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Galapagos seabirds' lice community: host hetero-specific interactions and parasite evolution

Jose Luis Rivera
University of Missouri-St. Louis, jose.rivera@umsl.edu

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by

Jose Luis Rivera-Parra

M.Sc., Ecology, University of Missouri-St. Louis, 2010

Lcdo., Biología, Pontificia Universidad Católica del Ecuador, 2007

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Dissertation Advisory Committee

Patricia Parker, Ph.D. (Advisor)

Kevin Johnson, Ph.D.

Robert Marquis, Ph.D.

Robert Ricklefs, Ph.D.

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Dissertation Abstract

Parasites comprise a significant proportion of world biodiversity. The diversification of parasite species depends on parasite species characteristics (e.g., dispersal ability, type of transmission) and the connectivity among host populations and host hetero-specific interactions. The specific speciation mechanisms described are: cospeciation, where a parasite follows the evolutionary track of its host; host-switching, where an isolate of the parasite population colonizes a new host species and follows a different evolutionary track. During my dissertation I focused in understanding the factors behind: the likelihood of colonization of a novel host species (host-switching) and the diversification of parasite species that infect multiple hosts.

I started by describing the Galapagos seabirds' host-parasite community, focusing on five species of seabirds (magnificent frigatebird, great frigatebird, Nazca booby, blue-footed booby and red-footed booby). I found nine species of ectoparasitic lice: five species of *Pectinopygus* ischnoceran lice, one infecting each host; two species of *Colpocephalum* amblyceran lice, one on each frigatebird species; and two shared amblyceran lice, *Eidmanniella albescens* found on Nazca and blue-footed boobies and *Fregatiella aurifasciata* found on the two frigatebirds. Using a combined approach of traditional statistical tests and multi-model inference I analyzed the relative importance of sex, body size, host, island, host family and breeding status, to explain parasite prevalence and

intensity of infection. Overall, inter-island differences possibly related to host-density explain the observed variation.

Using as focal species *Eidmanniella albescens* and *Fregatiella aurifasciata*, which infect multiple hosts, I analyzed how the spatial location within a mixed colony and the movement of host individuals between colonies relate to parasite diversification. I used three genetic markers (one mitochondrial, COI, and two nuclear, EF1-α and wingless) and maximum likelihood phylogenetic trees to test whether: (a) parasites show lineage sorting based on their host species; and (b) switching of lineages to the alternate host species depends on the spatial location of individual hosts within a colony. I found that host species identity was the only factor explaining patterns of genetic clustering in both parasite species. In the case of *Fregatiella aurifasciata*, the pattern of genetic divergence suggests a concordant evolutionary history with their hosts. In contrast, the genetic structure found in *Eidmanniella albescens* suggests a host-switching event, where Nazca booby parasites' colonized blue-footed boobies.

A major challenge when studying host-switching has been to define the original conditions that facilitated such events. So, taking advantage of this highly connected multi-host multi-parasite system and an extensive sampling effort, I analyzed the factors behind host-switching events, that are thought to start by successive arrival "straggling" parasites until establish a breeding population. I used a combination of classical morphology-based parasitology approaches with measurements of spatial distribution of hosts in mixed breeding colonies and

molecular genotyping to test: (a) the effect of local host community composition on straggling parasite identity; (b) effect of spatial location within a mixed colony on straggling frequency and parasite species identity; (c) limitations in straggling frequency depending on lice attachment specifics; and (d) evidence of breeding in cases where straggling adult lice were found. I analyzed more than 5,000 parasites and found a straggling rate of ~1%, with ~5% of host individuals having straggling parasites. I found that the presence of host and potential host in the same locality, together with the specifics of lice attachment are the main factors behind straggling frequency and, therefore, potential for successful host-switching. Moreover, this study suggests that successful host-switching depends on being transmitted to the next generation or across host individuals through physical interactions and the success of this process can be highly affected by stochastic events, such as the death of the host.

Host and parasite life histories are deeply intertwined, and therefore, parasite communities are structured based on host conspecific and heterospecifc interactions. Differences in nesting microhabitat may limit the potential for parasite exchange favoring divergence in parasite species that infect multiple hosts. Moreover, behaviors such as the kleptoparasitism observed in frigatebirds and something as specific as the way lice attach to the host feathers may drive which parasite species has the potential to colonize a novel host and possibly diverge into a different species.

Table of Contents

Chapter I

Comparative ectoparasite loads of five seabird species in the Galapagos Islands

Unpublished manuscript: J. L. Rivera-Parra, I. I. Levin and P. G. Parker. Comparative ectoparasite loads of five seabird species in the Galapagos Islands

ABSTRACT: We describe here the ectoparasitic lice (Insecta: Phthiraptera) found on five species of seabirds (magnificent frigatebird, great frigatebird, Nazca booby, blue-footed booby and red-footed booby) on the Galapagos Archipelago. We found 9 species of ectoparasitic lice: 5 species of *Pectinopygus* ischnoceran lice, 1 infecting each host; 2 species of *Colpocephalum* amblyceran lice, 1 on each frigatebird species; and 2 shared amblyceran lice, *Eidmanniella albescens* found on Nazca and blue-footed boobies; and *Fregatiella aurifasciata* found on the 2 frigatebirds. We tested the relative importance and interactions of: sex, body size, host, island, host family and breeding status and found that inter-island differences were the main driving factor determining prevalence and intensity. These differences could be related to host density and weather, but further investigation is needed.

Host-parasite interactions are ubiquitous, having effects on hosts that range from subtle to extreme impacts on fitness that can decimate populations

(Burrows et al., 1995; McCallum and Dobson, 1995; Daszak and Cunningham, 1999; Wyatt et al., 2008; Vredenburg et al., 2010). Understanding the mechanisms and factors that generate, maintain, and constrain these interactions can be relevant to broad areas of ecology and evolution (Brooks and Ferrao, 2005).

In this study, the parasites we focus on are chewing lice (INSECTA: PTHIRAPTERA), with representatives of 2 suborders, Amblycera and Ischnocera. These obligate ectoparasites rarely, if ever, leave the host except for transferring between parents and offspring (vertical transmission) or during direct contact between host individuals (horizontal transmission). Even when not highly pathogenic, these parasites can affect several aspects of avian life history, such as life expectancy (Brown et al., 1995; Clayton et al., 1999) flight performance (Barbosa et al., 2002), sexual selection (Kose and Moller, 1999; Kose et al., 1999) and metabolism (Booth et al., 1993). Body lice (Amblycera) feed on feather tissue and blood from the host and have better dispersal capabilities than ischnoceran lice, since they have been shown to abandon dying hosts and are fairly mobile (Clayton et al., 1992). On the other hand, feather lice (Ischnocera) are less mobile and tend to have closer associations with their hosts; thus, ischnoceran feather lice are thought to be more host-specific than amblyceran body lice (Price et al., 2003).

Parasite infections are highly varied in prevalence and intensity across individuals within populations (Reiczigel and Rozsa, 2005). General rules that

can explain the observed patterns of parasite infection have been elusive, particularly at the scale of the parasite community (Poulin, 2007). This has led to a general perception of host-parasite interactions being specific to the host and parasite species involved. In this study our goal was to determine if there are general rules that explain parasite infection in our study system that comprises 5 species of seabirds and ectoparasitic lice from 2 suborders and 4 genera. The factors that we considered were differences in local communities, host sex and breeding status, host body size, host life history and the interactions among these factors contribute to a host individual being infected and the intensity of such infection (Brooke, 2010; Matthee et al., 2010; Whiteman and Parker, 2004; Hamstra and Badyaev, 2009; Clayton and Walther, 2001; Felso and Rozsa, 2006). The specific objectives of our research were: A) describe ectoparasitic lice abundance and distribution on 5 species of seabirds on the Galapagos Archipelago; and, B) examine factors of the host and/or the parasites that contribute to general patterns of parasite infection.

MATERIALS AND METHODS

We sampled 5 species of seabirds on 7 different islands in the Galapagos Archipelago during the summer months of 2007 to 2011 (Table I; Fig. 1). Each bird was caught by hand when it was nesting or roosting on land. The processing of each individual included a standard morphometric measurement (unflattened wing chord), number of nests within 10 m (when nesting birds were captured),

blood sampling via brachial vein venipuncture (to be used in other concurrent projects) and dust ruffling for ectoparasite sampling (Walther and Clayton, 1997). All the procedures conformed to best practices for animal welfare (UM-St. Louis IACUC protocol number 11-05-06 and Galapagos National Park research permits).

Study system

The host community we studied comprised 5 species of seabirds (Pelecaniformes: Fregatidae and Sulidae) found on the Galapagos Islands. Specifically, we analyzed the ectoparasitic lice community found on the magnificent (*Fregata magnificens*) and great (*F. minor*) frigatebirds, and blue-footed (*Sula nebouxii*), Nazca (*S. granti*) and red-footed (*S. sula*) boobies. Seabirds feed entirely on fish and other creatures from the ocean and nest in colonies that range in size, from mono-specific to significantly overlapping colonies of several species. The 2 species of frigatebirds have reduced waterproofing of their feathers and therefore cannot dive in the water and nstead kleptoparasitize other seabirds to steal their food. Frigatebirds tend to nest in highly aggregated colonies. The 3 booby species are plunge divers, nesting in large dense colonies (red-footed and Nazca boobies), or in smaller more dispersed colonies (blue-footed boobies; del Hoyo et al., 1992).

We sampled 7 islands representing most of the geographic range of the archipelago: Wolf, Darwin, Genovesa, North Seymour, Daphne Major, San Cristobal and Española (Fig.1). There is variation in the host species composition

on the different islands (Table I) and in specific ecological characteristics such as humidity and vegetation. Another relevant factor might be the density of hosts found in each island. We evaluated this using a relative measurement of nest density, i.e., number of nests within 10 m of each sampled nest.

Dust ruffling

We followed a modified dust ruffling protocol (based on Walther and Clayton, 1997) using a pyrethrin-based flea and tick powder (Zodiac, pyrethrins 1%). We applied a standardized amount (~6g) of powder to each bird throughout the body and ruffled a maximum of 3 times. All the calculations and data considered for this study come only from individuals who were dust-ruffled 3 times. Between each ruffle we waited a standard time (2 min) and collected and counted the parasites in each bout. We dust ruffle the birds inside a plastic crate and wipe it clean with clean paper towels and alcohol. Due to animal welfare concerns in such extreme heat we did not dust-ruffle until the point of diminishing returns. Thus, our parasite loads do not represent absolute parasite numbers on each bird. Our standardized parasite load estimate is a relative and comparable measurement across species useful to gain insights into the population biology of these ectoparasites. We stored the collected ectoparasites for later identification in 95% ethanol.

We identified the different species present following the identification key and information of host-parasite association found in Price et al. (2003). Ricardo Palma (Museum of New Zealand, Te Papa Tongarewa) confirmed the

identifications and those specimens were used as reference throughout the counting and sorting of the samples. In the case of the ischnoceran parasites, we counted and sorted the parasites by sex and age class (nymphs and adults). We did not perform similar sorting for the amblyceran lice due to high morphological similarities among sexes and lifestages.

Molecular analysis

We confirmed the putative visual sexing of sexually monomorphic hosts using a PCR-based standard sexing technique that relies on the different lengths of introns found in the CHD-W and CHD-Z genes (Fridolfsson and Ellegren, 1999; Balkiz et al., 2007). In the case of the amblyceran and ischnoceran parasites found on the frigatebirds we confirmed the species identification using a mitochondrial genetic marker. We extracted DNA from individual lice using the voucher method (Cruickshank et al., 2001) using a Macherey-Nagel Tissue extraction kit (Macherey-Nagel, Co., Düren, Germany) with the following modifications to the protocol: we only made a partial cut between the head and the thorax, keeping the head attached to the body (J.Weckstein, pers. comm.); we used 20μl of proteinase K and incubated for 72 hr, and performed 2 sequential elutions each with 20μl of warm buffer (~70°C). We amplified a 300bp segment of the *cytochrome oxidase-I* (COI) gene following the protocol and primers by Hughes et al. (2007). In the case of the *Pectinopygus* species, we used sequences detailed in Hughes et al. (2007; GenBank accession numbers: *Pectinopygus gracilicornis* DQ482969, *Pectinopygus fregatiphagus* DQ489433)

as a reference. For the *Colpocephalum* species, we did not find reference sequences in any online database. Therefore, we relied on a maximum-likelihood analysis to find evidence of lineage sorting and measured the genetic distance between parasites from different hosts. All the analyses were done using MEGA v5.05 (Tamura et al., 2011) using a GTR + I evolutionary model (which was the best fitting model) and 1,000 bootstrap replications. The sequence alignment was done using Clustal W (Larkin et al., 2007) integrated in that software and corrected by hand.

Statistical analysis

We first grouped our analysis by parasite genus, considering this an appropriate level of resolution to look for general patterns underlying parasite infections. We grouped *Eidmanniella* and *Fregatiella* together due to their similarity in prevalence and intensity of infection (Table III). We also analyzed overall parasite loads by grouping all the parasite species from each host individual. To describe the infection of each parasite species in each host, we calculated the prevalence, mean intensity and median intensity using Quantitative Parasitology v3.0 (Reiczigel and Rozsa, 2005) with 1,000 bootstrap replications to calculate the confidence intervals. We were also interested in looking for general patterns of parasite population biology and possible effects of host life history on parasite infection. Thus, using Quantitative Parasitology v3.0 we performed a Fisher's exact test to compare prevalences and a Mood's test to compare median infection intensities. We decided to use the median as a central

tendency descriptor due the right-skewed and overdispersed distribution of parasite infections (Reiczigel and Rozsa, 2005). For the hypothesis regarding host family differences, we compared among grouped *Pectinopygus* (Ischnocera) species (frigatebird or booby), and between *Eidmanniella* and *Fregatiella* (Amblycera). We did not include *Colpocephalum* in this analysis due to the lack of a phylogenetically close relative and comparable counterpart in the boobies. In the case of the *Pectinopygus* parasites, we calculated sex (adult males vs. adult females) and age (nymphs vs. adults) ratios.

In order to better understand and find general patterns behind parasite infection (described by its prevalence and its intensity) we performed Generalized Linear Models (GLM) using SPSS v20 for Mac (SPSS Inc., Chicago, Illinois). For the models analyzing parasite prevalence, we used a binomial distribution on a variable coded as infected and uninfected; and for the models testing infection intensity, we used a negative binomial distribution (Alexander et al., 2000; Reiczigel and Rozsa, 2005). To select the model that best fit the data, we used the Akaike Information Criterion (AIC) and performed a Likelihood-Ratio test to choose between models in case the difference in AIC was less than 2 (Burnham and Anderson, 2002). We compared our models to a general model that consisted of the full factorial design including all the factors being tested. We tested the following factors: island (representing local community effects), host species, host sex, host breeding status (classified as breeding, non-breeding and juvenile), host family, and host body size (using unflattened wing chord as proxy).

The factors we analyzed and the specific hypotheses and predictions we tested were (the corresponding expression used in the generalized linear model analysis is given in the parentheses): 1) The relationship between each parasite with each host is the driving factor behind the observed differences in prevalence and/or infection intensity (Host-species); 2) Differences in the local communities of each island explain most of the observed variance in parasite load (Island); 3) Different host species respond differently to aspects of local communities that directly affect parasite loads (Island + Host-species + Island*Host-species); 4) Sex and breeding status exert a strong effect on parasite abundances and/or intensities (Sex +Breeding-status +Sex*Breeding-status); evidence suggests that males tend to have higher parasite loads than females (Brooke, 2010; Matthee et al., 2010) and studies of house finches and Galapagos hawks suggest that juveniles tend to have higher lice infection intensities than adults, and breeding hosts higher than non-breeding hosts (Whiteman and Parker, 2004; Hamstra and Badyaev, 2009). The major hypotheses also include: 5) Host body size explains a significant amount of the observed variation (Body-size); there will be a positive relationship between body size and intensity of lice infection (Clayton and Walther, 2001); 6) Host body size affects each parasite species differently on each host (Body-size +Host-species +Body-size*Host-species); 7) Differences between frigatebirds and boobies (e.g. diving vs. non-diving behavior) cause differences in parasite infections (Host-family; Felso and Rozsa, 2006); and 8) Differences between frigatebirds and boobies are relevant but the relationship is

species specific (Host-family + Host-species +Host family*Host-species); more distantly related hosts and parasites, and hosts with different life histories will tend to have different parasite prevalences and intensities (Clayton and Walther, 2001).

