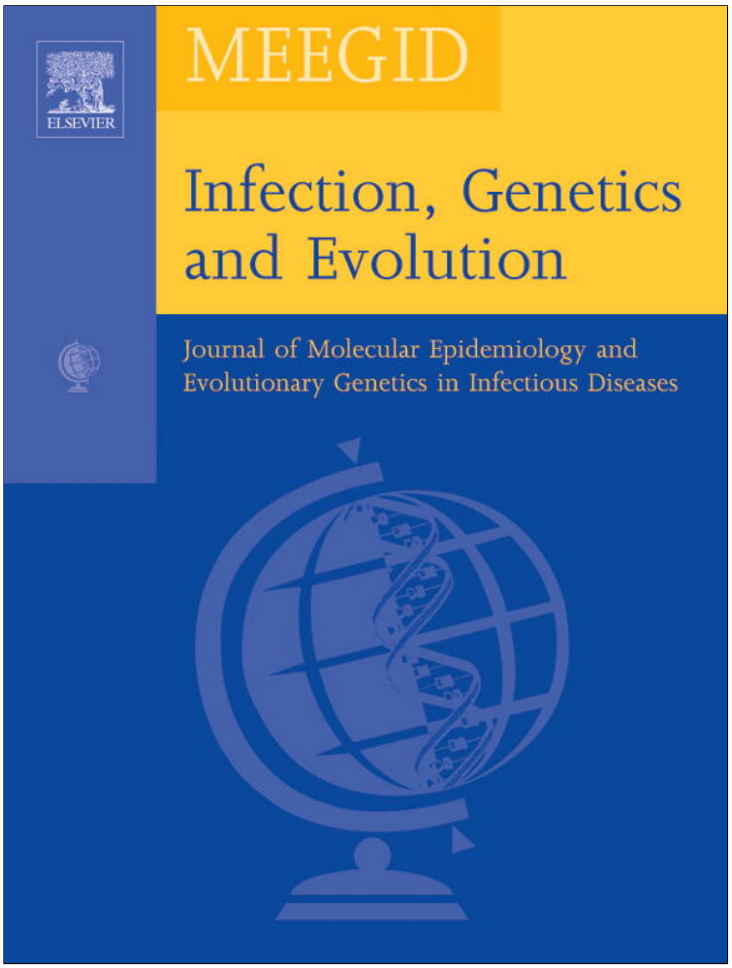

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Host selection and parasite infection in *Aedes taeniorhynchus*, endemic disease vector in the Galápagos Islands

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ABSTRACT

Host selection in blood-sucking arthropods has important evolutionary and ecological implications for the transmission dynamics, distribution and host-specificity of the parasites they transmit. The black salt-marsh mosquito (*Aedes taeniorhynchus* Wiedemann) is distributed throughout tropical to temperate coastal zones in the Americas, and continental populations are primarily mammalphilic. It is the only indigenous mosquito in the Galápagos Islands, having colonised the archipelago around 200,000 years ago, potentially adapting its host selection, and in the process, altering the dynamics of vector mediated pathogen interactions in the archipelago. Here, we use blood–meal analysis and PCR-based parasite screening approach to determine the blood-feeding patterns of *A. taeniorhynchus* in the Galápagos Islands and identify potential parasite transmission with which this mosquito could be involved. Our results show that *A. taeniorhynchus* feeds equally on mammals and reptiles, and only one avian sample was observed in 190 successful PCR amplifications from blood meals. However, we detected endemic filarial worms and *Haemoproteus* parasites known to infect various Galápagos bird species in mosquito thoraces, suggesting that feeding on birds must occur at low frequency, and that *A. taeniorhynchus* may play a role in maintaining some avian vector-borne pathogens, although more work is needed to explore this possibility. We also isolated three different DNA sequences corresponding to hemogregarine parasites of the genus *Hepatozoon* from mosquito and iguana blood samples, suggesting that more than one species of *Hepatozoon* parasites are present in Galápagos. Phylogenetic analysis of *Hepatozoon* 18sRNA sequences indicates that *A. taeniorhynchus* may have facilitated a recent breakdown in host–species association of formerly isolated *Hepatozoon* spp. infecting the reptile populations in the Galápagos Islands.

1. Introduction

Disease vectors colonising new areas where their primary host is naturally rare may come under pressure to modify their feeding behavior and accept feeding on novel hosts. The capacity and the direction of the change in host selection will depend on the genetic background of the vector and on the ecological characteristics of its new environment, such as habitat, phenology, host-availability and host-constraint of insects (Tompkins and Clayton, 1999; Stireman and Singer, 2003; Kilpatrick et al., 2007). Host selection in blood-sucking arthropods, and any modification of these selection patterns, will have important evolutionary and ecological implications for the transmission dynamics, distribution and host-specificity of the parasites they transmit.

The black salt-marsh mosquito (*Aedes taeniorhynchus* Wiedemann) is the only native mosquito in the Galápagos Islands, where it is widely distributed, having colonised the archipelago naturally around 200,000 years ago (Bataille et al., 2009a). Bataille et al. (2009a) have shown that *A. taeniorhynchus,* which feeds principally on large mammals and occasionally on birds in the continent (O'Meara and Edman, 1975; Anderson and Fortner, 1988), feeds also on reptile hosts in the Galápagos Archipelago, suggesting that *A. taeniorhynchus* could have experienced a selective pressure to broaden its host feeding range, as the Galápagos Islands were depauperate in mammals before the arrival of humans. However,

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it remains unclear whether, when presented with a choice, *A. taeniorhynchus* in Galápagos would now select to feed on the more abundant reptile hosts over other hosts.

In comparison to *A. taeniorhynchus,* far more ancient colonisation dates (>5 million years ago) characterise other endemic fauna, some of which arrived soon after the archipelago was formed (Parent et al., 2008). This relatively recent colonisation of *A. taeniorhynchus* compared to other Galápagos endemic fauna, suggests that its arrival into a system with no other mosquito disease vectors may have altered the dynamics of existing endemic vector-borne diseases, or allowed novel and established pathogens to invade immunologically naive host species. Moreover, current introduction of multiple mosquito species in Galápagos could provoke further alterations of host–parasite associations in Galápagos, potentially threatening its endemic fauna (Bataille et al., 2009b). Our knowledge of host–parasite associations in Galápagos is poor, but a recent focus on wildlife disease threats to Galápagos fauna furthered our understanding of some of these associations (Wikelski et al., 2004; Kilpatrick et al., 2006a; Parker et al., 2006; Bataille et al., 2009a). Some of the host–parasite associations discovered in Galápagos might involve transmission by arthropod vectors (e.g. ticks, mosquitoes, or hippoboscid flies) and thus could have been established or altered by the arrival of *A. taeniorhynchus.* Therefore, it is important to determine the host range and feeding patterns of *A. taeniorhynchus* in Galápagos to understand its role in the transmission of parasites in the archipelago.

Here, we aim to determine the host feeding range of *A. taeniorhynchus* in the Galápagos Islands by analysing blood-meals from engorged mosquitoes caught opportunistically across the archipelago. We also estimated host utilisation and host availability to measure host selection of *A. taeniorhynchus*. We also screened mosquitoes for hematozoan and filarial parasites to identify natural host–parasite associations. We discuss the implications of our results in the context of host seeking adaptations of *A. taeniorhynchus* and its role in endemic host–parasite systems.

