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Host specificity of *Hepatocystis* infection in short-nosed fruit bats (*Cynopterus brachyotis*) in Singapore

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ABSTRACT

Haemosporidians infect a wide diversity of bat genera and species, yet little is known about their transmission cycles or epidemiology. Though several recent studies have focused on the genus *Hepatocystis*, an Old World parasite primarily infecting bats, monkeys, and squirrels, this group is still understudied with little known about its transmission and molecular ecology. These parasites lack an asexual erythrocytic stage, making them unique from the *Plasmodium* vertebrate life cycle. In this study, we detected a prevalence of 31% of *Hepatocystis* in short-nosed fruit bats (*Cynopterus brachyotis*) in Singapore. Phylogenetic reconstruction with a partial *cytochrome b* sequence revealed a monophyletic group of *Hepatocystis* from *C. brachyotis* in Malaysia, Singapore, and Thailand. There was no relationship with infection and bat age, sex, location, body condition or monsoon season. The absence of this parasite in the five other bat species sampled in Singapore indicates this *Hepatocystis* species may be host restricted.

1. Introduction

Malarial parasites (Phylum: Apicomplexa, Family: Plasmodiidae), specifically *Plasmodium* spp., have long been recognized for their contribution to the global burden of human disease (Guinovart et al., 2006). However, over 500 related species from 15 genera in the order Haemosporidia infect other vertebrates using at least seven families of dipteran arthropod vectors (Levine, 2018). Ninety percent of the described haemosporidian species worldwide fall within four genera: *Plasmodium, Haemoproteus, Hepatocystis, and Leucocytozoon* (Martinsen et al., 2008). However, the genus *Hepatocystis* has fewer described species than the other three genera. Although these single-celled protozoan parasites share basic similarities in morphology and life cycle, they also vary greatly in life history, vertebrate host selection, and insect vectors (Perkins and Schaer, 2016).

Approximately 5% of haemosporidian species are in the genus *Hepatocystis* (Levine, 2018) and their life cycle and morphology, largely described from *Hepatocystis kochi* (Garnham et al., 1967), define them as a distinguishable genus. Among the four major genera, *Hepatocystis* parasites are unique in their development because they form merocysts within the liver parenchyma and schizonts are found within these bodies (Garnham and Pick, 1952; Landau and Adam, 1973). Merozoites are released from mature merocysts, which then infect erythrocytes and develop into gametocytes. These parasites are further distinguished by their narrow host-range, only infecting mammals, primarily Old World bats, non-human primates and squirrels (Lutz et al., 2016; Perkins and Schaer, 2016; Thurber et al., 2013).

Current bat parasite research aims to understand host-parasite relationships, with a particular focus on parasite diversity, the epidemiology of these infections (Gay et al., 2014), microparasite and

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macroparasite richness in bats (Bordes et al., 2008; Turmelle and Olival, 2009), and importantly, host switching of parasites (Duval et al., 2007). Bats are reservoir hosts for several species of haemosporidians, with recent research illustrating their high genetic diversity (Boundenga et al., 2018; Schaer et al., 2013). There have been few studies on blood borne parasites in the bats of Southeast Asia, even though this region is one of the most biologically diverse, with nearly 30% of all bat species (Kingston, 2010). Bat species richness and high level of endemism may result in high levels of haemosporidian parasite diversity (Kamiya et al., 2014).

Hepatocystis infections in bats from Southeast Asia were originally described using microscopy, with detection of *Hepatocystis pteropi* in *Pteropus* bats in Malaysia, an undescribed species of *Hepatocystis* from a *Hipposideros* bat in Malaysia and another parasite from *Cynopterus brachyotis* and *C. horsfieldi* bats in Indonesia (Garnham, 1966; Masbar et al., 1981; Mialhe and Landau, 1977). Molecular studies have contributed to our understanding of bat *Hepatocystis* diversity in this region, with sequence data generated from parasites in Cambodia, Malaysia, Singapore, and Thailand (Arnuphapprasert et al., 2020; Duval et al., 2007; Martinsen et al., 2008). However, little is known about bat blood parasite epidemiology in Singapore. Our study screened bat blood collected in Singapore from 2011 to 2014 with microscopy and PCR for the presence of bat haemosporidians. Here we describe the distribution, host specificity, epidemiology of infection and the phylogenetic relatedness of *Hepatocystis* parasites detected.

2. Materials and methods

2.1. Bat trapping and sample collection

All bats were trapped with permission from the National University of Singapore Institutional Animal Care and Use Committee (Permit #B01/12), National Parks Board-Singapore (NPRP11-011-3a), and the Agri-food and Veterinary Authority Singapore (AV 16.01.004.0004). Sex, age, weight, and forearm length were collected from each bat. Blood was collected by pricking the protopatagial or uropatagial veins, collecting with a pipette and then diluting 1:10 in 1xPBS solution. Thin blood smears were made if sufficient blood was available, and these were air dried in the field and fixed in 100% methanol before being brought back to the lab where they were stained with Giemsa stain at Singapore General Hospital. Ectoparasites were collected, stored in 90% ethanol, and identified as previously described (Lim et al., 2020). All samples were held at 4 °C until transported to Duke-NUS Medical School where the blood was centrifuged and the serum heat-inactivated at 56 °C for 30 min and stored at −80 °C.