For *Pectinopygus* and *Colpocephalum* we only tested the models regarding intensity of infection, because the variation in prevalence was so low that no models could be reliably tested. We found no evidence of over-parameterization (e.g. models fewer less parameters did not give lower AIC scores) when analyzing other mathematically possible permutations of the studied factors; thus our discussion and interpretation of contributing factors focuses just on the models originally proposed based on the hypothesis to be tested. We tested the models even when redundant to information obtained by previous tests (i.e. Mood's and Fisher tests) to compare AICs across our set of hypotheses. Moreover, the generalized linear models bring biological meaning to purely statistically significant differences found with our complementary analytical approach (Fisher's exact test, and Mood's test). We tested any other permutations of the target factors that seemed mathematically relevant to prevent over-parameterizing the original models.

RESULTS

We captured 318 individuals from 5 different host species across 7 islands, finding a total of 9 different parasite species (Table II). The parasite species found were from 2 suborders and 4 genera. *Pectinopygus* are ischnoceran lice, and *Colpocephalum*, *Eidmanniella* and *Fregatiella* are amblyceran lice.

In general terms, when considering all host species combined, we identified Española, Darwin, Wolf and Genovesa (11.41; 8.56; 5.28; 4.41 average number of nests within 10m respectively) as the islands with the densest concentrations of breeding birds. Low densities of breeding hosts were found on North Seymour, Daphne Major and San Cristobal (respectively 1.9; 1.25; 0.5 average number of nests within 10m; Table 1). North Seymour was a special case, because frigatebirds nested in high-density colonies (average of 2.39 nests within 10m), whereas in the same island, blue-footed boobies preferred to nest more dispersed (average of 1.25 nests within 10m).

For the genus *Pectinopygus* , we found that there is 1 species per host (Table II). The results of the genetic analysis of COI for the *Pectinopygus* found on the frigatebirds showed complete lineage sorting and a genetic distance of 16.7% between parasites of different hosts that matched the reference sequences tested. Thus, we used the species names *Pectinopygus fregatiphagus* (found on magnificent frigatebirds) and *Pectinopygus gracillicornis* (found on great frigatebirds; Table II; Price et al., 2003). *Colpocephalum* sp. parasites were found only on the 2 frigatebird species (Table II). The results of

the genetic analysis showed complete lineage sorting and 21.1% genetic distance between parasites from each frigatebird species. Therefore, we used the species names *Colpocephalum angulaticeps* (found on great frigatebirds) and *Colpocephalum spineum* (found on magnificent frigatebirds) following Price et al. (2003).

To describe the infection of these parasites, we estimated the prevalence, mean and median intensity of infection. Table III summarizes our findings and Figures 2 and 3 show them graphically. The prevalence for the *Pectinopygus* and *Colpocephalum* species is close to 100%, whereas *Eidmanniella albescens* and *Fregatiella aurifasciata* have significantly lower prevalence and intensities of infection (Table III; Fig. 2: Fisher's exact test P=0.001; Fig. 3: Mood's test P=0.001). The only parasite species shared by more than 1 host species were *F. aurifasciata*, found on both frigatebirds, and *E. albescens*, found on blue-footed and Nazca boobies. We did not find a single *E. albescens* on a red-footed booby (Table II).

Parasite species-level analysis

When analyzing *Pectinopygus* prevalences, we found significant differences within species across islands for the red-footed boobies where San Cristobal was the only island with prevalence less than 100% (prevalence in San Cristobal is 81%); and for *E. albescens* found on Nazca boobies, where we did not find any infected individuals on San Cristobal (Table IVa).

We found significant differences in intensity of infection within host species among islands only for Nazca boobies infected with *E. albescens* and *Pectinopygus annulatus*; and magnificent frigatebirds infected with *P. fregatiphagus*, where individuals from Daphne Major had higher intensities of infection than individuals from North Seymour. In the case of the Nazca boobies the intensity of infection for *P. annulatus* was lower in the individuals sampled on Wolf and San Cristobal, whereas for *E. albescens*, the individuals sampled on Darwin had higher intensities of infection. We did not find statistically significant differences between host species for the shared *E. albescens*, or for *F. aurifasciata* (Table IVb).

Parasite genus-level analysis

We did not find differences in prevalence across the 5 *Pectinopygus* species, but there were significant differences for the median intensity of infection (Table IVb). Further analysis found that the significant difference was found in the intensity of infection between magnificent and great frigatebirds, with magnificent frigatebirds having higher intensity of infection. There were no significant differences in intensity of infection among the 3 species of boobies (Table IVb; Fig. 2). The generalized model approach found that island, host-species and the interaction among these factors was the most plausible explanation for our findings regarding the intensity of infection (Table V). The host species showing the highest intensity of infection was the magnificent frigatebird, with the rest of the species being similar to each other (consistent with Fig. 3). The island with

the overall lowest intensities was San Cristobal, and individuals from Wolf showed the overall highest intensities of infection. There were no significant differences in intensities of *Pectinopygus* infection when frigatebrids were compared to boobies (Table IVc).

There were no statistically significant differences in prevalence or median intensity of infection of *Colpocephalum* parasites between frigatebirds (Table IVb). Our complementary analytical approach showed that the variation of intensity of infection was best explained by the model that includes the effect of breeding status and sex (Table V). This model showed that males present overall higher intensities of infection than females, and juveniles had slightly higher intensities than adults.

In the case of the 2 less common amblycerans, *E. albescens* and *F. aurifasciata,* we did not find statistically significant differences in prevalence or intensity of infection (Table IVc). The generalized model approach showed that for intensity of infection, 2 models were statistically indistinguishable (likelihood ratio test P=0.26). These models were the one that had host body size as the only factor and the one that had host family (frigatebirds vs. boobies) as a factor. These models show that larger birds tend to have higher intensities of infection than smaller birds, and overall, frigatebirds have slightly higher intensity of infections than boobies, even when these differences may not be statistically significant (Table IVc). For prevalence of infection, the model that best explained the variation was the one that had island and species as factors. This model

shows that Darwin, Wolf and Genovesa have higher parasite prevalence, whereas North Seymour and Daphne Major have the lowest. Great frigatebirds and Nazca boobies show higher prevalence than magnificent frigatebirds and blue-footed boobies, respectively.

All parasites combined

In the case of total parasite loads per host, we found that magnificent frigatebirds had significantly higher parasite loads than great frigatebirds and the 3 species of boobies (Mood's median test $P<0.0001$; Fig. 4). Prevalence did not differ across hosts, with all the species showing prevalence close to 100% (Fisher's exact test $P=0.32$). In the case of the generalized models, the hypothesis most supported by our data was that island differences explained most of the observed variance (Table V). This model estimates that the islands showing the highest parasite infection intensity were Darwin and Wolf and the one with the lowest intensities was San Cristobal.

DISCUSSION

The general trend that emerged across the levels of our analysis was the relevance of island as a factor to explain parasite infection. We included this factor as a proxy for local community effects on parasite loads; among such effects we analyzed if the local host density had a significant effect on lice intensity and prevalence of infection. Whiteman and Parker (2004) showed how host sociality and therefore density was driving the population biology of

ectoparasitic lice on the Galapagos hawk. For the seabirds we studied, islands such as Wolf, Darwin and Española showed high-density colonies, whether judging by host species or considering all species, whereas San Cristobal and Daphne showed low-density colonies. Our models support that the higher intensities of infection are seen on islands with high densities of breeding birds and lower intensities are consistently found on birds on islands with lower density breeding colonies.

We tested a correlation between mean intensity of infection and mean number of nests within 10m, first using overall breeding density measures and then specific to each host species. We found no significant relationship when analyzing overall breeding densities of all species combined (r=0.28; P=0.25). When looking at the specific relationships by host species we found a significant relationship in the case of blue-footed boobies, where higher parasite loads were seen at higher breeding densities of this bird species (r=0.9; $P < 0.001$). Moreover, the blue-footed booby was the host species that consistently showed more dispersed colonies, when compared to the other host species. Perhaps blue-footed boobies are highly susceptible to lice infections and their preference in nesting sites (sandy, flat, inshore areas) and low nesting density reduces their parasite load. In all other host species, the relationship between nesting density and parasite load was not significant (great frigatebird r=0.42; red-footed boobies r=0.52; Nazca boobies r=0.46; P>0.4 in all cases). However, evidence from inter-island comparisons at the species level pointed out that in cases where

there were significant differences, lower parasite loads were seen on low-density islands such as San Cristobal, further supporting local host breeding density as a possible relevant factor behind intensity of parasite infection.

Even though we found this suggestive trend of the relevance of breeding host-density to explain parasite infection, this relationship needs to be more fully explored and extended to include alternative factors not considered in this study (e.g., local weather conditions). The temperature in the islands is similar across the archipelago at sea level, but islands with the presence of highlands and eastern location within the archipelago such as San Cristobal and Española tend to be more humid (Jackson, 1993). Research by Moyer et al. (2002) shows evidence of local weather significantly affecting ectoparasitic lice loads; with higher parasite loads in more humid climates. We did not measure climatic variables at the specific sampling points (and to the best of our knowledge, no fine-scale weather data are available in any database), thus we cannot rule out possible effects of such factors. Therefore, we suggest this factor needs to be further explored, by measuring local weather conditions, particularly humidity and precipitation. Furthermore, we recommend analyzing this relationship using alternate measurements of host density (e.g. total host density instead of host breeding density) and possible interactions with local climatic conditions.

We found significant differences in the intensity of infection of *Pectinopygus* parasites; magnificent frigatebirds showed significantly higher intensities of infection than the rest of the hosts, including the great frigatebird

(Table IVb). The magnificent frigatebird was the species that seemed more prone to over-heating and stress during the handling process, limiting the number of individuals dust ruffled 3 times (*n*=8). Thus, further comparative research between these two host species is needed to understand the reasons behind these differences.

Colpocephalum lice were the only parasites in our system for which host breeding status and sex were relevant in explaining intensities of infection. Our results corroborate findings in other systems with males having higher intensities of infection than females, and juveniles higher than adults (Poulin, 1996; Perez-Tris et al., 2002; Morales-Montor et al., 2004; Badyaev and Vleck, 2007). Male frigatebirds have an elaborate courtship behavior in which they inflate their gular sack to attract females. Males spend considerable time and energy during courtship and this may make them more vulnerable to higher intensity infections than females, as males may face a trade-off in time allocation between attracting females and time spent preening (Hamstra and Badyaev, 2009).

One of the hypotheses we were interested in testing was whether there might be differences between frigatebirds and boobies, which have very different foraging strategies. We predicted that boobies might have lower parasite infections due to plunge diving behavior. However, we did not find any statistically significant differences in parasite prevalence or intensity of infection between frigatebirds and boobies (Table IVb, c). However, *Pectinopygus* parasites that presented a phylogenetically controlled test for this hypothesis did

not show differences attributable to diving behavior (Table IVc). *Eidmanniella albescens* and *F. aurifasciata* are amblyceran whereas *Pectinopygus* are ischnoceran lice; we hypothesize differences in their life histories (e.g. attachment and mobility) may be behind this discrepancy. Moreover, the fact that parasite intensity of infection seems so conserved within parasite genus and between parasites suborders, regardless of host species (Figs. 2, 3; Table IVb, c), may indicate that infra-population size might have a phylogenetic component. Further analysis relating parasite loads to parasite phylogenetic relationships is needed to understand what is behind this pattern. Even though we did not find any differences in parasite intensity of infection attributable to diving behavior, it is worth mentioning that the non-diving frigatebirds had one parasite species more than the diving boobies, which would be consistent to the findings by Felso and Rozsa (2006).

We used a DNA bar coding approach to determine the identity of morphologically similar lice species infecting seabirds of Galapagos, finding that the *Pectinopygus* and *Colpocephalum* parasites found on frigatebirds are completely sorted lineages. There is controversy over the taxonomic status of *Pectinopygus gracilicornis* and *P. fregatiphagus* (Price et al., 2003; R. Palma, pers. comm.). We found 16.7% difference in a ~300bp fragment of COI. There is a similar case with *Colpocephaulm angulaticeps* and *C. spineum*, where we found a genetic difference of 21.1% in a 300bp COI fragment. In both cases, our findings support the idea of 2 isolated lineages (within each genus) evolving

independently. However, this evidence needs to be further explored in order to make any taxonomic recommendations.

In the case of the multi-host parasites, both were found on 2 host species, *E. albescens* found on Nazca and blue-footed boobies, and *F. aurifasciata* found on great and magnificent frigatebirds. The distribution of *E. albescens* on Nazca boobies and blue-footed boobies, but not on red-footed boobies, which are hosts elsewhere (Price et al., 2003), was concordant to the finding by Palma and Peck (2013). We cannot venture to give explanations for this, since all 3 hosts overlap in parts of their ranges, and individuals infected with *E. albescens* were sampled on the same islands, but still not a single *E. albescens* was found on a red-footed booby. One possible explanation is a higher degree of specialization than originally thought, with specific parasite lineages found on each host. Thus, it could be possible that the red-footed boobies lost a parasite in the process of colonization of this archipelago. Red-footed boobies nest in trees, whereas Nazca and blue-footed boobies nest on the ground and are found nesting in overlapping areas. Thus, an alternate explanation might be that this spatial separation explains the absence of *E. albescens* on red-footed boobies. However, these hypotheses remain to be tested.

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TABLE I. Local host community composition and sample sizes of each host per each island.

Host Island	magnificent frigatebird	great frigatebird	Nazca booby	blue-footed booby	red-footed booby	Nest density*
Darwin	-	15	12	-	12	8.56
Wolf	-	13	10	-	13	5.28
Genovesa	-	26	25	-	30	4.41
N. Seymour	6	7	-	9	-	1.9
Daphne M.	2	-	-	3	-	1.25
San Cristobal	-	35	18	4	16	0.5
Española	-	11	33	18	-	11.41

* Average number of nests within 10m.

TABLE II. Parasite diversity and host breadth.

Host / Parasite	magnificent frigatebird	great frigatebird	Nazca booby	blue-footed booby	red-footed booby
Pectinopygus fregatiphagus	X				
Pectinopygus gracillicornis		X			
Pectinopygus annulatus			X		
Pectinopygus minor				X	
Pectinopygus sulae					X
Colpocephalum spineum	X				
Colpocephalum angulaticeps		X			
Fregatiella aurifasciata	X	X			
Eidmanniella albescens			X	X	

TABLE III. Summary of descriptive statistics of parasite infection by parasite species and host. Numbers in parentheses correspond to the 95% Confidence Interval.

Parasite	Host	N Hosts	N Parasites	Prevalence	Mean Intensity	Median Intensity
Pectinopygus annulatus	Nazca booby	98	1098	96.9 % (91.5 - 99.2)	11.3 (9 - 15.2)	8
Pectinopygus minor	blue-footed booby	34	463	97.1% (84.4 - 99.8)	13.6 (10.3 - 17.8)	10
Pectinopygus sulae	red-footed booby	71	1038	95.8% (88.2 - 98.8)	15.3 (12.3 - 20)	9
Pectinopygus fregatiphagus	magnificent frigatebird	8	165	87.5% (50 - 99)	23.7 (18 - 28.7)	24
Pectinopygus gracillicornis	great frigatebird	107	1130	97.2% (92.2 - 99.6)	11.6 (9.7 - 14.3)	7.5
Colpocephalum spineum	magnificent frigatebird	8	26	87.5% (50 - 99.4)	3.7 (1.6 - 5.3)	5
Colpocephaulm angulaticeps	great frigatebird	107	766	91.6% (84.7 - 95.7)	7.8 (6.4 - 9.6)	5
Fregatiella aurifasciata	magnificent frigatebird	8	4	37.5% (11.1 71.1)	1.3 (1 - 1.7)	1
Fregatiella aurifasciata	great frigatebird	107	91	34.6% (26.1 - 44.4)	2.46 (1.8 - 3.4)	1
Eidmanniella albescens	blue-footed booby	34	13	29.4% (15.7 - 47)	1.3 (1 - 1.5)	1
Eidmanniella albescens	Nazca booby	98	63	25.5% (17.2 - 35.2)	2.3 (1.6 - 4)	1

TABLE IV. Summary of results from Fisher's exact test for prevalence differences and Mood's test for differences in median intensity.

a) Within species tests for differences among islands

Parasite	Host	N Islands	Fisher's Exact test *p*	Mood's test *p*
ISCHNOCERA				
Pectinopygus fregatiphagus	magnificent frigatebird	2	0.25	0.429
P. gracillicornis	great frigatebird	6	0.132	0.001*
P. annulatus	Nazca booby	5	0.299	0.024*
P. minor	blue-footed booby	4	0.471	0.243
P. sulae	red-footed booby	4	0.019*	0.493
AMBLYCERA				
Colpocephalum spineum	magnificent frigatebird	2	0.25	1
C. angulaticeps	great frigatebird	6	0.088	0.501
Fregatiella aurifasciata	magnificent frigatebird	(samples only from one island)		
F. aurifasciata	great frigatebird	6	0.209	0.135
Eidmanniella albescens	blue-footed booby	3	0.267	0.067
E. albescens	Nazca booby	4	0.002*	0.008*

b) Differences in prevalence and intensity across host species

Contrast	Fisher's Exact test *p*	Mood's test *p*
ISCHNOCERA - *Pectinopygus*		
Across the five species	0.522	0.039*
Between frigatebird species	0.254	0.006*
Among booby species	0.888	0.376
AMBLYCERA		
Colpocephalum (between frigatebirds)	0.529	0.450
Fregatiella aurifasciata (between frigatebirds)	1	1
Eidmanniella albescens (between blue-footed and Nazca boobies)	0.658	0.709

c) Differences between frigatebirds and boobies

Contrast	Fisher's exact test *p*	Mood's test *p*
Pectinopygus – frigatebirds vs boobies	1	1
Fregatiella (frigatebirds) vs. *Eidmanniella* (blue-footed and Nazca boobies)	0.49	0.769

Table V. Summary of results of generalized linear models.

