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Dual-pathogen etiology of avian trichomonosis in a declining band-tailed pigeon population

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ABSTRACT

The Pacific Coast band-tailed pigeon (*Patagioenas fasciata monilis*) is a migratory game bird of North America that is at risk for population decline. Epidemics of avian trichomonosis caused by upper digestive tract infection with *Trichomonas* spp. protozoa in these and other doves and pigeons of the United States are sporadic, but can involve tens of thousands of birds in a single event. Herein, we analyze the role of trichomonosis in band-tailed pigeon mortality and relate spatial, temporal and demographic patterns of parasite transmission to the genetic background of the infecting organism. Infections were most common in adult birds and prevalence was high in band-tailed pigeons sampled at mortality events (96%) and rehabilitation centers (36%) compared to those that were hunter-killed (11%) or live-caught (4%). During non-epidemic periods, animals were primarily infected with *T. gallinae* Fe-hydrogenase subtype A2, and were less often infected with either *T. gallinae* subtype A1 (the British finch epidemic strain), *T. stableri* n. sp. (a *T. vaginalis*-like species), or *Tritrichomonas blagburni* n. sp.-like organisms. Birds sampled during multiple epidemics in California were only infected with *T. gallinae* subtype A2 and *T. stableri*. The non-clonal etiology of avian trichomonosis outbreaks in band-tailed pigeons and the risk of spill-over to raptor and passerine species highlights the need for additional studies that clarify the host range and evolutionary relationships between strains of *Trichomonas* spp. in regions of trichomonosis endemicity.

1. Introduction

Trichomonas gallinae, the primary etiologic agent of avian trichomonosis, is a globally-distributed, flagellated protozoal parasite that can infect a wide range of bird species (Forrester and Foster, 2008). Severe disease is characterized by the development of proliferative caseous lesions of the upper digestive tract that can block the animal's esophagus or trachea, ultimately leading to starvation or suffocation and death (Cole, 1999; Stabler, 1954; Stabler and Braun, 1975). Some species of columbids (e.g. rock pigeons, *Columba livia*) are generally asymptomatic carriers of the parasite, while other species including mourning doves (*Zeneida macroura*), wood pigeons (*Columba palumbus*) and Pacific Coast band-tailed pigeons (*Patagioenas fasciata monilis*) suffer from sporadic mortality events involving hundreds or even tens of thousands of animals (Cole, 1999; Haugen and Keeler, 1952; Höfle et al., 2004; Stabler, 1947; Stabler and Herman, 1951; Stromberg et al., 2008; USGS, 2013; Villanùa et al., 2006). Trichomonosis also is an emerging infectious disease of passerine birds that most likely become exposed to *T. gallinae* at artificial food and water sources shared by doves and pigeons (Forzán et al., 2010; Neimanis et al., 2010; Zadravec et al., 2012). Finch trichomonosis emerged in Great Britain in 2005, causing significant decline in breeding populations of greenfinches (*Carduelis chloris*) and chaffinches (*Fringilla coelebs*) (Lawson et al., 2012; Robinson et al., 2010). In 2008, the epidemic strain of *T. gallinae* from Great Britain spread into southern Fennoscandia, causing multiple trichomonosis mortality events in greenfinches and chaffinches over a two year period (Lawson et al., 2011a; Neimanis et al., 2010). In 2007 and 2008, the Maritime Provinces of Canada identified multiple trichomonosis outbreaks involving purple finches (*Carpodacus purpureus*) and American goldfinches (*Carduelis tristis*) (Forzán et al., 2010). Raptor species that prey on infected birds are also susceptible to trichomonosis, and significant mortality due to *T. gallinae* infections in Cooper's hawk (*Accipiter cooperii*) and Bonelli's eagle (*Aquila fasciata*)

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nestlings have been reported (Boal et al., 1998; Real et al., 2000). Decline of the sparrowhawk (*Accipiter nisus*) population in Great Britain may be linked to trichomonosis acquired from passerine species, which make up a large proportion of their diet (Chi et al., 2013).

While our ability to detect trichomonad infections in birds has been simplified and, perhaps, improved in the last 20 years with the use of the InPouch™ TF culture device (BioMed Diagnostics, White City, OR) (Cover et al., 1994), our understanding of the demographic, environmental, genetic, and behavioral factors that shape pathogen transmission and bird susceptibility remains incomplete, especially across the vast range of avian species at risk worldwide. Risk factors under investigation include the use of artificial water and food sources, crop feeding and billing behavior, climate, bird density during congregation, bird migration, consumption of infected prey, and parasite species or genotype (Bunbury et al., 2007; Cole, 1999; Girard et al., 2014; Höfle et al., 2004; Lawson et al., 2011a,b; Robinson et al., 2010; Stabler, 1947, 1948; Stabler and Herman, 1951). The relative roles of these risk factors are expected to vary temporally, spatially, and between host species.