2.2. Blood smear reading

Blood smears from PCR positive bats were read under 1000x oil immersion using a Leica DM 2000 compound microscope. For every slide, red blood cells (RBCs) were counted from 10 representative fields to generate an average per field. A total of 100 fields were viewed and early gametocytes, macrogametocytes, and microgametocytes were counted. Infection intensity, the number of parasites per RBC, followed previously established protocols (Warhurst and Williams, 1996).

2.3. DNA extraction, PCR and cloning

To detect DNA parasites in blood, 2 μL of a bat red blood cell pellet was dissolved in 200 μL PBS and proteinase K solution, followed by heat-inactivation for 10 min at 56 °C. DNA was extracted using a QIAxtractor (Qiagen). DNA was eluted in 75 μL of elution buffer and stored at −80 °C. Ectoparasites were pooled by the individual *C. brachyotis* they were collected from and the entire bat fly was homogenized with a Mini-Beadbeater-96 (BioSpec, Bartlesville, OK) using silicon-carbide particles (BioSpec, Bartlesville, OK). Samples were then centrifuged for 1 min at 13,000 rpm, and the supernatant removed for DNA extraction using a DNeasy Blood and Tissue kit (Qiagen). Extracted DNA from the blood and ectoparasites was screened for the presence of haemosporidia using the primers targeting the cytochrome *b* with a previously established protocol (DW2: 5′-TAATGCCTAGACGTATTCCTGATTATCCAG-3′ and 3932R: 5′-GACCCCAAGGTAATACATAACCC-3′) (Olival et al., 2007). PCR reactions were prepared using the QIAgility liquid-handling system (QIAGEN) and were amplified using a Veriti 96 well thermocycler (Applied Biosystems).

Amplified PCR products were visualized by agarose gel electrophoresis (1.5%) and GelRed Nucleic Acid Gel (Biotium) staining. Positive samples were purified using a QIAquick PCR purification kit and sent for direct sequencing. Select PCR-positives were cloned using a *pGEM®-T Easy* Vector (Promega) using a T4 DNA ligase. The plasmid was then transformed into TOP10 cells and purified with an EZNA® Plasmid Mini Kit (Omega Bio-Tek). Purified plasmids were restriction digested and sent for sequencing. To generate additional genetic information from the cytochrome *b* region, six positive samples were subjected to an additional PCR using the BatMalF3 (5′-GGATTTAATGTAATGCCTAGACGT-3′) and BatHepR2 (5′-AATGCTGTATCATACCCTAAAGGATT-3′) primers using an established protocol (Schaer et al., 2013) and treated as above for sequencing (Accession Numbers MW366837-MW366842).

2.4. Phylogenetic analysis

Over 300 sequences of the mitochondrial cytochrome *b* (Cyt-b) gene of *Hepatocystis* parasites were downloaded from the NCBI database, followed by multiple sequence alignments using MAFFT v.7 (Katoh and Standley, 2013) in Geneious (Biomatters Ltd). The final dataset consisted of 125 Cyt-b sequences comprising Cyt-b sequences isolated from various mammalian hosts (including bats, monkeys, and squirrels), six sequences of Singapore bat *Hepatocystis* parasites generated from this study, and two *Haemocystidium* sequences from squamates and two *Plasmodium* sequences from deer as outgroups. A maximum likelihood (ML) method was used to reconstruct the Cyt-b phylogeny of *Hepatocystis* parasites using RAxML v8.0.14 (Stamatakis, 2014), with the GTR + GAMMA model and 1000 non-parametric bootstrap replicates. Bootstrap support (BS) greater than 50% are indicated at major nodes.

2.5. Statistical analysis

Prevalence data was analyzed using Bayesian multi-level logistic models with the “rethinking” package version 2.01 in R version 4.0.2 (McElreath, 2016; Team, 2013). The outcome, positive *Hepatocystis* PCR status, was determined by the methods explained above and was coded as negative (0) or positive (1) for each bat. Variables included in the models were site, season, sex, age, and bat body condition (BBCI) (weight of individual/forearm length) (Lewis, 1996). Season was classified as “Northeast Monsoon” - (1 Dec - 15 March), “Inter-monsoon” - (16 March - 31 May & 1 Oct - 30 Nov), and “Southwest Monsoon” - (1 June - 30 Sept) (Meterological Service Singapore, 2020). Number of individuals sampled in each season is as follows; Northeast - 17, Inter-monsoon - 49, and Southwest - 34. Missing data on body condition was imputed (n = 14). Site was included in each model as a primary level variable except for the site level model where it was the sole variable. Model parameters are interpreted as providing evidence of an effect on the outcome if their 89% credible interval (CI) did not cross zero, with 89% CIs the default value in the “rethinking” package. We used adaptive priors by estimating an intercept for each site. Adaptive priors “learn” the prior that is common to all sites and we assume that sites are different, however, by using adaptive priors, each site helps estimate the infection rate for the other sites. Hyper-priors were used inside the adaptive priors. These priors use the data to estimate the amount of regularization. Weakly regularizing priors on the predictors assume that every individual has an equal probability of infection before including the effect of predictors. Flat priors on the probability scale

Table 1
Description of bat species, sampling site and PCR result in this study. The number of samples per species and the prevalence per site and per species is indicated.

