

# Biogeographical patterns and co-occurrence of pathogenic infection across island populations of Berthelot's pipit (*Anthus berthelotii*)

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**Abstract** Pathogens can exert strong selective forces upon host populations. However, before we can make any predictions about the consequences of pathogen-mediated selection, we first need to determine whether patterns of pathogen distribution are consistent over spatiotemporal scales. We used molecular techniques to screen for a variety of blood pathogens (avian malaria, pox and trypanosomes) over a three-year time period across 13 island populations of the Berthelot's pipit (*Anthus berthelotii*). This species has only recently dispersed across its range in the North Atlantic, with little subsequent migration, providing an ideal opportunity to examine the causes and effects of pathogenic infection in populations in the early stages of differentiation. We screened 832 individuals, and identified two strains of *Plasmodium*, four strains of

*Leucocytozoon*, and one pox strain. We found strong differences in pathogen prevalence across populations, ranging from 0 to 65%, and while some fluctuations in prevalence occurred, these differences were largely stable over the time period studied. Smaller, more isolated islands harboured fewer pathogen strains than larger, less isolated islands, indicating that at the population level, colonization and extinction play an important role in determining pathogen distribution. Individual-level analyses confirmed the island effect, and also revealed a positive association between *Plasmodium* and pox infection, which could have arisen due to dual transmission of the pathogens by the same vectors, or because one pathogen lowers resistance to the other. Our findings, combined with an effect of infection on host body condition, suggest that Berthelot's pipits are subject to different levels of pathogen-mediated selection both across and within populations, and that these selective pressures are consistent over time.

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Species–area relationship

## Introduction

Pathogens—disease-causing organisms—play a vital role in the ecology and evolution of their hosts. In wild animal populations, pathogens can affect individual fitness in a number of ways, such as increasing predation risk, reducing survival and reducing reproductive output (Anderson and May 1979; Gulland 1995; Johnson et al. 2008; Møller and Nielsen 2007). These effects can be observed at higher organizational levels, with pathogens playing a decisive role in host population dynamics and range distributions (Anderson and May 1981; Hudson et al. 1998; Ricklefs

2010), driving genetic variation (Acevedo-Whitehouse et al. 2003; Ortego et al. 2007; Spurgin and Richardson 2010) and sexual selection (Hamilton and Zuk 1982). Understanding how patterns of pathogen-mediated selection vary across populations may therefore provide new insights into the mechanistic processes behind adaptation and natural selection. However, before we can investigate how patterns of pathogen-mediated selection operate across populations, we first need to establish how and why pathogen regimes vary over spatiotemporal scales. Yet, determining the causes and consequences of pathogen distribution is likely to be extremely difficult in most cases, as in any given system an enormous array of pathogens may be present, and many different environmental, ecological and physiological variables may all influence pathogen distribution.

Island archipelagos have been described as “natural laboratories” for ecological and evolutionary research, as they contain multiple populations in geographically discrete yet ecologically variable locations (Whittaker 1998). The simplified nature of island systems has meant that they have been particularly useful for host–pathogen association studies, as the pathogen fauna on islands is generally less diverse than on mainland systems (Alcaide et al. 2010; Dobson 1988), simplifying analyses. Moreover, island archipelagos provide an opportunity to tease apart the different factors governing pathogen distribution across populations. In a scenario where host–pathogen associations are replicated across islands, “island effects” may arise due to differing ecological conditions, which may affect pathogen success due to differences in the availability of a specific habitat or vector for the pathogen (Apanius et al. 2000). Alternatively, differences in pathogen community composition between islands may occur as a result of temporal patterns and fluctuations in pathogen colonization and extinction, independent of island ecology (Fallon et al. 2004). For vector-borne pathogens, colonization and extinction are expected to play an especially important role, as the concurrent presence of both pathogen and vector is required for transmission. In addition to island effects, pathogen distribution may be constrained by factors related directly to the host. If the distribution of pathogens was determined solely by that of the host, one would expect the pathogen distribution to be homogeneous over the host’s range, even across islands (Apanius et al. 2000). Within-host factors such as age, sex, host behaviour or immune competence (McCurdy et al. 1998; Mougeot and Redpath 2004; Sol et al. 2003; Sorci 1996; Tompkins et al. 2010; van Oers et al. 2010) may also affect the observed patterns of infection. In reality, the most likely scenario is that the effects of hosts and islands will interact, resulting in unique outcomes of host–pathogen relationships, and therefore different selection regimes, across populations (Apanius et al. 2000; Fallon et al. 2003).