2. Material and methods

2.1. Sampling of blood-fed mosquitoes

Adult mosquitoes were collected in 2006 and 2007 at multiple sites across the Galápagos Islands (Fig. 1) with miniature UV light traps or with miniature incandescent light traps with photoswitch-controlled CO_2 release system (John W. Hock Company, Gainesville, Florida). Sites included locations sampled regularly as part of previous studies to assess population structure, temporal variation in abundance and dispersal capacity of *A. taeniorhynchus* (Bataille et al., 2009a, 2010, 2011) and locations sampled opportunistically during fieldwork for other projects to maximise geographic coverage.

Another field trip designed for this study was organised in January 2008 on the Bolivar Canal separating Fernandina and Isabela Islands (Fig. 1) to sample mosquitoes in sufficient numbers to generate reliable estimates of low prevalence pathogens and of host feeding patterns. This region of the archipelago was targeted because this uninhabited and relatively undisturbed area shelters many endemic species, notably an important part of the population of flightless cormorants (*Phalacrocorax harrisii*) and Galápagos penguins (*Spheniscus mendiculus*) (Jimenez-Uzcategui and Vargas, 2007).

After each field trip, samples were brought back to the Galápagos Genetics, Epidemiology and Pathology laboratory (GGEPL) where mosquitoes were separated from other insects, identified to species using morphological features and then stored at −20 °C. Mosquitoes with blood-engorged abdomens were noted and kept in separate collection tubes at −20 °C.

2.2. Blood-meal analysis

Each blood-fed mosquito was prepared using a sterile scalpel to separate the abdomen from the rest of the body; the latter was kept in 100% ethanol for parasite screening or for microsatellite genotyping. DNA was extracted from the abdomen using a salting-out extraction method with ammonium acetate (Nichols et al., 2000). A portion of the cytochrome *b* (*cytb*) gene was amplified by PCR using primers described by Lee et al. (2002). PCR was performed in a 30 μl volume containing 2–10 ng of genomic DNA with 0.5 μM of each primer, 0.2 mM of each dNTP, 1.0 unit of *Taq* DNA polymerase (BioLine UK Ltd., London, UK) and 3 mM of $MgCl_2$. The PCR program used was 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 45 s and 72 °C for 60 s, and finally 72 °C for 7 min. PCR products were sequenced using an ABI 3730 Automated Sequencer (PE Applied Biosystems Ltd., USA) at the Core Genetics services, University of Sheffield (for PCR analyses done at the University of Leeds) or at Macrogen USA (Rockville, MD; for PCR analyses done at the GGEPL). Sequences (size: 260–300 bp) were compared against the GenBank database using blastn 2.2.4 (Altschul et al., 1997) to identify the species on which each mosquito had fed (99–100% of similarity were always obtained).

Initial *cytb* analyses identified a high proportion of blood-meals from marine iguana (*Amblyrhynchus cristatus,* see results Section 3.2.2.). To improve efficiency and reduce costs for the remaining blood-meals, we used species specific primers targeting marine iguana mitochondrial control region (Hanley and Caccone, 2005). This allowed a rapid PCR-based test, assayed by the presence/absence of product with agarose gel electrophoresis, although it had the caveat to potentially miss the other component of mixed blood-meals involving iguanas. Only samples which did not yield a marine iguana product were subsequently analysed for *cytb*. PCR targeting the marine iguana mitochondrial control region was performed with a 30 μl volume containing 2–10 ng of genomic DNA with 0.2 μM of each primer, 0.2 mM of each dNTP, 1.0 unit of *Taq* DNA polymerase (BioLine UK Ltd., London, UK) and 2 mM of $MgCl_2$. The PCR program used was 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, and finally 72 °C for 10 min.

2.3. Statistical analysis of blood-feeding patterns

In order to estimate mosquito feeding patterns, one needs to know host abundance on the site of collection (Kilpatrick et al., 2006b). The effective range of light traps is usually considered to be less than five meters, but addition of CO_2 bait attract mosquitoes at a distance of over 16 m (Service, 1993). In addition, blood-fed mosquitoes usually do not travel much but tend to find a resting place close-by to digest their last meal (Nielsen and Nielsen, 1953). A recent study has determined that mosquitoes with fresh blood-meals are mostly found within 40 m of their host (Ejiri et al., 2011). Therefore, all potential mammal, reptile and bird hosts found in a 50 m radius around the trapping sites in the Bolivar Canal were identified and counted once during an hour before dusk (5 p.m.). Trapping was done for 5 h (until battery ran out) starting at 5 p.m. to coincide with mosquito peak activity (Nielsen and Nielsen, 1953), and with the time when vertebrate hosts were settling for the night (so with limited movement during trapping time). Galápagos fauna is usually not frightened by the presence of humans, and we did not observe any animal moving away from us during the whole time necessary to set the traps and count the animals. Results from the blood-meal analysis and data on animal counts from the three sites in the Bolivar Canal were used to analyse mosquito blood-feeding patterns following a method modified from Kilpatrick et al. (2006b).

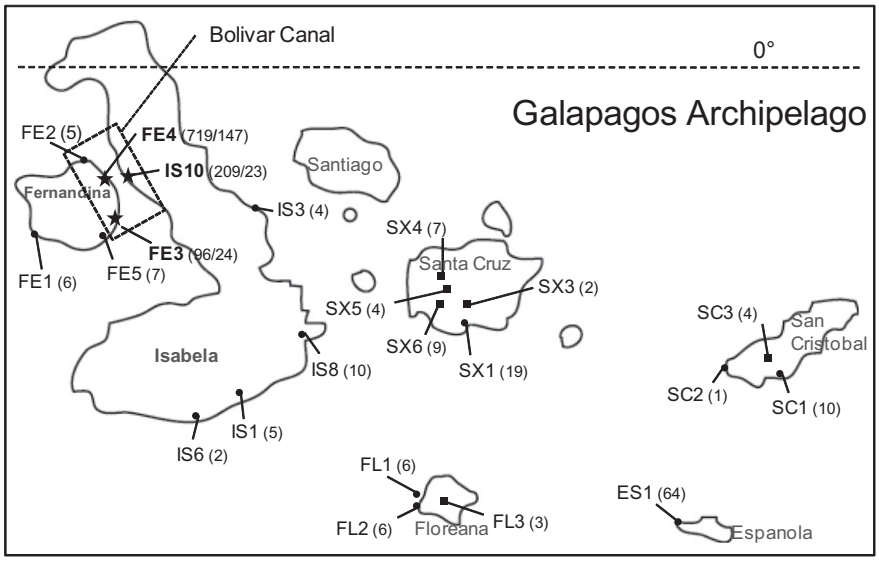

Fig. 1. Map of the sampling sites of *A. taeniorhynchus* in the Galapagos Islands. Numbers between brackets are the number of blood-fed specimens collected in each site. The sites used for blood-feeding patterns estimates and parasite screening are indicated in bold and by a star. Numbers between brackets for these sites are the total number of mosquitoes/the number of blood-fed mosquitoes collected, respectively.