Location	Bukit Timah Nature Reserve	Chek Jawa Wetlands	Dairy Farm Nature Park	Kent Ridge Nature Park	Rifle Range Road	Telok Blangah Hill Park	Total
Bat species	No. Positive/No. Sampled	No. Positive/No. Sampled	No. Positive/No. Sampled	No. Positive/No. Sampled	No. Positive/No. Sampled	No. Positive/No. Sampled	No. Positive/No. Sampled
Cynopterus brachyotis	–	0/1 (0%)	5/18 (27.8%)	18/57 (31.6%)	0/8 (0%)	9/17 (52.9%)	32/101 (31.7%)
Eonycteris spelaea	–	–	0/2 (0%)	–	0/115 (0%)	–	0/117 (0%)
Macroglossus minimus	–	0/1 (0%)	–	–	–	–	0/1 (0%)
Myotis muricola	–	–	0/1 (0%)	–	–	–	0/1 (0)%
Penthetor lucasi	0/52 (0%)	–	–	–	–	–	0/50 (0%)
Rhinolophus lepidus	0/29 (0%)	–	0/1 (0%)	–	–	–	0/30 (0%)
Total	0/81 (0%)	0/2 (0%)	5/22 (22.7%)	18/57 (31.6%)	0/123 (0%)	9/17 (53%)	32/300 (10.7%)

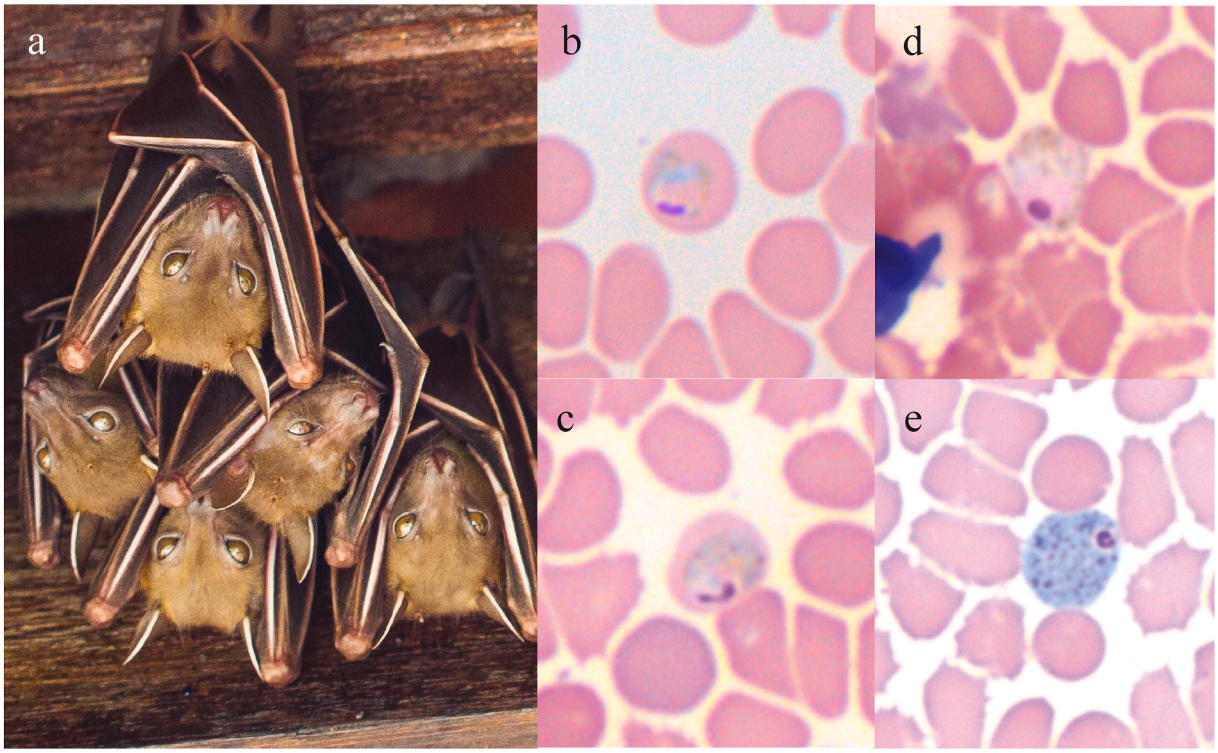

Fig. 1. Micrographs from Giemsa-stained blood smears from short-nosed fruit bats (*Cynopterus brachyotis*) (a) showing *Hepatocystis* parasites shown at 1000X magnification. Early-stage gametocytes (b) presented as a ring form with a chromatin dot, while more developed young gametocytes had a solid nucleus (c). Microgametocytes had a large nucleus that was absent of granules, with the majority stained light red and a smaller portion stained dark red (d). The macrogametocyte stained blue with pigment granules throughout the cytoplasm, had a comparatively smaller nucleus that stained purple (e). Photo of *C. brachyotis* by Lim Zong Xian.

would assume that over the data set all individuals would either be infected, or all would not and this would generate inference error. Convergence criteria such as effective sample sizes and Rhat values were used to check for appropriate model convergence throughout, and trace plots were inspected for signs of incomplete mixing when necessary. Model fit was assessed using WAIC (Watanabe and Opper, 2010).