INTENSITY OF INFECTION

MODELS	**AIC**	**ΔAIC**	**Log-likelihood**	**K**
Pectinopygus				
Island + Host-species + Island*Host-species	2245.85	-	-1097.93	25
Island	2251.16	5.31	-1118.58	7
Host-species	2272.65	26.80	-1131.33	5
Body-size +Host-species +Body-size*Host-species	2275.17	29.32	-1127.59	10
Host-family + Host-species +Host family*Host-species	2276.01	30.16	-1131.01	7
Host-family	2277.92	32.07	-1136.96	2
Sex +Breeding-status +Sex*Breeding-status	2278.35	32.50	-1133.18	6
Body-size	2344.70	98.85	-1171.35	1
General model including all the factors and interactions	2370.35	124.49	-1053.17	131
Colpocephalum*				
Sex +Breeding-status +Sex*Breeding-status	689.01	-	-338.51	6
Host-species	690.62†	1.61	-343.31	2
Body-size +Host-species +Body-size*Host-species	691.82†	2.81	-341.91	4
Body-size	692.52	3.51	-345.26	1
Island	693.45	4.44	-339.73	7
Island + Host-species + Island*Host-species	695.36	6.35	-339.68	8
General model including all the factors and interactions	718.93	29.91	-310.46	49
***Fregatiella* and *Eidmaniella*‡**				
Body-size	309.34	-	-153.67	1
Host-family	310.99§	1.65	-153.94	2
Host-species	312.94	3.60	-152.47	4
Sex +Breeding-status +Sex*Breeding-status	313.70	4.36	-150.85	6
Island	317.88	8.54	-151.94	7

Body-size +Host-species +Body-size*Host-species	319.89	10.55	-151.95	8
Island + Host-species + Island*Host-species	327.07	17.73	-147.54	16
General model including all the factors and interactions	386.88	77.53	-141.44	52
Overall Intensity of infection (all parasites combined)‡				
Island	2390.97	-	-1188.48	7
Island + Host-species + Island*Host-species	2391.80‖	0.83	-1172.90	23
Host-family	2402.19	11.22	-1199.09	2
Host-species	2405.57	14.60	-1197.79	5
Body-size +Host-species +Body-size*Host-species	2409.85	18.88	-1194.92	10
Body-size	2413.36	22.39	-1200.68	6
Sex +Breeding-status +Sex*Breeding-status	2431.46	40.49	-1214.73	1
General model including all the factors and interactions	2513.55	122.58	-1124.78	132
PREVALENCE OF INFECTION				
***Fregatiella* and *Eidmanniella*‡**				
Island + Host-species + Island*Host-species	250.85	-	-106.42	19
Host-family	264.09	13.24	-130.04	2
Sex +Breeding-status +Sex*Breeding-status	264.52	13.68	-126.22	6
Island	265.97	15.13	-125.99	7
Body-size	267.03	16.19	-132.52	1
Host-species	267.99	17.14	-129.99	4
Body-size +Host-species +Body-size*Host-species	272.17	21.32	-128.09	8
General model including all the factors and interactions	305.80	54.96	-52.90	100

*Models Host-group and Host-group + Species +Host Group*Species not tested. *Colpocephalum* was only found on frigatebirds.
†Models significantly different than the best fitting one (likelihood-ratio test $p<0.001$).
‡Model Host-family + Species +Host family*Species excluded.
§ Model not significantly different than the best fitting one (likelihood-ratio test $p>0.1$).
‖Model significantly different than the best fitting one (likelihood ratio test $p<0.001$).

Figure legends

FIGURE 1. Map of the Galapagos Archipelago. Only sampled islands are named.

FIGURE 2. Prevalence estimate for each parasite species. Error bars correspond to 95% CI. A) *Pectinopygus annulatus* (Nazca booby); B) *P. minor* (blue-footed booby); C) *P. sulae* (red-footed booby); D) *P. fregatiphagus* (magnificent frigatebird); E) *P. gracillicornis* (great frigatebird); F) *Colpocephalum spineum* (magnificent frigatebird); G) *C. angulaticeps* (great frigatebird); H) *Fregatiella aurifasciata* (magnificent frigatebird); I) *F. aurifasciata* (great frigatebird); J) *Eidmanniella albescens* (blue-footed booby); K) *E. albescens* (Nazca booby).

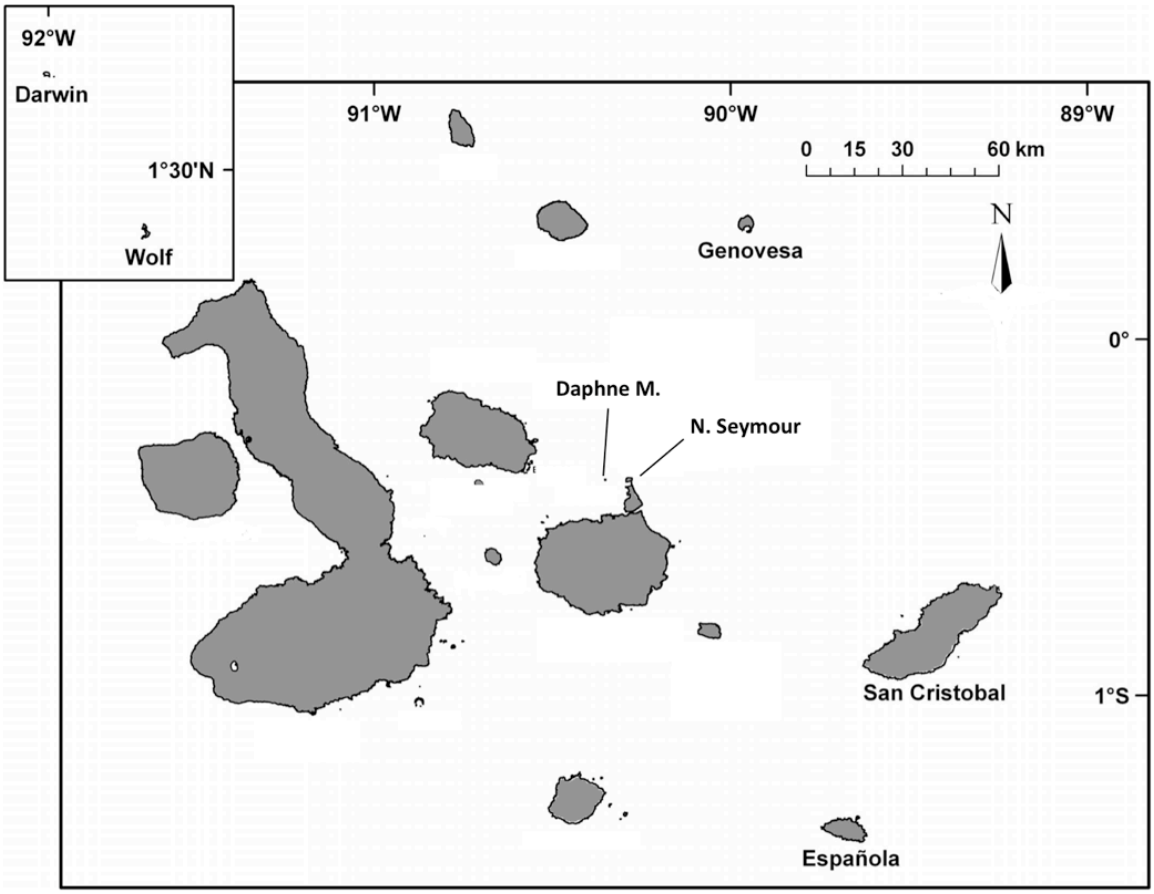
FIGURE 3. Intensity of infection estimates for each parasite species. Open circles represent the mean, black line represent the median and error bars correspond to 95%CI. A) *Pectinopygus annulatus* (Nazca booby); B) *P. minor* (blue-footed booby); C) *P. sulae* (red-footed booby); D) *P. fregatiphagus* (magnificent frigatebird); E) *P. gracillicornis* (great frigatebird); F) *Colpocephalum spineum* (magnificent frigatebird); G) *C. angulaticeps* (great frigatebird); H) *Fregatiella aurifasciata* (magnificent frigatebird); I) *F. aurifasciata* (great frigatebird); J) *Eidmanniella albescens* (blue-footed booby); K) *E. albescens* (Nazca booby).

FIGURE 4. Intensity of infection by host with all parasite species combined. Open circles represent the mean, black line represent the median and error bars correspond to 95%CI.

FIGURE 5. Sex ratios for the *Pectinopygus* (Ischnocera) parasites. Gray bars correspond to males and open bars correspond to females.

FIGURE 6. Proportion of nymphs vs. adults for the *Pectinopygus* (Ischnocera) parasites. Open bars correspond to nymphs and solid gray bars correspond to adults.

FIGURE 1


92°W
Darwin
1°30'N
Wolf
91°W
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0 15 30 60 km
N
Genovesa
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Daphne M.
N. Seymour
San Cristobal
1°S
Española

FIGURE 2

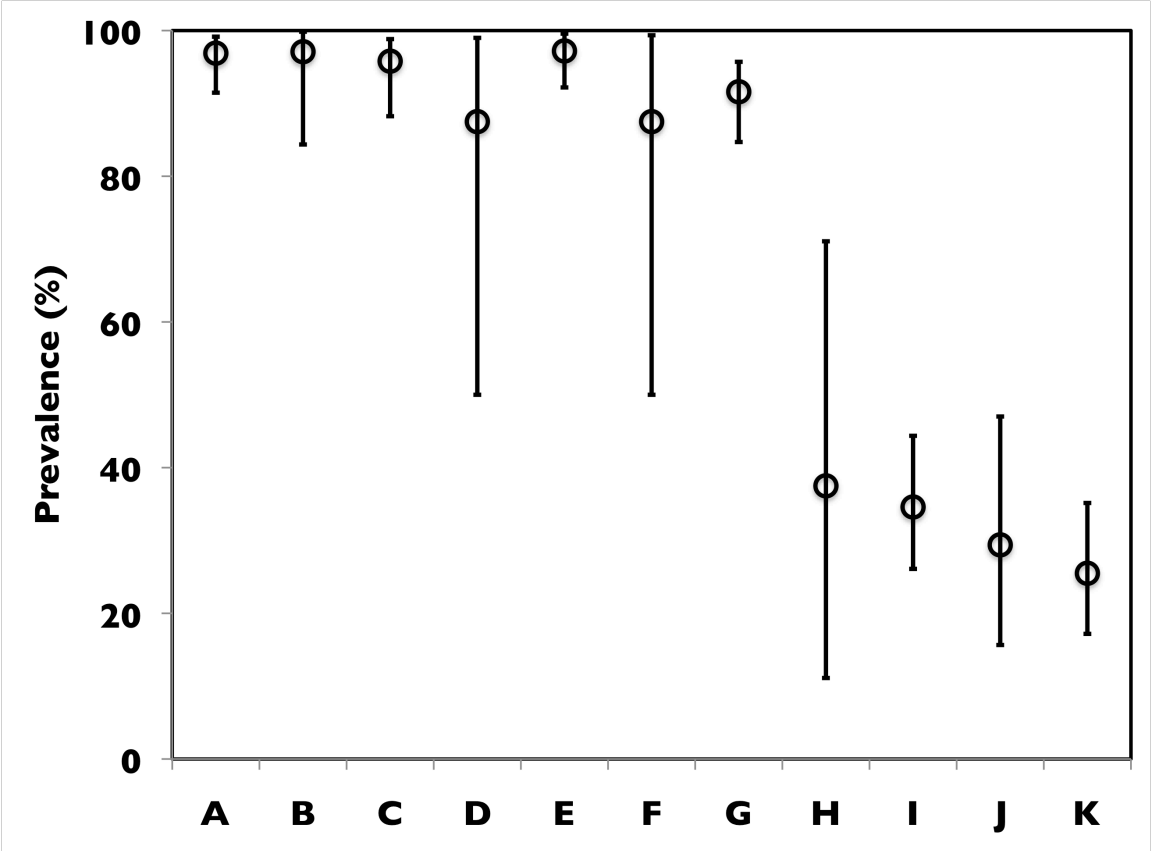
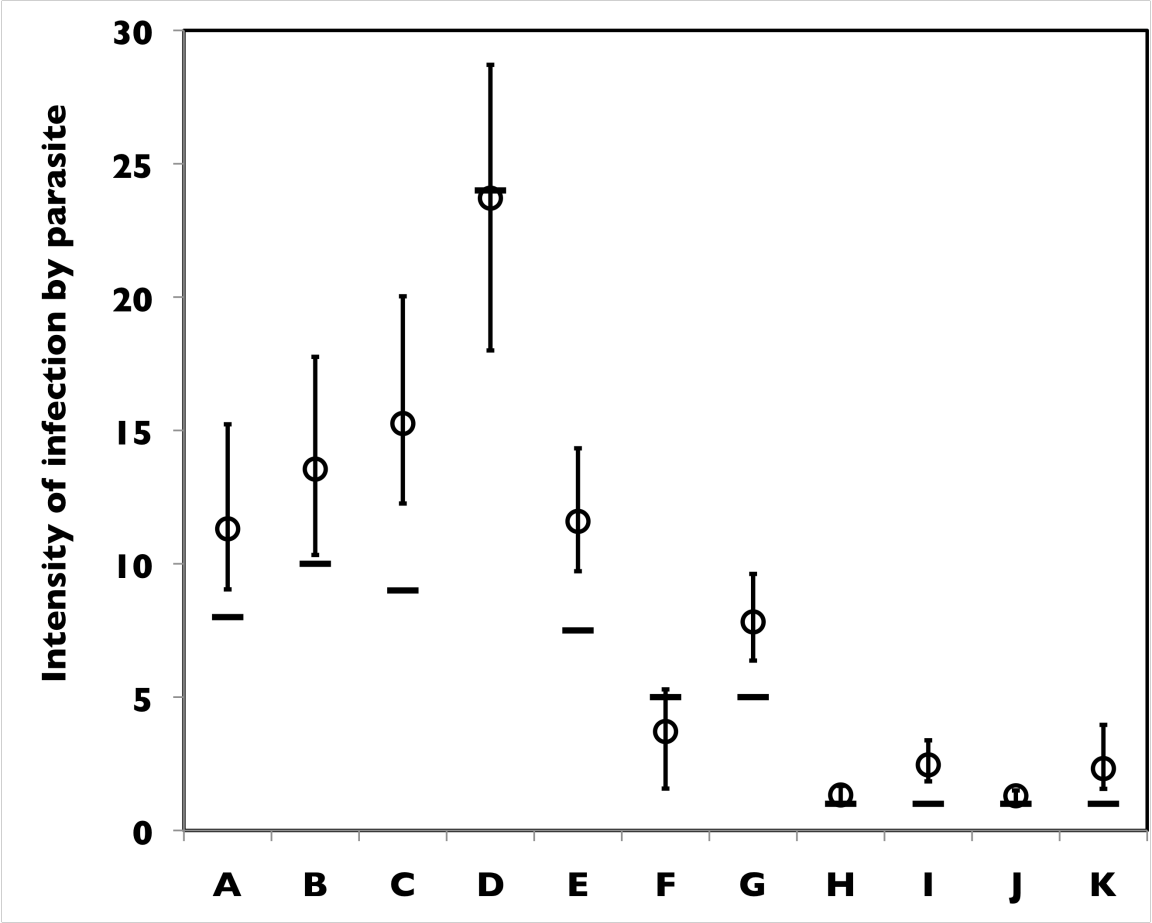


FIGURE 3


Intensity of infection by parasite
30
25
20
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10
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K