Spatiotemporal scale is a key factor to consider for host–pathogen association studies. For example, fine-scale ecological variations can result in marked differences in pathogen distribution within populations (Wood et al. 2007), meaning that effects of different biotic and abiotic variables on pathogen distribution may be obscured if the sampling regime is too coarse. Temporal variation in pathogen regimes, both seasonally and across longer time periods, also needs to be accounted for (Bensch and Åkesson 2003; Cosgrove et al. 2008; Fallon et al. 2004; Marghoob 1995). Without sampling over more than one time period, it is not possible to tell whether any observed patterns of spatial variation in the pathogen distribution represent consistent differences across populations, or whether they represent a “snapshot” of a rapidly changing pathogen community. This distinction is particularly important in the context of pathogen-mediated selection, as selection is only likely to produce observable differences among host populations if the pathogen regime is consistent within populations. Studies conducted over a range of spatiotemporal scales will provide the most comprehensive overview of what governs the pathogen distribution, and therefore variation in pathogen-mediated selection, in wild populations. However, such studies are, at present, few and far between.

In wild birds, the most widely studied pathogens are malarial species of the genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon* (Bensch et al. 2004; Eggert et al. 2008; Ishtiaq et al. 2008; Pérez-Tris et al. 2005; Ricklefs et al. 2008; van Riper et al. 1986; Vögeli et al. 2011). Malarial infection has been shown to have implications for host mate choice (Dale et al. 1996), parental investment (Wiehn et al. 1999), reproductive success (Dufva 1996), immune gene variability (Bonneaud et al. 2006; Westerdahl et al. 2005) and population or species persistence (van Riper et al. 1986). Other avian pathogens have received less attention in the ecological literature. For example, trypanosomes (*Trypanosoma* spp.) are also vector-transmitted blood pathogens that infect avian hosts worldwide, and are known to be detrimental to host growth and fitness (Apanius 1991). Yet the factors affecting trypanosome distribution within and across avian host populations have rarely been studied. Avian pox is a viral disease comprising numerous species in the genus *Avipoxvirus*. This pathogen is often fatal, and can be transmitted by vectors, directly by contact, or indirectly through contact with contaminated water (Ritchie 1995; Smits et al. 2005). Avian pox is being reported in an increasingly large number of wild bird species (Mondal et al. 2008; Saito et al. 2009; Smits et al. 2005; Tarello 2008; Van Riper and Forrester 2007), and has been highlighted as a threat to island bird populations (Kleindorfer and Dudaniec 2006; van Riper et al. 2002). Again, this pathogen has so far been largely overlooked in

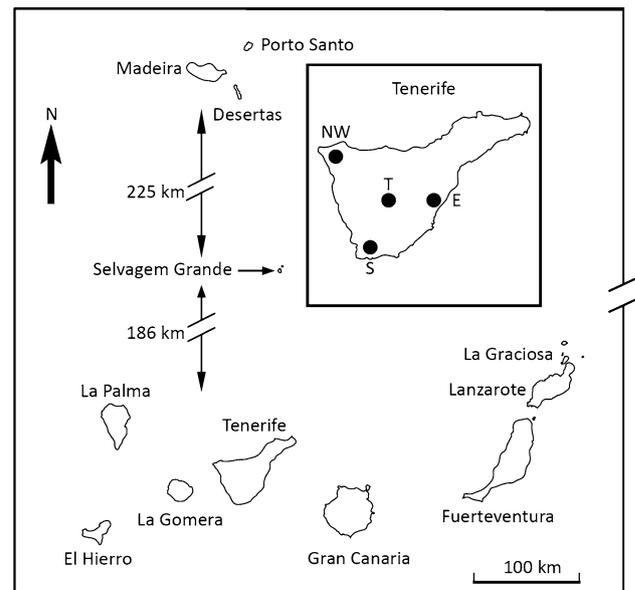
an ecological and evolutionary context (but see Carrete et al. 2009). Less well explored still is how these pathogens interact in wild populations. For example, avian malaria and pox have recently been shown to be positively associated in Hawaiian birds (Atkinson et al. 2005), yet the extent to which this occurs in other systems is not known.

Berthelot's pipit (*Anthus berthelotii*) is a passerine bird endemic to 13 island populations in the North Atlantic (Fig. 1). There is significant genetic structure across these populations, despite extremely low levels of genetic variation (Illera et al. 2007), suggesting that the pipit has only recently dispersed across its range, probably within the last 100,000 years (LGS, JCI and DSR, unpublished data), and that little migration occurs between populations. The islands differ greatly in size (ranging from approximately 2 to 2,000 km<sup>2</sup>) and isolation (Fig. 1). Thus, this species provides an ideal opportunity to study biogeographical patterns of pathogenic infection across populations. More generally, the pipit provides an excellent model for research into the adaptive and neutral processes involved in the early stages of differentiation. We use molecular techniques to screen for avian malaria, pox and trypanosomes in all populations over a 3 year time-period. We test two main hypotheses: first, that spatiotemporal variation in pathogen distribution can be explained by biogeographical factors (i.e., island size and isolation); and second, that there are significant associations between infection with different pathogens. We also explore whether, within an individual island, geographic structuring of pathogen infection occurs across subpopulations. The implications of our findings for host ecology and evolution are discussed.

