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Avian Malaria is Absent in Juvenile Colonial Herons (*Ardeidae)* but not *Culex pipiens* Mosquitoes in the Camargue, Southern France

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Abstract.-- Apicomplexan blood parasites *Plasmodium* and *Haemoproteus* (together termed "Avian malaria") *and Leucocytozoon* are widespread, diverse vector-transmitted blood parasites of birds, and conditions associated with colonial nesting in herons (*Ardeidae*) and other watering birds appear perfect for their transmission. Despite studies in other locations reporting high prevalence of parasites in juvenile herons, juvenile Little Egrets (*Egretta garzetta)* previously tested in the Camargue, Southern France, had a total absence of malaria parasites. This study tested the hypotheses that this absence was due to insufficient sensitivity of the tests of infection; an absence of infective vectors; or testing birds too early in their lives. Blood was sampled from juveniles of four species shortly before fledging: Little Egret (n = 40), Cattle Egret (*Bubulcus ibis*; n = 40), Black-crowned Night Heron (*Nycticorax nycticorax*; n = 40), and Squacco Heron (*Ardeola ralloides;* n = 40). Sensitive nested-PCR was used to test for the presence of parasites in both birds and host-seeking female mosquitoes captured around the colonies. No malaria infection was found of in any of the heron species. Four different lineages of *Plasmodium* were detected in pooled samples of female *Culex pipiens* mosquitoes, including two in potentially infective mosquitoes. These results confirm that the absence of malaria parasites previously demonstrated in Little Egret is not due to methodological limitations. Although the prevalence of infection in mosquitoes was low, conditions within the colonies were suitable for transmission of *Plasmodium*. These colonial heron species may have evolved strategies for resisting malaria infection through physiological or behavioural mechanisms.

Key words.-- avian malaria, *Culex pipiens*, colonial birds, herons, Plasmodium, *Plasmodium relictum*

Running Head.-- AVIAN MALARIA IN CAMARGUE HERONRIES

Many species of waterbirds breed colonially, a strategy that is expected to favour transmission of parasites, particularly since juvenile birds are often relatively sessile, immunologically naïve and naked (Baker 1975). Several species of *Ciconiiforme* birds, including *Ardeidae* (bitterns, herons and allies) nest in large mixed colonies, and some evidence suggests this can result in high parasite transmission (Telford *et al.* 1992; McKilligan 1996) including transmission of *Plasmodium* (Gabaldon and Ullua 1980). *Plasmodium* and *Haemoproteus* parasites of birds, the two genera of haematozoans generally termed avian malaria (Perez-Tris *et al.* 2005), are a widespread and diverse group of pathogens (Bensch *et al.* 2009). Despite increasing research, relatively little is known about the dynamics of avian malaria in wild host/vector transmission networks, particularly from non-passerine bird species (e.g. European *Ardeid* birds) and mosquitoes (Clarke *et al.* 2014). Avian malaria is transmitted to the avian host by arthropod vectors: *Plasmodium* by mosquitoes (*Culicidae)* and *Haemoproteus* by biting midges (*Ceratopogonidae;* Valkiūnas 2004). Although host immunity can explain some patterns of range size of avian malaria parasites (Medeiros *et al.* 2013), the prevalence of malaria parasites will be largely determined by frequency of infected vectors encountering hosts and is expected to vary spatiotemporally, with greater prevalence in places or times with greater vector activity (Childs *et al.* 2006). In the Camargue, the Rhone river delta in Southern France, *Ardeid* birds form large breeding colonies, with nest density up to 2.2 nests/m^2 (Tourenq *et al.* 2001). Typically these mixed colonies consist of Little (*Egretta garzetta*) and Cattle (*Bubulcus ibis)* egrets, Black-crowned Night-Heron (*Nycticorax nycticorax)* and Squacco Heron (*Ardeola ralloides)* (hereafter, herons; Hafner 1980). The Camargue is also notable for having a high diversity and abundance of mosquitoes and other vector species, including *Culex pipiens* (Ponçon *et al.* 2007; Balenghien *et al.* 2009) the putative vector for many *Plasmodium* infections

