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The pathology and pathogenicity of a novel *Haemoproteus* spp. infection in wild Little Penguins (*Eudyptula minor*).

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Abstract

One hundred and thirty four Little Penguin (*Eudyptula minor*) carcases found since 2004 in south west Australia were necropsied. The livers and spleens from ten of the penguins exhibited varying degrees of multifocal, randomly scattered areas of necrosis and varying numbers of parasites were associated with these areas. Hepatomegaly and splenomegaly was noted in many of these ten cases. Necrosis and parasites were also observed in the cardiac muscle of four of the cases and in the lung tissue in one of the penguins. Using PCR, the parasites were positively identified in four of the cases as *Haemoproteus* spp. and morphologically identical tissue stage parasites associated with histopathological changes were observed in all ten dead penguins. This is the first study to demonstrate both the *in situ* presence of the *Haemoproteus* parasite in any member of the Sphensicidae family and mortality due to its presence. We postulate the involvement of anomalous environmental conditions in a potential increase in local vectors.

keywords: Little penguins, *Haemoproteus*, wildlife; haemosporidian parasites

1. Introduction

The largest colony of Little Penguins (*Eudyptula minor*) in Western Australia, found on Penguin Island, has the highest conservation status of all the major Little Penguin colonies in Australia and New Zealand (Dann et al. 1996). However, they face an increasing range of natural and anthropogenic threats, such as reduction in food resources, collisions with watercraft, hyperthermia, pollution, and introduced predators (Cannell 2004). These threats, along with parasites and infectious diseases, are known causes of mortality for Little Penguins throughout Australia and New Zealand (Obendorf & McColl 1980, Harrigan 1992, Norman et al. 1992, Hocken 2000a, Clarke and Kerry 1993, Cannell unpubl. data).

The prevalence of the haemosporidian parasites of the genera *Plasmodium, Haemoproteus* and *Leucocytozoon* is generally low in seabirds (Peirce 2005, Quillfeldt et al. 2011), and worldwide, penguins appear to be more likely to be infected by *Plasmodium* than by species of *Haemoproteus* or *Leucocytozoon* (Levin et al. 2009, Quillfeldt et al. 2011). Infections of avian malaria, caused by *Plasmodium relictum, P. elongatum* and *P. cathemerium*, have been observed in many captive populations of penguins, often resulting in high mortality (Fleischman et al. 1968, Bennett et al. 1993a, Clarke & Kerry 1993, Graczyk et al. 1994, Jones & Shellam 1999a, Valkiunas 2005, Bueno et al. 2010). *Haemoproteus* spp. in penguins have only been reported so far in a study of haemoparasites of Galapagos penguins (*Spheniscus mendiculus*), where a single individual, out of 362 tested, was positive (Levin et al 2009). *Leucocytozoon* spp. have been observed in Fiordland crested penguins (*Eudyptes pachrhynchus*), African penguins (*Spheniscus demersus*) yellow-eyed penguins (*Megadyptes antipodes*), and Macaroni penguins (*Eudyptes chrysolophus*) (Valkiunas 2005, Peirce et al.

2005) and were associated with increased regional chick mortality with evidence of severe, disseminated megaloschizont formation in multiple tissues in the latter species (Hill et al. 2010). Haemoparasites have rarely been observed in Little Penguins (Jones & Shellam 1999a,b), though a "malaria-like parasite" was noted to have caused the mortality of two wild Little Penguins from New South Wales in 2000 (Rose 2001) and *Leucocytozoon takawi* was transferred from a Fiordland crested penguin into a juvenile Little Penguin (Allison et al. 1978).

Avian haemosporidians have a sexual phase that develops in a vector, a blood-sucking dipteran, and an asexual phase in birds (Valkiunas 2005). The abundance and geographic range of vectors and hence vector-borne disease may be impacted by climate change (Jones & Shellam 1999b, Harvell et al. 2002), while migratory birds are an important factor in the distribution of parasites and diseases (Jourdain et al. 2007) due to their movement over large areas. In addition to this normal large scale movement, the geographic range of many bird species is also affected by changes in climate. In Western Australia, tropical birds have moved into areas that once were the domain of temperate birds (Dunlop & Wooller 1986, Wooller et al. 1991), and haemosporidian parasites have been reported in a greater number of species of tropical compared to temperate seabirds (Quillfeldt et al. 2011). The expansion of tropical seabirds onto temperate islands such as Penguin Island could therefore result in the introduction or rise in prevalence of such parasites in endemic species.

Since 2004, 134 necropsies have been performed on many of the Little Penguins found dead on the South-western Australian coastal foreshores as well as Penguin Island itself. It is extremely rare to find wild birds that have died of haemoparasitic infections (Valkiunas 2005), as most dying birds are predated upon (Bennett et al. 1993b, Ladds 2009). In this

paper, we report on the first known cases of haemoproteosis caused by *Haemoproteus* spp. in wild Little Penguins in Western Australia, and describe the pathology and pathogenicity.

2. Materials and Methods

2.1 Specimen collection and gross examination

Necropsies were performed on 10 dead Little Penguins found on Penguin Island, Western Australia (32°18'S, 115°41'E) or at various locations in the southwest of Western Australia (Fig. 1) between November 2006 and May 2012. Eight of the dead penguins were frozen for 10-90 days prior to necropsy (following an unspecified interval between death and the discovery of the carcase), and two were necropsied within 24 hours of discovery and subsequent refrigeration, though the post-mortem interval was unknown. Gross external and internal examinations were performed and the body was weighed in all but one case. The liver and spleen were weighed in eight of the cases and abdominal fat pad in seven cases. The bodies were weighed using an Avery platform scale (resolution 10g) and internal organs were weighed using an A&D EK-410i scale (resolution 0.01g). The mass of each of the organs was compared with that reported for Little Penguins from New Zealand (Hocken 2000b). However, penguins from the Penguin Island colony are known to be larger than those from New Zealand (Wienecke 1993, Dann et al. 1996), and as Hocken (2000b) found a consistent organ-to-body mass ratio in penguins, the liver to body mass ratio was used to demonstrate hepatomegaly. The ratio of abdominal fat to body mass was also used to demonstrate the body condition of the penguins. Histopathology was performed on various organs, providing autolysis did not preclude such examination; all carcases were scored according to their degree of autolysis.

Fig.1 here

2.2 Screening of organs for tissue stages of haemosporidians

Tissue samples from the lung, spleen, liver and heart were fixed in formalin, cut into 4µm thick sections and stained with haematoxylin and eosin. 2µm thick sections stained with Martius Scarlet Blue were used to obtain photomicrographs of the organisms, which appeared more prominent using this technique. The samples were examined using light microscopy, including 1000x magnification with oil immersion. Impression smears were made for cytological examination of the liver and spleen in the final case, by rolling the cut surface of the fresh tissues onto glass slides, air drying, then staining with Wright-Giemsa.

2.3 Molecular screening for haemosporidians and sequence analysis.

DNA was extracted and purified from the liver of each bird using a Qiagen DNeasy Blood and Tissue Kit. Using polymerase chain reaction (PCR) a fragment of approximately 480 bp of the mitochondrial cytochrome-*b* (cyt-b) gene was targeted for *Plasmodium* and *Haemoproteus* using a nested PCR strategy. For each bird, at least three independent PCR amplifications were performed before being declared negative. Avian blood samples known to be positive for either *Haemoproteus* or *Plasmodium* were used as positive controls for the PCR. Samples were first amplified using the primers HaemNF (5'-CATATATTAAGAGAATTATGGAG-3') and HaemNR2 (5'-AGAGGTGTAGCATATCTATCTAC-3') described in Waldenstrom et al. (2004). A second nested PCR was performed using HaemNF1 (5'-CATATATTAAGAGAAITATGGAG-3') [I = a universal base, inosine] and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3') (Hellgren et al. 2004). PCR were set up as 15 µl reactions containing 50 ng template DNA, 0.75 units of AmpliTaq Gold (Applied Biosystems, Carlsbad, California), 1.5 µl of MgCl, 0.2mM of each dNTP, and 0.5mM of each primer. The HaemNF and HaemNR2 PCR were run using a program of 94°C for 8 minutes followed by 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds, and a final extension at 72°C for 10 minutes.

The conditions for HaemNF1 and HaemNR3 are identical except for an annealing temperature at 52°C. An additional positive control to assess DNA quality was performed using avian-specific cyt-*b* primers as described in Cicero and Johnson (2001).