## Materials and methods

### Study species and sampling

Berthelot's pipit is a small ( $\approx 16$  g), sedentary and insectivorous passerine that breeds on all of the main islands within the Atlantic archipelagos of the Canary Islands, Selvagens and Madeira (Illera 2007, Fig. 1). The pipit inhabits sparse xerophytic shrublands from sea level up to mountainous habitats at elevations of around 3,700 m. Representative samples (ca. 30 individuals) were obtained from each of the 12 main island populations. On Tenerife, a population occurs on an alpine plateau on the mountain of Teide more than 2,000 m above sea level. This population is separated from the rest of the Tenerife population by dense pine and laurel forests on the mountainsides, which the pipit does not inhabit. For this reason, Teide was sampled as a separate, thirteenth population. Samples were obtained during two field seasons, the first covering April 2005 (Selvagens), January–March 2006 (Canary Islands)



**Fig. 1** Distribution of Berthelot's pipits across the Atlantic islands. *Inset*: sampling locations of three coastal subpopulations (northwestern, southern and eastern), and the mountain of Teide (*T*) in Tenerife

and September 2006 (Madeira), and the second between January and April 2009 (for all islands). The three-year time period between screenings is likely to exceed the average lifespan of pipits (Coulson 1956), and thus the period over which selection can be expected to operate. Individuals were captured at multiple localities across each island to obtain a representative sample of the population as a whole. Nonetheless, fine-scale structuring of avian pathogens has been shown to occur (Wood et al. 2007). To explore this, in April 2010, one of the largest populations, Tenerife, was sampled more extensively, obtaining at least 30 individuals from three distinct subpopulations in the northwest, south, and east of the island, as well as from the top of Teide, which is located in the centre of Tenerife (Fig. 1). Note that the pipits are less common in the wetter northeastern peninsular of the island.

Birds were captured using spring traps baited with *Tenebrio molitor* larvae. Each bird was fitted with a unique numbered aluminium ring from the relevant Spanish or Portuguese ministries, or with a coloured plastic ring. Individuals were aged on the basis of feather moult pattern (Cramp 1985), and seven morphological measurements (wing length, tarsus length, bill length, height and width, head length and mass) were taken. Individuals were examined for pox lesions, which usually consist of growths on the feet, legs or face (Smits et al. 2005); where possible, small samples were taken with a sterile scalpel, diluted in 800  $\mu$ l of absolute ethanol in screw-cap microfuge tubes, and stored at room temperature. Blood samples (c. 40  $\mu$ l) were collected by brachial venipuncture, and likewise preserved in absolute ethanol.

## Molecular procedures

Genomic DNA was extracted from blood using a salt extraction technique (Richardson et al. 2001). DNA extraction techniques do not appear to affect the accuracy of malarial identification (Freed and Cann 2006). However, amplifying pox DNA from blood and lesions could potentially be problematic. In order to minimize the possibility of the DNA extraction technique affecting the amplification of pox DNA, we extracted DNA from lesions and from blood samples of birds on which lesions were found using both the salt extraction method and DNeasy blood and tissue kits (Qiagen), following the manufacturer's instructions. The quality of genomic DNA was visualized on 1.2% agarose gel after electrophoresis. Prior to pathogen screening, the extracted DNA was used to determine the sex of the birds using the molecular protocol described in Griffiths et al. (1998). Samples that did not produce strong amplicons for this sexing procedure were re-extracted or discarded. This ensured that only samples which contained amplifiable DNA went on to be used in the pathogen screening procedures.

Molecular methods were used to detect and characterize the strains of each pathogen. For avian malaria, a nested polymerase chain reaction (PCR) was used that amplifies a 422 bp fragment of the mitochondrial cytochrome *b* gene (Waldenstrom et al. 2004). For avian pox, primers developed by Lee and Lee (1997) were used, which amplify a 578 bp fragment of the *4b* gene. For both malaria and pox PCR reactions, the reagents and conditions described in Illera et al. (2008) were used. For trypanosomes, primers developed by Maslov et al. (1996) were used as well as the nested PCR reaction described in Sehgal et al. (2001), which amplifies a 326 bp fragment of the small subunit ribosomal RNA gene. To ensure the accuracy of the results, all samples were screened twice, and where results from two reactions were not concordant, samples were screened a third time. Given the low level of discrepancy between repeated PCRs (see “Results”), this was deemed to be a sufficient number of reactions. PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced on a PerkinElmer ABI PRISM 3700 automated sequencer. Only positive results that amplified twice and gave good sequences were counted as genuine infections. The quality of sequences was checked using FinchTV (<http://www.geospiza.com/finchtv/>), and sequences were aligned using BIOEDIT version 5.0.6 (Hall 1999), against homologous sequences published in the National Centre for Biotechnology Information (NCBI) genbank database. Malarial sequences were also searched for in the MalAvi public database for avian malaria sequences (Bensch et al. 2009) in order to identify if, when, and where strains had previously been found.