in European birds (Santiago-Alcaron *et al.* 2012). Many of these vector species (including *Culex pipiens*) are notably active in and around heron colonies and other aggregations of birds (e.g. henhouses and duck farms; Rageau and Mouchet 1967). It thus seems likely that conditions in these breeding colonies would encourage a high prevalence of malaria, however, two previous studies failed to detect haematozoan infections in nestling Little Egrets in this region (Ashford *et al.* 1994; Tourenq *et al.* 2001) despite juvenile herons species being infected in other locations (Telford *et al.* 1992) . Indeed, in Venezuela it has previously been suggested that almost all juvenile *Ciconiiforme* birds are infected with blood parasites (Gabaldon and Ullua 1980). Tourenq *et al.* (2001) sampled blood from Camargue Little Egret chicks between 6 and 21 days old, and examined blood smears before concluding that these chicks were not infected with avian malaria. It was suggested that this absence of infection could be related to the low age of the egrets, the absence of vectors, or a very low prevalence of malaria, or a low parasitaemia that was undetectable through slide examination.

In this study we sampled blood from juvenile birds of a range of colonial heron species in the Camargue. By testing blood samples from Little and Cattle egrets, Black-crowned Night-Herons, and Squacco Herons we hoped to assess whether the scarcity of infection in previous Camargue studies was specific to Little Egrets, or reflected an absence of infection in juvenile colonial herons in general. We only sampled chicks that were very close to fledging. The prepatent period of *Plasmodium* infection (the phase of infection when the parasite has invaded the body, but is not yet present in blood) can vary considerably (Valkiūnas 2004). Precise data is unavailable from Ardeids, but in experimentally infected passerine birds the prepatent phase of *Plasmodium relictum* and *Plasmodium ashfordi* differed, and depended on the host species, with a maximum length of 30 days (Palinauskas *et al.* 2011). This information is not available for *Ardeidae,*

though *Plasmodium relictum* has been documented in the blood of great blue heron juveniles, including nestlings < 20 days old in Florida, indicating a prepatent time less than 20 days (Telford *et al.* 1992). By sampling older chicks (> 30 days old) we wished to maximise the chances of finding infections that had progressed beyond the prepatent period, as has been demonstrated in other studies of similar species (Gabaldon and Ullua 1980). Importantly, we also used mosquito traps to collect host-seeking female mosquitoes in the vicinity of heron colonies. This allowed us to assess the abundance of potential vectors in these colonies and to test whether mosquitoes in this area are infected or infective with *Plasmodium* species. Finally, it has been shown that nested Polymerase Chain Reaction (PCR) techniques are more likely to detect avian malaria infection than microscopy (Waldenström *et al.* 2009), especially when parasitaemia is very low (as may be the case immediately following the prepatent period of development; Valkiūnas 2004). We used a nested PCR protocol to screen blood samples from birds and pools of female mosquitoes for infection with avian malaria, and sequenced any amplified parasite DNA to address the following specific questions: are juvenile colonial herons of any species infected with avian malaria in the Camargue?; Which mosquitoes species are active around heron colonies during the breeding season?; Are mosquitoes infected/infective with avian malaria in Camargue heron colonies?; Is *Plasmodium* prevalence found in potential vectors associated with the prevalence observed in juvenile herons?

METHODS

Study Area

This study was conducted from April - August 2010 in the Camargue, France. The Camargue is the main wetland area in the southeast of France and covers the Rhone River Delta (see Ponçon

et al. (2007)). It is considered one of the most important wintering and breeding grounds for waterbirds in Europe and the wetland region comprises natural habitats, salinas, agricultural lands including rice fields, and urbanized-industrial zones (Tourenq *et al.* 2001).The Camargue has a Mediterranean climate characterized by warm, dry summers and mild, wet winters. Total annual rainfall is typically 500–700 mm and occurs mainly in autumn; the annual mean temperature is 14 °C. From May (after mosquito emergence) we trapped mosquitoes at six historical heron colonies in the Camargue: Etang de Scamandre (43° 36' 26.25" N, 4° 20' 42.49" E), Etang de Redon (43° 28' 16.31" N, 4° 39' 9.75" E), Musette (43° 36' 19.51" N, 4° 13' 54.23" E), Tyrasses (43° 29' 33.69" N, 4° 38' 43.33" E), La Palissade (43° 22' 5.54" N, 4° 49' 20.09" E) and Mas d'Agon (43° 35' 28.03" N, 4° 31' 51.57" E). The Palissade colony was situated in a White Poplar (*Populus alba*) wood on an island in the Rhone River in the southeast part of the delta, located by the marshes of the Domaine de la Palissade reserve between the industrial complex of Fos-sur-Mer and the saltpans of Salin de Giraud. The Colonies at Etang de Redon, Mas d'Agon, and Tyrasses were located in the central part of the delta on Tamarisk (*Tamarix gallica*), and the colonies at Scamandre and Musette were located in the west of the Camargue and were on Tamarisk and mixed Narrow-leaved Ash (*Fraxinus exelsior*)-White Poplar forest. All were surrounded by a mosaic of rice fields, marshes, and salted dry land. In some areas of the Camargue, mosquitoes have been controlled, principally by the spraying of insecticides, since the 1960s. However, to preserve biodiversity, parts of the important local wetlands have been protected from spraying (Poulin 2012). In our study, Etang de Scamandre, Musette and Palissade were located in mosquito-controlled regions. Etang de Redon, Mas d'Agon and Tyrasses were located in the unsprayed region.