3. Results

A total of 300 bats with sufficient blood for screening were trapped from six different locations throughout Singapore from April 2011 to March 2014. Six bat species were trapped, including; *Cynopterus brachyotis, Eonycteris spelaea, Macroglossus minimus*, *Myotis muricola, Penthetor lucasi,* and *Rhinolophus lepidus* (Table 1). *Cynopterus brachyotis* (Fig. 1a) was the only bat species positive for any haemosporidia, with positive bats detected in four different sampling sites. PCR results revealed 31.7% (32/101) of *C. brachyotis* were positive for bat *Hepatocystis*. PCR products from six individual bats were sequenced resulting in four unique haplotypes sharing a nucleotide pairwise identity of 99.9%. All other species were negative by PCR (Table 1). A total of 76 *Leptocyclopodia ferarii* bat flies were collected from *C. brachyotis* and all were PCR-negative for bat haemosporidia. Eight blood smears from the *Hepatocystis* PCR-positive bats were of sufficient quality to be read with visible parasites (Fig. 1). Young gametocytes, microgametocytes and macrogametocytes were detected in the blood smears. The young gametocytes had a ring form with a chromatin dot (Fig. 1b), while more mature forms presented with a solid nucleus (Fig. 1c). Microgametocytes had a nucleus with no granules and the majority stained light red and a smaller portion dark red (Fig. 1d). The macrogametocytes (Fig. 1e) were stained blue with pigment throughout the cell and a small, purple-stained nucleus. The mean red blood cell (RBC) count for all the slides was 221.7 (STDEV 28.31). The highest intensity of infection was 7.2%,

Table 2
Parasitemia of *Hepatocystis* infections in *Cynopterus brachyotis* individuals. Mean red blood cell (RBC) counts per field and standard deviation are given per field for 10 fields counted. The intensity of infection was calculated with the number of RBC infected with gametocytes divided by the total RBC. An overall infection rate was calculated with the total number of parasites (early stage and mature gametocytes) divided by the total RBC.

Slide	Mean RBC/Field (Standard Deviation)	Early Stage Gametocytes	Microgametocytes and Macrogametocytes	Total Parasites	Intensity of Infection
Bat 11-2	224.7 (21.78)	84	78	162	7.210%
Bat 11-3	211.1 (22.61)	14	12	26	1.232%
Bat 11-4	239.3 (24.87)	0	22	22	0.919%
Bat 12-5	234.6 (21.35)	14	3	17	0.725%
Bat 12-7	176.6 (14.77)	0	7	7	0.396%
Bat 12-8	223 (26.56)	1	20	21	0.942%
Bat 12-10	244.5 (15.83)	0	5	5	0.204%
Bat 12-13	220 (17.32)	6	0	6	0.273%

Table 3
Model comparison results using WAIC (Watanabe–Akaike information criterion) for *Hepatocystis* prevalence. Prevalence was tested with a multi-level Bayesian logistic regression model. BBCI: Bat Body Condition Index.

Model	WAIC	SE	dWAIC	dSE	pWAIC	Model weight
Site + Age	130.3	10.66	0.0	NA	2.1	0.61
Site + Sex + Age + BBCI	132.1	11.00	1.9	15.61	3.0	0.24
Site + Season + Sex + Age + BBCI	135.2	11.52	5.0	15.92	4.3	0.05
Site	135.9	10.97	5.6	2.09	2.0	0.04
Site + BBCI	136.9	10.96	6.6	2.27	2.4	0.02
Site + Sex	137.5	11.01	7.2	2.16	2.8	0.02
Site + Season	138.5	11.10	8.2	15.44	3.3	0.01
Site + Season + BBCI	140.1	11.60	9.8	15.84	4.0	0.00

pWAIC: estimated effective number of parameters; **dWAIC**: relative difference between the value of WAIC for the top-ranked model and the value of WAIC for each model; **SE**: standard error for the WAIC computations; **dSE**: standard error of the differences between two values of WAIC.

while only one other bat had an infection rate greater than 1%, and the remaining six were less than 1% (Table 2).

The following is an overview of the results of the *C. brachyotis* individuals that were assessed in the sub-group analysis (n = 100). One bat was excluded from analysis as it was the only individual trapped at Chek Jawa wetlands and was PCR-negative. We defined eight models of *Hepatocystis* prevalence, including a full model with all covariates, and compared them using WAIC (Table 3). Based on the WAIC values, the two models that had the lowest out of sample deviance were the site + age model and the model that included all the life history characteristics. In other words, these models have a higher predictive performance than the other models. All the other models were equivalent in their out of sample deviance. Parameter estimates were then logit-transformed to display probability of prevalence. There was no significant difference among site parameters (Table 4) or the probability of *Hepatocystis* prevalence among sites (Fig. 3a), however *C. brachyotis* in Telok Blangah Hill Park were more likely to be infected compared to other sites. There was no difference in probability of prevalence among seasons (Fig. 3b). The site + age model had the highest model weight when compared to the other models and there was no difference between probability of *Hepatocystis* prevalence between age classes (Fig. 3c). Sampling distribution for the age variable was 10 juveniles (none of which were positive for *Hepatocystis*) and 90 adults. Even though there were no positive juveniles, the 89% CIs still overlapped. There was no difference between sexes (Supplementary Table 1) and no difference in probability of prevalence between sexes (Fig. 3d). There was no significant relationship between *Hepatocystis* prevalence and BBCI (Fig. 4), however, prevalence did decline slightly with increasing BBCI.