## Statistical analyses

At the island level, linear regression was used to test whether larger, less isolated islands harboured more pathogen species than smaller or more isolated ones. For the purpose of this analysis, individual pathogen strains were counted as “species” (Bensch et al. 2004), and thus pathogen “species richness” is, for our purposes, the number of pathogen strains found on an island. A common problem with this kind of analysis is that sampling effort might correlate with both island size and pathogen species richness (Walther et al. 1995). This is unlikely to be an issue in the present study, as sample size was roughly equal across all populations. Nonetheless, path analysis (Sokal and Rohlf 1995) was used to assess the direct and indirect effects of sampling effort (for details of methods, see Guégan and Kennedy 1996; Ishtiaq et al. 2010). Island isolation was calculated as both the total land area within a 100 km radius of the coastline of the focal island, and the distance to the nearest continental mainland (Europe or Africa), using Google Earth (<http://earth.google.com>). Island size was obtained from the Island Directory website (<http://islands.unep.ch/isldir.htm>). In all cases, least-squares regression was used on log-transformed variables. As some islands had no pathogens,  $n + 1$  was used for pathogen species richness (Cornell 1986; Hockin 1981).

Generalized linear models (GLMs) were used to test the factors affecting infection at the individual level. First, to test whether pathogen prevalence varied across space and time, GLMs were constructed for each pathogen using all individuals, with pathogen presence/absence as the response variable and island identity and year as explanatory variables. A second set of GLMs were then carried out to test for associations between pathogens while controlling for potentially confounding factors. For these models, only islands where pathogens were found in more than two individuals were included, as the presence of individuals from islands where pathogens are very rare or absent may confound results. Again, a separate GLM was carried out for each pathogen, this time including island, year, age and sex as explanatory variables. Presence/absence of infection with other pathogens were subsequently added as explanatory variables in order to test their independent explanatory power on likelihood of infection (Crawley 2007). For all GLMs, a quasi-binomial error structure was used, with a logit link function. To explore the effect of infection on body condition, mass was entered as the dependent variable in a general linear model (LM) with body size as a covariate—a preferable approach to using mass/length residuals (Green 2001). As an indicator of overall body size, the first component from a principal component analysis of the six morphometric measurements (excluding mass) was used (Freeman and Jackson 1990; Green 2001).

Age, sex, island, year and infection with each pathogen were entered into the LM as additional explanatory variables. All statistical tests were carried out in R version 2.12.2 (R Development Core Team 2008), and *P* values are two-tailed unless indicated otherwise.

## Results

### Molecular characterization and prevalence levels

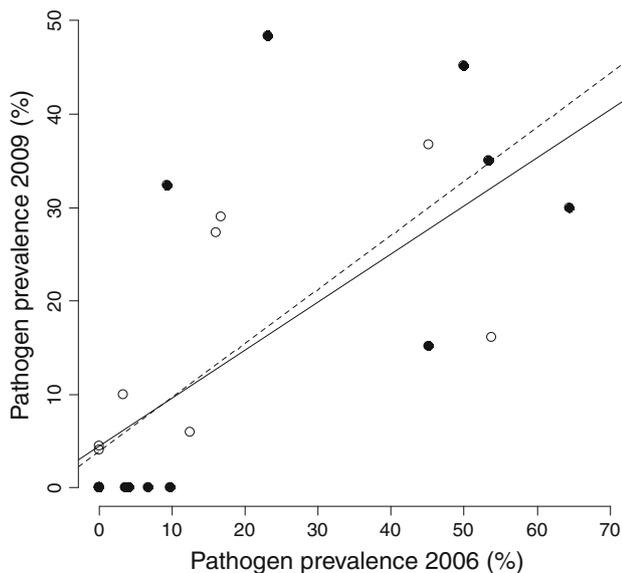
In total, 832 individuals were screened for pathogenic infection. We found 27 instances of nonconcordance between the two PCRs. In all but five cases, infection was confirmed by a third PCR. Those five cases were counted as negatives. In all cases, positive controls successfully amplified while negative controls did not. For avian malaria, no *Haemoproteus* was detected, but two *Plasmodium* strains were identified. These same strains were detected in Berthelot's pipits by Illera et al. (2008). In the present study, the most common *Plasmodium* strain, TF413, was found in all but two of the individuals infected with *Plasmodium*. The other strain, PAL282, was detected in two individuals—one from La Palma and one from El Hierro—in 2006, but was not found in any individuals in 2009. *Leucocytozoon* infection was rare (see below), though four different strains were detected. Three were identical in mitochondrial sequence to the previously described sequences RS4, REB11 and SYAT22 (Bensch et al. 2009), while the fourth strain, which we named ANBE1, has not previously been detected and appears to be unique to Berthelot's pipit. This strain has been submitted to GenBank (accession number JF803824.1). In the

2006 samples, *Leucocytozoon* infection was detected on three islands, with three strains on Porto Santo (REB11, RS4 and SYAT22), two (REB11 and ANBE1) on Gran Canaria, and one (REB11) on Tenerife. REB11 was the most common strain. In 2009, only REB11 was found, and only on Porto Santo. No evidence for trypanosome infection was found in any of our samples, despite the successful amplification of trypanosome DNA from positive controls. For avian pox, successful amplification was achieved in seven samples from 2006 (six from Porto Santo and one from Lanzarote), all of which gave identical sequences, apparently unique to Berthelot's pipit (Illera et al. 2008). We were unable to achieve amplifications from any 2009 samples (discussed later).