Mosquito Collection

We collected mosquitoes from May until July 2010 at each location to coincide with the heron nesting phase. The mosquitoes were trapped using four Centers for Disease Control (CDC) miniature light traps (John W Hock Company). The traps were baited using yeast-generated CO_2 (Saitoh *et al.* 2004). The CO_2 released at the trap mimics the respiration of host animals, and thus these traps are used to capture host-seeking individuals (those that, if infected, are responsible for transmission of disease). We prepared the yeast-sugar mixture in the afternoon at least 4 hours before setting up the traps. The mixture was placed in a 5 litre sealed plastic can. Plastic hosing was attached from the can containing the mixture with a small exit hole close to the CDC light trap entrance. The traps were equipped with a detector, that started the operation of the traps after dusk, and all traps were recovered shortly after dawn the following day.

In addition to investigating the mosquito species that were active around the heron colonies, we wished to compare the mosquito assemblage in insecticide sprayed, and unsprayed regions. Since we had four traps in total, we employed two at a specific site in the mosquito-controlled region, and two at a specific site in the unsprayed region on each night that mosquitoes were trapped. The contents of the two traps were pooled. However, some heron colonies failed (birds deserted the colony prior to breeding), and others became inaccessible during the breeding season. For this reason we focussed our efforts on the two major colonies: Scamandre and Redon, where we were sure to be able to capture juvenile herons. The traps were hung 1.5 m from the ground (the approximate height of the vegetation, and of heron nests) and were always located within 100 m of the heron colonies, though they were not placed directly in the colonies themselves. This was principally to avoid disturbing the birds regularly and risking nest desertion, and since evidence from heronries in other locations has also shown that mosquito traps can be ineffective when located immediately beside heron nests, presumably as birds are

more attractive than the light on the trap (Thiemann *et al.* 2011) However, evidence from the Camargue and other locations have shown that herons and their nests are very attractive to host-seeking mosquitoes (Rageau and Mouchet 1967; Burkett-Cadena *et al.* 2011). The traps were deployed in different locations at each site on different trapping nights to avoid capture biases restricted to single locations. The Camargue is characterised by windy conditions, and we found that our traps were completely unsuccessful on windy nights. To avoid having zeros in our dataset these nights were discarded from the analysis and traps were only hung on calm nights. In total this left at least two successful trapping nights for every location during the period of chick growth and development, and 8 trapping nights at Etang Redon and Etang de Scamandre for the duration of heron breeding. Traps were collected from each site and collection pots were stored in thermally isolated plastic chiller boxes at 4 °C until the mosquitoes were killed in the laboratory in a -20 °C freezer. Following this freezing, mosquitoes were quickly separated from other insects in the collection jars, and identified following morphological keys (Becker 2011). Since we were interested primarily in potential vectors of avian malaria, we chose only to perform subsequent molecular analyses on *Culex pipiens* (for other species encountered see Table 2). Other studies have shown that *Culex pipiens* is the main vector of *Plasmodium* in European study sites with a similar mosquito assemblage (Glaizot *et al.* 2012) and we have previously found no evidence of infections in any other mosquito species from the Camargue (S. D. Larcombe, unpubl. data). Following identification, all *Culex pipiens* females were dissected into abdomen and thorax, using two sterile needles under a dissection microscope. Detection of parasite DNA in mosquito thoraxes is suggested to reflect infective individuals (the presence of sporozoites in salivary gland) while positive amplification from abdomens reflects infected individuals; oocysts on the mid-gut wall (Kim *et al.* 2009). For each site and each trapping