We defined $u_{i,j}$ as the number of mosquitoes with blood from host species i in a site j. The density $v_{i,j}$ of host species i in a site j had been assessed by counting the number of vertebrate hosts which were on site, when the mosquito traps were collected. Not finding any mosquito fed on a given species does not necessarily mean that mosquitoes never feed on this species. It might just be a rare event that we were unable to capture due to the limitation of our sample size. Therefore, if no blood meals were identified from a host species i in a site j, we assumed $u_{i,j} = 0.5$.

The feeding selection for a host species i in a site j was then defined as the ratio between the proportion of mosquitoes blood-fed on host of species i in a site j, and the proportion of hosts of species i in a site j:

$$P_{i,j} = \frac{(u_{i,j}/U_j)}{(v_{i,j}/V_j)}$$

where $U_j = \sum_k u_{k,j}$ is the number of mosquitoes blood-fed in a site j, and $V_j = \sum_k v_{k,j}$ is the number of vertebrates in a site j. Mosquitoes selected to feed or not to feed on a host if $P_{i,j} > 1$ or $P_{i,j} < 1$ respectively. If $P_{i,j} = 1$, mosquitoes feed on the host species in proportion of their abundance (i.e. mosquitoes select these hosts at random).

Multinomial simulations were implemented in the statistical platform R v2.12.0 (R Development Core Team, 2011), using the package multinomRob (Mebane and Sekhon, 2004), to test if the observed $P_{i,j}$ was significantly different from 1. The R script is provided as supplementary material. First, the probability $p_{i,j}^{H_0}$ of a mosquito feeding from host species i in a site j, under the null hypothesis (H_0: mosquitoes are selecting resources at random) was defined as the proportion of vertebrates from this species:

$$p_{i,j}^{H_0} = \frac{v_{i,j}}{V_j}, \text{ such that } \sum_k p_{k,j}^{H_0} = 1$$

Then, the number of blood meals $u_{i,j}^{H_0}$ under H_0 was generated from a multinomial distribution, with parameters the total number of blood meals identified in a site j, and the probabilities $p_{i,j}^{H_0}$ (as defined above). By iterating this sequence 100,000 times we could then estimate the p-value, which was the proportion of simulations in which the number of blood meals was higher (for $P_{i,j} > 1$), or lower (for $P_{i,j} < 1$) than the observed one. The number of simulations was fixed to 100,000 in order to allow the p-value to converge within a small range of potential values. The feeding selection index and its associated p-value may be sensitive to inaccuracies in animal counts. In order to assess the robustness of our conclusions about the mosquito feeding selection for each host species, we ran additional simulations varying the animal counts and examined the impact on our results.

2.4. Parasite screening

Infection rates observed in mosquitoes are often very low (below 1%) for arboviruses, *Plasmodium* spp. and filarial infections (Plichart et al., 2006; Lardeux et al., 2008), so many specimens need to be collected and those are usually pooled to be screened in an efficient and cost-effective way (Gu, 1995). Parasite screening effort focused on vector-borne parasites previously detected in Galápagos endemic fauna comprising *Plasmodium and Haemoproteus* blood parasites infecting Galápagos penguins and other bird species (Padilla et al., 2006; Levin et al., 2009; Levin et al., 2011); filarial worms infecting birds and potentially mammals (Labarthe and Guerrero, 2005; Merkel et al., 2007); and hemogregarine parasites infecting Galápagos tortoises and iguanas (authors, unpublished data).

2.4.1. DNA extraction

Mosquitoes were dissected with sterile scalpels to separate head and thorax. The thorax and head of mosquitoes were screened separately, because the infective stage of filarial nematodes is found only in the head, which gives a direct estimate of the prevalence of infective mosquitoes (Yameogo et al., 1999). In contrast, infectious stage of haematozoa such as *Plasmodium* spp. and *Haemoproteus* spp. are found mainly in the salivary glands of the mosquito, which are situated in its thorax. Thoraces of specimens from the same site were pooled with a maximum of 20 specimens per pool. Heads were pooled with a maximum of 40 specimens per pool. Head pools and thorax pools were ground with micropestles in 200 µl of lysis buffer (QIAGEN Ltd., Crawley, UK), and DNA was extracted with the QIAGEN DNeasy kit (QIAGEN Ltd., Crawley, UK), following the manufacturer's instructions. The DNA samples were then tested for blood-borne parasites using PCR methods as described below. Nested or semi-nested PCR

Table 1
Identification of blood-meals from *A. taeniorhynchus* specimens collected in the Galapagos Islands during multiple field trips in 2006 and 2007.

	Number of mosquito blood-meals collected											
	Espanola	Fernandina	Floreana		Isabela	San Cristobal		Santa Cruz		All islands		
	Coast	Coast	Coast	Highland	Coast	Coast	Highland	Coast	Highland	Coast	Highland	All
No. blood meals tested by PCR	64	18	12	3	21	11	4	19	22	145	29	174
No. blood meals positive (%)	27	10	9	1	12	5	2	8	10	71 (49%)	13 (44.8%)	84 (48.3%)
Host species identified (common/*latin* name; %)												
Mammals												
Human/*Homo sapiens*	5	2	6	1	2	1	-	1	3	17 (23.9%)	4 (30.8%)	21 (25%)
Cow/*Bos Taurus*	–	–	–	–	–	–	–	–	1	–	1 (7.7%)	1 (1.2%)
Dog/*Canis canis*	–	–	–	–	7	–	–	–	–	7 (9.9%)	–	7 (8.3%)
Galapagos sea lion/ *Zalophus wollebaeki*	6	5	–	–	–	–	–	–	–	11 (15.5%)	–	11 (13.1%)
Pig/*Sus scrofa*	–	–	–	–	–	–	2	–	–	–	2 (15.4%)	2 (2.4%)
No. mammals	11	7	6	1	9	1	2	1	4	35 (49.3%)	7 (53.9%)	42 (50%)
Reptiles												
Marine iguana/*Amblyrhynchus cristatus*	16	3	3	–	1	–	–	3	3	26 (36.6%)	3 (23%)	29 (34.5%)
Galapagos tortoise/*Geochlone nigra*	–	–	–	–	2	4	–	4	3	10 (14.1%)	3 (23%)	13 (15.5%)
No. reptiles	16	3	3	–	3	4	–	7	6	36 (50.7%)	6 (46%)	42 (50%)

methods were used to increase the sensitivity of the parasite screening, as the quantity of parasite DNA can be very low in pooled mosquito samples. Mosquito blood-meals were also screened for parasites to investigate possible infection in the vertebrate hosts.

2.4.2. Detection of Haemoproteus spp. and Plasmodium spp

A modified version of the nested PCR protocol described by Waldenstrom et al. (2004), targeting a portion (size: 400–450 bp) of the cytochrome b (*cytb*) gene of *Haemoproteus/Plasmodium* parasites, was used to identify the presence of these parasites in the pooled samples. The first PCR reaction was performed in a 30 μl volume containing 2–10 ng of genomic DNA with 0.5 μM of each primer, 0.2 mM of each dNTP, 1.0 unit of *Taq* DNA polymerase (BioLine UK Ltd., London, UK) and 3 mM of $MgCl_2$. The PCR program used was 94 °C for 5 min, followed by 20 cycles of 94 °C for 30 s, 50 °C for 45 s and 72 °C for 45 s, and finally 72 °C for 7 min. The second PCR reaction was done in exactly the same way, but 0.5 μl of products from the first PCR round was used instead of genomic DNA and the PCR was run for 35 cycles instead 20. Positive PCR products were sequenced using an ABI 3730 Automated Sequencer (PE Applied Biosystems Ltd., USA) at the Core Genetics services, University of Sheffield.

