gayathri and Hegde, 2006; Donovan et al., 2008; Olias et al., 2011; Waite et al. 2012b; Ghosh et al. 2014, Valkiūnas and Iezhova, 2017). Further studies are needed for better understanding of whether the prepatent period of infection can markedly vary in same parasite lineage and what factors are critical for such variation during development in the same avian host.

It has been shown that infection by haemosporidians, e.g. *Plasmodium gallinaceum* (de Macchi et al., 2013) or *Haemoproteus* spp. (Cannell et al., 2013) has a direct effect on haematocrit values causing anaemia or sometimes death (Farmer, 1964; Donovan et al., 2008; Cannell et al., 2013). Previous reports on haematocrit values in healthy pigeons vary between 41 and 50% (Gayathri and Hegde, 2006; Glomski and Pica, 2016). Our results, however, show two opposite cases. The first pigeon, No. 68, at the time of its sacrifice had a parasitaemia of 70.8% and its haematocrit was 50%. The second pigeon, No. 871, showed very low values of haematocrit over several days of the infection with parasitaemia <2%. This probably indicates differences in immune responses of the hosts. It is possible that such a grade of infection in the pigeon No. 68 does not cause anaemia, and probably, if this infection can cause disease or death in nature, it would be associated with the rupture of exo-erythrocytic meronts, which can induce an uncontrolled

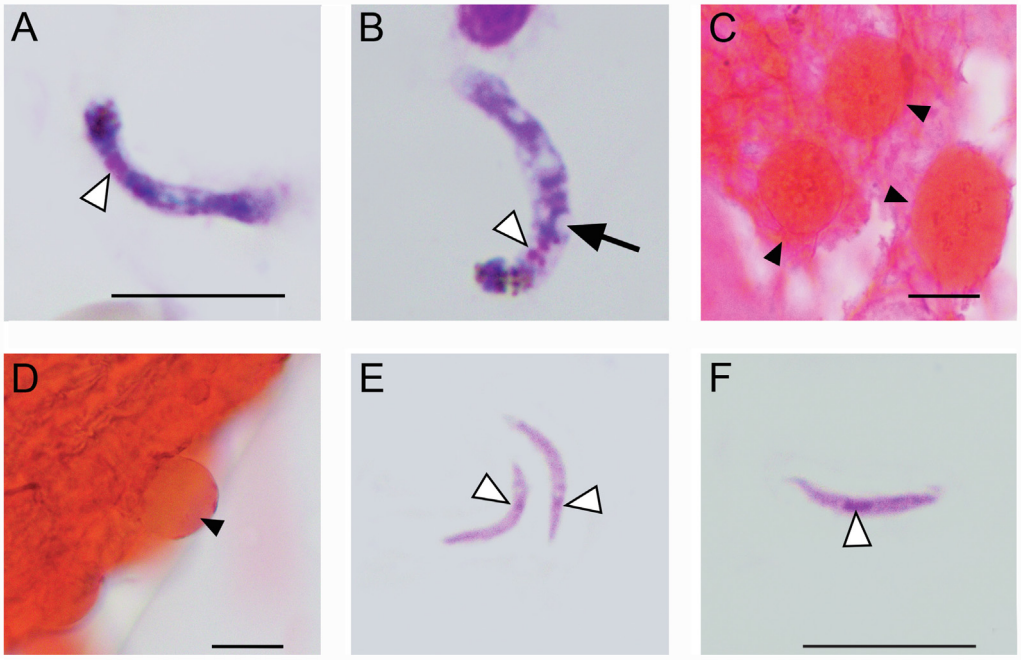

Fig. 4. Sporogonic stages of *Haemoproteus columbae* in experimentally infected louse flies. Methanol-fixed and Giemsa-stained preparations of ookinetes (A, B) and sporozoites (E, F). White arrowheads – parasite nuclei; black arrow – vacuole. Scale bar (A, E, F) = 10 μm. Formalin-fixed and Erlich's hematoxylin stained (C) and mercurochrome stained fresh preparations of midguts (D) showing developing oocysts. Black arrowheads indicate oocysts. Scale bar (C, D) = 20 μm. Note gathering of pigment granules at the distal end of ookinetes (A, B), and heterogenous structure of developing oocysts (C).

Table 2
Comparison of measurements (in μm) of ookinetes, oocysts and sporozoites of *Haemoproteus columbae* reported in different studies. Extrinsic incubation periods (EIP) until the parasite reaches the salivary glands are provided.