Considering all samples, *Plasmodium* prevalence was 19.2% in 2006 and 17.1% in 2009, *Leucocytozoon* prevalence was 0.02% in 2006 and 0.01% in 2009, and pox prevalence (determined from the presence of lesions) was 9.2% in 2006 and 11.2% in 2009. The low overall prevalence of *Leucocytozoon* was due to it being very rare or absent from all populations other than Porto Santo, where it was abundant in both years (Table 1). Indeed, the prevalence of all pathogens differed markedly across populations, ranging from 0 to 65% (Table 1). Temporal stability in pathogen abundance was observed: considering all populations, there was a strong correlation between population-level prevalence across the two sampling years for both malaria and pox (Pearson correlation: malaria,  $R = 0.71$ ,  $P = 0.007$ ; pox,  $R = 0.73$ ,  $P = 0.005$ , Fig. 2). The central and eastern Canary Islands, as well as Porto Santo, had consistently moderate to high levels of pathogens in both years. Other islands had consistently low prevalence levels, while three islands (Madeira, Deserta

**Table 1** Prevalence (percentage of individuals infected) of blood pathogen infection in 13 populations of Berthelot's pipit across Macaronesia

Archipelago	Island	<i>Plasmodium</i>		<i>Leucocytozoon</i>		Pox		Sample size	
		2006	2009	2006	2009	2006	2009	2006	2009
Madeira	Deserta Grande	0	0	0	0	0	0	31	4
	Madeira	0	0	0	0	0	0	33	29
	Porto Santo	64.5	30	25.8	13.3	45.2	36.7	31	30
Selvagens	Selvagem Grande	0	0	0	0	0	0	34	42
Canary Islands	La Graciosa	4.2	0	0	0	0	0	24	26
	Lanzarote	23.1	48.4	0	0	53.8	16.1	13	31
	Fuerteventura	50	45.2	0	0	16.7	29	12	31
	Gran Canaria	45.2	15.2	6.5	0	16.1	27.3	31	33
	El Teide	6.7	0	0	0	0	4	30	25
	Tenerife	9.4	32.4	3.1	0	12.5	5.9	32	34
	La Gomera	53.3	35	0	0	3.3	10	30	20
	La Palma	3.6	0	0	0	0	4.5	28	22
El Hierro	9.7	0	0	0	0	0	31	30	

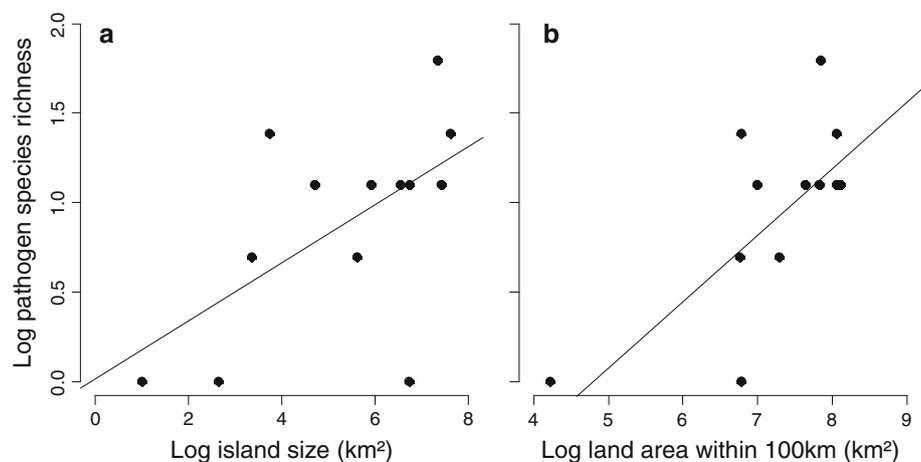


**Fig. 2** Temporal patterns of pathogen prevalence (percentage of individuals infected) across island populations of Berthelot's pipit. The filled circles and solid line represent malaria, and the open circles and dashed line represent pox. Both relationships are significant (Pearson's correlation: malaria,  $R = 0.71$ ,  $P = 0.007$ ; pox,  $R = 0.73$ ,  $P = 0.005$ )

Grande and Selvagem Grande) remained free of all screened pathogens in both years (Table 1). After removing populations with no parasites, this relationship was no longer significant (malaria,  $R = -0.22$ ,  $P = 0.69$ ; pox,  $R = 0.40$ ,  $P = 0.43$ ), suggesting that a degree of temporal fluctuation in prevalence occurred in populations with moderate to high pathogen levels (Fig. 2).