FIGURE 4

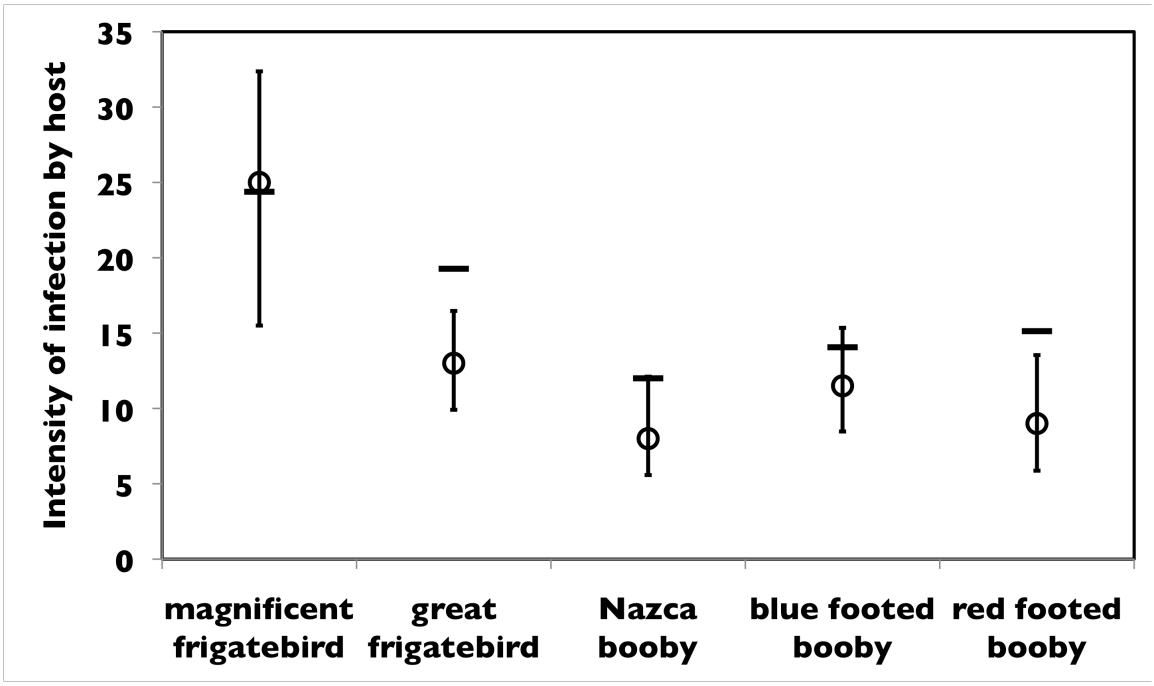

Intensity of infection by host
35
30
25
20
15
10
5
0
magnificent frigatebird
great frigatebird
Nazca booby
blue footed booby
red footed booby

FIGURE 5

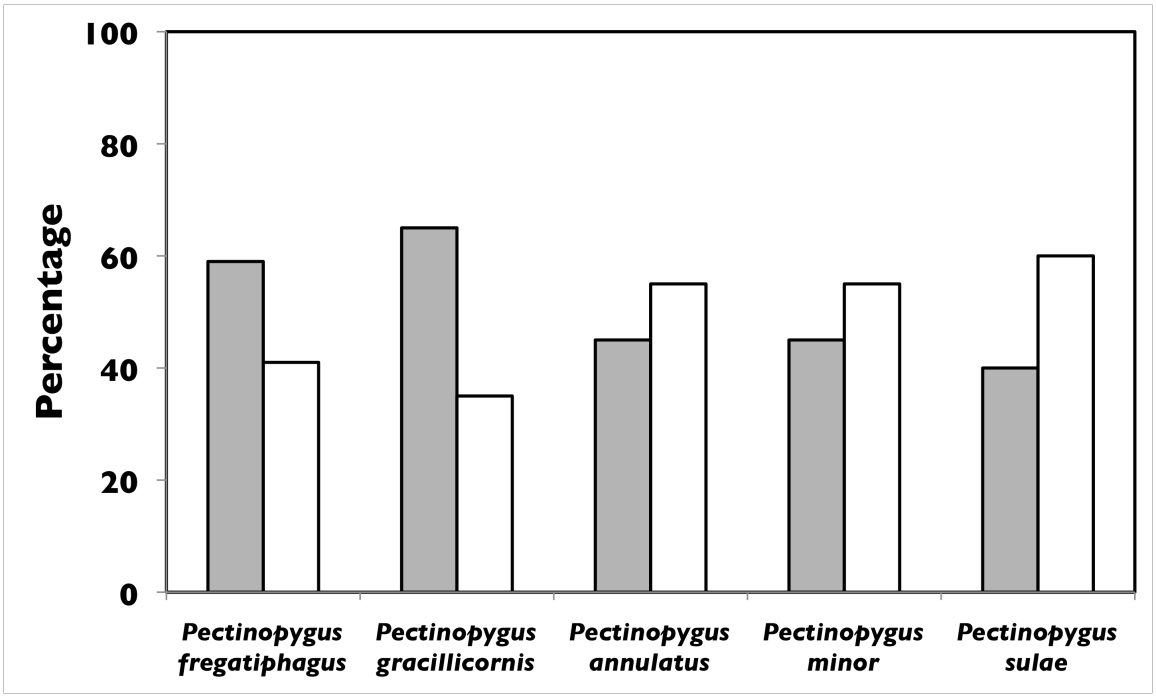
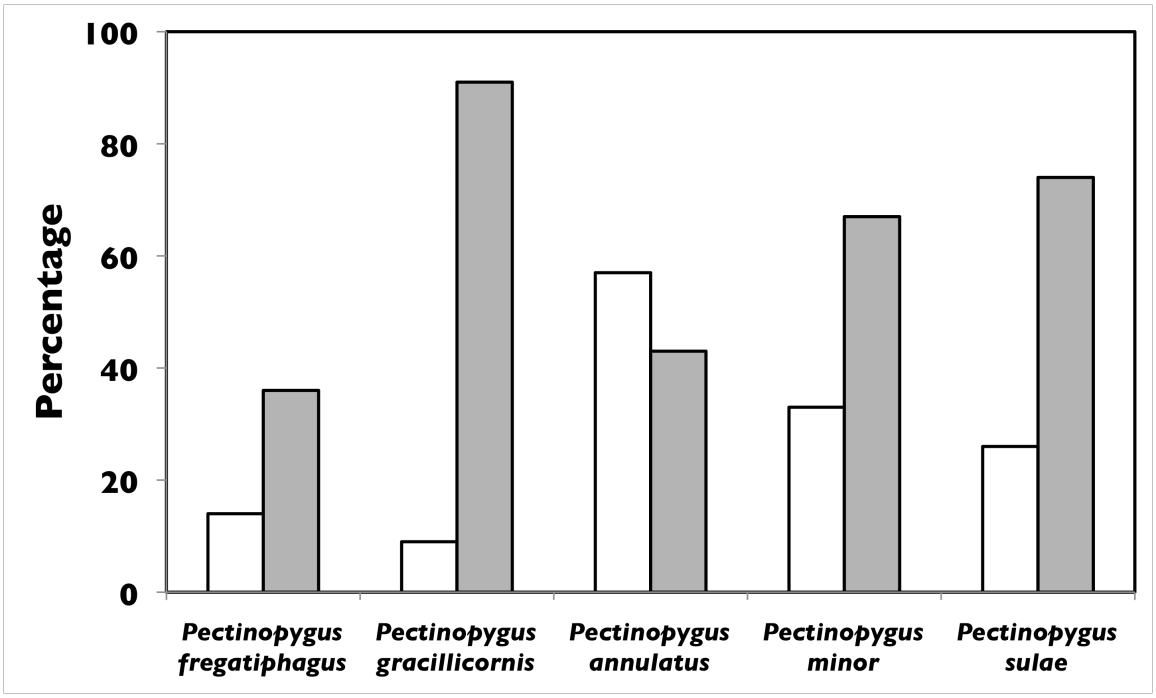

100
80
60
40
20
0
Percentage
Pectinopygus fregatiphagus
Pectinopygus gracillicornis
Pectinopygus annulatus
Pectinopygus minor
Pectinopygus sulae

FIGURE 6


100
80
60
40
20
0
Percentage
Pectinopygus fregatiphagus
Pectinopygus gracillicornis
Pectinopygus annulatus
Pectinopygus minor
Pectinopygus sulae

Chapter II

Lineage sorting in multi-host parasites: *Eidmanniella albescens* and *Fregatiella aurifasciata* on seabirds from the Galapagos Islands

Unpublished manuscript: J. L. Rivera-Parra, I. I. Levin, K. P. Johnson and P. G. Parker. Lineage sorting in multi-host parasites: *Eidmanniella albescens* and *Fregatiella aurifasciata* on seabirds from the Galapagos Islands

Abstract

Parasites comprise a significant percentage of the biodiversity of the planet and represent arenas to test evolutionary and ecological hypotheses. In this study we analyze the effect of host species identity and spatial location within mixed species colonies of nesting seabirds on patterns of genetic clustering within two species of multi-host ectoparasitic lice. We use three genetic markers (one mitochondrial, *COI,* and two nuclear, *EF1-*α and *wingless*) and maximum likelihood phylogenetic trees to test whether: (a) parasites show lineage sorting based on their host species; and (b) switching of lineages to the alternate host species depends on the spatial location of individual hosts within a colony. Specifically, we examine the genetic structure of two louse species: *Eidmanniella albescens,* infecting both Nazca and blue-footed boobies, and *Fregatiella aurifasciata*, infecting both great and magnificent frigatebirds. We found that host species identity was the only factor explaining patterns of genetic structure in

both parasites. Moreover, in the case of *Fregatiella aurifasciata,* the pattern of genetic divergence is consistent with a concordant evolutionary track with their host, showing significant differentiation in the gene regions tested. Thus, a revision of the taxonomy of this species is needed. In contrast, the genetic structure across host species within *Eidmanniella albescens* suggests a host-switching event, with parasites from Nazca boobies colonizing blue-footed boobies. These species do show evidence of lineage sorting by host species, and one possible explanation is low louse migration rates between host species, related to fine-scale spatial separation within mixed colonies and low parasite population numbers. This study contributes to the understanding of parasite diversity, and to the general understanding of the effect of population connectivity in naturally fragmented landscapes on biodiversity maintenance and generation.

Key words: chewing lice, cryptic speciation, lineage sorting, parasites, seabirds.

Introduction

Parasites comprise a significant percentage of the planet's biodiversity (Koh *et al.* 2004; Whiteman and Parker 2005). There is variation in the nature of these relationships, with an extreme of complete dependence of the parasite on the host, such as malarial protozoan parasites and ectoparasitic lice and mites (Price *et al.* 2003; Valkiunas 2005). This paper reports our studies of ectoparasitic chewing lice, which are obligate parasites that depend on the resources and microclimate of the host to survive (Price *et al.* 2003). Parasites

with a life history strongly tied to the host have proven to be excellent systems in which to pose questions on the generation and maintenance of diversity and on mechanisms of speciation (Whiteman and Parker 2005; Whiteman *et al.* 2007; Hughes *et al.* 2007; Johnson *et al.* 2003). Moreover, because their populations are fragmented into small infrapopulations, with varying degrees of connectivity depending on both host and parasite dispersal capabilities, permanent parasites can be good models in which to examine island biogeography and meta-community dynamics (Weckstein, 2004; Banks *et al.* 2005; Whiteman and Parker 2005; Whiteman *et al.* 2007).

Johnson *et al.* (2003) and Huyse *et al.* (2005) summarized the modes of parasite speciation as: (a) co-speciation, where speciation in parasites follows speciation in the hosts; (b) host-switching, where a parasite colonizes a novel host and limited gene flow leads to later speciation; and (c) parasite duplication, where structure in the within the host population limits gene flow in the parasites. Among these, the most studied mechanism is co-speciation. Studies such as Hughes *et al.* (2007) and Kaewmongkol *et al.* (2011) have provided examples of parasites matching the evolutionary history of their hosts. Thus, restriction of host gene flow can similarly limit parasite gene flow, resulting in parasite speciation. Studies analyzing such co-evolutionary patterns have inferred host-switching when incongruent phylogenetic trees of hosts and parasites are observed (e.g. Hughes *et al.* 2007). Studies focusing on parasite duplication, or parasite differentiation, even when the host has not differentiated to the point of separate

species designation, are rare. Whiteman *et al.* (2007) found that in the Galapagos hawk, which has a significantly structured population across the archipelago, parasites show higher genetic differentiation and genetic isolation than the hawks themselves, which may be early steps of lineage sorting and later speciation. The situation becomes more complex in cases where a parasite species is infecting more than one host species. Few studies of parasites have examined parasite divergence in this latter kind of case.

In groups of parasites where most species infect only one host species (Johnson *et al.* 2002; Barret *et al.* 2008), there are examples of parasites infecting multiple host species (e.g. avian malaria in African forest birds, Njabo *et al.* 2011; dove feather lice, Johnson *et al.* 2002). One possible scenario is that these are cases of cryptic species where parasites are morphologically identical and there are host-specific lineages (Poulin and Keeney 2008). Cryptic species of parasites might be relatively common, and estimates of host-specificity might change if genetic studies of multi-host parasite species were performed. McCoy *et al.* (2003; 2005) analyzed a common and shared tick species, which infects seabirds, and found clear evidence of lineage sorting (or race formation) based on the host that they were infecting; such separation depended negatively on the extent and type of interactions among individuals within and between host species (McCoy *et al.* 2005). Thus, overlapping host species with a relatively high degree of interaction (e.g. nesting next to each other in a mixed colony) have the potential to limit the genetic differentiation of the parasites. In this paper

we analyze the way host-parasite interactions can shape parasite diversity, by focusing on obligate parasites that depend entirely on their hosts for survival and transmission (Clayton *et al.* 1992; Price *et al.* 2003; Huyse *et al.* 2005; Nieberding and Olivieri 2007).

We studied the genetic structure of two multi-host ectoparasites: *Eidmanniella albescens* parasitic on boobies (*Sula* spp.), and *Fregatiella aurifasciata* parasitic on frigatebirds (*Fregata* spp.). Both parasite species do not show any morphological differentiation between populations on different host species. Populations of these parasites were studied on host populations that occur in the Galapagos Archipelago (Figure 1), because island biogeography provides another geographically informative layer over which to study genetic differentiation. Both parasites, *Eidmanniella albescens* and *Fregatiella aurifasciata*, are obligate ectoparasitic lice (Phthiraptera) from the suborder Amblycera, members of which have relatively high dispersal capabilities and feed from tissue and blood of the host (Price *et al.* 2003). Both parasites are relatively uncommon, with a prevalence of 35% for *F. aurifasciata* and 27% for *E. albescens,* and a median intensity of infection of 1.8 individuals per infected host for both parasites (Rivera-Parra *et al. submitted*). *F. aurifasciata* is found on both species of frigatebirds in the archipelago (Palma and Peck 2013), the magnificent frigatebird (*Fregata magnificens*) and the great frigatebird (*Fregata minor*). *Eidmanniella albescens* is found on two of the three species of boobies in the archipelago (Palma and Peck 2013), the blue-footed booby (*Sula nebouxii*) and

the Nazca booby (*Sula granti*), but it is not found on the Red-footed booby (*Sula sula*; Rivera-Parra *et al. submitted*), even though it is reported from this host elsewhere (Price *et al.* 2003) and *S. sula* is sympatric with the other hosts on several islands.

Regarding hosts population genetic structure (which is a proxy for host intra-species inter-island connectivity), Levin and Parker (2012) found no genetic structure in the great frigatebird among five island populations within the archipelago, similar to the findings of Taylor *et al.* (2011) on three colonies of blue-footed boobies. On the other hand, in five island populations of Nazca boobies, there is evidence of genetic structure between several pairs of islands, resulting in three distinct genetic clusters (Levin and Parker 2012). To the best of our knowledge, there are no studies describing the intra-archipelago genetic structure of the magnificent frigatebird. All the host species overlap in parts of their range and have different degrees of spatial overlap in mixed nesting colonies.

The goals of our research were to test whether: (a) multi-host parasites in a potentially highly connected system are the same species or if there is evidence of lineage sorting based on the host species; and (b) the degree of spatial overlap of potential hosts explains patterns of genetic clustering. Our specific predictions were that: (1) there will be evidence of lineage sorting depending on the host species; and (2) such evidence will be weaker on islands where the hosts overlap spatially in mixed colonies.

Materials and methods

Ectoparasite collection - Dust ruffling

We followed a modified dust ruffling protocol (based on Walther and Clayton 1997). We applied a standardized amount (~6g) of powder to each host throughout the body, ruffling a maximum of 3 times, and waited a standard time (2 minutes) between bouts of ruffling. We stored the collected ectoparasites in 95% ethanol. Louse identification followed the key and information of host-parasite association found in Price *et al.* (2003) and Palma and Peck (2013).

Furthermore, from each sampled host we recorded the relative spatial location within a colony by recording: the identity of and distance to the nearest neighbor and the species composition of nests within 10m. This measure was used as an estimate of inter-species interaction and a measure of breeding density. Figure 1 summarizes the islands sampled and the local host species composition relevant to this study.