PCR products were cloned using the pCR4-TOPO TA kit following the manufacturers recommended protocol (Invitrogen, Carlsbad, CA). Cloning rather than direct sequencing of PCR products was performed initially to avoid problems that may arise due to presence of mixed infection. Multiple clones from each positive PCR amplification were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Invitrogen, Carlsbad, CA). Sequences from independent clones obtained from the same bird were always identical indicating absence of variation due to PCR and/or cloning artifacts. Sequences obtained were viewed and manually edited using Sequencher ver 5.0 (GeneCodes, Ann Arbor, MI) and aligned using ClutalX (Larkin et al. 2007). Sequences were compared to those in the GenBank database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov). Novel sequences generated in this study have been deposited in GenBank under accession numbers KC121053-KC121056

Parameters for phylogenetic analysis and rooting were based on that of Outlaw and Ricklefs (2011). A maximum likelihood phylogeny was generated using MEGA 5.0 (Tamura et al. 2011) and a GTR+ gamma model of nucleotide substitution with 1,000 bootstrap iterations, and was rooted between mammalian *Plasmodium* and avian *Plasmodium*. Accession numbers for additional sequences used in the phylogenetic analyses are: Avian *Haemoproteus* spp: *Spheniscus mediculus*, JX679087 and KC121057; *Heteromyias albuspecularis*, AY714147; *Turdus merulus*, DQ630013; *Sylvia atricapilla*, GU784854; *Malimbus rubricollis,* HQ386243; *Quelea quelea*,EF117230; *Fringilla coelebs*, DQ368340; *Phylloscopus trochilus,*

AF254972; unknown avian host, EF032812. Avian *Parahaemoproteus* spp: *Dumetella carolinensis*, GU252002; *Vireo olivaceus*, GU252005; *Falco sarverius*, GQ141558. Avian *Plasmodium* spp.: *Seiurus aurocapillus*, HM222481; *Parus major*, DQ658590 and JQ778277; *Passer domesticus*, AF069611. Mammalian *Plasmodium* sp.: *Gorilla gorilla*, GU045322; *Grammomys surdaster*, DQ414646; *Mandrillus sphinx*, JF923750.

3. Results

3.1 History and body condition of penguins cases

Three of the ten cases were adults that had previously been banded or microchipped. These marked penguins had been observed 11-45 days prior to being found dead (Table 1). Of the other seven cases, one was an unmarked fledgling and six were unmarked adults, none of which had previously been observed (Table 1). Penguin 11/624 had just completed rearing two chicks, which were last observed in the nest on 14th September 2011, i.e. approximately 2 months prior to 11/624 being found dead.

The body condition of the penguins was variable; however none were in poor condition, all having an appreciable layer of subcutaneous adipose tissue and a reasonable to adequate intra-abdominal fat pad. The penguins of known ID were all lighter than their average body mass, however the body mass of 11/624 had actually increased from the time it was last observed alive (Table 1).

3.2 Gross pathological findings

3.2.1 Liver

Hepatomegaly with rounded lobar edges was evident in several of the penguins. The livers were weighed in eight penguins and ranged from 58.6-79.7g (Table 2), with an average of

65.8 ± 4 g for males and 65.7 ± 3.3 g in females. This is notably greater than that of healthy Little Penguins in New Zealand (males: 42.4 ± 14.6g, females: 34.2 ± 10.9g Hocken 2000b). The liver mass, as a percentage of body mass, averaged 5.3 ±0.5 for males and 5.5 ± 0.3 in females (Table 3). In this study, the ratio of liver to body mass for all penguins (5.4±0.3) was substantially greater than that obtained by Hocken (2000b) for healthy Little Penguins (3.67±0.8). Thus, all eight individuals in which the liver was weighed had demonstrable hepatomegaly. Pinpoint flat white to off-white spots were disseminated over the hepatic capsular surfaces in 11/626, 11/627, 11/628, and 12/362 (Fig. 2). A focal area of metallic yellow speckling was noted on the capsular surface of the right liver lobe of 08/1075 (interpreted as urate precipitate).

Fig. 2. here

3.2.2 Spleen

The spleen was enlarged in all cases (8/10) in which it was measured (Table 2). The average for both males (6.1±1.0g) and females (5.2±1.2g) was notably greater than that found by Hocken (2000b) for healthy males (1.5±1.0g) and females (1.2±0.7g). Pinpoint white to off-white spots were scattered throughout the splenic parenchyma and over its capsular surface in the case of 11/624.

3.2.3 Abdominal Fat Pad

The abdominal fat pad was weighed in six of the penguins and ranged from 4.1-16.1g (Table 2), with an average of 8.6±2.3 g for the males and 9.9±3.2g for the females. The mass of the abdominal fat pad as a percentage of body mass varied from 0.3-1.3%. The average for the

males was 0.7±0.2% and 0.8±0.2% for the females. This was lower than that for healthy males (1.4±0.9%) and slightly lower than that for females (0.9±0.8%) (Hocken 2000b).

3.3 Histopathological findings

The degree of autolysis ranged from minimal to moderate-marked and rendered histopathological examination of some tissues difficult in some cases (however parasites were obvious in all ten cases).

The livers from all penguins exhibited varying degrees of multifocal, randomly scattered areas of hepatocellular necrosis (Table 4), which in the worst affected cases was multifocal to coalescing. Associated with these areas were varying numbers of parasites, present in all ten cases. The protozoa were present both extracellularly (scattered amongst necrotic tissue) and intracellularly within macrophages/Kupffer cells, hepatocytes and endothelial cells (the latter was difficult to confirm, given the host cell was significantly enlarged/distorted; however they were often in an intracellular location immediately adjacent sinusoids) at the periphery of the necrotic areas. Each parasite varied from 2-4μm diameter and was round to ovoid with a dark basophilic (in haematoxylin & eosin stained sections) paracentral to eccentric nucleus and pale amphophilic cytoplasm (cytomere). When present intracellularly they were often clustered in small groups (approximately 20μm diameter) of up to 18 cytomeres contained within a thin walled vacuole (meronts containing cytomeres) (Fig. 3). Increased numbers of macrophages were scattered throughout the liver (particularly associated with areas of necrosis) in five cases, with two of these also exhibiting heterophilic infiltrates (Table 4). Scattered hepatocytes and Kupffer cells (particularly at the periphery of necrotic areas) contained small amounts of intracytoplasmic pigment in four cases (Table 4). This material appeared to be comprised of two components mixed together in varying amounts, depending

on the area within the section. The first was opaque, golden-brown and granular (in haematoxylin & eosin stained sections), staining bright blue with Perl's Prussian Blue histochemistry, confirming its identity as haeme pigment (from erythrocyte degradation, given the extensive areas of necrosis with accompanying haemorrhage seen); rather than volutin pigment (which can be present in gametocytes of *Haemoproteus* spp.). This is because the latter is intraerythrocytic and also (being protein bound) does not react positively with Perl's Prussian Blue histochemistry. The second was refractile, appearing pale golden to tan (in haematoxylin & eosin stained sections; it was negative for Perl's Prussian Blue); it occasionally (but not reliably) polarised with the use of a polarising lens. Its morphology was interesting in that it was not granular, but rather linear and occasionally formed a lining around apparently clear vacuoles within the affected hepatocytes and Kupffer cells, often in close proximity to individual extracellular merozoites / cytomeres. Its exact identity is unknown; however it was felt it may represent the residual hyaline walls of ruptured meronts having released merozoites / cytomeres.

Fig. 3. here

Multifocal areas of splenic parenchymal necrosis of varying size were observed in all penguins, the worst affected cases exhibited multifocal to coalescing necrosis. Cytomeres identical to those described in the liver were observed scattered extracellularly throughout the necrotic debris. Single to small groups (up to 8) of identical organisms, possibly meronts, were observed intracellularly within both macrophages as well as what appeared to be reticuloendothelial cells on the periphery of the necrotic areas. Rare intraerythrocytic organisms (consistent with intraerythrocytic merozoites prior to their development into young gametocytes) were observed in an impression smear of the spleen of 12/362 (Fig. 4). Accompanying the necrosis and intralesional parasites was a moderate to marked, multifocal

to coalescing histiocytic and heterophilic (the latter often appearing degenerate) inflammatory infiltrate in five cases, with another two cases exhibiting solely a histiocytic infiltrate (Table 4). Pigment of mixed morphology as previously described in the liver was observed within the cytoplasm of scattered macrophages in the spleen of one case (12/338).

Fig. 4. here

The lungs of 08/1075 and 11/624 exhibited increased numbers of inflammatory cells, predominantly macrophages; however they appeared to be confined within circulation, rather than being representative of a true inflammatory infiltrate. It is unknown whether this was related to a leukocytosis, as pre-mortem haematology was not performed; alternatively, it could have occurred secondary to stress-related leukocyte sequestration within the pulmonary vasculature. Rare parasites resembling merozoites (≤ 1 μm) were observed within alveolar macrophages in the case of 06/1172. Rare merozoite-like organisms were also found within the pulmonary interstitium in three cases (06/1172, 08/1075 and 11/624), but were not observed in the other cases.

Cardiac muscle from four cases (06/1172, 08/1075, 11/628 and 12/362) exhibited rare small focal to multifocal areas of cardiac myocyte degeneration and necrosis. 06/1172 additionally exhibited a focal histiocytic and lymphoplasmacytic periarteritis. Associated with these foci of necrosis and inflammation were sparse individual parasites resembling merozoites (each 1-2 μm), which appeared largely extracellular and scattered throughout the affected tissue.

3.4 PCR Results and Sequence Analysis

Of the ten Little Penguin samples obtained from 2006 to 2012, only four (11/626, 12/020, 12/021 and 12/338) were positive for haemoparasite cyt-*b* (Fig. 5). When multiple PCR

products from each positive bird were cloned and sequenced, each bird was found to harbour a single haemoparasite cyt-*b* sequence (not shown). When compared between birds, the sequences from 11/626, 12/021 and 12/338 were identical. The sequence from 12/020 differed by only a single nucleotide (not shown). When compared to public databases of cyt-*b* genes, the sequences were found to have 86 to 97% nucleotide identity to sequences from parasites previously identified as *Haemoproteus* subgenus *Parahaemoproteus*, and 80 to 84% identity to *Haemoproteus* subgenus *Haemoproteus.* The isolated sequences only shared 79 to 83% nucleotide identity to avian *Plasmodium* isolates. Further confirmation of the *Haemoproteus* spp. nature of the sequences was established using phylogenetic analysis (Fig. 6). The sequences obtained from the Little Penguin samples were aligned to representative haemosporidian parasite cyt-*b* sequences obtained from GenBank. A phylogeny based on these alignments reveals that the Little Penguin sequences are clearly within a clade containing avian *Haemoproteus* spp., distinct from *Plasmodium* spp. Within the *Haemoproteus* clade they are most related to the *Haemoproteus* of the subgenus *Parahaemoproteus* as would be expected based on the nucleotide sequence identity. Previously isolated *Haemoproteus* sequences from Galapagos penguins were also in the *Parahaemoproteus* subgenus, however these are distinct from those detected in the Little penguins.