night, we made pools of five abdomens or five thoraxes. The needles were changed between each mosquito to avoid potential contamination. All of the mosquitoes were inspected for signs of blood meals, to ensure that the samples contained only unfed individuals. Thus any parasite DNA detected in the mosquitoes did not reflect infected blood from a previous blood meal. Female mosquitoes generally require one complete blood meal for egg production, after which no further blood is required. After a blood meal females no longer seek new hosts, but rest during egg formation, prior to seeking a suitable site for egg deposition. For this reason CO_2 - baited CDC traps do not generally trap blood-fed individuals and female mosquitoes caught in these traps may be considered "host-seeking". Occasionally, blood fed females will enter light traps, for example after incomplete blood meals. However, these are easily detectable under microscopy. In the absence of signs of blood in the abdomen, it is therefore highly unlikely that any parasite DNA detected is from undigested blood. Each pool was frozen at -80 °C before subsequent DNA extraction and molecular analyses. In total we analysed 102 pools, representing 521 individual *Culex pipiens* females.

Bird Captures

We captured 20 juveniles of each species at each site; both Redon and Scamandre resulting in a total of 40 juveniles for each species. The species caught were Little Egret, Cattle Egret, Black-crowned Night Heron, and Squacco Heron. We wished to capture nestlings immediately prior to fledging, to maximise the chances of infection being detectable. The exact age of the chicks was unknown, and fledging age does vary between species, but by sampling birds that were old enough to have left the nest and stretch their wings, but not to fly away, we hoped to sample only birds of > 30 days old. Since chicks of each species hatched at different times in each colony, we made several trips to each colony to ensure only older birds were

sampled. A small volume of blood (< 50 µl) was taken from the brachial vein of each bird and was immediately stored in ethanol for subsequent DNA analysis.

DNA Extraction and Molecular Identification of Haemosporidians

Mosquito Samples.--Each pooled sample was removed from the freezer and allowed to equilibrate to room temperature. We used Qiagen DNeasy tissue kits to extract DNA following the modifications previously used for mosquitoes (Plichart *et al.* 2006), with one exception: prior to commencement of DNA extraction we homogenized each pooled sample in 200 µl of PBS for 30 s using a Qiagen Tissuelyser. The extracted DNA samples were then used for subsequent nested PCR reactions. For the mosquito samples we used the a nested PCR to identify plasmodium/haemoproteus infections (Waldenström *et al.* 2009). We did not test for leucocytozoon infections in mosquito samples. Both reactions were performed in 25 µl volumes. The first reaction contained 2 µl of genomic DNA, 0.2 µM of each primer (HaemNF [5'-CATATATTAAGAGAATTATGGAG-3'] and HaemNR2 [5'-AGAGGTGTAGCATATCTATCTAC-3']) and 12.5 µl BiomixRed PCR mastermix. The second reaction contained 1 µl of product from reaction 1 with 0.2 µM of each primer (HaemF [5'-ATGGTGCTTTCGATATATGCATG-3'] and HaemR2 [5'-GCATTATCTGGATGTGATAATGGT-3']) and 12.5 µl BiomixRed PCR mastermix.

Avian Samples.--In the laboratory we thoroughly dried a small fragment of the ethanol preserved blood before extracting the DNA using DNeasy blood and tissue kits (Qiagen). We encountered some problems with clotted and dried blood failing to lyse properly in the initial step of DNA extraction, so each dried blood pellet was homogenized for 30 s in 200 µl of PBS using a Tissuelyser (Qiagen) prior to extraction. The DNA was then extracted according to the manufacturer's guidelines. For each extracted DNA sample we used a nested PCR approach

(Hellgren *et al.* 2004) to identify *Plasmodium/Haemoproteus* or *Leucoytozoon* infections. Both reactions were performed in 25 µl volumes. The first reaction contained 2 µl of genomic DNA, 0.2 µM of each primer (HaemNF1 [5'-CATATATTAAGAGAAZTATGGAG-3'] and HaemNR3 [5'-ATAGAAAGATAAGAAATACCATTC-3']) and 12.5 µl BiomixRed PCR mastermix.The second reactions contained 1µl of product from reaction 1 with 0.2 µM of each primer (HaemF [5'-ATGGTGCTTTCGATATATGCATG-3'] and HaemR2 [5'-GCATTATCTGGATGTGATAATGGT-3'] or HaemR2L [5'-CATTATCTGGA TGAGATAATGGZG-3'] and HaemFL [5'-ATGGTGTTTTAGATACTTACATT-3']) and 12.5 µl BiomixRed PCR mastermix.