2.4.3. Detection of Hepatozoon spp

Two pairs of primers targeting a 480–500 bp portion of the 18S rRNA gene in *Hepatozoon* spp. were designed using aligned 18S rRNA sequences of *Hepatozoon* spp. and other Apicomplexa species available in the GenBank database (data not shown). These primers were used to perform a nested PCR for the detection of *Hepatozoon* parasites in mosquitoes. The first PCR was performed using primers Hepa18SF (5′-ATTCTACAGCTAATACATGAGC-3′) and Hepa18SR (5′-ACAAATCTAA GAATTTCACCTCTGACAGTTA-3′) in a 30 μl volume containing 2–10 ng of genomic DNA with 0.5 μM of each primer, 0.2 mM of each dNTP, 1.0 unit of *Taq* DNA polymerase (BioLine UK Ltd., London, UK) and 3 mM of $MgCl_2$. The PCR program used was 94 °C for 5 min, followed by 20 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 60 s, and finally 72 °C for 7 min. The second PCR reaction was done in exactly the same way, but we used primers Hepa18SF2 (5′-TTTCGACGGTATGGTATTG-3′) and Hepa18SR2 (5′-TGTCCCTATCAATCATTAATTT-3′), and 0.5 μl of products from the first PCR round instead of genomic DNA, and the PCR was run for 35 cycles instead of 20.

2.4.4. Detection of microfilariae

A semi-nested PCR protocol targeting a portion (size: 340–360 bp) of the cytochrome oxidase subunit I (*COI*) gene of filarial nematodes was used to detect the presence of these parasites in mosquitoes. The first PCR was performed with primers designed by (Casiraghi et al, 2001), COIintF (5′-TGATTGGTGGTTTTGGTAA-3′) and COIintR (5′-ATAAGTACGAGTATCAAT ATC-3′), in a 30 μl volume containing 2–10 ng of genomic DNA with 0.5 μM of each primer, 0.2 mM of each dNTP, 0.5 unit of *Taq* DNA polymerase (BioLine UK Ltd., London, UK) and 3 mM of $MgCl_2$. The PCR program used was 94 °C for 5 min, followed by 20 cycles of 94 °C for 30 s, 52 °C for 45 s and 72 °C for 60 s, and finally 72 °C for 7 min. The second PCR reaction was done with the primer COIintF and a new primer COIintRn (5′-CATAAAAAGAAGTATTAAAATTACG -3′) designed for this study using aligned *COI* sequences (data not shown). This second PCR was performed with the same conditions as the first PCR, but here 0.5 μl of products from the first PCR round were used as template instead of genomic DNA, and using 35 thermal cycles instead of 20.

Table 2
Number of vertebrates counted in the mosquito sampling sites in the Bolivar Canal in January 2008.

Species (common/*latin* name)	Vertebrates counted on site			
	Fernandina Site FE3	Fernandina Site FE4	Isabela Site IS10	Total
Galapagos sea lion/*Z. wollebaeki*	1	2	–	3
Marine iguana/*A. cristatus*	45	86	12	143
Flightless cormorant/*P. harrisii*	–	16	9	20
Total	46	104	21	166

2.4.5. Identification of the parasites

Positive PCR products from the parasite screenings were sequenced using an ABI 3730 Automated Sequencer (PE Applied Biosystems Ltd., USA) by the Core Genetics services, University of Sheffield. The sequences obtained were compared to sequences available in GenBank using blastn 2.2.4 (Altschul et al., 1997) to confirm that they corresponded to sequences of parasites targeted.

Confirmed parasite sequences were aligned with sequences of closely related blood parasites collected from GenBank using ClustalW (Thompson et al., 1994) as implemented in BioEdit software (Hall, 1999). For *Hepatozoon* sequences, the nucleotide substitution model that best fitted the alignment was determined with the software MODELGENERATOR (Keane et al., 2006). Phylogenetic relationships between our parasite sequences and other blood parasites sequences were inferred using a Bayesian inference approach implemented in MrBayes v.3.1.1. (Huelsenbeck and Ronquist, 2001). Multiple simulations were run for 10,000,000 generations with the first 200,000 discarded as burn-in period after confirming the convergence of chains. Trees were sampled every 1000 generations and a consensus tree was constructed from the results.

Prevalence of parasite infections in mosquitoes was calculated along with confidence intervals by estimating the proportion of infected individuals in the pooled samples using a maximum likelihood approach implemented in the CDC software PooledInfRate (Biggerstaff, 2006). This method determines the most likely infection rate given the testing results and an assumed binomial distribution of infected individuals in a positive pool.

3. Results

3.1. Mosquito sampling and analysis of blood-meals

The results of the blood-meal analyses are summarised in Table 1 and Table 2. A total of 174 blood-fed mosquitoes were collected in 13 coastal sites and 6 highland sites across five islands of the Galápagos Archipelago in 2006 and 2007 (Fig. 1). Successful amplification of PCR products was achieved for 84 of 174 (48.3% of success, Table 1). The remainder failed to amplify after multiple attempts. Of those that yielded PCR products, fifty percent of the mosquitoes had fed on reptile blood: 34.5% on marine iguanas (*A. cristatus*) and 15.5% on Galápagos tortoises (*Geochelone nigra*). The remaining fifty percent of the mosquitoes had fed on mammal blood (25% on human beings, 13.1% on Galápagos sea lions, *Zalophus wollebaeki*, and 11.9% on domestic animals; Table 1). In coastal areas, 71 of 145 blood meals were identified, of which 36.6% were marine iguana, making this the most common host species for mosquitoes in coastal sites (Table 1). From highland sites, 13 of 29 blood-meals were identified, six of which were reptile: three giant tortoises and three marine iguanas (Table 1). No avian blood meals were identified from any site.

A total 1024 *A. taeniorhynchus* mosquitoes were caught in the Bolivar Canal between the 12th and the 15th of January 2008, among which 194 were blood-fed (Fig. 1). No other blood-feeding arthropod species (e.g. mosquito, hippoboscid fly, tick) were caught in our traps. Most of the mosquitoes (719, 147 of them blood-fed) were collected in a site South of Punta Espinoza in Fernandina Island (Site FE4, Fig. 1). Another 96 mosquitoes were caught in Punta Mangle (Site FE3), 24 of which were blood-fed. A total of 209 mosquitoes (23 blood-fed) were collected in the site Caseta in Isabela Island (Site IS10). A total of 106 out of 194 blood-meals were positively identified (54.6% of success, Table

Table 3
Identification of blood-meals from *A. taeniorhynchus* specimens collected in the Bolivar Canal in January 2008.