The maximum likelihood phylogeny of *Hepatocystis* parasites based on the Cyt-b gene can be segregated into two supported clades (clade 1 and 2 in Fig. 2). Clade 1 (BS ML = 94%) of *Hepatocystis* species infects squirrels from Borneo and Laos, whereas clade 2 (BS ML = 79%) is predominantly found in bats from South East Asia to Africa and Australia. Our results indicate that all six novel *Hepatocystis* sequences from Singapore bats (*C. brachyotis*) are phylogenetically clustered within clade 2 (Fig. 2) and most related to two other *Hepatocystis* sequences (MT136146 and DQ396146) from the same bat species, *C. brachyotis,* as previously reported in Thailand and Malaysia (Arnuphapprasert et al., 2020). They shared a high level of nucleotide similarity (99.7%–100%) and formed a strongly supported clade (BS ML = 100%). The Cyt-b sequences of our bat *Hepatocystis* parasites are also related to a sequence found in *C. brachyotis* reported from Singapore in 2008 (Martinsen et al., 2008). However, this clade (with a strong bootstrap support of 98%) appears to be phylogenetically distinct from two *Hepatocystis* parasites (DQ396526 and AY099030) of *C. brachyotis*, exhibiting lower nucleotide similarity (92.4–93.5%) among them.

4. Discussion

In this study, we report on the high rates of infection of *Hepatocystis* parasites in *Cynopterus brachyotis* (short-nosed fruit bats) and the absence of haemosporidia in five other bat species in Singapore. There was also no evidence of *Hepatocystis* infection in bat fly ectoparasites (*Leptocyclopodia ferarii*) collected from these bats. This study detected that there was no significant difference in the probability of *Hepatocystis* infection by sampling site, body condition, sex, age or season.

Bat *Hepatocystis* infections have only been detected in species in the suborder Yinpterochiroptera, including members of the families Pteropodidae (fruit bats), Craseonycteridae (Kitti's hog-nosed bat), Hipposideridae (Old World leaf-nosed bats) and Rhinolophidae (horseshoe bats) (Arnuphapprasert et al., 2020; Boundenga et al., 2018; Martinsen et al., 2008; Schaer et al., 2017). Short-nosed fruit bats (*C. brachyotis*) are pteropodids with previous *Hepatocystis* detections in Malaysia, Singapore, and Thailand (Arnuphapprasert et al., 2020; Martinsen et al., 2008). *Hepatocystis* has also been detected in several *Pteropus* species (flying foxes), including an individual *Pteropus hypomelanus* in Malaysia (Olival et al., 2007) and three species (*P. alecto*, *P. poliocephalus*, and *P. scapulatus*) in Australia (Schaer et al., 2019). Bat *Hepatocystis* host range is highest in sub-Saharan Africa with pteropodid members of the genera *Eidolon*, *Epomophorus*, *Epomops*, *Hypsignathus*, *Micropteropus*, *Myonycteris*, and *Nanonycteris* all being suspect reservoirs (Boundenga et al., 2018; Schaer et al., 2013, 2017).

Hepatocystis prevalence in *Pteropus* in Australia varies greatly, with 100% of *P. scapulatus* infected (albeit with a small sample size) to only 3% of *P. poliocephalus* infected (Schaer et al., 2019). In West and Central Africa, prevalence in pteropodids differed across species, with *Hypsignathus* ranging from 5% to 100% and *Epomops* from 13 to 90% (Boundenga et al., 2018; Schaer et al., 2013). In South Sudan, prevalence in *Epomophorus* was 94% in 138 bats tested, demonstrating the hyperendemicity of this parasite in specific regions (Schaer et al., 2017). On average, parasitemia in individuals tends to be less than 1%, but in two individual bats (*Epomophorus* and *Micropterus*), it was greater than