Molecular Analysis

We extracted DNA from individual lice using the voucher method (Cruickshank *et al.* 2001) using a Macherey-Nagel Tissue extraction kit (Macherey-Nagel, CO., Düren, Germany). We followed the kit protocol, with the following modifications: we used 20μl of proteinase K and incubated the whole body for 72 hours after making a partial cut between the head and the thorax, keeping the head attached to the body (J. Weckstein, pers. comm.), and

performed 2 sequential DNA elutions each with 20μl of warm buffer. We amplified the three gene regions using 1μl of total genomic DNA in a 25μl PCR reaction with TaKaRa *Ex Taq* polymerase and reagents. The specific conditions were: 1X $MgCl_2$ free Buffer (2.5 μl; Takara), 1.5 mM of $MgCl_2$ (1.5 μl; Takara), 0.2 mM of each dNTP (2 μl; Takara), 0.08mg/mL of BSA (0.2; Promega) and 0.625 units of Takara *Ex Taq* DNA Polymerase (0.125 μl; Takara). We amplified *COI* using the primers L6625 (5'-COG GAT CCT TYT GRT TYT TYG GNC AYC C-3') and H7005 (5' –CCG GAT CCA CAN CRT ART ANG TRT CRT G-3'; Hafner *et al.* 1994). The specific amplification conditions were initial denaturation at 94°C for 2min, then 35 cycles of: 94°C for 30s, 46°C for 30s and 72°C for 30s, and then a final extension at 72°C for 7min. For EF1-α we used the primers EF1-For3 (5'-GGN GAC AAY GTT GGY TTC AAC G-3') and Cho 10 (5'-AC RGC VAC KGT YTG HCK CAT GTC-3'; Danforth and Ji 1998). The specific PCR conditions were an initial denaturation for 4min at 94°C, then 35 cycles of: 94°C for 20s, 45°C for 30s, and 72°C for 50s, and then a final extension for 5min at 72°C. In the case of *wingless* we used the primers Lep wg1a (5'-GAR TGY AAR TGY CAY GGY ATG TCT GG-3') and Lep wg2a (5'-ACT ICG CAR CAC CAR TGG AAT GTR CA-3'; Hughes *et al.* 2007; Danforth *et al.* 2004), with reaction conditions of initial denaturation for 4min at 94°C, then 35 cycles of: 94°C for 45s, 5o°C for 45s, and 72°C for 45s, and then a final extension for 5min at 72°C.

Phylogenetic analysis

We used MEGA v5.0 (Tamura *et al.* 2011) to build maximum likelihood

trees for each gene. We tested for the best fitting model using MEGAv5.0. We constructed maximum likelihood trees using a T92+I evolutionary model when analyzing *COI,* Jukes-Cantor for *EF1-*α and a T92+G model for *wingless*, with 1000 bootstrap replications. In order to root the *Eidmanniella albescens* trees for *COI* and *EF1-α,* we used a sequence from *Fregatiella aurifasciata* from the same genes. We did the same for the *Fregatiella aurifasciata* trees, using *Eidmanniella albescens* sequences to root them. *Fregatiella aurifasciata* and *Eidmanniella albescens* are considered closely related species that used to be part of the same genus (Ryan and Price 1969). In the case of the *E. albescens* tree for *wingless* we used reference sequences from GenBank of species from the same family (Menoponidade), specifically from *Heteromenopon psittacum* (GU569387.1; Yoshisawa and Johnson 2010) and *Trinoton querquedulae* (GU569385.1; Yoshisawa and Johnson 2010).

Results

Spatial distribution of hosts

In the case of frigatebirds, the only island where both species breed in sympatry is North Seymour (n=30), where the great frigatebird nests in colonies that have an average of 1.8 nests within ten meters of the sampled nest, of which 0.6 nests correspond to magnificent frigatebirds and 1.2 nests correspond to conspecifics (great frigatebirds). On the other hand, the magnificent frigatebirds

on North Seymour (n=20) have an average of 4.0 nests within ten meters, of which one is a nest of great frigatebirds and 3.0 are magnificent frigatebirds.

The two booby species are sympatric on Española, San Cristobal, and Daphne Islands (Figure 1). On Daphne and San Cristobal the nests of blue-footed and Nazca boobies are not closely associated (no Nazca boobies nest within ten meters of a sampled blue-footed booby nest and vice versa). On Española, the Nazca boobies (n=39) had an average density of 4 nests within ten meters of the focal nest, but none of these nests are of blue-footed boobies. The blue-footed boobies (n=15) have an average of 6.82 nests within ten meters, of which 0.67 belong to Nazca boobies and 6.15 correspond to other blue-footed boobies.

Eidmanniella albescens

COI - We sequenced 87 individuals of *Eidmanniella albescens* and found complete lineage sorting by host species, thus revealing a Nazca booby lineage and a blue-footed booby lineage within these parasites (Figure 2). The genetic distance between these lineages is 13.0% or 39bp in the sequenced 300bp fragment. There was no genetic variation within either haplotype cluster at this region of COI.

EF1-α - There was no genetic variability in *EF1-α* across 270bp of sequence among all the individuals of *Eidmanniella albescens* (Figure 2). Thus, it was not possible to detect any lineage sorting by host species at this locus.

Wingless - To further test the results found in *EF1-α,* we sequenced 348 bp *wingless* fragment in a subsample of 42 individuals of *E. albescens* (14 found on blue-footed boobies and 28 found on Nazca boobies, which corresponded to the overall proportion of sampled parasites). Unlike *EF1-α,* we did find evidence of lineage sorting using this nuclear marker (Figure 2). Sequences of parasites on different host species differed by 0.4% genetic distance, i.e. a single difference across 348 bp. A transition in the position 77 of the amplified fragment sorted lice from Nazca booby versus blue-footed booby. The mean within-lineage genetic variability found in the Nazca booby lineage and the blue-footed booby lineage was 0.1% (GenBank accession numbers XXXXXX).

Fregatiella aurifasciata

COI - We sequenced 115 individuals of *Fregatiella aurifasciata*, finding clear evidence of lineage sorting by host species (Figure 3). The observed lineages from great frigatebird and magnificent frigatebird are 14.7% divergent (or 44bp in a 300 bp fragment). The magnificent frigatebird lineage showed a mean genetic variation of 0.5%, whereas the great frigatebird lineage showed no genetic variation.

EF1-α - Sequences of *EF1-α* also clustered lice according to host species (Figure 3). There was 1.9% genetic distance between these two groups (or 5bp in a 270bp fragment sequenced). The specific lineages showed no within-lineage genetic variability (GenBank accession numbers XXXXXX).

In both species of multi-host parasites, consistently across markers, we did not find evidence of parasites from one lineage on the alternate host. Moreover, we did not find evidence of clustering based on island where the host was sampled, nor intra- species clustering by geography within any of the host-specific lineages.

Discussion

Two species of seabird lice from the Galapagos showed evidence of cryptic speciation and lineage sorting, even in the cases where the potential for host-switching and gene flow is high. In *Fregatiella aurifasciata* two very distinct and genetically divergent monophyletic lineages differ in the host species that they infect. The genetic differentiation found in *Fregatiella aurifasciata* is suggestive of concordant speciation with the host that needs to be further explored including other parts of the hosts' ranges and other related species not present in the Galapagos Archipelago. Studies done on the *Pectinopygus* genus of Ischnoceran ectoparasitic lice in the same hosts are consistent with our results, in which ectoparasite diversification appeared to follow a pattern of co-speciation with the host species (Hughes *et al.* 2007). Results in these cases suggest that these host species are isolated enough such that distinct parasite lineages could emerge.

Similar to *Fregatiella aurifasciata*, *Eidmanniella albescens* individuals showed clear lineage sorting when the mitochondrial marker was analyzed, but

such differentiation was not as evident in the nuclear markers, which showed very little divergence overall. Moreover, the clustering pattern on the phylogenetic trees of *COI* and *wingless* suggest host-switching may have occurred, from *E. albescens* found on the Nazca boobies colonizing blue-footed boobies. Studies of deeper evolutionary relationships of the *Eidmanniella* clade that include samples from hosts elsewhere and directly test the timing of divergence will clarify this pattern and have the potential to distinguish between host-switching and other scenarios.

In general both species of these lice have substantial cryptic genetic variation that sorts according to host species. The case of *F. aurifasciata* may be an oversight of classical taxonomic studies (Ryan and Price 1969), where lack of morphological divergence led to classifying this taxon as a single species. Thus, we recommend a revision of the taxonomic classification of this species, and recommend further analysis to include *Fregatiella* individuals from other frigatebird species and other locations, to examine the possibility that at larger spatial scales host races may emerge. *Eidmanniella* may need revision as well with larger series to detect the structure of morphological variation between populations on different host species.

Our results highlight the importance of genetic studies to understand and describe cryptic biodiversity of parasites (Poulin and Keeney 2008). Furthermore, detailed studies on the evolutionary history of these populations or species may lead to a better understanding of local adaptation and population dynamics, and

can provide relevant information to define management units to conserve not only taxonomic biodiversity but unique evolutionary histories (phylogenetic diversity) as well (Waples and Gaggiotti 2008; Paisbøll *et al.* 2007).

In both parasite species, geography and spatial location within a colony were irrelevant to patterns of genetic structure. We initially predicted that geography would be a significant factor in genetic clustering of parasites. It is important to understand the relative importance of intra-host population dynamics and inter-host interactions on parasite evolution. Whiteman *et al.* (2007) showed how comparable ectoparasitic lice showed a stronger pattern of differentiation than their fragmented host population. In our case, we were expecting that *Eidmanniella albescens* found on the Nazca boobies would show genetic structure across islands similar to or stronger than that found in its host (Levin and Parker 2012). However, such a pattern was not observed. One possible explanation for these results is that while Nazca boobies are highly philopatric (Huyvaert and Anderson 2004; Levin and Parker 2012), they are moving more than their genes are revealing (Levin and Parker, *submitted*). Contact between individuals from different islands at sea may facility parasite transmission.

The lineages of *Eidmanniella albescens* from the blue-footed boobies and *Fregatiella aurifasciata* from great frigatebirds showed no clustering based on geography, which is consistent with the evidence from host population genetics (Taylor *et al.* 2011; Levin and Parker 2012). Even though in the case of the magnificent frigatebird there is no population genetic study for comparison, our

study suggests that individuals are moving across the two sampled (nearby) islands. Overall, our study suggests that even when these highly mobile seabird species are isolated from their counterparts across their range (Hailer *et al.* 2011; Taylor *et al.* 2011) there is no evidence of intra-archipelago isolation or differentiation.

We did not find any cases where an individual from one genetic lineage was found on the alternate host either in *Fregatiella aurifasciata* or in *Eidmanniella alsbescens*. McCoy *et al.* (2005) found that local composition of a colony had no effect on the genetic differentiation of the analyzed parasite, which is consistent with our findings. We did not find host switching even on islands where host colonies have some overlap such as Española in the case of the boobies, and North Seymour in the case of the frigatebirds. A caveat is that our sampling effort is a snapshot in a highly dynamic system, where seabird colonies in the Archipelago are reported to change in species composition significantly across years (Valle C. *personal communication*). Furthermore, we found that at fine scale, even on islands where the hosts are sympatric, there is low spatial overlap of nests across species. Both parasites are relatively rare, with relatively low intensities of infection (Rivera-Parra *et al. submitted*). Thus, this fine-scale spatial separation together with few parasite individuals that can "jump" from one host to the other may explain this lack of parasites from one lineage on the alternate host. Low louse population numbers and higher contact within host species than between host species could explain the pattern of lineage sorting by

host observed in both parasite species and the low intra-lineage genetic diversity. Moreover, the whole life cycle of these parasites is about 3 weeks (Price *et al.* 2003); thus, low potential for gene flow and short generation times may further reinforce the isolation pattern detected in this study.

Rivera-Parra *et al.* (*submitted*) and Palma and Peck (2013) did not find any *E. albescens* on red-footed boobies from the Galapagos Archipelago, even though this seabird species is a documented host for *E. albescens* elsewhere (Price *et al.* 2003). Our results suggest the potential for additional lineage specificity and red-footed boobies might have lost this parasite lineage in the colonization process. Genetic evidence suggests an isolation of red-footed boobies in the Galapagos Archipelago population from those elsewhere in the world (Baiao and Parker *unpublished data*), supporting the idea of few individuals founding the population. This, together with the relatively low prevalence of these parasites, is suggestive that the red-footed booby lineage from *Eidmanniella albescens* did not colonize the archipelago with founding red-footed boobies. Moreover, the red-footed boobies are the only booby species that nests in trees (del Hoyo *et al.* 1992), which may offer fewer opportunities for inter-specific transmission even on islands where they are sympatric (see also Johnson *et al.* 2011). Thus, this relative isolation from potential sources of colonization and the relatively low numbers of this parasite species could have prevented them from colonizing this host species. Alternatively, since many Amblycera consume blood (Price *et al.* 2003), there may be species-specific immune interactions that

prevent survival of lice across multiple host species. Other studies on parasites infecting birds from Galapagos and comparing them to parasites infecting sister species or populations in the mainland have shown a decrease in parasite diversity in the Galapagos Archipelago (Sari *et al.* 2013). Our findings may add to the list of parasite lineages that did not make it to the islands. Further analysis of *Eidmanniella albescens* including parasites from the red-footed boobies should answer if there is a specific lineage for this host species.

Conclusion

Our study shows how detailed genetic studies on multi-host parasite species can greatly increase our comprehension of biodiversity and speciation even when morphological differences are not evident (Smith *et al.* 2007). Furthermore, parasite diversity seems to depend primarily on host diversity rather than on geography or the spatial location of the host. Our study suggests that this intimate host-parasite relationship prevents gene flow across parasites found on different host species, promoting the divergence of host-specific lineages. This study shows snapshots of this process, with one parasite showing marked genetic divergence (*Fregatiella albescens*) in both mitochondrial and nuclear markers; and another in the early steps of differentiation, showing strong evidence of lineage sorting in the presumably faster evolving mitochondrial marker and only in one out of two more slowly evolving nuclear gene regions (*Eidmanniella albescens*).

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Figure legends

Figure 1. Map of the Galapagos Archipelago showing the sampled islands and the local host community composition. Sampled Islands include: Darwin, Wolf, Genovesa, North Seymour, Daphne Major, Española and San Cristobal. Species codes are as follows: GREF (*Fregata minor*); MAFR (*Fregata magnificines*); NABO (*Sula granti*); BFBO (*Sula nebouxii*). In parenthesys is listed the number of parasites tested from each island for each host.

Figure 2. Maximum likelihood phylogenetic trees for the tree genetic markers, *COI, EF1-*α and *wingless* for *Eidmanniella albescens*. Number of parasites analyzed from each population are noted in parenthesis next to the island name.

Figure 3. Maximum likelihood phylogenetic trees for the two genetic markers, *COI* and *EF1-*α for *Fregatiella aurifasciata*. Number of parasites analyzed from each population are noted in parenthesis next to the island name.