Genetic analysis of isolates infecting finches during outbreaks in Great Britain and Fennoscandia indicates that a single clonal strain of *T. gallinae* was responsible for widespread mortality in both regions (Lawson et al., 2011b). Epidemics in North American doves and pigeons are similarly hypothesized to be caused by particularly virulent strains of *T. gallinae* (Cole, 1999; Stabler, 1948; Stabler and Herman, 1951) and laboratory experiments in homing pigeons (*Columba livia*) and band-tailed pigeons support the notion that differences exist in the virulence of parasite strains (Stabler, 1948; Stabler and Braun, 1975). However, a rigorous analysis of parasite genetic background during epidemic and non-epidemic transmission cycles in wild columbids of North America that are at risk of disease has yet to be described. Preliminary analysis of a small number of trichomonad isolates from mourning doves and band-tailed pigeons of the western U.S. indicate that outbreaks in these species may actually involve more than one genetically distinct trichomonad species (Gerhold et al., 2008; Girard et al., 2014).

The Pacific Coast band-tailed pigeon (henceforth, "band-tailed pigeon") is a migratory upland game species distributed in coastal and inland coniferous forests between southeast Alaska and Baja California (USA) (Sanders, 2012). The North American Breeding Bird Survey, Mineral Site Survey, and other sources indicate that this population has been in decline for the past 40 years (Keppie and Braun, 2000; Sanders, 2012). In California, this decline is reflected in recent reductions in hunter take and restricted hunting opportunities (California Department of Fish and Wildlife, 2013). Epidemics of trichomonosis in band-tailed pigeons have been recorded since 1945, with morbidity and mortality involving thousands of individuals often in the wintertime or during spring migration (Cole, 1999; Stabler and Braun, 1979; Stromberg et al., 2008; USGS, 2013). In the present study, we sampled band-tailed pigeons and sympatric species in California for trichomonad infections using active, opportunistic, and syndromic surveillance techniques. We genetically characterized protozoa isolated in diseased and asymptomatic birds and analyzed spatial, temporal, and demographic patterns during epidemic and non-epidemic transmission periods.

2. Materials and methods

2.1. Study population

Surveillance of free-ranging band-tailed pigeons was conducted from February 1, 2011 to August 31, 2012 coinciding with two seasons of winter and spring band-tailed pigeon migration (December–April) and two seasons of band-tailed pigeon nesting and breeding (May–September). Included in the study were 475 band-tailed pigeons and 28 birds from three sympatric species: mourning doves, rock pigeons and California quail (*Callipepla californica*).

2.2. Live-caught and hunter-killed bird sampling

Band-tailed pigeons and occasionally mourning doves and California quail were trapped at backyard bird feeders between June and October of 2011, and between March and August of 2012 at 11 locations in California (supplementary data Fig. S1). Funnel entrance traps were placed on the ground under feeders where pigeons and doves regularly fed. Captured birds were removed from the trap and placed in a plastic game bird crate or cloth bag prior to sampling and processing. Each bird was examined physically for obvious signs of disease and clinical features of trichomonosis, and age and sex were recorded. Hunter-killed birds were sampled at check stations near Whiskeytown National Recreation Area, Shasta County and Chew's Ridge and Bottcher's Gap in Los Padres National Forest, Monterey County during the split northern (September) and southern (December) California band-tailed pigeon hunting seasons (supplementary data Fig. S1). Birds were aged and sexed, and were examined for obvious signs of disease.

All birds admitted to the study were assigned an 8-digit barcode beginning with CA0-. Bird capture, handling and sampling procedures were approved under the U.S. Fish and Wildlife Service Scientific Collecting Permit (No. MB191637-0) and the U.S. Geological Survey Federal Bird Banding Permit (No. 23539) issued to the University of California, Davis Wildlife Health Center.