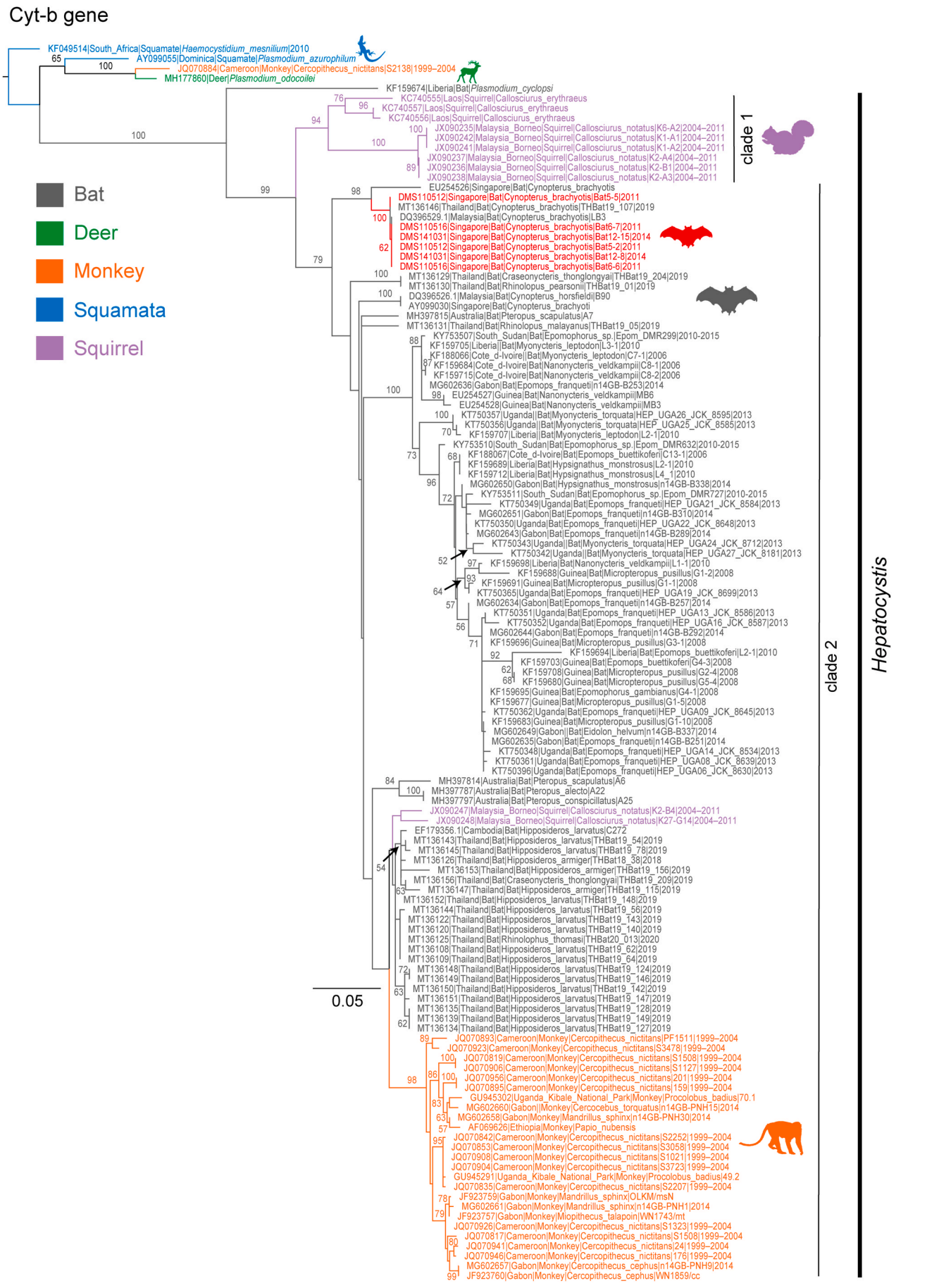

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Fig. 2. Evolutionary relationships of the mitochondrial cytochrome B (Cyt-b) gene sequences of *Hepatocystis* parasites detected in different mammalian hosts. The tree phylogeny was reconstructed using the maximum likelihood method in RAxML. Coloured branches denote different host species. Bootstrap values above 50% are indicated at the major nodes. Scale bar indicates the number of nucleotide substitutions per site. Red fonts represent novel *Hepatocystis* sequences generated from this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