	Number of mosquito blood-meals collected			
	Fernandina Site FE3	Fernandina Site FE4	Isabela Site IS10	Total
Total tested by PCR	24	147	23	194
Total positively identified	15	73	18	106
% of success	62.5%	50.3%	78.3%	54.6%
Host species identified (common/*latin* name)				
Galapagos sea lion/ *Zalophus wollebaeki*	–	7	–	7 (6.6%)
Marine iguana/ Amblyrhynchus cristatus	15	66	17	98 (92.4%)
Flightless cormorant/ *Phalacrocorax harrisii*	–	–	1	1 (0.9%)

Table 4
Calculation of *A. taeniorhynchus* blood-feeding preferences in three different study sites in the Bolivar Canal in January 2008.

Site	Host species	$u_{i,j}$	$v_{i,j}$	$P_{i,j}$	*p*-value
Fernandina Site FE3	Marine iguanas	15	45	0.99	0.282
	Sea lions	0.5	1	1.48	0.282
Fernandina Site FE4	Marine iguanas	66	86	1.09	0.048[a]
	Sea lions	7	2	4.95	<0.001[a]
	Cormorants	0.5	16	0.04	0
Isabela Site IS10	Marine iguanas	17	12	1.65	<0.001[a]
	Cormorants	1	9	0.13	<0.001[a]

Name codes of sites correspond to codes given in the text and in Fig. 1; $U_{i,j}$, number of mosquitoes with blood from host species *i* in a site *j*; $V_{i,j}$, density of host species *i* in a site *j*; $P_{i,j}$, feeding selection of mosquitoes for a host species *i* in a site *j*. Mosquitoes will show a selection for or against a host if $P_{i,j} > 1$ or $P_{i,j} < 1$, respectively. If $P_{i,j} = 1$, mosquitoes feed on the host species in proportion of its abundance. Multinomial simulations were generated to test if $P_{i,j}$ obtained were different from 1. See Section 2 for details.

[a] Values of $P_{i,j}$ significantly different from 1.

3). The vast majority of the mosquitoes (92.4%) had fed on marine iguanas. Seven mosquitoes caught in site FE4 had fed on Galápagos sea lions and one mosquito from site IS10 in Isabela Island had fed on flightless cormorant.

3.2. Assessment of host feeding patterns

A total of 166 vertebrates were found on the sites of mosquito sampling on the Bolivar Canal (Table 2). The majority (143) were marine iguanas. Sixteen flightless cormorants were counted in site FE4 and nine in site IS10. Two Galápagos sea lions were sighted in site FE4 and another one in site FE3.

Feeding selection indices are shown for the three sites in Table 4. $P_{cormorants,FE4}$ and $P_{cormorants,IS10}$ were significantly lower than 1 (p-value < 0.001), indicating a strong mosquito feeding avoidance of flightless cormorants at both sites FE4 and IS10. These results were robust to variations in animal counts (Fig. 2B, E), as the p-value associated to $P_{cormorants,FE4}$ and $P_{cormorants,IS10}$ remained lower than 0.05 even after a drastic increase in the number of marine iguanas, or decrease in the number of cormorants. At site FE3, where only marine iguanas and sea lions were observed, there were no significant mosquito selection for any of these host species. This result was robust to changes in the number of hosts at site (Fig. 2A). Although $P_{marine\ iguanas,FE4}$ and $P_{sea\ lions,FE4}$ were both significantly higher than 1, suggesting mosquito feeding selection for marine iguanas and sea lions at site FE4, this result was highly sensitive to variations in the number of hosts. Increasing the number of flightless cormorants by one unit, and the number of sea lions by two units increased the p-value associated to $P_{marine\ iguanas,FE4}$ and $P_{sea\ lions,FE4}$ above 0.05, respectively (Fig. 2.C,D). While mosquitoes seemed to avoid feeding on flightless cormorants, they may not have any feeding selection for sea lions and marine iguanas.

3.3. Parasite screening

A total of 872 *A. taeniorhynchus* caught on the Bolivar canal in 2008, comprising 33 thorax pools (20 specimens per pool), and 18 head pools (40 specimens per pool) were screened for the presence of *Hepatozoon* spp., *Haemoproteus* spp./*Plasmodium* spp. and filarial nematodes (Table 5). PCR products positive for *Hepatozoon* spp. were obtained in six pools of mosquito thoraces and three pools of mosquito heads, all from site FE4. One positive thorax pool and one positive head pool originated from the same group of mosquito samples, so the two positives could be coming from the same individual(s). The presence of *Hepatozoon* spp. parasites was also detected in 25 out of 194 blood-meals across the three sites. These 25 blood-meals had been positively identified as marine iguana blood. One PCR product positive for *Haemoproteus* spp./*Plasmodium* spp. and one positive PCR product for filarial nematodes were obtained, each coming from a different pool of mosquito thoraces from site FE4 (Table 5).

The nine PCR products from mosquito pools positive for *Hepatozoon* spp. and eight from the blood-meal samples were sequenced for a 485 bp portion of the 18S rRNA gene. An identical nucleotide sequence (named Hepatozoon_MIG1, GenBank accession ID: JQ080302) was obtained from six products from blood-meals, four from mosquito thoraces, and two from mosquito heads. The two remaining products from marine iguana blood-meals and one from a thorax pool shared another nucleotide sequence (Hepatozoon_MIG2, GenBank accession ID: JQ080303). A third sequence (Hepatozoon_MIG3, GenBank accession ID: JQ080304) was ob-