Fig 5. here

Fig 6. here

4. Discussion

Haemoproteus spp. were identified by PCR in four dead penguins in 2011 and 2012, and morphologically identical tissue stage parasites were observed in all ten dead penguins associated with significant pathological changes sufficient to result in acute death. It is worth noting that parasites were not seen in histologically normal tissue, only in areas affected by significant pathological changes. Given that the birds were still in adequate to good body condition (as evidenced by adequate subcutaneous and intra-abdominal adipose); it is unlikely that the penguins died from starvation. This, combined with the fact that significant hepatic and splenic necrosis associated with *Haemoproteus* spp. was seen indicates that disseminated haemoproteosis was responsible for their deaths. These constitute only the second recorded cases of *Haemoproteus* spp. identified in any member of the Spheniscidae family (Levin et al. 2009), however this report is the first to demonstrate the presence of the parasite *in situ* associated with overt pathological changes. Moreover, it is the first time that *Haemoproteus* spp. infection has been implicated in the mortality of Little Penguins.

Although all ten birds tested here for haemosporidian infection using DNA analysis exhibited similar histopathological findings, six of the birds were negative by PCR. Previous investigators have reported similar false negatives using PCR based methodology (Richard et al. 2002, Beadell et al. 2004, Valkiunas et al. 2006). Inability to amplify haemosporidians from DNA extracted from microscopically positive samples may be the result of degradation of the DNA sample over time; however this does not seem to be the case here since positive controls performed support the quality of the DNA. Alternatively, due to variation in tissue sampling, parasite DNA may be underrepresented in some samples. Microscopy alone can also result in false negatives due to low infection levels and often does not provide species level information on the parasite. For example, it is difficult or impossible to use the morphology of the preerythrocytic tissue stages alone for accurate diagnosis of

Haemoproteus spp. infection (Atkinson, 2008). Hence the value in the use of both molecular as well as microscopic methods in identifying infection (Valkiunas et al. 2008a) is supported by the results presented here.

The molecular methodology used in this study has been used previously to identify both *Haemoproteus* and *Plasmodium* spp. (Hellgren et al. 2004, Waldenstrom et al. 2004). Indeed, the close phylogenetic relationship between *Plasmodium* and *Haemoproteus* spp. makes it difficult to design primers specific for one that would not amplify the other (Beadell and Fleischer, 2005). Only *Haemoproteus* was identified in the Little Penguins tested here, however it is impossible to rule out the presence of other infections based on negative results. Mixed infections can result in preferential amplification of DNA from the parasite with the greatest level of parasitemia and/or tissue burden (Valkiunas et al. 2006). Therefore PCR based methods can underestimate the occurrence of mixed *Haemoproteus* and *Plasmodium* spp. infections. One method to reduce such underestimation is by sequencing multiple independent clones from more than one independent PCR reaction, increasing the chances of identifying dual infections, as was performed here.

As the primers used only amplify the *Plasmodium* or *Haemoproteus* spp. cyt-*b* gene, we cannot rule out the possibility that the penguins could have also harboured *Leucocytozoon* spp. infections. In addition, the clinical observations of splenomegaly, hepatomegaly and histiocytic infiltrates in the current study not only correlate with previous reports of *Haemoproteus* spp. infections (Atkinson and Van Riper III 1991, Ladds 2009) but also those of *Plasmodium* and *Leucocytozoon* spp. infections (Fix et al. 1988, Graczyk et al. 1994, Valkiunas 2005, Ko et al. 2008). However, the intraerythrocytic stage observed in 12/362 (noting that this case was PCR negative but histologically positive), did not deform the host

cell nucleus, as would be expected of even young *Leucocytozoon* gametocytes (Valkiunas 2005). Furthermore, the intralesional haemosporidians observed in the six "PCR-negative" cases were morphologically identical by light microscopy to that of the four PCR positive cases. However, many of the necropsy findings could also apply to infection with *Babesia* spp., and concurrent infection with *Babesia peircei* cannot be excluded. This piroplasm has been identified from Little Penguins in Australia (Peirce, 2000), and from Jackass Penguins (*Spheniscus demersus*) in South Africa (Erlé et al, 1993). Schizonts and trophozoites of *B. peircei* are difficult to differentiate from early intraerythrocytic stages of haemoproteids, the main difference being the absence of pigment in species of *Babesia* (Homer et al, 2000). However, refractile hyaline pigment of unknown significance was present in the livers of four out of the ten birds in this study, including one which was negative for *Haemoproteus* spp by PCR (11/624). In addition, no organisms consistent with *Babesia* were noted in the splenic impression from 12/362 (P. Irwin pers comm.), even though intraerythrocytic merozoites were present in this case. It should be noted that the molecular techniques that we used for haemosporidia do not detect *Babesia* spp; therefore, the potential remains for mixed infections. However, ticks are vectors of *Babesia* spp, and although they are usually associated with nesting bird colonies, none were found on Little Penguins or in their nests in this study. Therefore we conclude that a diagnosis of *Haemoproteus* spp. is likely the case for all 10 dead penguins.

Haemoproteus spp. are normally considered benign in birds (Bennett et al. 1993b, Quillfeldt et al. 2010), although it has also been postulated that heavy infections of *Haemoproteus* spp. may delay bird migration (Valkiunas and Iezhova 2001). In addition, tissue stages of the parasite have been reported to cause necrosis of pectoral muscle fibres surrounding megaloschizonts in several avian species (Atkinson and Forrester 1987, Atkinson 2008, Olias

et al. 2011); pneumonia-like symptoms (Valkiunas 2005) and haemorrhage and necrosis associated with cyst rupture (Ferrell et al. 2007). However, all but one of these cases were from captive or laboratory animals, and the apparent lack of pathogenicity in the wild could be artefactual, arising from sampling birds that are in apparent good condition (i.e. aclinical/asymptomatic), and not those found ailing or dead (Valkiunas 1998). Indeed, Ladds (2009) notes that the severity of the disease may be underestimated in wild birds.

The exact life cycle and pathogenesis of the *Haemoproteus* spp. identified is unknown; however it is assumed that they follow the general scheme of the haemoproteid life cycle as elaborated by Valkiunas (2005). Unlike the members of the genus *Plasmodium*, to which they are closely related, haemoproteids undergo merogony (asexual reproduction) in tissues, rather than circulating erythrocytes. It is these developing preerythrocytic tissue stages that have, albeit in a limited number of cases, been reported to be pathogenic, causing significant myonecrosis (Ferrell et al. 2007, Atkinson, 2008, Olias et al. 2011) as they mature and rupture to release merozoites capable of invading erythrocytes. No gross signs of skeletal myonecrosis were noted in our cases, and unfortunately skeletal muscle was not examined histologically. Four of the ten cases did exhibit small foci of cardiac myonecrosis associated with sparse intralesional merozoites.

No haemoparasites were previously found in blood smears taken from penguins on Penguin Island in 1992 (Jones and Shellam 1999a), nor from any of the other 124 penguins from the Perth region that have been necropsied since 2004. This, and the fact that only a limited number of preerythrocytic tissue stages (and rare intraerythrocytic merozoites in the case of the spleen of 12/362) were seen in all individuals, suggests that these cases represent the outcome of lethal abortive development of *Haemoproteus* spp. in a non-adapted penguin

population (Valkiunas et al. 2011). This is similar to the cases reported by Olias et al. (2011) in which non-adapted exotic parrots of various species in Europe died acutely; with PCR identifying parasites with 99-100% homology to *Haemoproteus* spp. known to be highly prevalent in wild European songbirds (without causing overt disease or mortality).

It is assumed, similar to the situation in human malaria (particularly that associated with *Plasmodium falciparum*) (Clark 2006); that the birds, having had heavy parasite burdens (i.e. disseminated infection), died acutely as a result of the excessive release of pro-inflammatory cytokines (in particular TNF and IL-1; "cytokine storm") secondary to the damage caused by the tissue stages (and therefore before gametocytes were able to develop), leading to systemic inflammatory response syndrome (SIRS). It is unclear whether these birds had a significant anaemia which might also have contributed to disease and death by way of exacerbating tissue ischaemia. It is also unclear how much of a role the pathogen-associated molecular pattern (PAMP) glycosylphosphatidylinositol anchor (GPI) plays in the pathogenesis of *Haemoproteus*-associated disease; this highly conserved molecule is present in the membranes of many parasitic protozoa and elicits the synthesis of pro-inflammatory cytokines as well as nitric oxide (Ropert, 2000); thus having been implicated in human malaria via its recognition by host cell toll-like receptor 2 (TLR2) (Oakley, 2011).