Figure 1

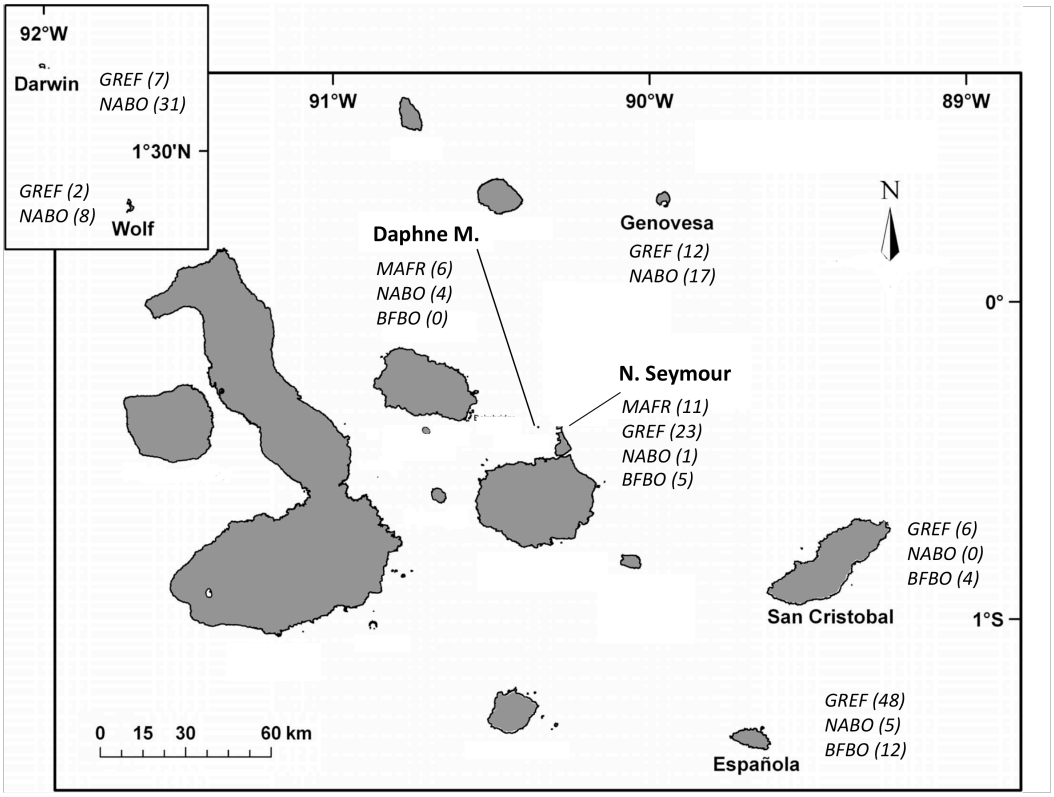

92°W
Darwin
GREF (7)
NABO (31)
1°30'N
GREF (2)
NABO (8)
Wolf
91°W
90°W
89°W
N
Daphne M.
MAFR (6)
NABO (4)
BFBO (0)
Genovesa
GREF (12)
NABO (17)
0°
N. Seymour
MAFR (11)
GREF (23)
NABO (1)
BFBO (5)
GREF (6)
NABO (0)
BFBO (4)
San Cristobal
1°S
0 15 30 60 km
GREF (48)
NABO (5)
BFBO (12)
Española

Figure 2

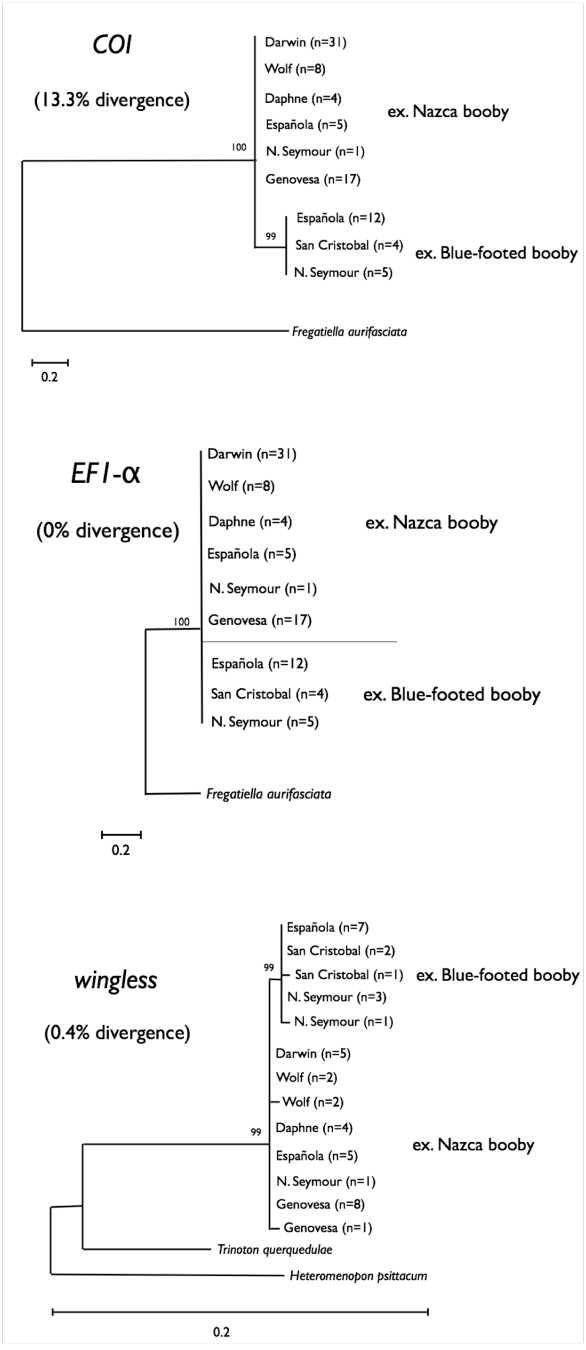
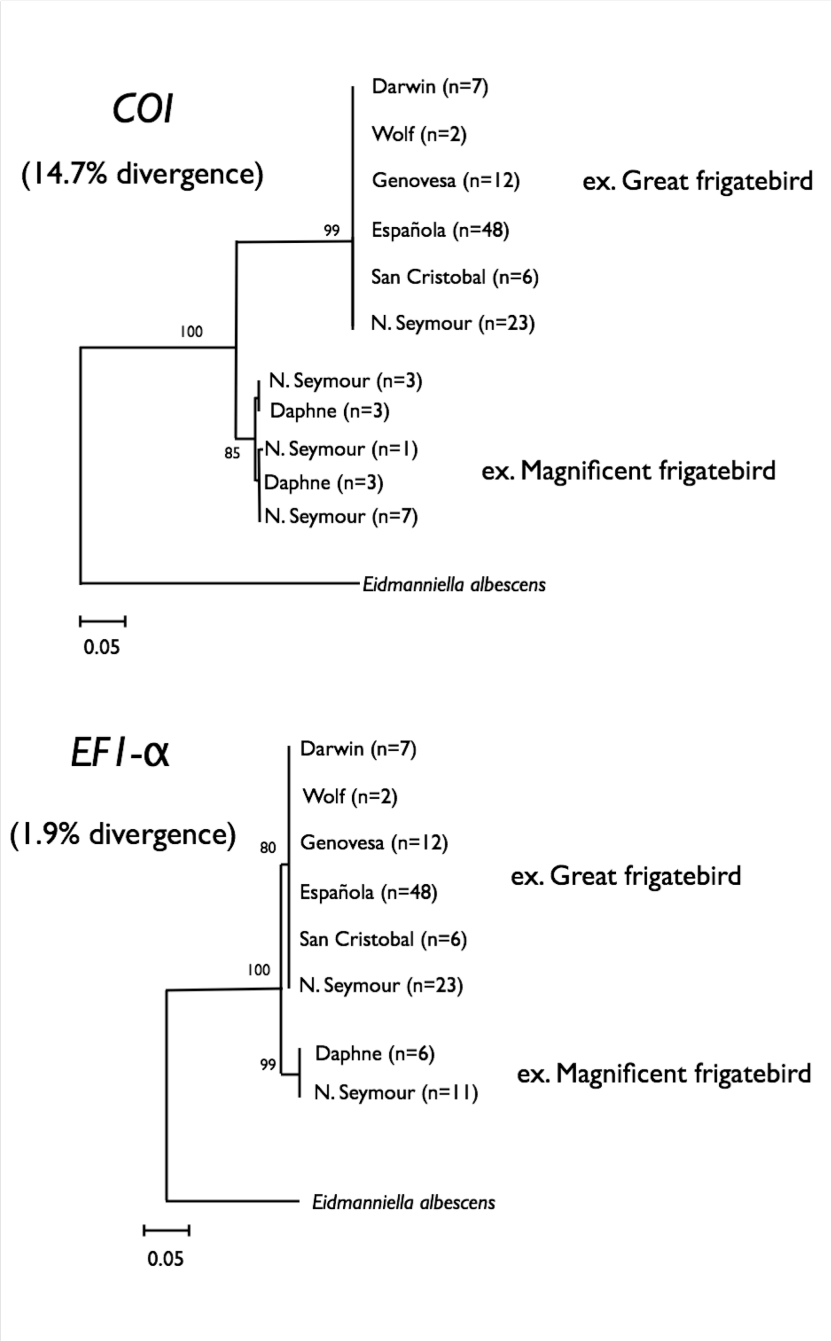

COI
(13.3% divergence)
Darwin (n=31)
Wolf (n=8)
Daphne (n=4)
Española (n=5)
N. Seymour (n=1)
Genovesa (n=17)
ex. Nazca booby
100
Española (n=12)
San Cristobal (n=4)
N. Seymour (n=5)
99
ex. Blue-footed booby
Fregatiella aurifasciata
0.2
EF1-α
(0% divergence)
Darwin (n=31)
Wolf (n=8)
Daphne (n=4)
Española (n=5)
N. Seymour (n=1)
Genovesa (n=17)
ex. Nazca booby
100
Española (n=12)
San Cristobal (n=4)
N. Seymour (n=5)
ex. Blue-footed booby
Fregatiella aurifasciata
0.2
wingless
(0.4% divergence)
Española (n=7)
San Cristobal (n=2)
San Cristobal (n=1)
N. Seymour (n=3)
N. Seymour (n=1)
99
ex. Blue-footed booby
Darwin (n=5)
Wolf (n=2)
Wolf (n=2)
Daphne (n=4)
Española (n=5)
N. Seymour (n=1)
Genovesa (n=8)
Genovesa (n=1)
99
ex. Nazca booby
Trinoton querquedulae
Heteromenopon psittacum
0.2

Figure 3


COI
(14.7% divergence)
Darwin (n=7)
Wolf (n=2)
Genovesa (n=12)
ex. Great frigatebird
99
Española (n=48)
San Cristobal (n=6)
N. Seymour (n=23)
100
N. Seymour (n=3)
Daphne (n=3)
85
N. Seymour (n=1)
Daphne (n=3)
ex. Magnificent frigatebird
N. Seymour (n=7)
Eidmanniella albescens
0.05
EF1-α
(1.9% divergence)
Darwin (n=7)
Wolf (n=2)
80
Genovesa (n=12)
ex. Great frigatebird
Española (n=48)
San Cristobal (n=6)
100
N. Seymour (n=23)
99
Daphne (n=6)
N. Seymour (n=11)
ex. Magnificent frigatebird
Eidmanniella albescens
0.05

Chapter III

Factors behind straggling rate and host-switching likelihood in a highly connected multi-host multi-parasite system

Unpublished manuscript: J. L. Rivera-Parra, I. I. Levin, K. P. Johnson and P. G. Parker. Factors behind straggling rate and host-switching likelihood in a highly connected multi-host multi-parasite system

Abstract

Parasite lineages commonly split when host lineages split. However, even when large clades of hosts and parasites are analyzed and co-speciation is inferred to be common, host-switching can still be another major diversification mechanism. In this study we analyze the initial stages of host-switching events, focusing on conditions associated with straggling events. Straggling is the infrequent occurrence of parasites on a host species other than their "usual" host. We use five species of colonially nesting seabirds from the Galapagos Archipelago and their highly specific ectoparasitic lice *Pectinopygus* spp and *Colpocephalum* spp to examine the occurrence of these straggling events. We use a combination of classical morphology-based parasitology approaches with measurements of spatial distribution of hosts in mixed breeding colonies and molecular genotyping to test: a) the effect of local host community composition on straggling parasite identity; b) effect of spatial location within a mixed colony

on straggling frequency and parasite species identity; c) limitations to straggling frequency as they relate to how lice attach to their hosts; and d) whether there is evidence of breeding in cases where straggling adult lice were found, which separates straggling events from the initial stages of host-switching. We analyzed more than 5,000 individual parasites and found a straggling rate of ~1%, with ~5% of host individuals having straggling parasites. We found that the presence of the usual host and the potential host in the same locality together and the specifics of louse attachment are the two main factors correlated with straggling frequency and parasite identity. Parasites most likely to be found on alternate hosts are smaller than the typical parasite of that host. This suggests that parasites at the extreme of Harrison's rule, the larger parasites infecting larger hosts, are less able to colonize other hosts. Moreover, our study further supports the general perception that successful colonization of a novel host is extremely rare. We suggest that host-breadth expansion (and thus potential for evolutionary host-switching) start by straggling lice establishing a breeding population on a single host and being transmitted to the next generation or across host individuals through physical interactions. The success of this process is likely to be strongly affected by stochastic events such as the death of the host.

Key words: host-breadth, host-switching, lice, parasite speciation, seabirds.

Colonization of novel environments can lead to the interruption of gene flow and the origin of novel species (Feder et al. 2012; Schluter 2009; Ogden and Thorpe 2002). Fragmented and isolated habitats, such as oceanic archipelagos like the Galapagos or Hawaiian islands have been of central importance in our understanding of the mechanisms of adaptive radiation and speciation by genetic drift (Grant and Grant 2002). Parasite populations are fragmented naturally the host's body serves as a discrete patch of habitat. Thus, understanding what conditions limit host breadth of parasites and under which circumstances parasites can overcome these barriers is key to understanding parasite diversification. Furthermore, this information is fundamental to understand how parasites might adapt to local host community changes and the risk of co-extinction with their host.

Two major processes affect parasite speciation as it relates to their hosts. One major mechanism for parasite speciation is co-speciation (Huyse et al. 2005; Hughes et al. 2007; Cooper et al. 2012; Demastes et al. 2012), which occurs when a parasite lineage diversifies in more or less a simultaneous pattern with its host (Huyse et al. 2005). Another second major mechanism that can generate parasite diversity is host-switching (Johnson et al. 2002a; Clayton and Johnson 2003), in which a subset of a parasite population successfully colonizes a new host species and then diverges because of isolation and selection on that new host species. In the parasitic chewing lice of birds both cospeciation (Hughes et al. 2007) and host switching (Johnson et al. 2002b), have been

shown to be important mechanisms generating parasite diversity. A challenge in studies of host-switching using co-phylogenetic analysis is to pinpoint the conditions under which the host-switching events began.

Host-switching likely first starts by expansion of host breadth, in which straggling individuals establish a breeding population on a novel host and later colonize other individuals in the novel host population (Norton and Carpenter 1998; Ricklefs et al. 2004; Paterson and Gray 1997). Straggling parasites are considered individuals that ended up in the "wrong host" by different circumstances but will not survive or establish breeding populations on that host (Rozsa 1993). Whiteman et al. (2004) provided insights on how straggling parasites from goats and Galapagos doves occur on Galapagos hawks (*Buteo galapagoensis*), suggesting that the scavenging behavior of hawks on goat carcasses and predation on doves provided the opportunities for parasites to end up on this atypical host. In the current study, we performed a comprehensive analysis of the conditions behind parasite straggling events in a highly connected and phylogenetically closely related multi-host multi-parasite system and looked for evidence of cases where breeding populations of parasites were established in atypical hosts.

Our study focuses on ectoparasitic lice infecting five species of seabirds in the Galapagos Islands, including both the ischnoceran *Pectinopygu*s spp. feather lice, as well as the amblyceran *Colpocephalum* spp. body lice. These two groups of lice are obligate ectoparasites that complete their life cycle on their host.

Ischnoceran lice feed on feathers, are considered poor dispersers, and are generally highly host specific (Price et al. 2003). Amblyceran lice are considered better dispersers than ischnoceran lice and often less host-specific (Clayton et al. 1992). Amblycerans feed on skin tissue and may rupture the skin to feed on blood, where they might interact with the immune system of the host (Johnson et al. 2005; Johnson et al. 2011). The main mechanism that avian hosts use to combat these parasites is preening (Johnson et al. 2005; Bush et al. 2006; Bush and Clayton 2006). In both Ischnocera and Amblycera, the way these parasites escape from host preening is by firmly attaching to different components of the host feathers. Johnson et al. (2005) and Bush et al. (2006) found that, in the case of ischnoceran lice, the match between inter-barb space of the feathers and louse body width was critical for the ability of these to effectively escape host preening. In the case of amblyceran lice that live closer to the skin, these lice escape preening by attaching with their mandibles to filamentous barbs of the down feathers, but the specific relationship between feather components and lice attachment is not as clear as for ischnoceran lice (Johnson et al. 2005). These lice may also run over the skin to escape host preening, unlike Ischnocera, which have more limited locomotory capabilities.

In studying straggling events, we can start to understand how host-switching events begin and therefore what factors are behind the speciation and diversity of parasites, particularly ectoparasitic lice. The specific objectives of this

study were to: a) describe the occurrence of straggling events across mixed seabird breeding colonies; b) analyze the effect of the local host species composition on the frequency of straggling events; c) test the effects of the specific location within a mixed seabird colony on the prevalence of straggling lice; d) test for directionality in the frequency of straggling events, related to louse attachment efficiency; and e) test for evidence of breeding on a novel host in cases where adult straggling lice were found.

Materials and Methods

Seabirds from the Galapagos Islands and their ectoparasitic lice

Our study focused on seabird lice in the Galapagos Islands, in the Pacific Ocean. We sampled seven islands across the archipelago, which represent the major breeding colonies for all of the relevant host species. Specifically, we sampled the northern islands of Darwin, Wolf, and Genovesa; the central islands of North Seymour and Daphne Major; and the eastern islands of Espanola and San Cristobal. Figure 1 summarizes the sampled islands, local host-community composition and sampled hosts from each island. Sampled hosts include three species of boobies: blue-footed (*Sula nebouxii*), Nazca (*S. granti*), and red-footed (*S. sula*);and two frigatebirds: great (*Fregata minor*) and magnificent (*F. magnificens*). All of these species are colonial breeders and they differ in key aspects of their natural history. Frigatebirds are kleptoparasites of other birds, which they harass to steal their catch, whereas boobies are plunge-diving fishers.