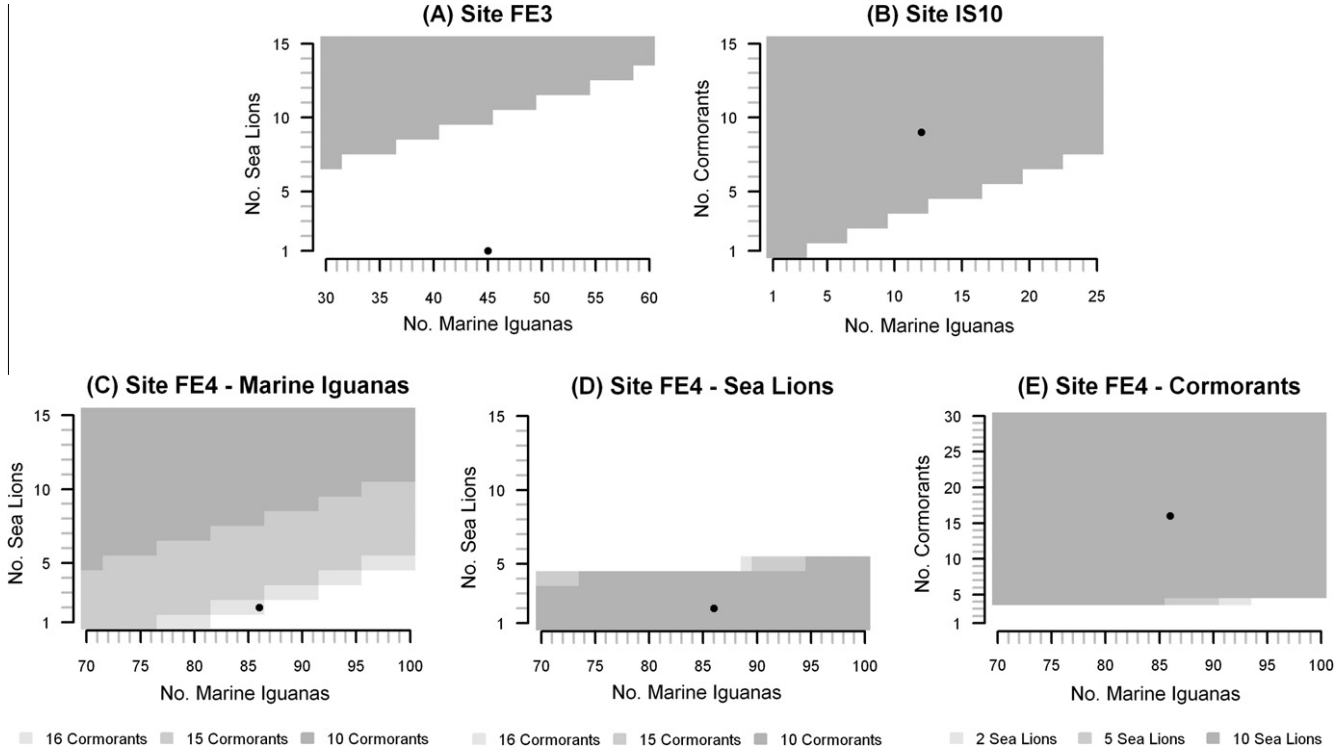

Fig. 2. Mosquito feeding selection as a function of animal host abundance. For all graphs, the number of hosts observed at sites is shown (black dot). The white area shows values of host abundance for which there is not any mosquito selection for or against a type of host (i.e. $P_{i,j}$ are not significantly different from 1). (A) Grey area: Host abundance for which there is a mosquito feeding selection for marine iguanas, and against sea lions, at site FE3 ($P_{marine\ iguanas,FE3}$ and $P_{sea\ lions,FE3}$ significantly higher and lower than 1, respectively). (B) Grey area: feeding selection for marine iguanas and against cormorants at site IS10. (C) Feeding selection for marine iguanas at side FE4, when there are 10 (dark grey), 15 (medium grey) and 16 (light grey) cormorants. (D) Feeding selection for sea lions at site FE4, when there are 10 (dark grey), 15 (medium grey) and 16 (light grey) cormorants. (E) Feeding selection for cormorants at side FE4, when there are 10 (dark grey), 5 (medium grey) and 2 (light grey) sea lions.

Table 5
Number of positive *A. taeniorhynchus* pools (heads and thoraces were tested separately) and positive blood-meals identified for each parasite and each site tested in the Bolivar Canal in January 2008.

	Fernandina Site FE3	Fernandina Site FE4	Isabela Site IS10	Total
Total number of mosquitoes tested by PCR	32	660	180	872
Nb thorax pools tested	2	33	9	44
Parasites identified				
Hepatozoon sp.	–	6	–	6
Haemoproteus sp.	–	1	–	1
Nematode sp.	–	1	–	1
Nb head pools tested	1	18	5	24
Parasites identified				
Hepatozoon sp.	–	3	–	3
Nb blood-meals tested	24	147	23	194
Parasites identified				
Hepatozoon sp.	2	18	5	25

Thoraces of mosquitoes were pooled by maximum 20 specimens; heads were pooled by maximum 40 specimens.

tained from a pool of mosquito heads and a pool of mosquito thoraces. There was no sign of the presence of multiple sequences within the same pool or blood-meal (absence of ambiguous peaks in the sequencing chromatograms), although cloning the PCR products obtained would be necessary to completely rule out mixed infections. The three sequences shared between 94.3% and 95.7% of sequence identity and were similar to published sequences of *Hepatozoon* spp. with 96–98% of similarity in the BLAST search. A Bayesian phylogenetic tree (Fig. 3) based on the three sequences and 18 other *Hepatozoon* sequences obtained from GenBank (accession numbers given in Supplementary Table S1), shows that the three new sequences fit within the *Hepatozoon* cluster with a posterior probability of 0.98, but seem to be derived from two distantly related groups. The most common sequence in the dataset (Hepatozoon_MIG1) clustered with the sequence Hepatozoon_MIG3 with a support of 0.77. The two sequences are included within a group containing *Hepatozoon* spp. infecting reptiles, amphibians and rodents with a posterior probability support of 0.88. The sequence hepatozoon_MIG2 is isolated from the other two sequences and clusters with a group of reptilian *Hepatozoon* species, although with a relatively low posterior probability (0.46). Overall the tree obtained did not offer a good resolution of the phylogenetic relationships within the *Hepatozoon* genus and between the outgroup sequences included in the tree, suggesting longer sequences may be necessary for future studies.

Analysis of the sequence from the filarial nematode PCR product yield a 100% similarity match in BLAST with Nematoda sp. NKW-2006 isolates (GenBank accession ID DQ838571-DQ838574), the filarial nematodes infecting flightless cormorants and Galápagos penguins described by Merkel et al. (2007). The positive match for *Haemoproteus/Plasmodium* spp. was 100% similar to an *Haemoproteus* (*parahaemoproteus*) sequence isolated from blue-footed boobies *Sula nebouxii* (GenBank accession ID JF833060) described in Levin et al. (2011).

Infection rate of *Hepatozoon* parasites in mosquitoes caught at site FE4 was 0.98 infected individuals per 100 tested (95% CI: 0.66–2.71) in thoraces, 0.48 (CI: 0.13–1.33) in heads and 1.41 (CI: 0.66–2.05) when results from both mosquito parts were grouped. *Hepatozoon* parasites were also recovered from marine iguana blood identified in mosquito blood-meals (Table 5). Assuming that each marine iguana has the same (and constant) probability of being a host, the infection rate of *Hepatozoon* parasites in marine iguanas can be estimated to be 29.4 infected individuals per 100 tested (CI: 11.9–53.3) in the site IS10, 13.3 (CI: 2.5–36.9) in the site FE3 and 27.3 (CI: 17.6–38.9) in the site FE4. Infection rate of *Hepatozoon* parasites in marine iguanas was not significantly different between sites. Infection rate of *Haemoproteus* and microfilariae in mosquitoes at site FE4 was of 0.15 infected individuals per 100 tested (CI: 0.01–0.74) for each parasite.

4. Discussion

4.1. Blood-feeding patterns of A. taeniorhynchus

A. taeniorhynchus feeds principally on large mammals and occasionally on birds in the continent (O'Meara and Edman, 1975; Anderson and Fortner, 1988). Our results show that, in the Galápagos Islands, half of the engorged *A. taeniorhynchus* that yield PCR products had fed on reptiles, supporting the previous results obtained in Bataille et al. (2009a). Reptile blood-meals were found across all the islands sampled and across most environments, including a site in Espanola Island where birds were more abundant than reptiles, suggesting that feeding on reptile blood is a wide-spread behaviour of *A. taeniorhynchus* in the Galápagos Islands. Estimation of blood-feeding patterns in the Bolivar Canal shows that *A. taeniorhynchus* select reptile and mammal blood over avian blood. However, it is not clear if mosquitoes select to feed on reptiles or mammals. It is possible that the selection between these two hosts is more opportunistic. The power of this analysis was limited by the small mosquito sample sizes and potential caveats regarding representativeness of the vertebrate counts. Animals were counted only once before setting the traps and it is possible that these numbers changed before the end of the trapping period. This is especially the case for sea lions that are much more mobile than the other animals sighted. On the other hand, the gregarious behaviour of marine iguanas and the sedentary lifestyle of flightless cormorants render the counts made more reliable. Moreover, the sensitivity analysis showed that some results were robust to changes in host abundance, and especially the strong mosquito selection not to feed on cormorants.