It is probable that dead penguins found elsewhere originated from Penguin Island, as shown by previous radio and satellite tracking data (Cannell unpubl. data). The penguin located near Garden Island (12/021) could have been either from the colony at Garden Island or from Penguin Island, as penguins from both colonies forage in Cockburn Sound (Cannell unpubl. data). As penguins are unlikely to be bitten by competent vectors of *Haemoproteus* spp. while at sea, and the penguins do not land on the mainland, they were probably infected whilst at

their colony. The competent vectors of *Haemoproteus* spp. include biting midges (Ceratopogonidae) and louse flies (Hippoboscidae) (Valkiunas 2005). The presence of louse flies on Little Penguins has rarely, if ever, been observed (Cannell pers. obs.), with fleas, lice and ticks being the listed ectoparasites on these birds (Obendorf and McColl 1980). In contrast, biting midges are likely to be on Penguin Island and are therefore the most likely competent vector for *Haemoproteus* spp.

Apart from the 10 cases in this study, no haemosporidians have been previously found in Little Penguins in this region (Cannell unpubl. data, Jones and Shellam 1999a), nor from colonies elsewhere in Australia and New Zealand (Quillfeldt et al 2011). However, the distribution, density, biology, physiology and transmission rates of vectors are influenced by changes in climate and habitat (Harvell et al. 2002, Rogers & Randolph 2006). In addition, the growth rate of the haemoparasite can also change with altered climatic conditions (Harvell et al. 2002). An anomalously strong La Niña from September 2010 and throughout 2011 was responsible for above average sea surface temperatures along the West Australian coast (Pearce et al. 2011, Pearce and Feng 2013) and high tides causing inundation of coastal wetlands (City of Mandurah 2011, 2012). It also affected other climatic conditions, with the mean minimum and maximum daily temperature well above average throughout the year resulting in the hottest year on record, with a record number of days ≥ 32 °C. This temperature increase was combined with above average rainfall during the austral spring as well as heavy rainfall in the first two weeks of December (beginning of the austral summer) (Australian Bureau of Meteorology). It is therefore possible that these climatic changes contributed to a sudden increase of vectors present on the island and hence in the cases of avian haemosporidiosis documented.

Even if prevailing climatic conditions are suitable for an increased presentation of the haemoparasite, it must first be available to be transmitted and for it to shift hosts. There is evidence to suggest that some *Haemoproteus* spp. have a low host specificity and thus can be transmitted between birds belonging to different families (Fallon et al. 2005, Križanauskiené et al. 2006). However, Beadell et al. (2004) found that the stability of jumps between different host families in tropical songbirds is low, though this could be due to the type of birds they sampled as well as their isolation. Fallon et al. (2005) found that one lineage of *Haemoproteus* was found in different bird families, dependent on the geographic region. Furthermore, the families of birds in one region were migrants that overwintered in another region. There are several species of migratory birds on Penguin Island. For example, bridled terns (*Sterna anaethetus*), caspian terns (*Hydroprogne caspia*) and crested terns (*S. bergii*) breed on Penguin Island, but migrate from the island after breeding. The caspian and crested terns are likely to move to wetland areas within the localized region (Cannell 2004), whereas bridled terns migrate to the Sulawesi Sea for the austral winter (Dunlop & Johnstone 1994). Further research needs to be undertaken to determine if any of these species are reservoir hosts for the *Haemoproteus* spp. found in the Little Penguins.

The dramatic increase in penguins presenting with disseminated, fatal haemosporidiosis is of concern. It is known that island hosts tend to be more susceptible to pathogens compared to mainland hosts due to lower exposure to pathogens (Levin et al. 2011 and refs within Valkiunas 2005). In addition, the prevalence of *Haemoproteus* parasites has been shown to increase in other bird species when stressed (Quillfeldt et al. 2010). Whilst the penguins that died from *Haemoproteus* spp. infections were not in poor body condition, the average weight of the fat pad as a percentage of body weight was lower than that identified by Hocken (2000b), particularly for the males. However, it is unknown if the Hocken (2000b) study

included penguins in a premoult or moult stage, which would carry a large reserve of fat. Nevertheless, in this study, the infected penguins for which we have body-mass data were lighter than their mean body mass, and a greater than average number of penguins were found to have died as a result of starvation in 2011 (Cannell unpubl. data). It is therefore likely that the infected penguins in the present study were in a state of stress and perhaps thus more vulnerable to succumbing to the infection.

Inclusion of the *Haemoproteus* sequences from Little Penguins continues to support two main haemoparasite clades, one containing mammalian *Plasmodium* and the other avian parasites. The avian clade is further subdivided into *Plasmodium* and *Haemoproteus* clades. Previous classification divided the genus *Haemoproteus* into two subgenera, *Haemoproteus* and *Parahaemoproteus,* that form sister clades in phylogenetic analyses (Valkiunas et al. 2008b; Levin et al. 2011). Martinsen et al. (2008) found *Haemoproteus* and *Parahaemoproteus* each form distinct monophyletic clades based on limited gene sequence analysis and suggested raising *Parahaemoproteus* to be a distinct genus. The addition of the sequences isolated from the Little Penguins continue to support the conclusion that *Parahaemoproteus* and *Haemoproteus* are distinct sister clades with the penguin parasites in the *Parahaemoproteus* subgenus.

5. Conclusion

Fatal infection by haemosporidian parasites, identified by molecular means in conjunction with histopathological findings as *Haemoproteus* spp., is reported in four of ten Little Penguins in Western Australia. Additionally, the other six cases exhibited identical histopathological findings with the presence of morphologically identical intralesional parasites. One of these also had rare intraerythrocytic merozoites. This population has

previously been free of infection. Recent changes in climate, ocean currents and the presence of other species of birds which could introduce *Haemoproteus* spp. to the area could all be contributing factors ultimately leading to these infections. Further studies are needed to ascertain the epidemiology and pathology of this parasite; and indeed, to investigate the possibility of mixed apicomplexan infections in this population.

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Table 1.

Details for each of the penguins. [a] adult, [b] fledgling. Area found – PI = Penguin Island, PB = Preston Beach, PK = Port Kennedy, CS = Cockburn Sound, WS = Warnbro Sound. The number of times each penguin was weighed to determine the average body mass is noted in parentheses (note an average was not possible for 11/628).

Table 2.

Mass of the liver, spleen and abdominal fat pad in each case. Note that the organs were not weighed in all cases. [a] adult, [b] fledgling.

Table 3.

Liver mass as a percentage of body mass. [a] adult, [b] fledgling.

Table 4.

Summary of histopathological findings.

Legends to Figures

Fig. 1. The location of dead penguins in Western Australia: on Penguin Island and the foreshores of Cockburn Sound, Warnbro Sound, Port Kennedy and Preston Beach. Penguin Island is approximately 50 km south of Perth, the capital city of Western Australia.

Fig. 2. Liver, penguin 12/362. H = heart, L = liver, I = intestines. Note the rounded hepatic lobar edges (arrow) and the multitudinous disseminated white to off-white flat pinpoint capsular discolourations (arrowheads).

Fig. 3 Liver, penguin 12/362. x400 magnification, 2µm thick, Martius Scarlet Blue stain. The arrow indicates a meront containing numerous cytomeres at the periphery of a necrotic focus.

Fig. 4 Splenic impression smear, penguin 12/362. X1000 (oil immersion) magnification, Wright-Giemsa stain. The arrow indicates an intraerythrocytic merozoite prior to its development into a young gametocyte.

Fig. 5 Visualisation of PCR results of the four positive (11/626, 12/020, 12/021 and 12/338) and one representative negative (11/628) birds. Each sample was run in triplicate except for the positive controls, which were run in duplicate. H_2O was included as a negative control.

Fig. 6 Phylogenetic tree based on mitochondrial *cytochrome b* sequences of haemosporidians obtained from the liver of Little Penguins along with representatives obtained from GenBank.

Table 1.

Details for each of the penguins. [a] adult, [b] fledgling. Area found – PI = Penguin Island, PB = Preston Beach, PK = Port Kennedy, CS = Cockburn Sound, WS = Warnbro Sound. The number of times each penguin was weighed to determine the average body mass is noted in parentheses (note an average was not possible for 11/628).

Case no.	Sex	Area found	Date found dead	Date last seen alive	Body mass - dead (g)	Body mass - last seen alive	Av. body mass(g)
06/1172[a]	M	PI	8/11/06	n/a	unknown	n/a	n/a
08/1075[a]	M	PI	28/7/08	n/a	1300	n/a	n/a
12/021[a]	M	CS	6/10/11	n/a/	1300	n/a	n/a
11/627[a]	M	PI	3/11/11	23/9/11	1160	1220	1323 (3)
11/624[a]	M	PI	6/11/11	25/10/11	1370	1210	1410 (24)
11/628[a]	F	PB	8/11/11	23/9/11	1225	1450	1450 (1)
12/020[a]	F	PK	8/11/11	n/a	1230	n/a	n/a
11/626[b]	F	PI	9/11/11	n/a	1105	n/a	n/a
12/338[a]	M	WS	30/04/12	n/a	1190	n/a	n/a
12/362[a]	M	PI	30/05/12	n/a	1240	n/a	n/a

Table 2.

Mass of the liver, spleen and abdominal fat pad in each case. Note that the organs were not weighed in all cases. [a] adult, [b] fledgling.