Both frigatebird species and red-footed boobies nest in trees, whereas Nazca and blue-footed bobbies nest on the ground. Blue-footed bobbies prefer nesting sites further inland if possible and in more sandy areas, whereas Nazca boobies favor rocky areas near cliffs. Previous research has found evidence of significant movement of most host species (there is no information regarding magnificent frigatebirds) within the archipelago (Taylor et al. 2011; Baiao and Parker *unpublished data*). Only Nazca boobies show some evidence of some population differentiation within the archipelago (Levin and Parker 2012).

On these hosts, we found a total of seven ectoparasitic lice (Phthiraptera) species from two different suborders: Ischnocera and Amblycera. Table 1 summarizes typical host-parasite association and overall sample numbers from each parasite and each host (based on Price et al. 2003; Rivera-Parra et al. *submitted*). For the purposes of this paper we define as "typical" the host-parasite association commonly reported in the literature (Price et al. 2003); for example, the typical host of *Pectinopygus annulatus* is the Nazca booby (Table 1).

We sampled five host species across seven islands in the Galapagos Archipelago (Figure 1). We captured the birds by hand and performed a modified dust-ruffling protocol to collect the ectoparasites (Rivera-Parra et al. *submitted*). We used a pyrethrin-based flea powder (Zodiac, pyrethrin 1%) and ruffled the bird a maximum of three times. We applied a standard amount of flea powder

(~6g) and waited a standard time (1min) between ruffling bouts. We recorded the species of each bird and sex, and later we confirmed this putative identification using molecular techniques (detailed below). In cases where we sampled a bird that was nesting, we recorded the number of nests within ten meters of the focal nest and the species identity of each of the neighboring nests.

We stored the collected ectoparasites in leakproof tubes with 95% ethanol for later identification. We used specimens identified by R. Palma as reference and the identification key found in Price et al. (2003) to sort the collected lice to the species level. In cases where there were no conspicuous morphological differences, e.g. *Pectinopygus gracilicornis* and *P. fregatiphagus*, we used a molecular identification approach to confirm the species identification.

We extracted DNA following the voucher method (Cruickshank et al. 2001), using a Mackerey-Nagel tissue extraction kit. We incubated each individual louse, which had previously been cut between the head and the thorax, in proteinase K for 72 hours at 55°C, then followed the extraction protocol from the kit, with two sequential elutions, each with 20 μl of warm buffer at 70°. We sequenced a 300bp fragment of the mitochondrial gene *cytochrome oxidase subunit I* (COI), using the primers L6625 (5'-COG GAT CCT TYT GRT TYT TYG GNC AYC C-3') and H7005 (5' –CCG GAT CCA CAN CRT ART ANG TRT CRT G-3'; Hafner *et al.* 1994). The specific PCR cocktail conditions were 1X $MgCl_2$, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.08mg/mL of BSA, 0.625 units of DNA Polymerase and 1μl of stock DNA. The specific amplification conditions were

initial denaturation at 94°C for 2min, then 35 cycles of: 94°C for 30s, 46°C for 30s and 72°C for 30s, and then a final extension at 72°C for 7min. PCR products were visualized in a 1.5% agarose gel, and then cleaned using ExoSap (USB Scientific, Cleveland , USA). We sequenced both chains of the products using BigDye terminator kit v3.1 (Applied Biosystems, Foster City, USA). Sequencing products were run in an automatic sequencer ABI 3130xl. Sequences were checked for quality and contigs were assembled using SeqManII v.4(DNAStar, Madison, USA). Sequences were aligned using Clustal W, part of Mega V5.05 (Tamura et al. 2011). In the case of the *Pectinopygus* spp. parasites, we used reference sequences from Hughes et al. (2007; GenBank accession numbers: *Pectinopygus gracilicornis* DQ482969, *P. fregatiphagus* DQ489433, *P. annulatus* DQ482970; *P. minor* DQ482966; *P. sulae* DQ482971) for each parasite species. We followed Rivera-Parra et al. (*submitted*) for the identification of the *Colpocephalum* spp. parasites. We tested for the best fitting evolutionary model (T92 + G for *Pectinopygus* spp. parasites and T92 for *Colpocephalum* spp. lice) and then constructed maximum likelihood trees with 1000 bootstrap pseudo replicates using MEGA V5.05 (Tamura et al. 2011). To test for presence of nymphs corresponding to the same species of straggling adults, we followed the same protocol described above and confirmed the species identity of each individual nymph based on the clustering pattern.

We calculated descriptive statistics of prevalence and distribution of straggling events based on host species, parasite species, and island. After

using both morphological and molecular techniques to confirm species identity, we performed chi-square tests with 10000 Montecarlo samples in SPSS v13.0 for Mac (SPSS Inc., Chicago, USA) to test for the effect of island local community composition, spatial location within a mixed breeding colony, and louse body size on the frequency of straggling events. We conducted Spearman's rho correlations with 1000 bootstrap replicates to test for the association between presence of straggling lice with distance to the nearest nest, number of con-specific nests within 10m of the focal nest, and number of hetero-specific nests within 10m of the focal nest.

Results

We sampled a total of 436 host individuals; of those, 26 had straggling adult lice (5.65%), 14 had only straggling Ischnocera, 9 had only straggling Amblycera, and 3 had straggling parasites from both groups. From the parasite perspective, we analyzed 3564 *Pectinopygus* spp lice (Table 2), and found 23 straggling individuals (0.65%). The median of individual straggling *Pectinopygus* found on each host was 1 (n hosts= 17; mean =1.35), and no more than 3 straggling *Pectinopygus* were found on a single host. In the case of the *Colpocephalum* spp. parasites (Table 3), out of 970 analyzed lice, 15 straggling lice were found (1.55%). The median of straggling *Colpocephalum* per host was 1 (n hosts = 11; mean=1.36) and the maximum straggling *Colpocephlum* found in a single host was 3.

We found that host nests were generally widely spaced, even in mixed breeding colonies. On average the closest nest was at 11.5m in blue-footed boobies, 4.4m in great frigatebirds, 3.8m in Nazca boobies, 3.7m in red-footed boobies, and 2.27m in magnificent frigatebirds. The average number of nests from conspecifics within 10m was 8.6 for Nazca boobies, 5.3 for great frigatebirds, 3.2 for red-footed boobies, 2.5 for magnificent frigatebirds, and 1.1 for blue-footed boobies. The average number of nests of heterospecifics (any other host species sampled in this study) within 10m of the focal nest was 1.6 for red-footed boobies, 1.4 for great frigatebirds, 1.1 for magnificent frigatebirds, 0.5 for blue-footed boobies, and 0.3 for Nazca boobies. The islands that showed the highest degree of overlap among host species were Darwin, where red-footed boobies and great frigatebirds overlap considerably, and Wolf, where Nazca and red-footed boobies were nesting highly mixed with each other.

We found significant effect of local community composition in explaining straggling parasite frequency. First, we analyzed all the straggling lice and found that 19 out of 23 ischnoceran straggling events happened on islands where the typical host was present (χ^2= 9.78, df = 1, p = 0.002 ± 0.001 95%CI). In the case of amblyceran lice, 13 out of 15 straggling events happened on islands where the typical host was present (χ^2 = 8.07, df = 1, p = 0.006 ± 0.002 95%CI). When combining both types of lice, 32 out of 38 events occurred on islands where the typical host was present (χ^2 = 17.79, df = 1, $p < 0.0001$ ± 0 95%CI). We did not

find any significant relationship between the presence of straggling lice and distance to the nearest nest ($p = 0.95$), number of conspecific nests within 10m ($p = 0.106$), or number of heterospecifc nests within 10m ($p = 0.676$).

We had seven host individuals that were breeding at the time of sampling and had straggling lice. We tested if the specific spatial location within a mixed breeding colony would have an effect on the species identity of these straggling lice on breeding birds. Specifically, we asked if the species identity of the straggling lice could be explained by the presence of the typical host within 10m of the sampled host (where the straggling lice was found). We found that the presence of the typical host within 10m of the sampled host did not explain the presence of straggling ischnoceran lice ($\chi^2 = 1.8$, df = 1, $p = 0.377 \pm 0.012$ 95%CI), amblyceran lice ($\chi^2 = 1.8$, df = 1, $p = 0.375 \pm 0.012$ 95%CI), or for a straggling event of either group (both parasites combined: $\chi^2 = 4.5$, df = 1, $p = 0.64 \pm 0.06$ 95%CI).

Straggling events may also be directional, in which one host species is the donor more often than others. The ability of ischnoceran wing lice, such as *Pectinpygus*, to escape from host preening defenses is related to the match between louse width and interbarb space of the wing feathers (Johnson et al 2005; Bush et al 2006). Lice may not be able to insert between feather barbs if they straggle to smaller hosts. We predicted that if the lice attachment has a significant effect, then only parasites smaller than the typical parasite of each host would be found as stragglers. When the parasite species are ranked based

on their head width, thorax width and abdomen width, they rank as follows, largest to smallest: *Pectinopygus annulatus* (Nazca booby), *P. minor* (blue-footed booby), *P. sulae* (red-footed booby) and the parasites that infect frigatebirds *P. fregatiphagus* (magnificent frigatebird) and *P. gracilicornis* (great frigatebird). Of 23 straggling lice, 20 were found on a host that usually harbors larger-bodied parasites (χ^2= 12.56, df =1, p = 0 ± 0 95%CI), supporting this hypothesis.

We found 12 individual birds that had straggling adult lice as well as nymphs. We examined a total of 58 nymphs and found one case of one nymph (out of two, the other corresponded to the typical parasite) from the straggling adult louse species on the novel host. Specifically, we found adults and a nymph of *Pectinopygus gracilicornis* (which is found on great frigatebirds) on a Nazca booby from Genovesa.

Discussion

We have documented widespread and prevalent straggling events in the parasite communities of seabirds in the Galapagos Archipelago. Moreover we have found evidence of the presence of adults that are stragglers on a novel host and, in one case, a nymph of a straggling species on the atypical host, which may indicate reproduction by the straggling adult lice. This might indicate the early steps in successful host breadth expansion. However, it is also possible that numphs can disperse between host species on their own. We also found

that the likelihood of survival on a novel host might be directly driven by specific eco-morphological adaptation to escape from host defense in ischnoceran lice.

We originally predicted that straggling events would happen during nesting and therefore would be positively related to host density in mixed colonies and the nearby (within 10m of sampled nest) presence of the typical host of the straggling lice. We did not find significant effects of distance to the nearest nest, number of conspecific nests, or number of heterospecific nests on the presence of straggling lice. We had seven cases where it was possible to test the relationship of the nearby (within 10m of the sampled nest) presence of alternate hosts with cases of straggling lice and the relationship was not significant. Thus, we suggest straggling events may be happening during any physical contact between host species, e.g. landing and bumping into other hosts, roosting together, or kleptoparasitism by frigatebirds. Furthermore, the typical (original) host was present on the island for a significant proportion of straggling cases, further supporting that the "jump" to an atypical host happens within or near the specific island. Most of the straggling ischnoceran lice corresponded to *Pectinopygus fregatiphagus* or *P. gracilicornis* (Table 2), which infect great and magnificent frigatebirds respectively, and most of these lice were found on red-footed boobies. Moreover, most of the *Colpocephalum* amblyceran lice that commonly infect frigatebirds were found on red-footed boobies as well (Table 3). Frigatebirds are kleptoparasites that harass other birds to steal their catch (del Hoyo et al. 1992). Observations during our field work suggest that among the

three booby species considered in this study, the most heavily parasitized by frigatebirds are red-footed boobies, which are the smallest of the three booby species (see also Le Corre and Jouventin 1997). Specifically, the way frigatebirds harass other birds is by pecking and plucking feathers from above while both are in flight (Osorno et al. 1992); during these "bumping" events it is likely that parasites can fall to the bird being parasitized by the frigatebirds. This may also explain why the amblyceran *Colpocephalum* spp showed higher percentage of straggling than ischnocera *Pectinopygus*. The ischnoceran lice are adapted for strong attachment to the host feathers and considered much less mobile than the amblyceran lice. Thus it is likely that during strong physical interactions amblyceran lice are more easily dislodged than ischnoceran lice that are firmly attached to the host feathers (see also Johnson et al. 2011).

There were few cases in which the typical host of the straggling lice was not found on the same island where the host was sampled. Specifically we found one Nazca booby sampled on Daphne Major that had *Pectinopygus sulae*, which is typically found on red-footed boobies, and two cases of magnificent frigatebirds, one that had *P. sulae* and other that had *P. gracilicornis* (which typically infects great frigatebirds). Both hosts, red-footed booby and great frigatebird, were not found in Daphne Major during our fieldwork nor have they been reported as present on the island (Swash and Still, 2005; Valle C. *personal communication*). Daphne Major is a small island in the middle of the archipelago, separated by ~10km from North Seymour, where there is another large colony of

magnificent frigatebirds sympatric with a colony of great frigatebirds (Anderson 1989; Valle C. *personal communication*, observations from this study). There are no studies on the connection between these colonies, but it is likely that highly vagile birds such as magnificent frigatebirds move between these nearby islands. Thus, the great frigatebird lice found on a magnificent frigatebird on Daphne Major may have come originally from a great frigatebird from North Seymour. More intriguing are the cases where we found *P. sulae*, which typically infects red-footed boobies, on a Nazca booby individual from Daphne Major. Nazca boobies and red-footed boobies overlap on several islands (Darwin, Wolf, Genovesa and San Cristobal), and the closest breeding colony of red-footed boobies is on Genovesa, which is ~85km away from Daphne Major. Genetic evidence suggests that red-footed boobies and Nazca boobies move significantly within the archipelago (Levin et al. 2012; Baiao and Parker *unpublished data*). Thus, the straggling lice may have been acquired during these movements.

Besides the presence of the typical host on the island, the other factor that significantly explained the observed straggling events in ischnoceran lice relates to the eco-morphology of lice attachment. Bush et al. (2006) and Johnson et al. (2005) documented how lice bigger than the space between wing feather barbs had lower survivorship than parasites the same width or smaller than this space. We found that straggling events happen significantly more often if the straggling louse is smaller than the typical parasite of a given host, or at least we are more likely to detect such straggling events. There is the possibility that parasites

bigger than the typical parasite have a similar rate of straggling, but they do not survive long enough to be detected. However, even if this was the case, parasite size is still an important component of straggling and eventual host switching. Parasites on the upper extreme of Harrison's rule, that is the largest bodied parasites found on the largest bodied hosts in the community, may be at an evolutionary dead end, where they cannot effectively survive on or successfully colonize any other host in the community. Thus, such parasite species are at greater risk of co-extinction with their host (Koh et al. 2004). The relationship between feather structures and the way amblyceran lice attach to their hosts and avoid death during preening is less well understood than for the ischnoceran lice (Johnson et al. 2005). It is generally believed that amblyceran lice burrow and run through the feathers or entangle themselves in the downy feathers closer to the host body. Frigatebirds when compared to boobies have overall fewer feathers and fewer inner downy feathers (*personal observation*), but they also differ in their feeding behavior. Unlike boobies, frigatebirds do not plunge dive. The *Colpocephalum* of frigatebirds likely could not survive the dislodging forces during plunge diving of boobies. Therefore, if individual *Colpocephalum* individuals straggle to boobies (particularly red-footed boobies) they would likely be removed by plunge diving. Thus, any *Colpocephalum* found on boobies might have been recently acquired during the approach to the island (and consequent harassment by frigatebirds).

A question in studies that analyze extensive samples of ectoparasitic lice has been how to define a straggler versus a successful host-switch or host-breadth expansion (Rosza 1993; Whiteman et al. 2004). Evidence of reproduction on an atypical host is a cutting point between straggling and successful host-breadth expansion. We found evidence of nymphs from a *P. gracilicornis* on a Nazca booby together with adults of the same species, which might be suggestive of presence of a breeding population of this parasite species on this host individual. However, it is also possible that nymphs may straggle to a host, so evidence of reproduction needs to be documented in more detail. An overall prevalence of straggling lice of ~1% suggests that these parasites can often end up "on the wrong host". One proposed speciation mechanism through host-switching starts with a population of the parasite species colonizing a novel host, expanding its host-breadth, and then due to lack of gene flow or differential selection diverging from the original species (Clayton and Johnson 2003; Rosza 1993). Moreover, for a successful host breadth expansion and later speciation, the transmission of this emerging parasite lineage to subsequent host generations is fundamental, followed by limited secondary contact with the original parasite population. Parasite populations are fragmented and have a relatively high risk of extinction (Nieberding and Olivieri 2007); when the host dies the whole parasite population resident on that host effectively goes extinct (unless it is a mobile parasite and/or with free living phases). Transmission to other individuals, in the case of parasites, can be vertical (to offspring) or it is

possible that it might be horizontal through social interactions such as during mating or territorial disputes (Whiteman and Parker 2004; Clayton et al. 1992). Horizontal transmission might be limited by the presence of the typical parasite on the specific host (Bush and Malenke 2008; Johnson et al. 2009; Johnson et al. 2011). Thus, parasite-free recently hatched chicks would be colonized by whichever parasite species is found on their parents. Then depending on the population size, isolation of the population, and stochastic events (e.g. death of hosts), something that started as a straggling event that established a breeding population on the novel host may lead to the displacement of the original typical parasite and by isolation from the source population it can lead to parasite speciation (Clayton and Johnson 2003; Johnson et al. 2002a). This means such events are often geographically restricted and therefore it explains cases where parasite distribution differs across host range (Price et al. 2003). Moreover, this suggests that parasite diversity and specificity is maintained by stochastic events during transmission, where the most common parasite is the one that is transmitted to the next generation and across individuals.