Pathogen species richness was positively related to island size (linear regression,  $r^2 = 0.35$ ,  $P = 0.034$ ; Fig. 3a), as well as to the total land area within a 100 km radius of the coastline ( $r^2 = 0.45$ ,  $P = 0.012$ ; Fig. 3b), suggesting that smaller and more isolated islands harbour fewer pathogens. It is possible that the latter of these two

**Fig. 3a–b** Pathogen species richness in island populations of Berthelot's pipit in relation to **a** island area and **b** land area within 100 km of the coast of each island (isolation). Both relationships are significant (linear regression: size,  $r^2 = 0.35$ ,  $P = 0.034$ ; isolation,  $r^2 = 0.45$ ,  $P = 0.012$ )



relationships was driven by a single point, Selvagem Grande, which is highly isolated (Fig. 1; bottom-left point in Fig. 3b). The regression was therefore performed again while excluding this population, and the relationship remained significant ( $r^2 = 0.36$ ,  $P = 0.039$ ). There was no significant relationship between pathogen species richness and distance to the nearest continental mainland ( $r^2 = 0.15$ ,  $P = 0.19$ ). Path analysis revealed no direct or indirect effect of sampling effort on pathogen species richness ( $P > 0.05$ ). There was no relationship between population level prevalence of *Plasmodium* or pox and island size or isolation (all  $P > 0.05$ ).

On Tenerife, a total of 217 samples were collected from the three coastal subpopulations and the mountain population of Teide, of which 62 were from 2006, 59 from 2009 and 97 from 2010. Moderate levels of *Plasmodium* infection were observed in the southern and eastern subpopulations, though this pathogen was rare on Teide and absent from the northwestern subpopulation (see Electronic supplementary material 1—ESM 1). There was even more pronounced geographic structuring of pox infection; high pox prevalence was observed in the southern subpopulation, but no pox infection was detected anywhere else other than in one individual on Teide (ESM 1). The only individual found to be infected with *Leucocytozoon* was from the eastern subpopulation.

#### Individual-level analyses

As *Leucocytozoon* infection was largely restricted to a single population, GLMs including all individuals were only carried out for pox and *Plasmodium*. For both pathogens, there was a significant effect of island identity on infection, but not one of year (Table 2a). There was a significant island by year interaction for *Plasmodium*, and a near-significant interaction for pox (Table 2a). The second set of more detailed models revealed that infection with

**Table 2** Results of generalized linear models showing (a) the effect of island identity and sampling year on pathogen load across all populations of Berthelot’s pipit, and (b) within infected islands, the effect of other blood pathogens on the likelihood of infection after controlling for island, year, sex, and age

	<i>df</i>	Deviance	Residual deviance	<i>P</i>
<b>(a) All islands</b>				
Pox				
Null			531.21	
Island	12, 819	148.09	383.12	<b>&lt;0.001</b>
Year	1, 818	0.11	383.01	0.66
Island × year	12, 806	11.1	371.9	0.07
<i>Plasmodium</i>				
Null			796.7	
Island	12, 818	206.88	589.83	<b>&lt;0.001</b>
Year	1, 817	0.78	589.04	0.28
Island × year	12, 805	28.93	560.12	<b>&lt;0.001</b>
<b>(b) Infected islands only</b>				
Pox				
Null			405.26	
Island	5, 412	35.89	369.37	<b>&lt;0.001</b>
Year	1, 411	0.17	369.2	0.68
Sex	1, 410	0.003	369.2	0.96
Age	1, 409	0.76	368.43	0.39
<i>Plasmodium</i>	1, 408	10.71	357.72	<b>&lt;0.001</b>
<i>Plasmodium</i>				
Null			537.06	
Island	5, 412	16.51	520.55	<b>0.01</b>
Year	1, 411	0.14	520.41	0.71
Sex	1, 410	0.15	520.27	0.71
Age	1, 409	1.57	518.7	0.22
Pox	1, 408	10.85	507.85	<b>0.001</b>
<i>Leucocytozoon</i>				
Null			56.76	
Year	1, 57	3.093	53.667	0.069
Age	1, 56	5.578	48.09	<b>0.015</b>
Sex	1, 55	0.909	47.181	0.325
<i>Plasmodium</i>	1, 54	1.233	45.948	0.252
Pox	1, 53	1.367	44.582	0.227

Significant values (*P* < 0.05) are highlighted in bold

*Plasmodium* had a significant effect on pox infection, while controlling for age, sex, island, and year (Table 2b). This association was positive; pox prevalence was 30% in individuals with *Plasmodium*, compared to 17% in those individuals without *Plasmodium*. Similarly, infection with pox was associated with an increased likelihood of *Plasmodium* infection (Table 2b); prevalence of *Plasmodium* in individuals with pox was 52%, compared to 33% in individuals without pox. For *Leucocytozoons*, the individual-level analysis was restricted to individuals from Porto Santo (*n* = 60), the only island where it was found at

**Table 3** Results of a generalized linear model showing the effect of intra-island variation on pathogen load in a single population (Tenerife) of Berthelot’s pipit