Penguin case no.	Liver mass (g)	Spleen mass (g)	Abdominal fat pad
06/1172[a]	Not weighed	Not weighed but splenomegaly noted	Not weighed
08/1075[a]	69.4	Not weighed	Not weighed
11/627[a]	79.7	5.0	8.3
11/624[a]	Not weighed	9.6	Not weighed
11/628[a]	63.7	4.2	16.1
11/626[b]	61.2	7.5	5.4
12/020[a]	72.1	3.8	8.3
12/021[a]	58.6	3.3	4.1
12/338	62.4	6.9	6.7
12/362	59.0	5.9	15.0

Table 3.

Liver mass as a percentage of body mass. [a] adult, [b] fledgling.

Case No	Liver	Gender
08/1075[a]	5.4	Male
11/627[a]	6.9	Male
12/021[a]	4.5	Male
12/338	5.2	Male
12/362	4.8	Male
Mean±SD	5.3±0.5	
11/626[b]	5.5	Female
11/628[a]	5.2	Female
12/020[a]	5.9	Female
Mean±SD	5.5±0.3	
All penguins	5.4±0.3	

Table 4.

Summary of histopathological findings.

Case no.	Degree of autolysis	Hepatocellular necrosis	Pigment in Kupffer cells/ hepatocytes	Splenic necrosis	Cardiac necrosis	Histiocytic infiltrate		Heterophilic infiltrate	
						Spleen	Liver	Spleen	Liver
06/1172	Minimal	✓		✓	✓				✓
08/1075	Moderate	✓		✓	✓	✓			
11/627	Moderate-marked	✓		✓		✓	✓	✓	
11/624	Mild-moderate	✓	✓	✓		✓	✓	✓	✓
11/628	Moderate-marked	✓		✓	✓	✓	✓	✓	
12/020	Mild	✓	✓	✓		✓	✓	✓	
12/021	Moderate	✓	✓	✓					
11/626	Moderate	✓		✓		✓		✓	
12/338	Moderate	✓	✓	✓					
12/362	Minimal	✓		✓	✓	✓	✓		✓

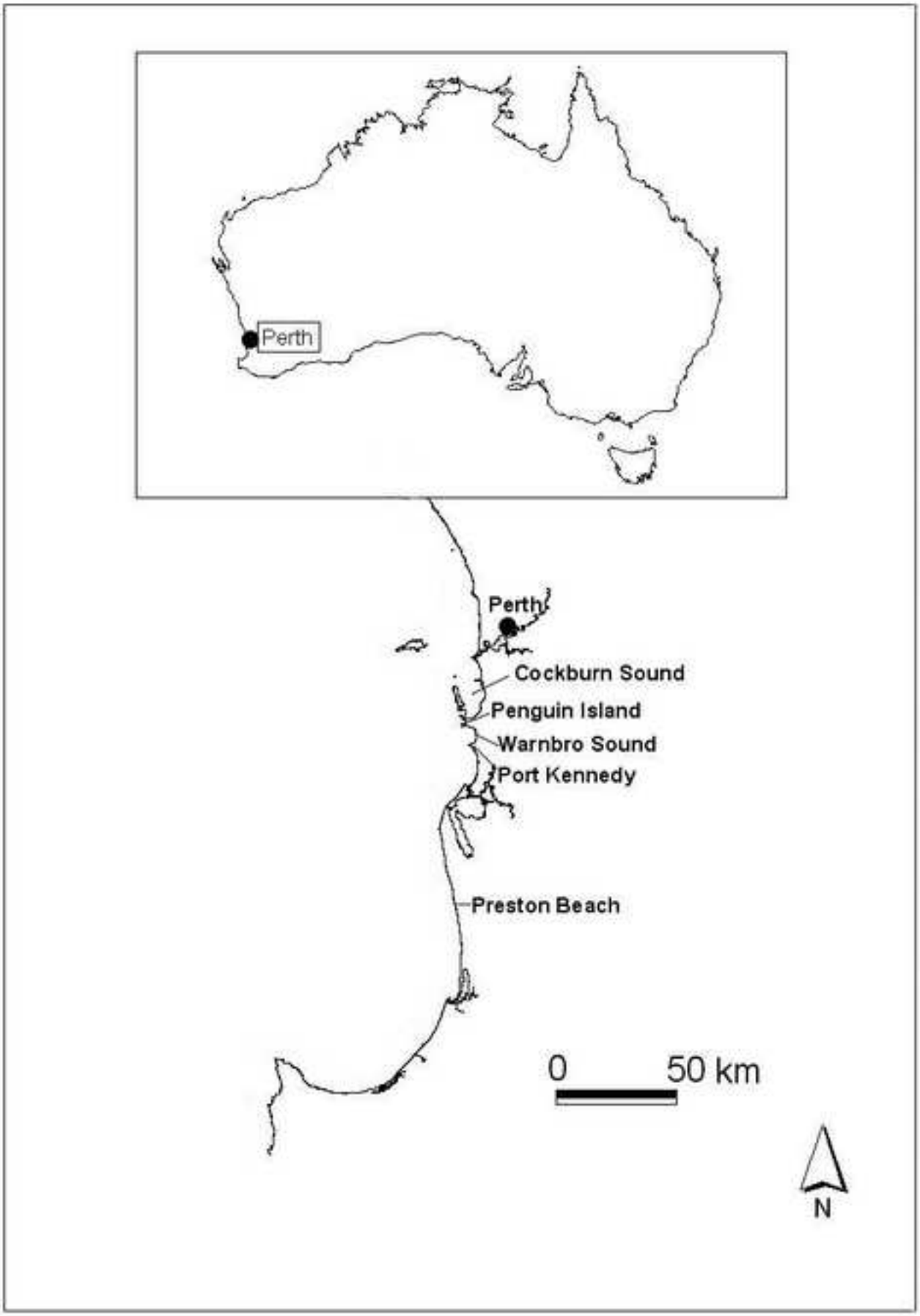


Figure 2 b&w

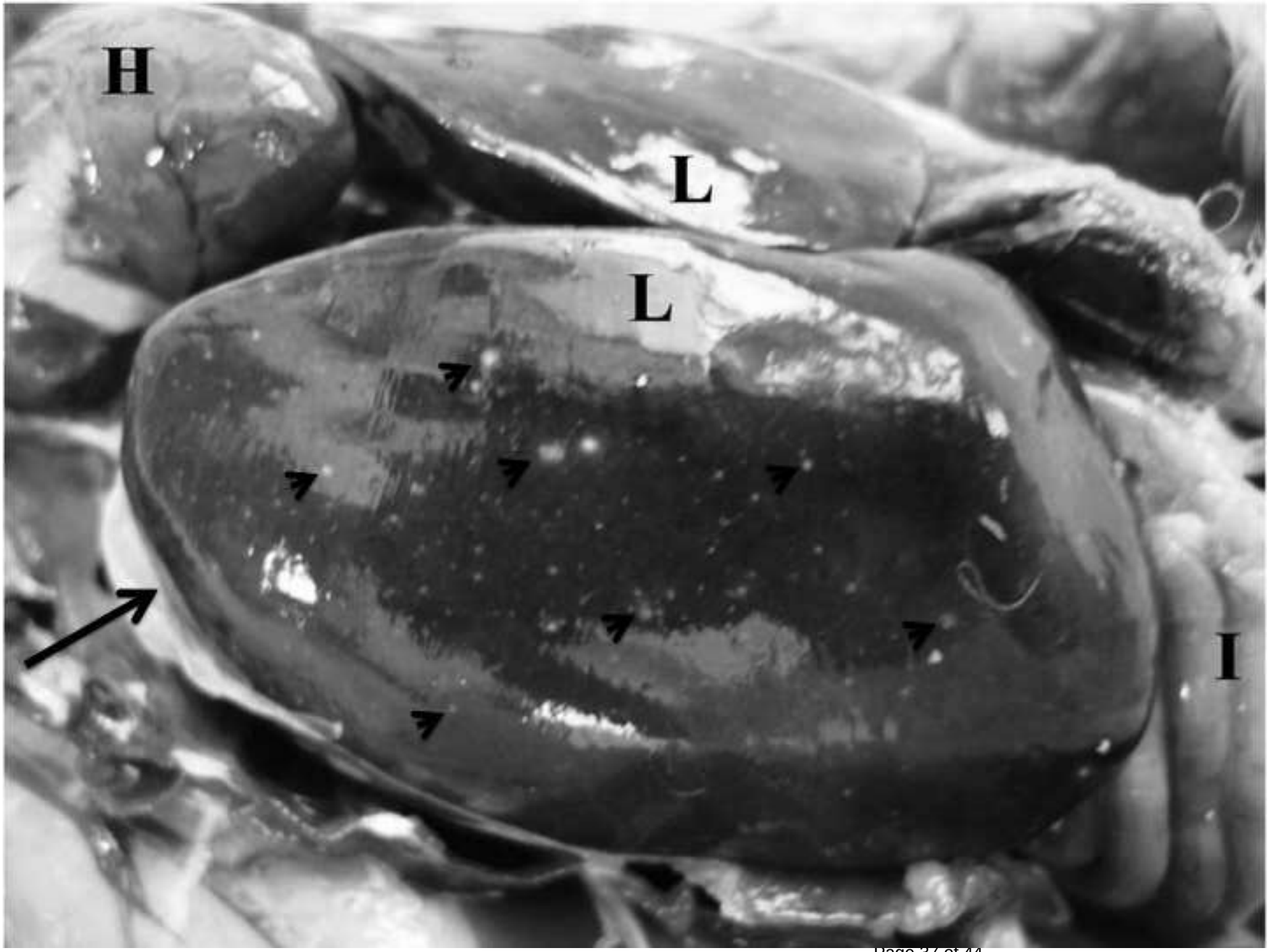


Figure2 colour (online)