The bird "avoidance" observed in our results of *A. taeniorhynchus* is surprising, considering that this mosquito has been observed harassing nesting birds in seabird colonies to a point that they sometimes abandon their eggs (Anderson and Fortner, 1988). Moreover, results from the parasite screening identified mosquitoes infected by bird parasites (*Haemoproteus* sp. and microfilariae), suggesting that they do feed on birds. A possible explanation for this contradiction is that *A. taeniorhynchus* is indeed an opportunistic feeder, more successfully feeding on hosts with lowest capacity of mosquito avoidance. Birds are usually more mobile than reptile, and therefore may be more successful at avoiding swarms of biting mosquitoes. Birds could be more

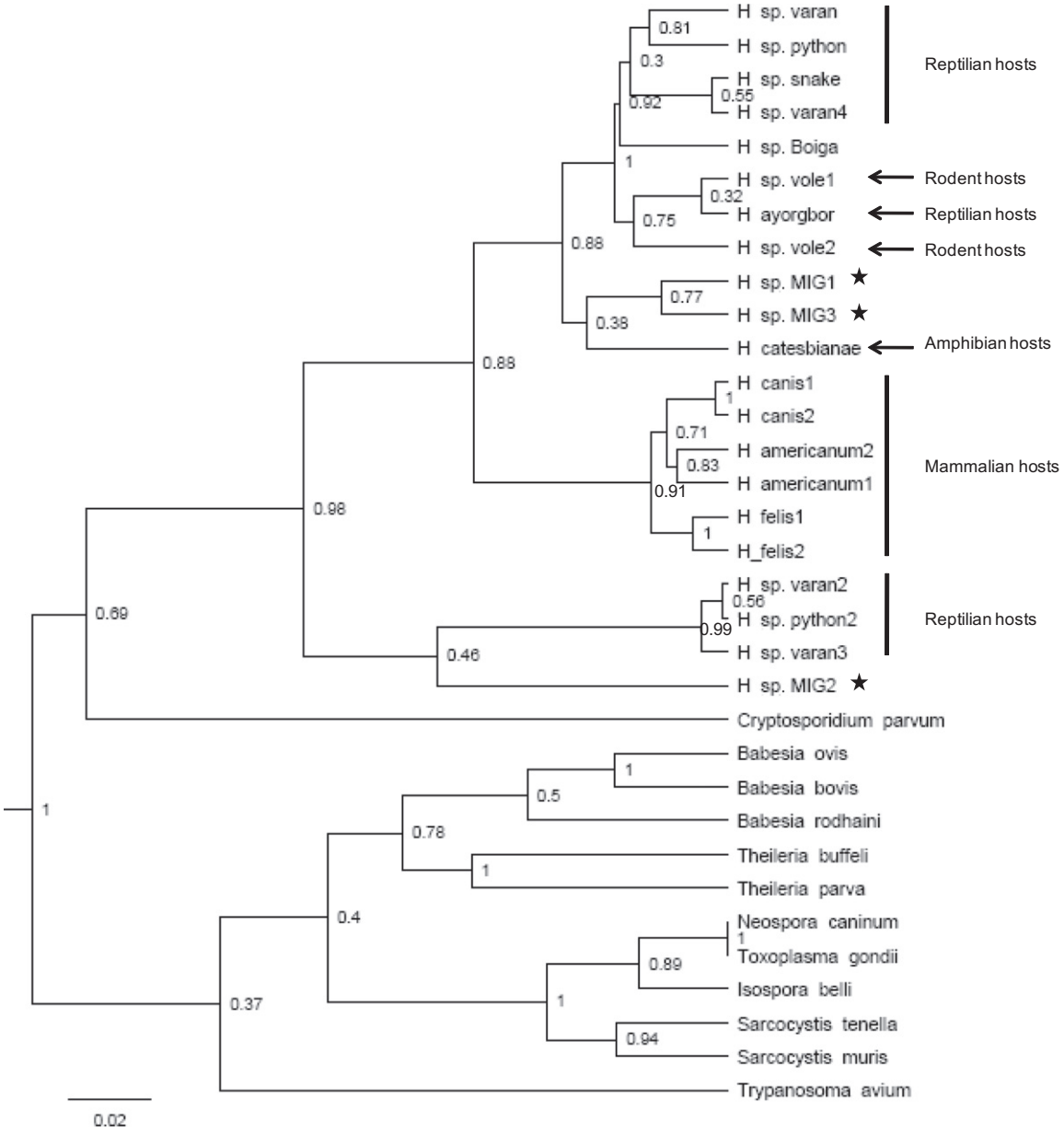

Fig. 3. Phylogenetic relationships between *Hepatozoon* parasites identified in Galapagos mosquitoes and other *Hepatozoon* spp. based on DNA sequence variation of the 18S rRNA gene. The tree was constructed with an alignment of 485 bp using a Bayesian inference approach implemented in MrBayes. Numbers beside branches indicate supports for the nodes of the trees from posterior probability. New sequences obtained from mosquito pools and blood-meals are indicated with a star. The type of vertebrate host infected by each species of *Hepatozoon* is indicated. Accession numbers for sequences of the parasites included in the phylogenetic analysis are indicated in Supplementary Table S1.

vulnerable to mosquitoes during the breeding season when adults and/or juveniles have to remain in the nest. It is worth noting that the only mosquito found to have fed on cormorant blood was trapped at a site where a juvenile cormorant was observed in a nest. However, the type of trap used in this study should be especially efficient at collecting hosts with strong anti-mosquito avoidance. The anti-mosquito behaviour hypothesis could be investigated further by sampling mosquitoes in the immediate vicinity of birds during the breeding season (such as at seabird nesting colonies) and again out of the breeding season (at the same seabird colonies) to determine if the frequency of mosquitoes feeding on birds increases during the nesting season.

Only half of the blood-meals collected could be identified using a PCR method. This low success rate may have been due to DNA degradation, notably because many mosquitoes were found dead at the time of collection. The level of blood digestion could also influence the success of the PCR, although it has been shown that blood-meals can still be identified 3 days after feeding (Ngo and Kramer, 2003). It is also possible that the primers used failed to amplify certain type of hosts, although preliminary tests showed that these primers could successfully amplify DNA from all class of vertebrate. Another potential source of bias in our results is the exclusive use of CO_2-light trap for the collection of blood-fed mosquitoes. These traps typically attract host-seeking (therefore, only with partial blood meals) mosquitoes, so the results obtained may be biased towards hosts with stronger anti-mosquito behaviour (Thiemann and Reisen, 2012). However, we have collected *A. taeniorhynchus* individuals with what appeared to be complete blood meals (fully red and highly extended abdomen) in our traps over 3 years of field work in Galápagos, suggesting that CO_2-light

traps may be used successfully to collect engorged *A. taeniorhynchus* seeking a resting place. Other studies have reported the same success with other mosquito species (e.g. Pappa et al., 2011). However, trap bias in our blood-meal results cannot be totally ruled out, and further studies using multiple types of traps should be carried out to confirm the results obtained in this study.