	<i>df</i>	Deviance	Residual deviance	<i>P</i>
Pox				
Null			114.377	
Region	3, 214	27.466	86.911	<b>&lt;0.001</b>
Year	1, 213	0.677	86.234	0.226
Region × year	3, 210	6.026	80.209	<b>0.005</b>
<i>Plasmodium</i>				
Null			213.236	
Region	3, 213	47.03	166.206	<b>&lt;0.001</b>
Year	1, 212	3.662	162.544	<b>0.022</b>
Region × year	3, 209	5.009	157.535	0.067

Significant values (*P* < 0.05) are highlighted in bold

anything but very low levels. Here, we found no effect of *Plasmodium* or pox on infection while controlling for other variables (Table 2b). A GLM restricted to individuals from Tenerife confirmed the intra-island variation, with a highly significant effect of region on infection with both pox and malaria. In this analysis, there was a less strong but nonetheless significant region × year interaction for pox, and an effect of year for *Plasmodium* (Table 3).

Effects on body condition

Analyses of body condition were restricted to the six islands where pathogens were present in more than two individuals. There was a significant effect of both pox and *Plasmodium* infection on mass, while controlling for body size, age, sex, island and year (pox, *F* = 5.15, *P* = 0.024, *Plasmodium*, *F* = 6.32, *P* = 0.012, ESM 2). Infected individuals were, on average, heavier than uninfected individuals: mean ± S.D. mass for infected and uninfected individuals, respectively, was 16.9 ± 0.8 and 16.2 ± 0.7 g for pox, and 16.7 ± 0.3 and 16.2 ± 0.7 g for malaria.

Discussion

Our study is one of the first to examine the distributions of multiple pathogens over a range of spatiotemporal scales across populations of a wild animal. The evidence indicates that, in Berthelot’s pipit, there are strong population-level differences in pathogen distribution, and that pathogen species richness is related to island size and isolation. These broad differences in distribution were stable over the three-year time period of this study. However, across some of the islands where the pathogens were present, prevalence levels varied considerably over the two sampling periods. Within a single population, we observed marked

differences in pathogen presence and prevalence across subpopulations. Analysis at the individual level further supported the island effect, and we also detected a positive association between pathogens. Finally, pathogenic infection appeared to have an effect on the body condition of Berthelot's pipits.

Over the three-year time period of our study, which roughly corresponds to the lifespan of pipits (Coulson 1956), we observed a high degree of temporal stability in pathogen presence at the population level (Table 1). In all populations where a pathogen was observed in one year but not another, the pathogen occurred in less than three individuals in the infected year (Table 1). This suggests that our failure to detect them in both years may have been due to them being very rare and not picked up in our sample, rather than absent. In other words, pathogen load on some islands is consistently low (or zero), and consistently moderate to high on others. Little work has been done on the long-term temporal stability of avian pathogens, though recent evidence from Hawaii suggests that avian pox variants have been maintained in populations for over 100 years (Jarvi et al. 2008). Similarly, the presence of avian malarial lineages has been shown to be relatively stable within populations over periods of up to a decade (Fallon et al. 2004). Over these sorts of time periods, however, marked fluctuations in the prevalence of these pathogens are expected to occur (Fallon et al. 2004). This was the case in our study, where temporal shifts in prevalence did occur within a few of the populations where pathogens were present at moderate to high levels (Fig. 2). However, with only two sampling periods, we have to be cautious in interpreting the extent to which pathogen load varies over time. In order to do so more fully, long-term datasets, ideally from multiple populations, are now needed.

Island biogeography theory predicts that smaller, more isolated islands will exhibit lower species richness than larger, less isolated islands due to lower rates of colonization and higher rates of extinction (MacArthur and Wilson 1967). Biogeographic studies of pathogens have mostly considered hosts as the "islands" (Dritschilo et al. 1975; Kuris et al. 1980). However, island size itself may also affect patterns of pathogen distribution, though evidence for this in the literature is currently limited, and has yielded mixed results. In a recent study, Ishtiaq et al. (2010) examined species–area relationships in *Plasmodium* and *Haemoproteus* lineages infecting white-eyes (*Zosterops* spp.) in 16 southwest Pacific islands. Significant species–area relationships were found for *Plasmodium*, but not for *Haemoproteus*. In Darwin's finches (*Geospiza fuliginosa*), a positive relationship between pathogen (pox and ectoparasite) abundance, but not diversity, was observed (Lindström et al. 2004). In *Anolis* lizards, no relationship was found between island size, elevation or rainfall and the presence of malaria (Staats and Schall 1996). In our study,

we observed significant effects of both island size and isolation on pathogen species richness across islands. One would predict island size and isolation to be especially important for vector-borne pathogens, as screened for here, as transmission to the host requires both the pathogen and vector to be present at a given point in time. Nonetheless, our population-level data suggest that colonization and extinction may have roles to play in determining pathogen distribution in our study system, and provide an explanation for why patterns of pathogen distribution are temporally stable across populations.