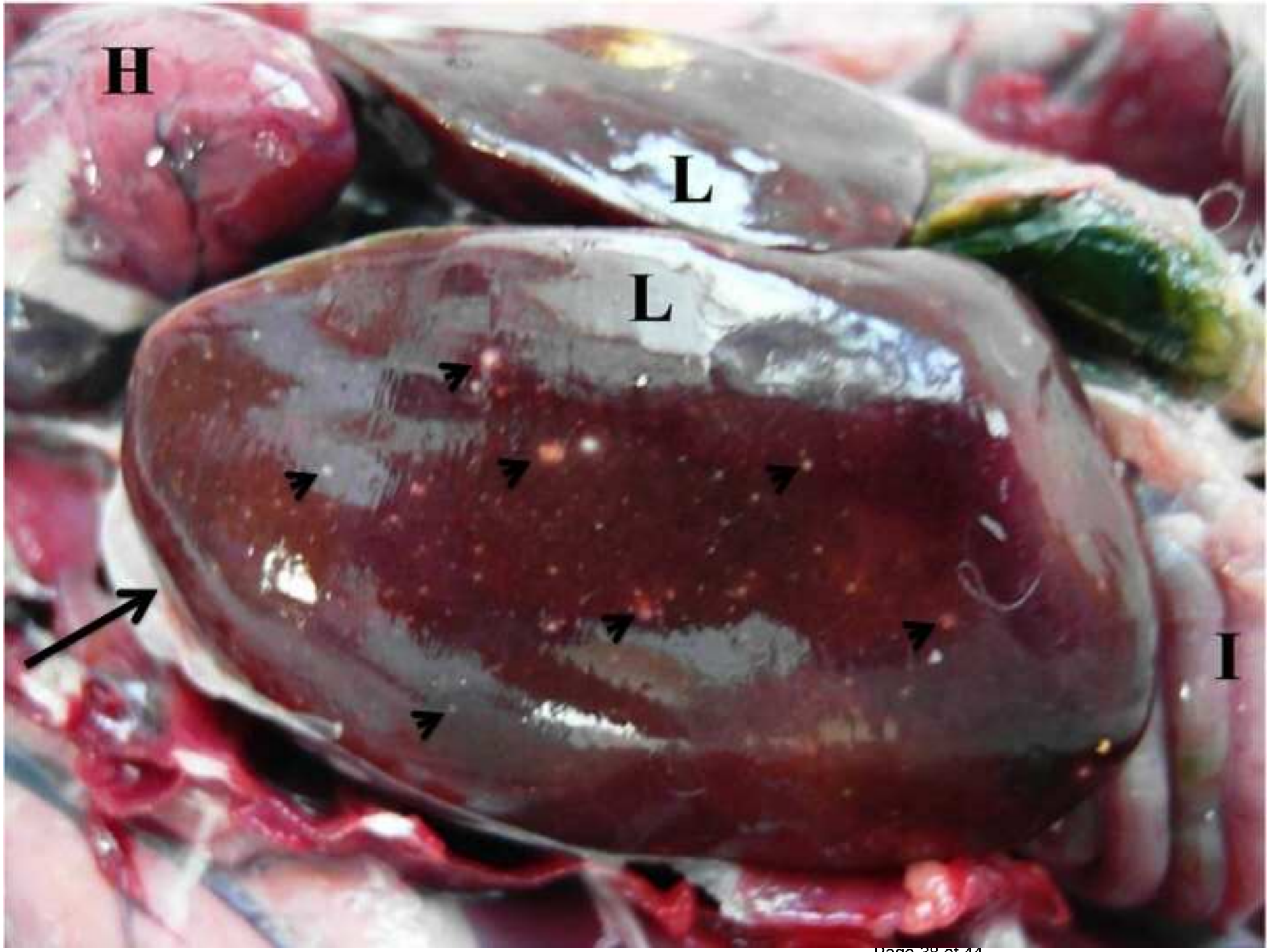


Figure 3 b&w

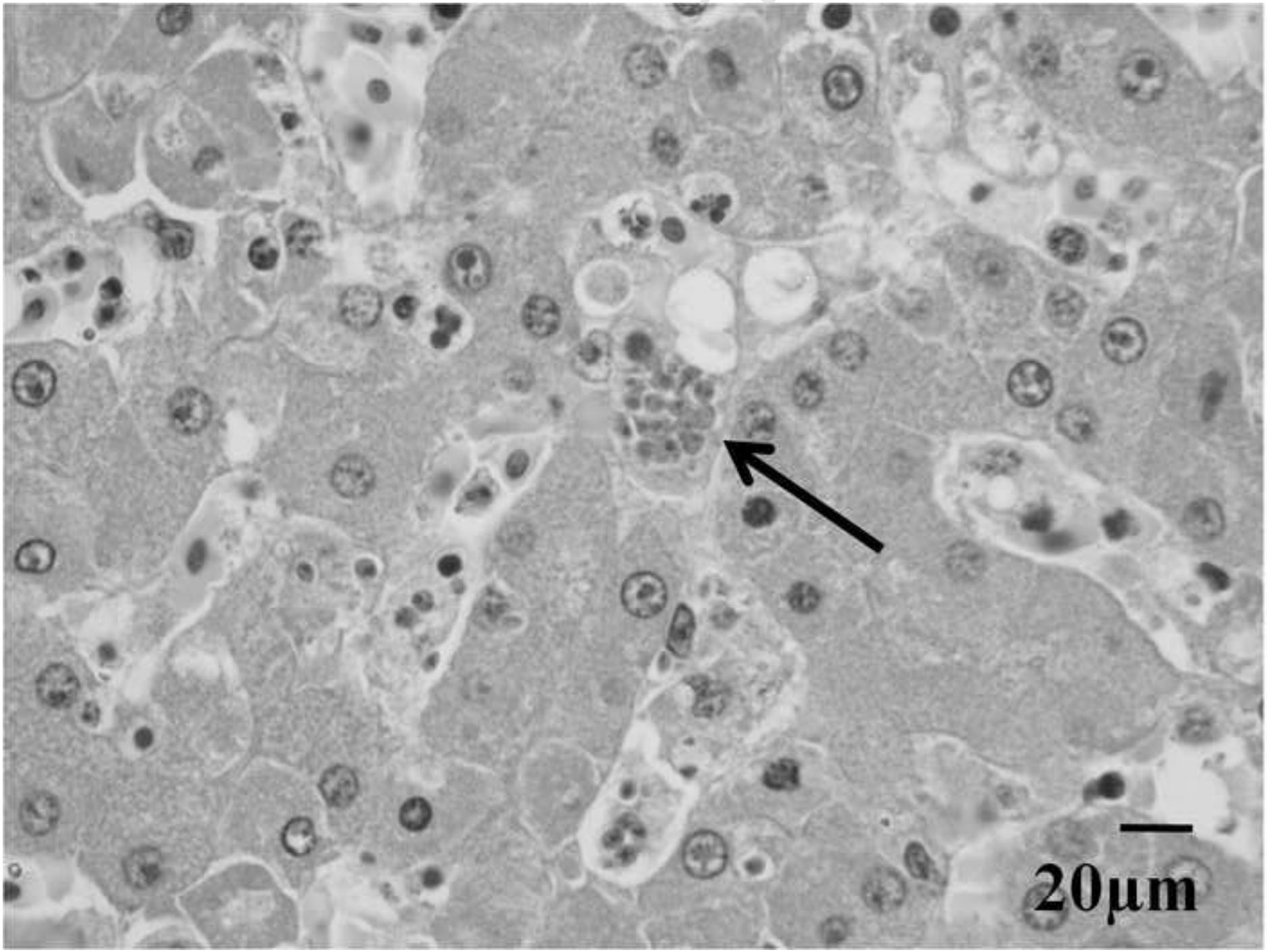


Figure 3colour (online)