Features	Measurements (μm)		
	H. columbae/ *Pseudolynchia canariensis* This study	*H. columbae*/ *Ornithomyia avicularia* (Baker, 1957)	*H. columbae*/*P. canariensis* (Adie, 1915)
Ookinetes	*n* = 29		
Length	9.06–19.86 (15.73 ± 2.33)	20.00	–
Width	1.36–2.45 (1.904 ± 0.28)	2.25	–
Oocysts	*n* = 30	*n* = 3	
Diameter	12.33–43.76 (26.64 ± 9.19)	36–46 (41.67 ± 5.13)	36.5
Sporozoites	*n* = 8	*n* = 3	
Length	6.74–9.45 (8.69 ± 0.87)	7.5–8.5 (7.8 ± 0.58)	7–10
Width	0.95–1.26 (1.06 ± 0.12)	0.5	–
EIP	13 days	–	10 days

immune response (Lee et al., 2016). It is essential to keep in mind that the individual host immune condition also could affect the course and the final result of the infection (parasitaemia burden).

Recently, significant advances have been made in characterization of the sporogonic development of haemosporidia parasites infecting birds. For the particular case of *Haemoproteus* (*Haemoproteus*) spp. that are transmitted by louse flies, sporogony takes from 6.5 to 7 days (*H. palumbis*; Baker, 1966) or even 10 days (*H. columbae;* Adie, 1915). Nevertheless, it has been reported that this process for parasites transmitted by louse flies may take longer, since oocysts are large and thus need more time to mature (Valkiūnas, 2005). Despite this, our results contrast with those reported by Adie (1915), where the sporogony of the same parasite species takes approximately 10 days to be completed in "*Lynchia maura*" (*P. canariensis*) (Table 2). Probably the aviary and difference in environmental conditions in different studies might be an explanation. However, Baker (1966) questions the environmental temperature variation as a probable cause of variation in the duration of the parasite sporogonic development because louse flies remain on birds in close contact with their skin for a long time, thereby they are under a more constant temperature condition.

Adie (1915) reported that parasite sporogony was completed within 9–10 days when insects were maintained on an infected bird. In our study, the insects did not remain on the infected host (where reinfection of the insect is possible), but instead the louse flies were transferred to an uninfected bird after 24 h of contact with the infected pigeons. The complete sporogony in our experiment took between 13 and 16 days p.i. Some environmental features or stress due to manipulation of the insects or host could also cause these changes.

Vector competence may fluctuate according to the strain of the vector (Collins et al., 1986), the parasite lineage, or the presence of endosymbionts (Moreira et al., 2009; Weiss and Aksoy, 2011; Zélé et al., 2014). Our current knowledge about the impact of such variables in this system is limited, and further studies on these matters need to be developed.

Previous studies have demonstrated a deleterious effect of the high gametocytaemia (over 1% of mature gametocytes) of *Parahaemoproteus* parasites on blood-sucking *Culicoides* biting midges and mosquitoes (Bukauskaitė et al., 2016). In this study, such gametocytaemia seems not to be harmful in louse flies, probably because biting midges and mosquitoes are tiny insects in comparison to louse flies. This issue requires additional investigation.

This study showed numerous exo-erythrocytic meronts in infected birds, with particularly high intensity in the lungs. Moderate focal granulomatous inflammation in the spleen, necrosis with central classification necrosis, and moderate generalised hemosiderosis (overload of iron deposition associated with hemolysis due to malaria infection; MacDonald, 1963) were observed. Recent reports have confirmed the virulence of infection of haemo-