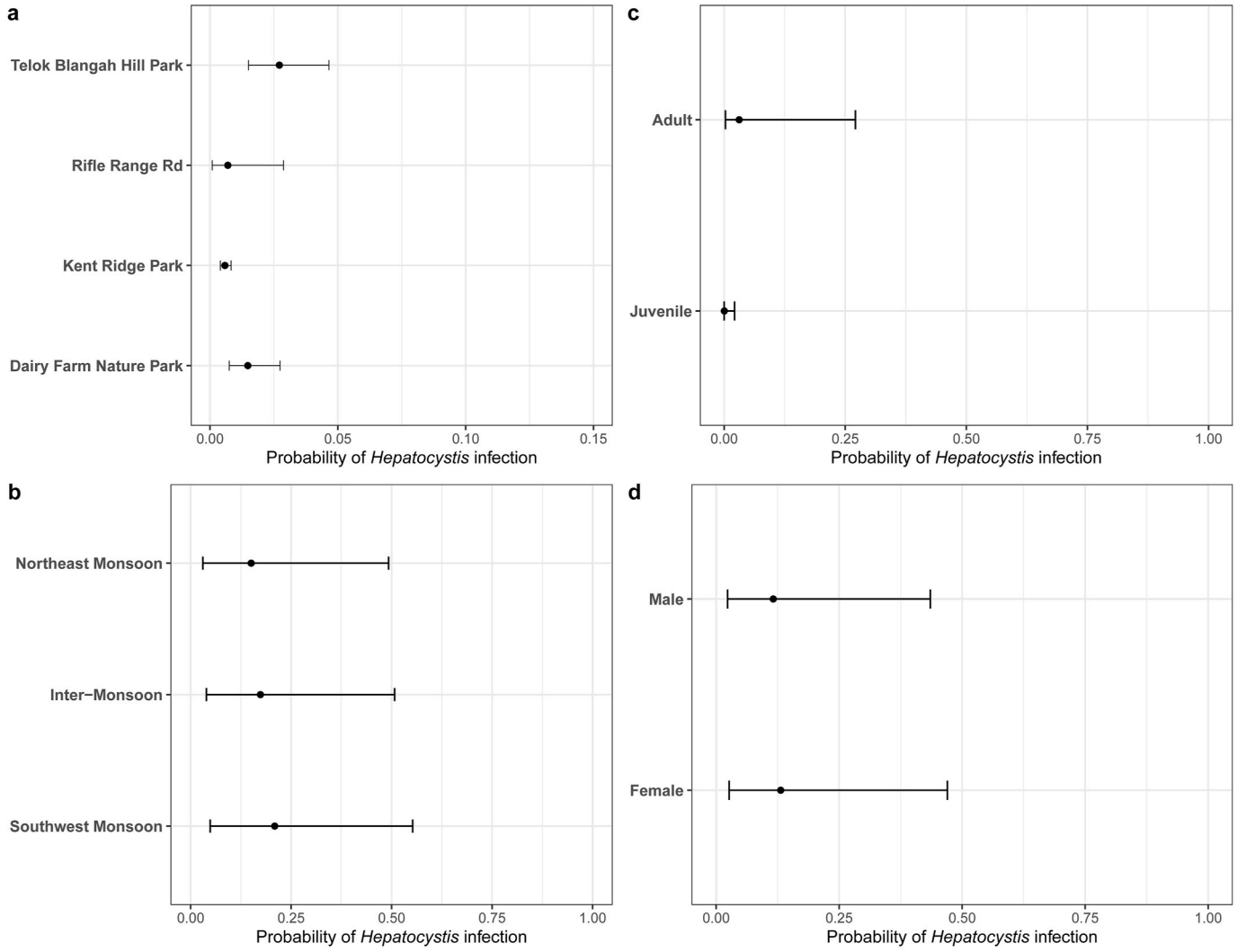

Fig. 3. Co-variate analysis for the probability of infection in *Cynopterus brachyotis* in relation to site, age, season, and sex. Error bars are 89% CI. (a) Logit-transformed model estimates of *Hepatocystis* prevalence among four sampled sites in Singapore; (b) Logit-transformed model estimates of *Hepatocystis* prevalence among seasons; (c) Logit-transformed model estimates for probability of infection by age; (d). Logit-transformed model estimates for probability of infection by sex.

4% (Schaer et al., 2017). This is similar to the parasitemia found in our study, though blood slides were unavailable for all infected individuals.

The results of our study indicated that bat sex, age, and body condition were not significant risk factors for *Hepatocystis* infection. No juveniles were positive for *Hepatocystis* and this may represent an age sampling bias as juvenile pteropodids in South Sudan have been found infected (Schaer et al., 2017). Robust sampling of juveniles in Singapore is important as age may play a role as a risk factor in other bat parasites (Mendenhall et al., 2017). There was a weak relationship between bat body condition and probability of prevalence, which appears to play a role in bat helminth infections and Hendra virus RNA status (Edson et al., 2019; Warburton et al., 2016). Stressors such as pregnancy, lactation, parasite load and resource availability can impact immune responses against parasites (Plowright et al., 2016). However, this may not be applicable in bats as they tolerate infection by reducing inflammatory immune responses (Banerjee et al., 2020) and *Hepatocystis* also does not undergo blood schizogony, potentially reducing malarial byproducts and reducing pyrexia (Greenbaum and FitzGerald, 2009). Additionally, *Hepatocystis* was not biased to either sex in our study population, thus partitioning females by reproductive status (dry, pregnant, lactating, carrying a pup) may also provide insight into the female life cycle and its role in maintaining pathogens.

Though there were no significant differences in infection rates among sites, Telok Blangah Hill Park had more infected individuals than the other three sites. Given that these bats roost in small harems in palm trees, it is likely that these roosts are nearby to each respective site (Francis and Barrett, 2008). Singapore is a relatively small island (~700 km^2) so these bats may be using multiple roosts during the night which would explain why there are no significant differences in infection (Campbell, 2008). Climatic variables may be useful in explaining variation in prevalence over time, however, our data indicate that these variables did not affect prevalence. We did not measure variables such as temperature, rainfall, and relative humidity, but did classify seasons in Singapore by monsoon seasonality. Our model demonstrated no differences in prevalence among seasons, which may represent an absence of dry season that would impact the presumed dipteran vector.