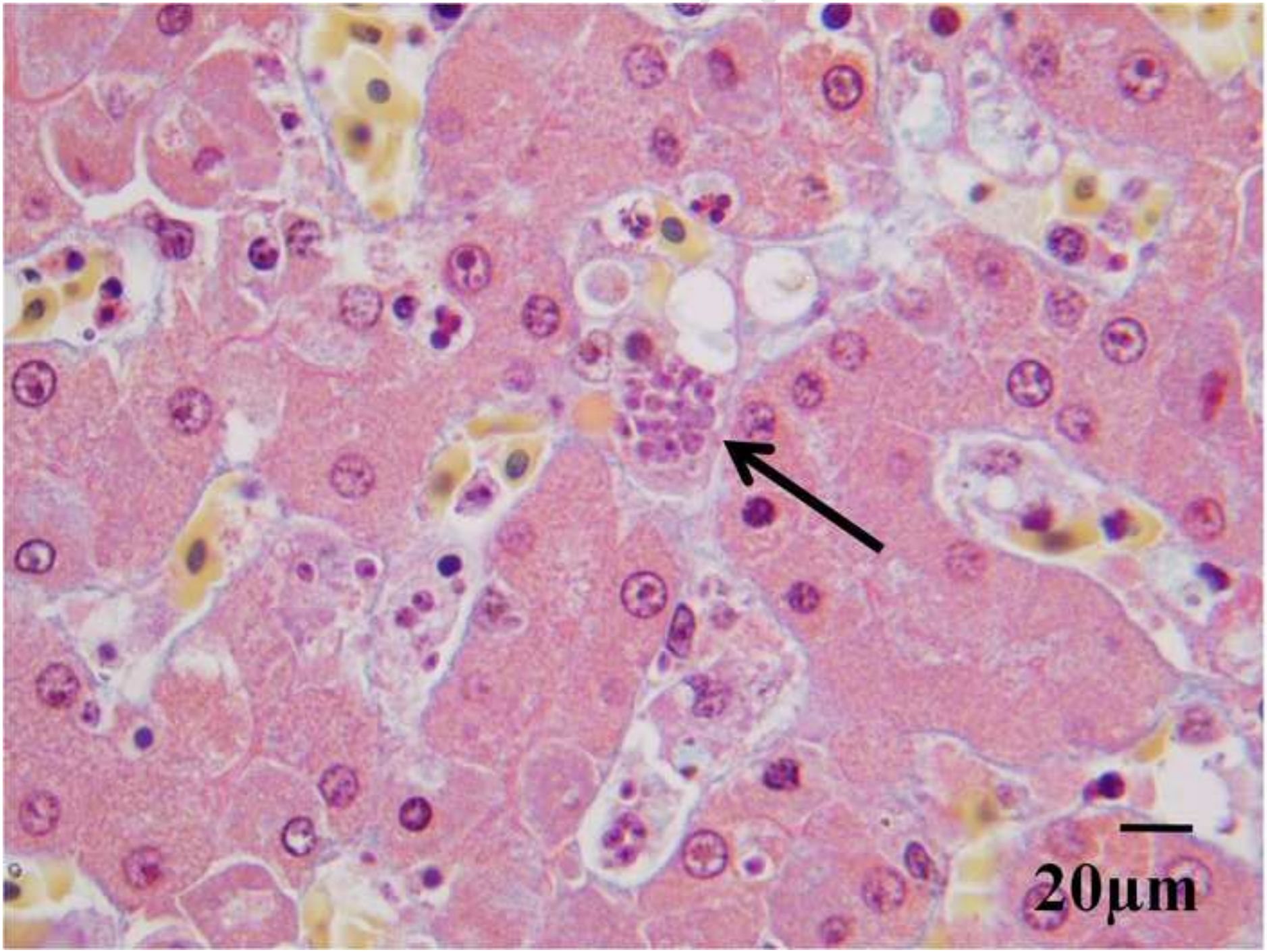


Figure 4 b&w

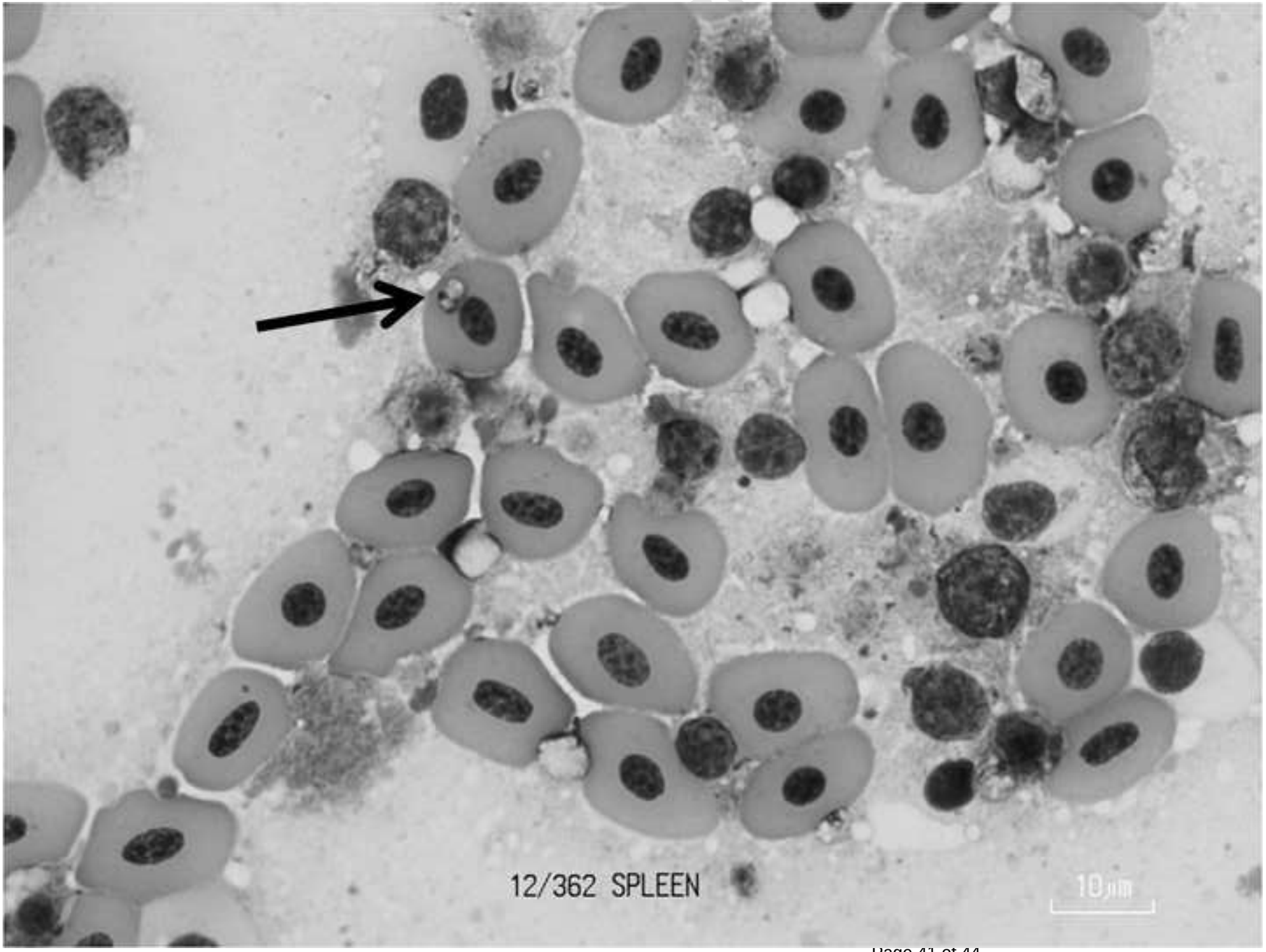


Figure 4 colour(online)