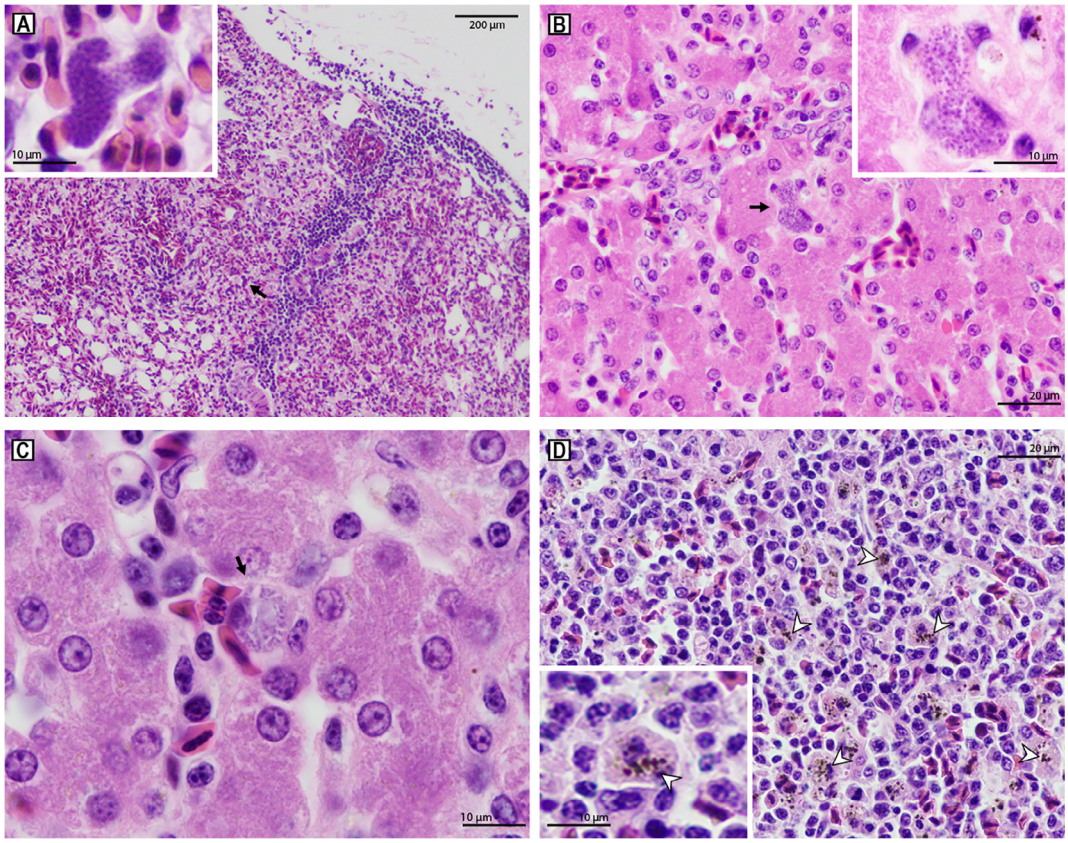

Fig. 5. Exo-erythrocytic meronts of *Haemoproteus columbae* in lungs (A), liver (B, C) and spleen (D) of experimentally infected Rock Pigeon (No. 68) 33 days p.i. Black arrows: meronts; white arrowheads: hemosiderosis. Note the branching shape of meronts in lungs (A).

Table 3
Measurements in µm of meronts found in lungs, kidneys and spleen of Rock Pigeon GERPH-UN868. Length, width, area and the number of *Haemoproteus columbae* merozoites are given.

	Meronts in lungs (*n*: 46)		Meronts in kidneys *n* = 3	Meronts in spleen *n* = 3
	Sagittal section *n* = 36	Transversal section *n* = 10		
Lenght	15.1–44.7 (27.5 ± 9.1)	9.1–15.4 (13.3 ± 1.8)	10.6–23.6 (15.04 ± 7.42)	13.5–27.5 (20.3 ± 6.9)
Width	4.6–17.14 (11.8 ± 3.6)	6.4–9.5 (7.7 ± 1.05)	8.7–11.9 (10.6 ± 1.7)	4.4–10.7 (7.4 ± 3.2)
Area	59.6–216.4 (137.1 ± 52.5)	47.97–91.5 (65.05 ± 17.8)	59.9–136.4 (107.6 ± 41.5)	83.8–303.6 (164.6 ± 120.1)
N° of merozoites	34–115 (63.82 ± 24.40)	24–65 (40.1 ± 11.9)	31–74 (46.7 ± 23.7)	27–96 (57 ± 35.4)

proteids that for several decades was considered benign. That is probably because it is difficult to follow infections in the wild, and sick avian hosts are easy prey for wildlife, so are difficult to sample during field work. A few studies discuss in detail the pathological mechanism underlying the infection or mortality caused by these parasites. Earlé et al. (1993) analysed histopathological sections of sick pigeons due to possible *H. columbae* infection and found numerous meronts and multi-lobular megalomeronts in several organs, particularly in the striated muscles. It was concluded that extensive fibre necrosis produced by rupture of megalomeronts could be the cause of mortality. Ortiz-Catedral et al. (2019) found that *Haemoproteus minutus* cause lethal infections in Australasian and South American parrots, even in the absence of parasites in peripheral blood. The infections were associated with multifocal extended haemorrhages caused by disrupted meronts in the heart and gizzard muscle. More research is needed for better understanding of the virulence of avian haemoproteids.

In our study, there was evidence of extensive damage, mainly in pigeons' lungs (Fig. 5A). However, in contrast to the report of Earlé et al. (1993), megalomeronts were not observed in this study (meronts in Fig. 5A–C). The meronts observed in our study were more similar to those reported in the lungs by Aragão (1908) in Brazil; they were of irregular shape in both studies. Differences in the host species or even the parasite lineage might explain such changes (Atkinson et al., 1995; Valkiūnas, 2005).

Finally, this study has developed, tested and used an experimental model host-parasite system to study haemosporidian parasites belonging to the subgenus *Haemoproteus* at all stages of their life cycle in avian hosts and insect vectors. This experimental methodology provides opportunities to collect biological parasite material to answer various research questions. Due to relatively easy access to different parasite life cycle stages (gametocytes, gametes, ookinetes, oocysts, sporozoites), this methodology enables the testing of hypotheses or solving of questions related to various morphological, physiological, immunological or genetic issues in studies aimed at better understanding haemosporidian infections. Moreover, the model allows precise monitoring of the course of infection under different and well-controlled biotic and

abiotic variables. Data on the life cycle of widely distributed *H. columbae* (lineage HAECOL1) were obtained, and this facilitates extrapolation of results from different geographical regions. Both sporogony and merogony of *H. columbae* can be readily followed under laboratory conditions, providing opportunities to obtain valuable information about this infection at any time using samples either from the vector or the vertebrate host.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.07.003.

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