All PCR runs contained several known positive controls (from sparrows or *Culex pipiens*) and negative controls (water). Infections were identified by the presence or absence of bands following the PCR, and positive controls successfully amplified 100% of the time. To check that absence of bands was definitely a lack of infection, we used another PCR with universal primers to test that the DNA in each sample successfully amplified (Cytb-2RC and Cytb-Wow; Dumbacher *et al.* 2003), and any negative samples for malaria infection were repeated twice to confirm the diagnosis.

All positive samples were sequenced with reverse primer HaemR2 (LGC Genomics, Berlin, Germany), and sequences were then edited using Genious software (Kearse *et al.* 2012). All edited sequences were compared to known avian malaria sequences in GENbank and the MalAVi database (Bensch *et al.* 2009).

Statistical Analysis

In order to investigate factors affecting the abundance of different mosquito species we analysed mosquito count data using a generalized linear mixed model (GLMM) with a Poisson distribution (SAS Institute, Inc. 2005). We included site of capture and night of capture as random factors in the model to control for non-independence of mosquitoes caught at a particular location and on a particular day respectively. Since we wished to determine if mosquito control programmes in the Camargue impact abundance at heron colonies we also included insecticide treatment as a fixed factor in each model.

RESULTS

Avian Malaria in Heron Blood Samples

None of the blood samples for Little Egrets (n = 40), Black-crowned Night Herons (n = 40), Cattle Egrets (n = 40) or Squacco Herons (n = 40) tested positive for *Plasmodium*, *Haemoproteus* or *Leucocytozoon* infection. However, positive controls indicated the PCR worked as intended.

Mosquito Diversity and Malaria Prevalence in Mosquitoes

In our pooled *Culex pipiens* samples we found only five pools that tested positive for infection (from a possible 102 pools, representing 521 individual mosquitoes). We estimated prevalence (± SE) using Epitools to be 0.01 ± 0.004 (Sergeant 2009). We compared the sequences of these infections to reference sequences on Genbank and the MalAvi database and found all were different lineages of *Plasmodium*, and all have previously been described in mosquitoes or avian hosts (Table 1). Most of these lineages have been found in a variety of avian host species, suggesting they are host generalists. Two of these lineages were found in thorax

277 only, while the others were found in abdomens only. We caught a total of nine different
278 mosquito species near the heron colonies. The most abundant species was *Culex pipiens,*
279 followed by *Ochlerotatus caspius* (Table 2).
280 Night of capture was the most important determinant of the number of mosquitoes
281 captured (GLMM covariance parameter estimates: intercept = 1.90 ± 0.77; site = 1.21 ± 0.93;
282 day = 1.60 ± 0.65), probably because the strength of the wind in the Camargue is highly variable
283 and the lack of wind cover in this flat landscape means even minor fluctuation in wind speed
284 may affect the number of circulating mosquitoes on different nights. Furthermore, since we
285 trapped at 2 sites on any given night, insecticide -spraying treatment was confounded by site, and
286 we lacked a large enough sample size from each location to disentangle site differences and
287 insecticide treatment differences (GLMM: insecticide treatment $F = 0.17$, $P = 0.7$). The exact
288 timing of any larvicidal spraying in the study period was unknown to us, but spraying
289 programmes have been ongoing for decades, with lasting impacts on mosquito populations. To
290 separate these possibly confounding effects, ideally mosquitoes would have been trapped at
291 every site each trapping night. Analysing data from Scamandre and Redon, the two largest sites
292 for which we had maximum coverage showed that Etang Redon, in the non-sprayed region, had
293 significantly more mosquitoes and more *Culex pipiens,* than Etang Scamandre in the insecticide
294 sprayed region (GLMM total mosquitoes: intercept = 2.83 ± 0.28; insecticide-treatment df = 24,
295 $F = 45.27$, $P < 0.0001$; Night of capture covariance parameter estimate = 1.17 ± 0.47. GLMM
296 total *Culex pipiens*: intercept = 2.08 ± 0.35; insecticide-treatment df = 24, $F = 12.53$, $P < 0.01$;
297 Night of capture covariance parameter estimate 1.17 ± 0.47).
298
299 DISCUSSION