4.2. Role of A. taeniorhynchus in parasite transmission

Results obtained in this study represent a first exploration of the potential involvement of *A. taeniorhynchus* in host–parasite interactions in Galápagos. We detected the presence of *Haemoproteus* parasites and filarial worms in the thorax of mosquitoes. We also identified three different DNA sequences matching with *Hepatozoon* parasites, among which one was distantly related to the other two, suggesting that more than one *Hepatozoon* parasites are present in Galápagos Islands. Further morphological and molecular studies should be carried out to provide a detailed description of this parasite. These results do not provide direct evidence for the role of *A. taeniorhynchus* as a vector of these parasites; however, this study warrants additional experiments to provide more definitive evidence. Detection of the parasites in salivary glands (for *Hepatozoon* and *Haemoproteus* parasites) or in the proboscis of mosquitoes (for filarial nematodes), and laboratory transmission experiments would be necessary to resolve this question.

The transmission of *Hepatozoon* spp. to the vertebrate host typically occurs via ingestion of the infected invertebrate host (Smith, 1996), but the presence of infective stages of an *Hepatozoon* species in the proboscis of infected mosquitoes has been reported before, suggesting the possible direct transmission of the parasite by mosquito bite to vertebrate hosts (Telford et al., 2001). However, ticks are the most probable major vector of *Hepatozoon* in Galápagos reptiles, as those are infested by ticks (Gadsen and Guerra, 1991) which are known vectors of *Hepatozoon* parasites (Telford et al., 2001). Mosquitoes could represent a dead-end host in the *Hepatozoon* transmission cycle. However, sporadic accidental or intentional ingestion of infected mosquitoes by Galápagos reptiles cannot be totally ruled out, so these mosquitoes could still play a substantial role in *Hepatozoon* host–parasite transmission dynamics, even if they are not able to transmit it by bite. Careful observations in the field would be necessary to determine if Galápagos reptiles feed on mosquitoes.

In the case of *Haemoproteus* parasites in Galápagos, hippoboscid flies are currently considered the most likely vector for the *Haemoproteus* haemoproteus subgenus infecting doves and seabirds (Levin et al., 2011). However, vector relationships with *Haemoproteus* (parahaemoproteus) parasites identified here is unknown and our work and the work of Ishtiaq et al. (2008) on avian blood parasites in New Caledonia suggest that a role of mosquitoes in *Haemoproteus* transmission should not be totally ruled out. Finally, the important role of *A. taeniorhynchus* in the transmission of the nematode *Dirofilaria immitis* on the American continent (Labarthe et al., 1998; Manrique-Saide et al., 2008) suggests that this mosquito has the capacity to transmit the Galápagos microfilariae, although we failed to identify microfilaria DNA in mosquito proboscis. Both the very low infection rate (0.15 infected individuals per 100 tested) of filarial infection found in *A. taeniorhynchus* and the apparent avoidance of this mosquito to feed on birds indicate that *A. taeniorhynchus* might not be an important vector of this parasite. However, such low filarial infection rate seems to be quite common in mosquitoes, as many species that are vectors of *D. immitis* in USA show infection rates lower than estimated here (Manrique-Saide et al., 2008).

Data on the abundance of other arthropod vectors such as ticks and hippoboscid flies in our sampling sites would be necessary to better evaluate the potential role of *A. taeniorhynchus* in parasite transmission. We did not observe any hippoboscid fly in our traps and ticks are not attracted by the type of traps we were using in this study. It would have been necessary to catch the animals and inspect their skin for presence of ticks and hippoboscid flies to totally rule out the presence of other vector species on our sampling sites. This method is very invasive, which is something we wanted to avoid to obtain more reliable vertebrate counting for our blood-feeding selection analysis. Also, our field work took place in an area devoted of introduced mosquito species (*Culex quinquefasciatus* and *Aedes aegypti*), and it is possible that the role of *A. taeniorhynchus* in parasite transmission in areas where competition between mosquito species occurs will be altered.

4.3. Alterations of pre-existing host–parasite interactions

The arrival time of the *A. taeniorhynchus* in Galápagos (around 200,000 years ago) generates two main scenarios for the effect of its arrival on Galápagos host–vector borne parasite systems. Firstly, some vector-borne pathogens (capable of transmission by mosquitoes) were already present in Galápagos prior to arrival of the mosquito, but were maintained by other vectors, and the mosquito acquired them after its arrival - this scenario would potentially provoke changes in the pathogen's distribution and host range across the archipelago. Second, some pathogens were introduced to the archipelago with, or subsequent to arrival of the mosquito, and the presence of the mosquito allowed their establishment in the islands. Although human mediated, an analogous situation would be the establishment of avian malaria in Hawaii following introduction of *C. quinquefasciatus* (Warner, 1968; van Riper et al., 1986). In both scenarios the evolutionary history of such pathogens in Galápagos would be linked with that of *A. taeniorhynchus* (and other potential vectors). Comprehensive population genetic or phylogenetic analysis of mosquito-borne pathogens in Galápagos could shed light on the impact of mosquito arrival on disease dynamics in the archipelago.

Our *Hepatozoon* phylogenetic tree shows that the most common *Hepatozoon* sequence isolated in our study is distantly related to the other two sequences, suggesting that the corresponding parasite species may have derived from a separate colonisation event by a distinct vertebrate and/or invertebrate host. The two remaining sequences may represent two closely related parasite species that would have diverged from a common ancestor in another host–vector system. The presence of these three *Hepatozoon* species in a same area suggests that all three are now circulating simultaneously in the marine iguana and mosquito populations. *A. taeniorhynchus* may have broken down host boundaries of *Hepatzoon* species formerly isolated in different reptilian host species, to give a mixed *Hepatozoon* population in marine iguanas, and possibly other Galápagos reptiles given *A. taeniorhynchus* also feeds on giant tortoises (Bataille et al. 2009a). Although this hypothesis is speculative at this time, our results suggest that further work on the *Hepatozoon*-vector-reptile system could shed light on the effect of *A. taeniorhynchus* arrival on pre-existing host–parasite interactions in Galápagos, and may be a useful natural model system in this regard.

Emerging infectious diseases with wildlife host reservoirs have been highlighted as threats to human health, the global economy and biodiversity (Daszak et al., 2000; Jones et al., 2008). Wildlife endemic to isolated oceanic islands is particularly susceptible to the introduction of infectious diseases (van Riper et al., 1986; Wyatt et al., 2008). Our results highlight the importance of deter-

mining host range and feeding patterns of disease vectors to understand pathogen transmission dynamics. This knowledge is critical to assess the epidemiology of emerging vector-borne diseases and to develop efficient mitigation measures to protect human and wildlife health.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2012.07.019.

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