Parasites depend on their host to survive and therefore their evolutionary history and survival through time are deeply intertwined with that of the host. In this study we have analyzed how parasite diversity might be generated and maintained by evolutionary cases of host-switching, given current patterns of specificity. This process is driven by the interaction between hosts and potential

host species and the transmission of the most common parasite lineage or species to the next host generation and across conspecific individuals.

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Table 1. Summary of typical host-parasite associations. In parenthesis is indicated the overall sample size of each host and parasite species.

HOST	ISCHNOCERA	AMBLYCERA
great frigatebird (*Fregata minor*) – (138)	*Pectinopygus gracilicornis* (1,505)	*Colpocephalum angulaticeps* (914)
magnificent frigatebird (*F. magnificens*) - (27)	*P. fregatiphagus* (405)	*C. spineum* (56)
Nazca booby (*Sula granti*) – (122)	*P. annulatus* (1,195)	
blue-footed booby (*S. nebouxii*) – (72)	*P. minor* (763)	
red-footed booby (*S. sula*) – (77)	*P. sulae* (1,055)	

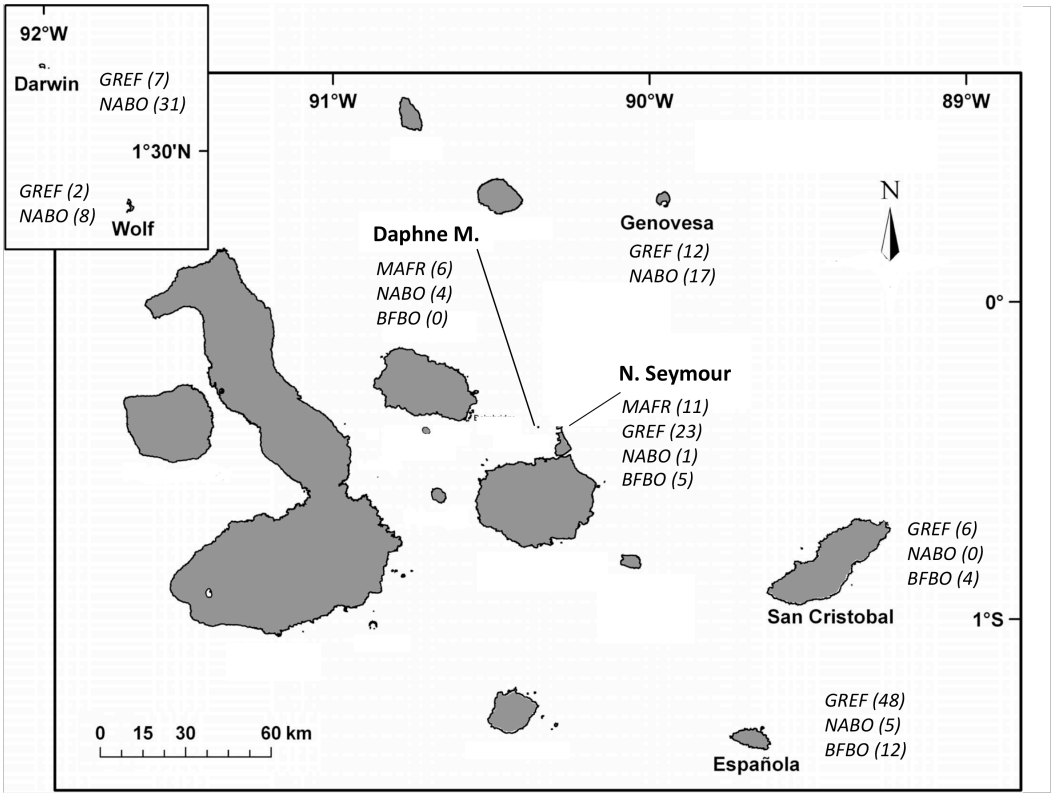
Table 2. Summary of straggling ischnoceran lice, showing the number of hosts with straggling lice on them in each island and, in parenthesis, the number of *Pectinopygus* parasites found on each host in each island and its species identity. PFREG = *P. fregatiphagus*, PGRA = *P. gracilicornis*, PMIN = *P. minor* and PSUL = *P. sulae.*

	Sula granti	*Sula nebouxii*	*Fregata magnificens*	TOTAL
Darwin	3 (2 PSUL, 1 PGRA)			3 (2 PSUL, 1 PGRA)
Wolf	3 (5 PSUL)			3 (5 PSUL)
Genovesa	4 (4 PSUL, 1 PGRA)			4 (4 PSUL, 1 PGRA)
Daphne M.	1 (2 PSUL)		3 (1 PSUL, 1 PMIN, 1 PGRA)	4 (3 PSUL, 1 PMIN, 1 PGRA)
N. Seymour	1 (3 PGRA)	2 (2 PFRE)		3 (3 PGRA, 2 PFRE)
TOTAL	12 (13 PSUL, 5 PGRA)	2 (2 PFRE)	3 (1 PSUL, 1 PMIN, 1 PGRA)	17 (14 PSUL, 6 PGRA, 2 PFRE, 1 PMIN)

Table 3. Summary of straggling amblyceran lice, showing the number of hosts with straggling lice on them in each island and, in parenthesis, the number of *Colpocephaulm* parasites found on each host in each island and its species identity. CANG = *C. angulaticeps*, CSPI = *C. spineum*.

	Sula granti	*Sula nebouxii*	*Sula sula*	*Fregata magnificens*	TOTAL
Wolf			1 2 CANG		1 2 CANG
Genovesa			1 1 CANG		1 1 CANG
Española		1 1 CANG			1 1 CANG
S. Cristobal		1 1 CANG	3 3 CANG		4 4 CANG
Daphne M.				1 2 CANG	1 2 CANG
N. Seymour	1 3 CANG	2 1 CANG, 1 CSPI			3 4 CANG, 1 CSPI
TOTAL	1 3 CANG	4 3 CANG, 1 CSPI	5 6 CANG	1 2 CANG	11 14 CANG, 1 CSPI

Figure 1. Map of the study area indicating the local host community composition and the number of hosts sampled in each island.



Chapter IV

Haemoproteus iwa distribution explained by ectoparasitic lice phylogenetic relationships

Unpublished manuscript: J. L. Rivera-Parra, I. I. Levin and P. G. Parker. *Haemoproteus iwa* distribution explained by ectoparasitic lice phylogenetic relationships

Abstract

Although both species of frigatebirds from the Galapagos Archipelago show evidence of long-term population isolation, they share hemoparasites with frigatebirds from other parts of their range. This study further explores the transmission distribution of *Haemoproteus iwa* using evidence from the phylogenetic relationships of ectoparasitic feather lice infecting the host species across their range. Our study suggests that only magnificent frigatebirds move outside the Galapagos Archipelago and potentially get infected with *Haemoproteus iwa* elsewhere, facilitating gene flow across parasite lineages preventing parasite divergence.

Key words: isolation, feather lice, frigatebird, Galapagos Archipelago, genetic differentiation

The Galapagos Islands have fascinated scientists since Darwin's time. Species such as *Geospiza* spp. finches and *Mimus* spp. mockingbirds are well - documented and clearly distinguishable endemics (Petren et al., 1999; Arbogast et al., 2006). However, recent molecular studies have documented the genetic isolation of Galapagos populations of highly mobile seabird species that had not been previously considered divergent from mainland populations (Hailer et al., 2011; Hailer et al., *personal communication*). Species that have a history of isolation may be more vulnerable when exposed to novel pathogens (Dobson and Foufopoulus 2001). Thus, it is critical to understand the routes of arrival and transmission dynamics for parasites already present in the archipelago to determine the likely modes of arrival of parasites of greater concern.

Hailer et al. (2011) found that magnificent frigatebirds (*Fregata magnificens*) populations from the Galapagos Archipelago are genetically distinct from conspecifics from elsewhere across their range. Similarly, Hailer et al. (*personal communication*) found evidence of isolation for the Galapagos population in the great frigatebirds (*Fregata minor*). Both species are strong flyers known for long flights across oceans (Dearborn et al. 2003) and even across ocean basins (across the Panama Isthmus; Hailer et al., 2011), but the information in their genes tells a story of long-term genetic isolation for the Galapagos populations. However, recent analysis on the *Haemoproteus* blood parasites infecting both species of frigatebirds in the Galapagos and elsewhere showed no differentiation in their lineages, suggesting gene flow between

parasite populations from the Galapagos Archipelago frigatebirds and parasite populations from elsewhere in the world (Levin et al., 2011). *Haemoproteus iwa* parasites in Galapagos frigatebirds are most likely vectored by the hippoboscid fly *Olfersia spinifera* (Levin et al., 2011; Levin and Parker, 2012). Such results suggest that frigatebirds from the Galapagos Archipelago might move outside the islands, where they exchange ectoparasites, but are philopatric breeders (Levin and Parker, *in press*).

This study further explores the distribution of *Haemoproteus iwa* using evidence of phylogenetic relationships in obligate ectoparasitic feather lice (Phthiraptera : Ischnocera) from the Galapagos frigatebirds in relation to conspecifics from elsewhere in the world. Specifically, we analyzed theischnoceran louse *Pectinopygus gracilicornis,* which infects great frigatebirds (*Fregata minor*; Price et al., 2003; Rivera-Parra et al., *submitted*), and *Pectinopygus fregatiphagus*, which infects magnificent frigatebirds (*F. magnificens*; Price et al., 2003; Rivera-Parra et al., *submitted*). We hypothesized that if the Galapagos frigatebirds have close interactions (e.g. roosting together, kleptoparasitizing the same non-frigatebird species) with conspecifics from elsewhere in their range, it is likely that some parasites would "jump" between hosts, thus preventing genetic divergence in parasite species. The objective of this study was to further explore the distribution of *Haemoproteus iwa* between the Galapagos archipelago and elsewhere.

We sampled 27 magnificent frigatebirds and 138 great frigatebirds on seven islands across the archipelago, representing the major breeding colonies of both species. To sample the ectoparasites we used a modified dust ruffling-protocol using pyrethrin-based flea powder (Walther and Clayton, 1997; Rivera-Parra et al., *submitted*). Results on specific parasite loads are published elsewhere (Rivera-Parra et al., *accepted for publication*). We stored collected parasites in 95% ethanol for later identification and DNA extraction. We followed the voucher method for DNA extraction (Cruickshank et al., 2001) using a Macherey Nagel Tissue extraction kit (Macherey-Nagel, CO., Düren, Germany). We followed the kit's extraction protocol with the following modifications: initial cut between the thorax and the head of individual lice, whole body incubation in buffer with 20μl of proteinase K for 72 hours and two sequential elutions, each with 20μl of warm buffer (~70°C).

We amplified a 300bp fragment of the mitochondrial gene *cytochrome oxidase subunit 1* (COI). We used the primers L6625 (5'-COG GAT CCT TYT GRT TYT TYG GNC AYC C-3') and H7005 (5' –CCG GAT CCA CAN CRT ART ANG TRT CRT G-3'; Hafner et al*.,* 1994), in a 25μl PCR reaction that included 1μl total genomic DNA, 1X $MgCl_2$ free Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.08mg/mL of BSA and 0.625 units of DNA Polymerase. The specific thermal cycling was initial denaturation at 94°C for 2min, then 35 cycles of: 94°C for 30s, 46°C for 30s and 72°C for 30s, and then a final extension at 72°C for

7min. The sequencing reaction was a 9μl reaction using BigDye terminator v3.1 cycle sequencing kits (Applied Biosystems, Carlsbad, CA); specifically the sequencing reaction included 2 μl of BigDye Terminator buffer, 2 μl of 1mM forward or reverse primer, 1 μl of Big DYE and 3 μl of deionized sterile water. Sequencing products were run in an ABI (3100) automated sequencer. The sequences were assembled using SeqManII v. 4 (DNASTAR, Inc.) and then aligned using Clustal W (Larkin et al., 2007) part of MEGA v5.05 (Tamura et al., 2011; this software was used throughout the rest of the phylogenetic analysis). We tested for the best fitting evolutionary model, finding T92 + I as the best fitting one. Then we constructed maximum likelihood phylogenetic trees with 1000 bootstrap pseudo-replications. We estimated within-group mean genetic distance and between-groups mean genetic distance (Galapagos parasites vs. reference sequences from elsewhere) using the best fitting evolutionary model (T92+I). We sequenced a total of 35 *Pectinopygus fregatiphagus* individuals and 168 *P. gracilicornis* from the Galapagos Archipelago (sequences deposited in GenBank with accession numbers XXXXXXX). To test for isolation of the Galapagos frigatebirds' parasites, we used sequences of *Pectinopygus fregatiphagus* found on a magnificent frigatebird (*F. magnificens*) from Louisiana, USA; and one individual of *Pectinopygus gracilicornis* found on a great frigatebird (*F. minor*) from Hawaii, USA (GenBank accession numbers: *P. gracilicornis* DQ482969, *P. fregatiphagus* DQ489433; Hughes et al. 2007). These reference lice sequences are from the same geographical areas (i.e., Hawaii and Louisiana) where Levin et

al. (2011) sampled and sequenced *Haemoproteus iwa,* finding no divergence among parasite lineages.

The phylogenetic analysis shows a highly supported distinction between *Pectinopygus gracilicornis* from great frigatebirds in Galapagos from the reference Hawaii sequence*;* in the case of *Pectinopygus fregatiphagus* from magnificent frigatebirds, however, there is no support for distinguishing the parasites from Galapagos from the reference Louisiana sequence. The genetic differentiation in the case of *Pectinopygus fregatiphagus* from Galapagos, when compared to the sequence from an individual from Louisiana, is 0.6% ± 0.1% (95%CI) with a mean genetic distance within Galapagos of 0.4% ± 0.03% (95%CI); whereas for *Pectinopygus gracilicornis* there was a divergence of 10.1% ± 0.3% (95%CI) between Galapagos' parasites and the reference sequence from an individual from Hawaii (in the same Pacific Ocean basin as Galapagos); the mean genetic distance for *P. gracilicornis* collected within Galapagos was 0.1% ± 0.001% (95%CI).

Our results suggest that the *Pectinopygus gracilicornis* infecting great frigatebirds in Galapagos are isolated from at least some parasites elsewhere, whereas the *Pectinopygus fregatiphagus* infecting magnificent frigatebirds might not be isolated from *P. fregatiphagus* from other parts of the world. Thus, our results support the genetic isolation of the great frigatebirds in Galapagos from

populations of the rest of the world (Hailer et al., *personal communication*). On the other hand, our results suggest that the magnificent frigatebirds move outside the Galapagos Archipelago and interact with conspecifics from elsewhere probably during movements in the non-breeding season, as suggested by Levin et al. (2011). These interactions between Galapagos and non-Galapagos magnificent frigatebirds favor gene flow across ectoparasitic lice, preventing their divergence, contrary to what was observed in the lice found on great frigatebirds in of the Galapagos, where lack of host interactions across their range may explain such a significant parasite divergence.

Levin et al. (2011) proposed that both frigatebird species are moving outside the Galapagos Archipelago to explain their findings of shared haemoparasites between both species of frigatebirds from Galapagos with frigatebirds from other parts of their ranges (including Hawaii, the Gulf of Mexico and the Atlantic Ocean basin). Our study further clarifies the distribution of *Haemoproteus iwa* (Levin et al., 2011) across its range, suggesting that the magnificent frigatebirds from Galapagos move outside the archipelago and therefore act as link between Galapagos and elsewhere, carrying lice and hemoparasite lineages across its range. Furthermore, the transmission of *Haemoproteus iwa* would happen within Galapagos (and in other parts of the world) by the effect of the hippoboscid fly *Olfersia spinifera* infecting both species of frigatebirds (Maa, 1969; Levin et al., 2011; Levin and Parker, 2012).

This study contributes to a better understanding of how parasites can arrive to Galapagos. Specifically, it has pointed to the magnificent frigatebirds as potential carrier of pathogens from other parts of the world to the seabird community of the Galapagos Islands, despite evidence of their genetic isolation from birds that breed outside of the Galapagos.

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