We used sensitive nested PCR protocols to test for avian malaria or *Leucocytozoon* infection in blood samples of juvenile Little and Cattle egrets, Black-crowned Night Herons, and Squacco Herons. We found no evidence for infection with *Plasmodium, Haemoproteus* or *Leucocytozoon* in any of the four heron species tested. We can rule out the possibility that absence of infection in nestling herons previously reported in the Camargue was due to the inability to detect low numbers of the parasite in the blood using microscopy methods (Ashford *et al.* 1994; Tourenq *et al.* 2001). Some studies have identified avian malaria in members of the *Ardeidae* in North America (Telford *et al.* 1992), South America (Durrant *et al.* 2006) and Asia (Silva-Iturriza *et al.* 2012), though sample sizes were generally too small to conclude whether malaria was prevalent or not in these birds. However, a large study on a range of *Ciconiiforme* birds in Venezuala suggested that malaria infection was holendemic in juveniles (Gabaldon and Ullua 1980). Data on avian malaria of heron species in Europe is mostly limited to the absence of malaria in Camargue herons, though other haematozoa (*Leucocytozoon*) have been detected infecting up to 74% of the sampled chicks in some Italian heronries (Prigioni and Sacchi 1993). We sampled birds when they were likely to be > 30 days old, to maximise the chances of detecting infections beyond the prepatent phase. There is a considerable degree of variation in the length of prepatent phase for different genera and species of haematozoans (Valkiūnas 2004) and this may depend on host species (Palinauskas *et al.* 2011). *Plasmodium relictum* and *Plasmodium vaughani* (lineages SGS1 and SYAT05 in this study) have previously been shown to have prepatent phases between 0-6 and 8-10 days respectively in experimental infections of passerines (Iezhova *et al.* 2005; Palinauskas *et al.* 2011) though without knowing which (if any) parasites infect these herons it is impossible to rule out the possibility that prepatent infections were present. Unfortunately, we could not capture adult birds during the course of this

experiment, to determine whether the absence of infection was limited to juveniles. However, the assumption that colonial nesting is likely to favour malaria prevalence is largely based on the idea that naked, immunologically naïve hosts will be concentrated in a confined environment leading to increased transmission between birds. In this case, juvenile birds show no signs of blood stage parasites immediately prior to fledging, irrespective of their true infection status. Due to the time at which we sampled birds, all of the juveniles will have left the breeding site very soon after sampling. Thus, the expected prevalence of malaria in this colonial species may become apparent after leaving the colony. This possibility is highlighted by the fact that when juvenile *Ciconiiforme* birds were taken into (vector free) captivity in Venezuela, the number of detectably infected birds doubled in a 30-day period (Gabaldon and Ullua 1980). However, in that study almost a third of juvenile birds already displayed patent infections in the nest.

One suggested reason for the lack of infections in young colonial herons is that vectors are absent in their colonies, or malaria is not prevalent in potential vector species (Tourenq *et al.* 2001). Increasing interest in the vectors of avian malaria (Kim *et al.* 2009; Kimura *et al.* 2010; Ejiri *et al.* 2011) including on potential vectors in European populations (Glaizot *et al.* 2012; Ferraguti *et al.* 2013; Lalubin *et al.* 2013) have implicated *Culex* spp. as principal vectors of avian *Plasmodium* infections. We found that *Culex pipiens* females were very active near the heron colonies, the most active of any mosquito species, and this species has been implicated as a potential vector for at least 35 different *Plasmodium* lineages (Inci *et al.* 2012; Kim and Tsuda 2012). In this study we found the estimated prevalence of infection (0.01%) was much lower than has been reported from other studies of European *Culex pipiens* collected in light or CO_2-baited traps (Glaizot *et al.* 2012; Ferraguti *et al.* 2013). We did find four different *Plasmodium* lineages in host-seeking mosquitoes, two of which were present in the thorax (SGS1 and