Within a single island, Tenerife, we observed a high degree of structuring in pathogen distribution, suggesting that in addition to the observed island-level effects, intra-island level factors also play an important role. Recent evidence from blue tits (*Cyanistes caeruleus*) has shown that pathogen lineages can be restricted to defined spatial regions, and that changes of up to 50% in malarial prevalence can occur at distances of less than 1 km (Wood et al. 2007). Our study confirms that local spatial variation in host–pathogen systems can occur. It is difficult to speculate about how variations at the inter- and intra-island levels may interact. One possibility is that larger islands are more likely to contain within-population variation in the pathogen distribution, and higher pathogen species richness as a result. However, more fine-scale sampling is now needed to determine the factors underlying within-population spatiotemporal variation, the scale at which it occurs, and its effects on population-level distribution.

At the individual level, we detected a positive association between avian pox and *Plasmodium*. This is somewhat surprising, as one may expect to find a low number of individuals with multiple infections either because of competitive interactions between pathogens, or due to the potential fitness costs incurred to the host (Balmer et al. 2009; Beadell et al. 2004; Haukisalmi and Henttonen 1993). However, positive associations between pathogens can occur, and associations between avian malaria and pox have recently been detected in birds from Hawaii (Atkinson et al. 2005). There are a number of possible explanations for such findings. First, it could be that infection with one pathogen reduces host resistance and makes birds more susceptible to the other, or that a third, unknown pathogen makes the birds more susceptible to both malaria and pox. A number of pathogens, including malaria, are well known to have immunosuppressive effects, and this can often lead to positive associations between multiple pathogens (Cox 2001). Alternatively, the two pathogens could be transmitted by the same vector. This is also possible; for example, *Culex* mosquitoes have been demonstrated to transmit both pox and malaria to wild birds (Akey et al. 1981). Unfortunately, however, little is known about the distribution of invertebrate hosts across the North Atlantic

archipelagos, and less still is known about the relationships between pathogens and invertebrate hosts across this region. More research in this area is now needed (see, for example, Hellgren et al. 2008; Njabo et al. 2011). Finally, it could be that the two pathogens are restricted to the same areas, and that the observed effect has arisen from sampling over multiple subpopulations (i.e., some with both pathogens and some with neither). Our data from Tenerife suggest that the latter of these explanations is unlikely to be the case in Berthelot's pipits, as we found a subpopulation with only one of the two pathogens (ESM 1). Such a finding would, if anything, obscure positive associations. In contrast, there was evidence of a positive association between the two pathogens in the southern subpopulation, the only one in which both pathogens occurred ( $\chi^2 = 5.37$ ,  $P = 0.02$ ), suggesting that *Plasmodium* and pox co-occur on a very local scale.

Research into the impact of avian diseases on host body condition has generally shown that, as one may expect, infected individuals present poorer body conditions than uninfected individuals (Marzal et al. 2008; Valkiunas et al. 2006). However, in our study, we found the opposite: infected individuals had better body conditions than uninfected individuals. One possible explanation for this is that there is variation in both size and immunocompetence within populations (i.e. larger subpopulations are less immunocompetent due to higher investment in growth). Another possible explanation is that infection kills low-quality individuals, and that our sample consisted of the high-quality individuals that have been able to cope with infection. This is in line with the fact that we were, for the most part, unable to amplify pox DNA from the pox lesions we sampled, nor from the corresponding blood samples. Scars from pox lesions can last on birds for months (Ritchie 1995), making it possible that individuals in 2009 had retained lesions from a previous infection but were no longer infected. Alternatively, it may be that some of the pox-like lesions were caused by a different, unknown pathogen, although this seems highly unlikely given the similarity in appearance to the pox lesions observed in pipits by ourselves and others (Smits et al. 2005). Similarly, avian malaria can remain in bird blood at chronic levels for long periods of time after an initial, acute infection (Atkinson et al. 2001; Kilpatrick et al. 2006; Valkiunas 2005). If infection with pox and malaria does kill low-quality individuals, this implies that infection with the pathogens studied here confers severe fitness costs to the hosts. However, we cannot rule out the possibility that individuals with good body conditions are more susceptible to infection due to decreased immunocompetence, rather than the survivors of infection. An assessment of infection levels using qPCR (e.g. Knowles et al. 2010), as well as data on the

effects of pathogenic infection on survival and reproduction, would help to confirm fitness costs.

As Berthelot's pipit has only recently dispersed across its range, with little subsequent migration between populations (Illera et al. 2007), the differential levels of pathogenic infection observed are likely to constitute an important selective force for promoting differentiation across populations. Moreover, because spatial variation in the pathogen regime appears to be constrained, at least in part, by biogeographical factors, these differential selective pressures are consistent over time, at least at scales comparable to the lifespan of this species. This is an important point, as spatial variation in selection is only likely to produce detectable effects upon host populations if it is consistent over time. Thus, our findings provide a foundation for further research into the genetic, physiological and behavioural consequences of these differential selective pressures.

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