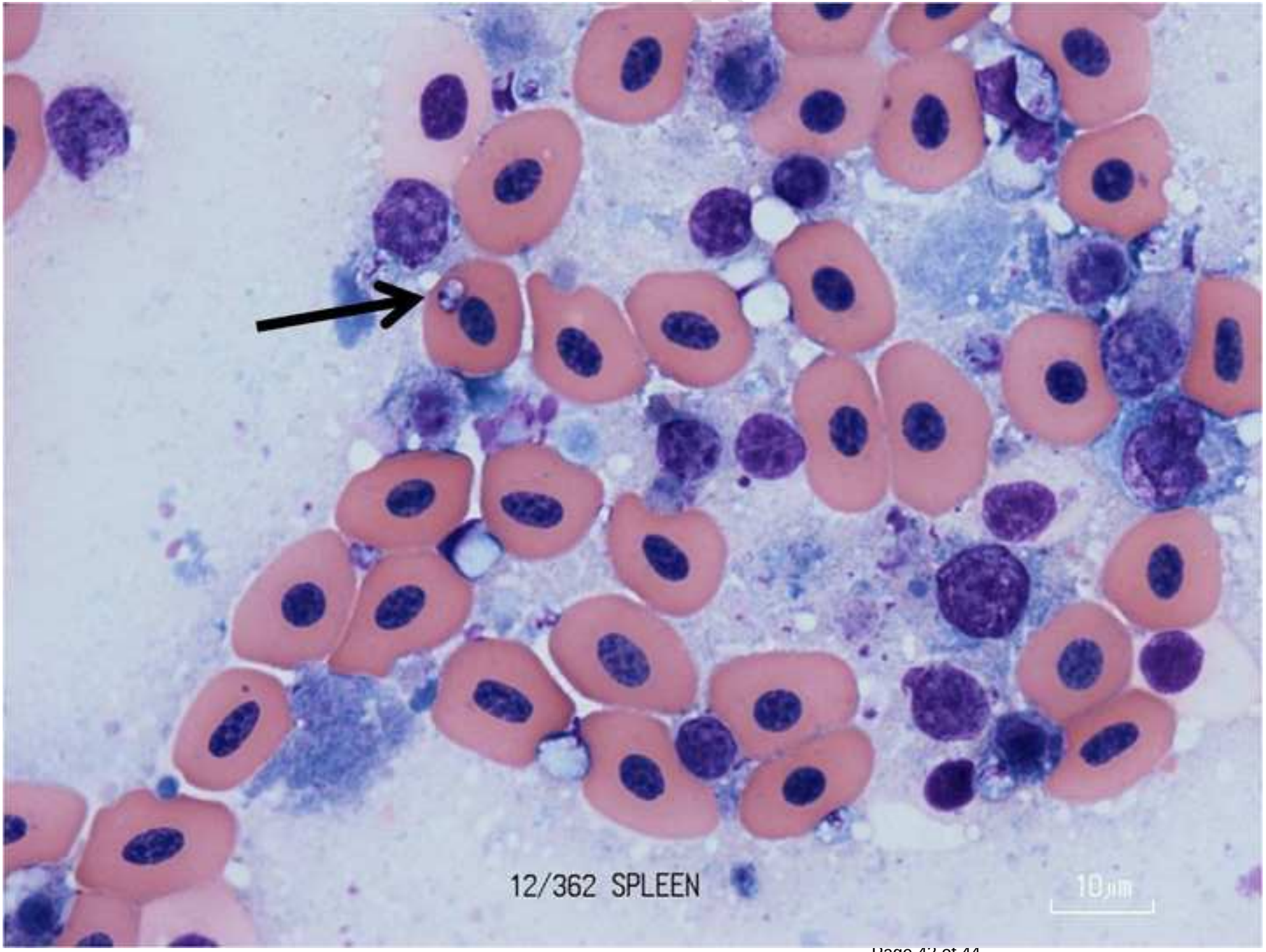


Figure 5

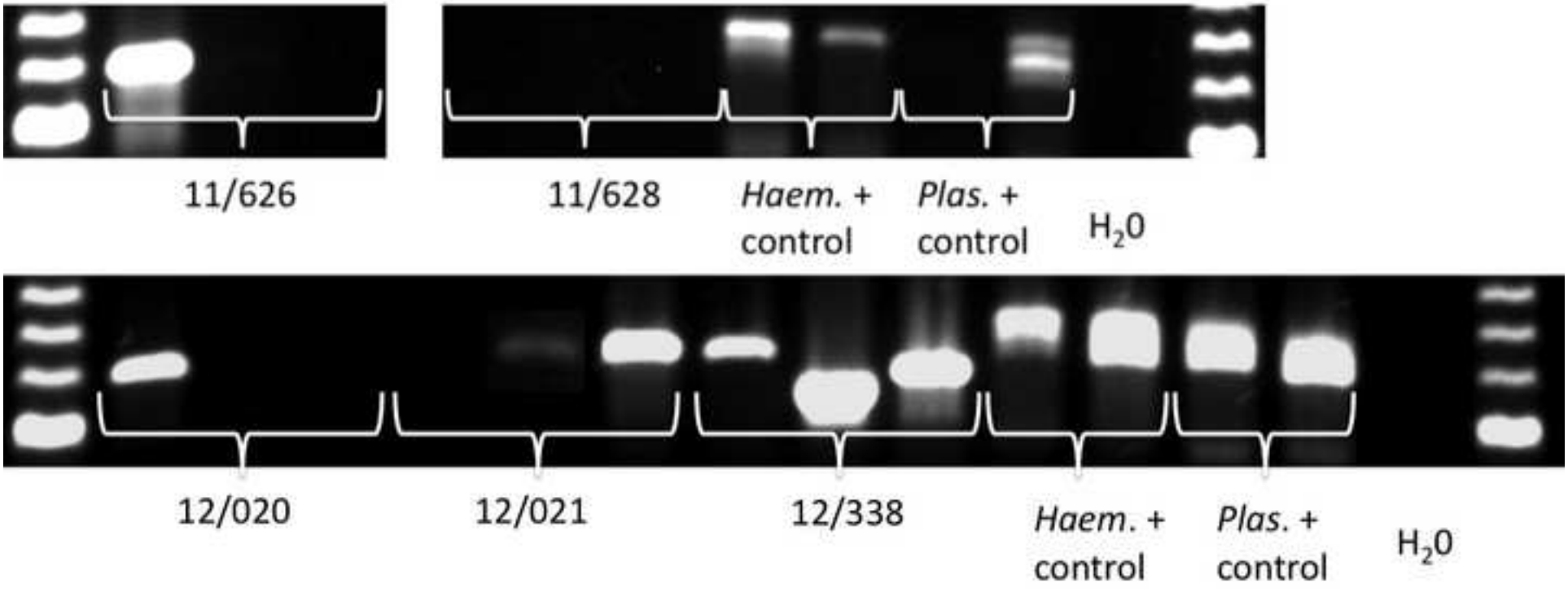
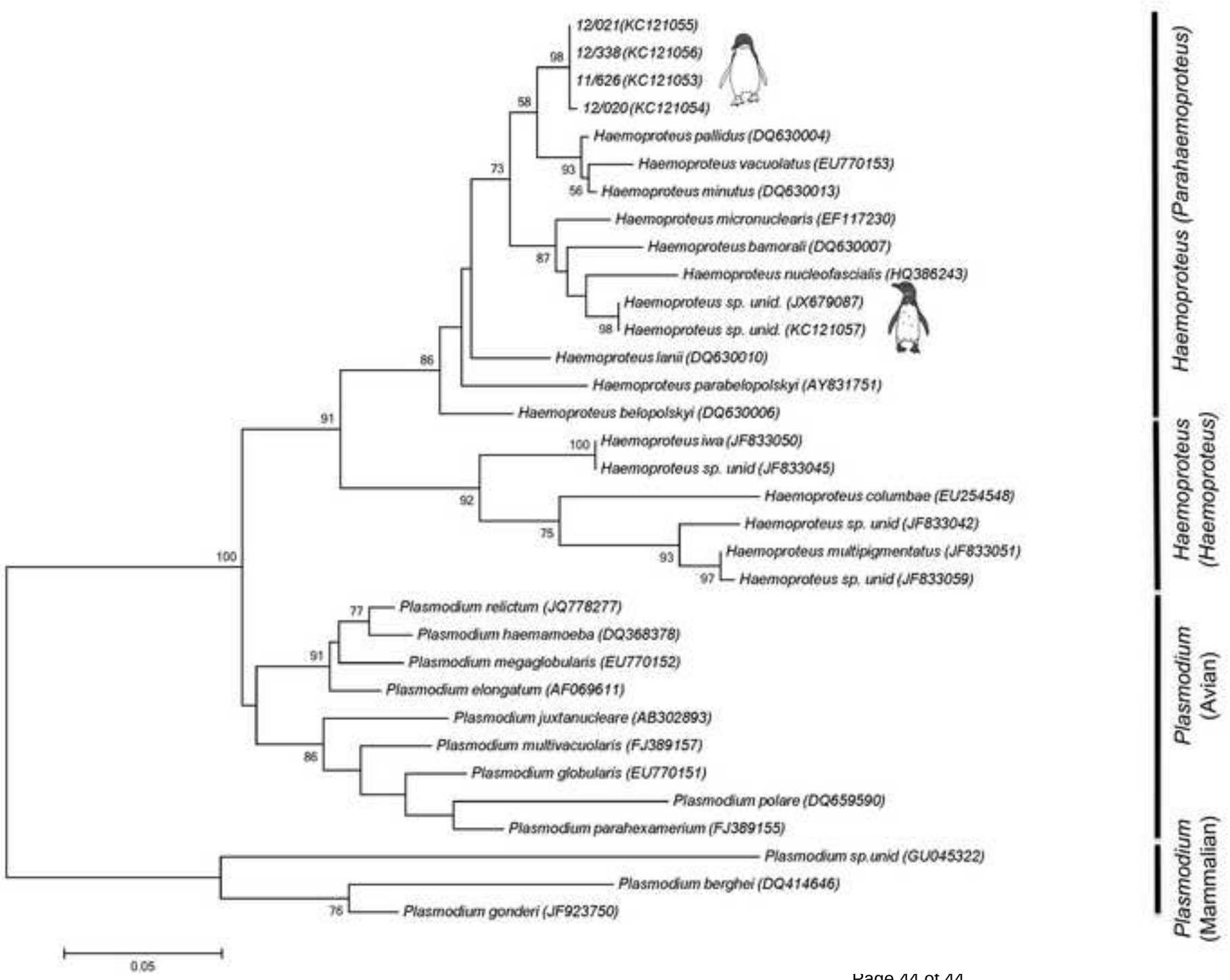

11/626
11/628
Haem. + control
Plas. + control
H20
12/020
12/021
12/338
Haem. + control
Plas. + control
H20

Figure 6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12/338(KC121056)
11/626(KC121053)
12/020(KC121054)
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Haemoproteus vacuolatus (EU770153)
Haemoproteus minutus (DQ630013)
Haemoproteus micronuclearis (EF117230)
Haemoproteus bamorali (DQ630007)
Haemoproteus nucleofascialis (HQ386243)
Haemoproteus sp. unid. (JX679087)
Haemoproteus sp. unid. (KC121057)
Haemoproteus lanii (DQ630010)
Haemoproteus parabelopolskyi (AY831751)
Haemoproteus belopolskyi (DQ630006)
Haemoproteus iwa (JF833050)
Haemoproteus sp. unid (JF833045)
Haemoproteus columbae (EU254548)
Haemoproteus sp. unid (JF833042)
Haemoproteus multipigmentatus (JF833051)
Haemoproteus sp. unid (JF833059)
Plasmodium relictum (JQ778277)
Plasmodium haemamoeba (DQ368378)
Plasmodium megaglobularis (EU770152)
Plasmodium elongatum (AF069611)
Plasmodium juxtanucleare (AB302893)
Plasmodium multivacuolaris (FJ389157)
Plasmodium globularis (EU770151)
Plasmodium polare (DQ659590)
Plasmodium parahexamerium (FJ389155)
Plasmodium sp.unid (GU045322)
Plasmodium berghei (DQ414646)
Plasmodium gonderi (JF923750)
0.05
Haemoproteus (Parahaemoproteus)
Haemoproteus (Haemoproteus)
Plasmodium (Avian)
Plasmodium (Mammalian)