346 SYAT05) suggestive of infective individuals, whereas the other lineages were only detected in
347 the abdomens. Even positive PCR from the thorax of mosquitoes cannot guarantee an infective
348 specimen (Valkiūnas 2011), and much further work is required to demonstrate the competency of
349 specific mosquito species as vectors for a given lineage of parasite. Despite this, our results
350 indicate that while there is evidence that vectors are very active in the colonies, and carry a range
351 of *Plasmodium* parasites, the prevalence of malaria is not especially high in mosquitoes around
352 the heron colonies. The biological importance of a prevalence of 0.01% in host-seeking *Culex*
353 *pipiens* for the transmission dynamics of *Plasmodium* is unclear. In mosquito traps baited with
354 live ducks in the Camargue, it has been demonstrated that as many as 120 separate *Culex pipiens*
355 will feed on a single bird in one day (Balenghien *et al.* 2009), so a very low prevalence will not
356 necessarily impede transmission. Indeed, we have also found a similarly low prevalence of
357 *Plasmodium* infection in *Culex pipiens* around sparrow colonies in the Camargue, which is
358 adequate to support transmission to almost all of the birds, including juveniles of similar or
359 younger ages than the herons sampled here (S. D. Larcombe, unpubl. data). There are many
360 human populations where malaria infections are holoendemic but where prevalence of infection
361 is extremely low, or even undetectable, in mosquitoes. Similarly, for West Nile Virus, a virus
362 transmitted to avian and mammalian hosts by *Culex* mosquitoes, a prevalence of 0.07% in
363 mosquitoes has been shown to impact transmission (Hayes *et al.* 2005). Together, this data
364 suggests that lack of infective vectors does not explain the absence of blood parasites in juvenile
365 herons in the Camargue, and that they may have some mechanism for avoiding or resisting
366 infection (Martinez-Abrain *et al.* 2004). This may be due to immunity to the strains of
367 *Plasmodium* that are common in other birds, though it has also been shown that some heron
368 species have developed a range of mosquito avoidance behaviours that severely restrict the

ability of mosquitoes to feed (Webber and Edman 1972), including shaking, scratching and rubbing with the bill or feet. Investigating the reasons for the absence of infection in juvenile herons is a challenge for future research.

We wished to test whether the absence of blood stage avian malaria parasites in nestling Little Egrets was specific to this species, or more general to colonial heron species in the Camargue. In agreement with some earlier studies, we found no evidence for infection with *Plasmodium*, *Haemoproteus*, or *Leucocytozoon* in any of the four heron species we tested. We found that potential vector species were present around the heron colonies, and that the prevalence of malaria infection in mosquitoes was low. However, conditions in the heron colonies certainly appear suitable to support transmission of avian malaria to birds. Future experiments will be required to confirm whether Camargue heron species are susceptible to avian malaria, and to elucidate the potential mechanisms behind the apparent ability of these birds to resist infection.

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Table 1. *Plasmodium* lineages found in thorax or abdomens of *Culex pipiens* at heronries of Little Egret, Cattle Egret, Black-crowned Night Heron, and Squacco Herons in the Camargue.

Site	Insecticide Treatment	Abdomen/ Thorax	Date	Lineage
Scamandre	Sprayed	Thorax	17/6/10	SYAT05
Scamandre	Sprayed	Abdomen	24/6/10	COLL1
Scamandre	Sprayed	Abdomen	21/7/10	YACHO1
Mas d'Agon	Unsprayed	Thorax	8/6/10	SGS1
Mas d'Agon	Unsprayed	Abdomen	8/6/10	YACHO1

Table 2. Mean counts of mosquito species caught per trap - night at Camargue heron colonies.

Site	Insecticide Treatment	*Culex pipiens*	*Uranotaenia unguiculata*	*Culex Modestus*	*Ochlerotatus caspius*	*Aedes vexans*	*Ochlerotatus detritus*	*Anopheles maculipennis*	*Anopheles hyrcanus*	*Culiseta annulata*
Redon	Unsprayed	25	4	1	4	0	0.5	1	0.5	1.5
Tyrasses	Unsprayed	10.5	14	0	0	0	0	0	0	0
Agon	Unsprayed	38	0	0.5	7	0	0	0.5	0	0
Scamandre	Sprayed	13	1.5	0.5	2	0	0	0.5	0	0
Musette	Sprayed	44	0	0	0	0	0	0	0	0
Palisade	Sprayed	6	0	0	40	0	0.5	0	0	0