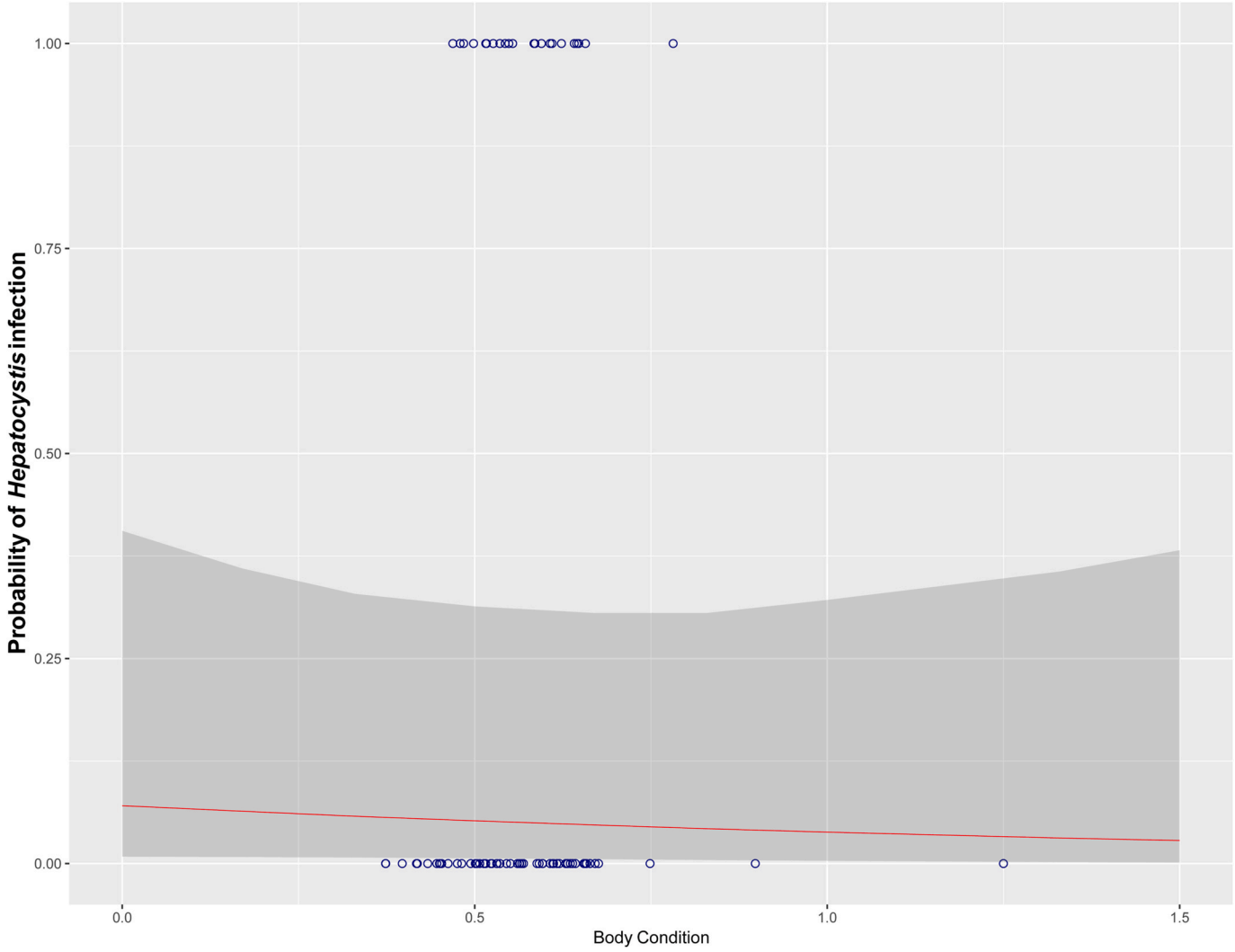

Fig. 4. Relationship of body condition (weight of individual/forearm length) to probability of *Hepatocystis* infection. Open blue circles are the observed data. The red line is the estimated linear relationship, and the shaded area represents the 89% CI. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Transmission of haemosporidian parasites may have seasonality depending on the availability of competent dipteran vectors, however little is known about *Hepatocystis* vector ecology (Reiner et al., 2015). In South Sudan, *Hepatocystis* parasitemia was markedly reduced in the dry season, but prevalence was nearly 90% in one species indicating long term infections (Schaer et al., 2017).

Sequence data generated from *C. brachyotis* in this study confirms that the *Hepatocystis* sampled is likely the same undescribed species as previously detected from the same bat species in Malaysia, Singapore and Thailand (Arnuphapprasert et al., 2020; Martinsen et al., 2008). The *Hepatocystis* parasites in *C. brachyotis* from Singapore are predominantly clustered within a clade without intermixing of other bat species, indicative of host specificity or under-sampling of hosts. *Hepatocystis* in *Cynopterus* species in other regions of the tree demonstrates the proclivity of this group to host switch (Duval et al., 2007). Although *Hepatocystis* in African bats and monkeys formed distinct clades, *Hepatocystis* is not monophyletic at the bat genus level (e.g. *Epomops franqueti*, *Micropterpus pusillus* and *Nanonycteris veldkampii*) and monkey genus level (e.g. *Cercopithecus nicitans* and *Procolobus badius*). Collectively, these results suggest that bat *Hepatocystis* parasites are unlikely host-specific at the host generic level and may undergo host switching, consistent with previous studies (Arnuphapprasert et al., 2020; Schaer et al., 2017).

The taxonomic assignment of *Hepatocystis* has long been uncertain, but multiple phylogenetic approaches have placed this monophyletic genus in a sister group with *Plasmodium* (*Vinckeia*) that infects rodents and bats (Galen et al., 2018). However, the absence of blood replication stages (erythrocytic schizogony) and the lost genes in the Reticulocyte Binding Protein family, required by *Plasmodium* for red blood cell entry, may necessitate taxonomic reorganization (Aunin et al., 2020). *Hepatocystis* sequences derived from infected African bats demonstrate little genetic structure and there are several closely related groups that infect multiple species, indicating an absence of host specificity (Boundenga et al., 2018; Schaer et al., 2013, 2017). A study in Central Africa found four *Hepatocystis* clades in bats, but there was no geographic delineation for these parasites (Boundenga et al., 2018).

This study is the first on the epidemiology of *Hepatocystis* in bats in Singapore. *Hepatocystis* is believed to be transmitted by biting midges (Diptera: Ceratopogonidea), specifically from the genus *Culicoides* unlike members of the genus *Plasmodium* which is transmitted by anopheline mosquitoes (Garnham et al., 1961). Transmission of bat *Hepatocystis* is driven by unknown vectors, though *Polychromophilus* haemosporidian species may be transmitted by bat flies (Mer and Goldblum, 1947). However, dissections of Nycterbiidae bat flies from *Pteropus* did not reveal *Hepatocystis* infection (McGhee, 1949). In avian *Plasmodium*, host compatibility is integral for transmission and this may apply to bat

Hepatocystis (Medeiros et al., 2013). Certain bat species may be refractory to infection, demonstrated by the absence of *Hepatocystis* infection in *Eonycteris spelaea* in Singapore and Thailand (Arnuphapprasert et al., 2020). However, *Nycteria* parasites were detected in *E. spelaea* in Thailand, though not in Singapore (Arnuphapprasert et al., 2020). Our study motivates further exploration into *Hepatocystis* host specificity, parasite diversity and how the host-vector-parasite relationship drives transmission. The absence of correlates of infection makes targeted surveillance difficult, but much work remains to be done to understand the natural transmission of bat haemosporidians in Southeast Asia.

Declaration of competing interest

The authors declare that there is no conflict of interest with this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2021.04.001.

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