2.3. Wildlife rehabilitation center sampling

Nine licensed wildlife rehabilitation centers throughout California were recruited to sample band-tailed pigeons admitted to their center during the study period, regardless of health status (supplementary data Fig. S2). Rock pigeons and mourning doves with oral lesions suggestive of trichomonosis also were sampled at centers. Birds were sampled for trichomonad parasites prior to treatment, euthanasia or release at the rehabilitation center. Intake and collection date, disposition, presence or absence of lesions in the oral cavity, age, sex, duration of stay and fate were recorded. Birds that died were submitted for post-mortem examination at either the California Animal Health and Food Safety Laboratory (CAHFS, 620 West Health Science Drive, Davis, California 95616) or the California Department of Fish and Wildlife (CDFW) Wildlife Investigations Laboratory (1701 Nimbus Road Suite D, Rancho Cordova, CA 95670). Six dead or dying band-tailed pigeons were submitted directly to the study by members of the public or CDFW biologists and were included in study-wide analyses in Sections 3.1 and 3.6. Their collection locations are shown in supplementary data Fig. S2.

2.4. Mortality event sampling

During winter and spring migration seasons of 2010–2011 and 2011–2012, rehabilitation centers, CDFW wildlife biologists and contacts living or working in band-tailed pigeon habitat were asked to report increased band-tailed pigeon morbidity and mortality. California Department of Fish and Wildlife responded to these reports and collected carcasses for post-mortem examination as described above. Carcasses were kept at 4 °C until shipped and oral swabs were collected when carcasses were in fresh condition. Necropsy and histologic examination of tissues were conducted on all birds in fresh condition and cause of death and co-morbid conditions were determined by a veterinary pathologist. Identification

of caseonecrotic lesions in the upper digestive tract was suggestive of trichomonosis, and wet-mount and/or immunohistochemistry (see Girard et al., 2014) confirmed the post-mortem diagnosis of trichomonosis. Lesion tissues were sampled and frozen at −80 °C.

2.5. Parasite isolation

Live and recently dead birds were sampled for trichomonad infection in the oral cavity using the InPouch™ TF culture device (BioMed Diagnostics, White City, OR, USA), and positive cultures were subcultured as previously described (Gerhold et al., 2008; Girard et al., 2014). InPouch™ TF culture devices inoculated in the field were kept at 25 °C during transport to the laboratory or at ambient temperature if shipped overnight.

2.6. Molecular characterization

All DNA extractions were carried out on tissues (⩽25 mg) and trophozoite cultures using the DNeasy Blood and Tissue kit (Qiagen, USA) as previously described (Girard et al., 2014). Published primers were used to amplify the 5.8S rRNA region and flanking internal transcribed spacers (ITS1/5.8S/ITS2) and the hydrogenosomal Fe-hydrogenase region of trichomonadid protozoa (Ecco et al., 2012; Felleisen, 1997; Lawson et al., 2011a). Primers TFR3 and TFR4 (Felleisen et al., 1998) were used to test for the presence of *Tritrichomonas foetus* in bird samples in which tritrichomonad DNA was amplified using primers TFR1 and TFR2 (Felleisen, 1997). Purified PCR products (QIAquick PCR Purification Kit) were sequenced by the DNA Sequencing Facility at UC Davis (Davis, CA). Forward and reverse sequences were assembled and aligned as previously described (Girard et al., 2014). Pairwise genetic distance values were determined through uncorrected-*p* distance matrices generated in PAUP* (Sinaeur Associates Inc.) using prepared alignments of 269 bp for ITS1/5.8S/ITS2 and 763 bp for Fe-hydrogenase. Posterior sets of phylogenetic trees (PSTs) were generated using either MrBayes in Geneious Pro v.5.3.4 or MrBayes v3.1 run for 500,000–2,000,000 generations, sampled every 1000 (Huelsenbeck and Ronquist, 2001). Markov chain Monte Carlo convergence of PSTs was confirmed using the Are We There Yet software (Nylander et al., 2008) and 25–50% of trees were discarded from the initial burning-in period. For the ITS1/5.8S/ITS2 alignment, the best fit nucleotide substitution model selected by MrModelTest2 v2.3 (Nylander, 2004) was general time reversible with gamma distributed rate variation among sites. For Fe-hydrogenase, we applied the codon-based model of nucleotide substitution in MrBayes. All nucleotide sequences generated from isolates in this study have been deposited in GenBank http://www.ncbi.nlm.nih.gov/genbank/: KC215387–KC215393 (ITS/5.8S/ITS2), and KC244200–KC244203, KC249971, KC660123–KC660128 (Fe-hydrogenase).

2.7. Statistical analysis

Statistical analyses including Pearson's chi-squared test, Fisher's exact test and two-sample proportion tests were performed using Stata 11.0 (StataCorps, 2011) and values of $P < 0.05$ were considered statistically significant. Maps were prepared using ArcGIS v.10 (ESRI, Redlands, CA, USA).

3. Results

Throughout the 18-month surveillance period spanning from February 1, 2011 to August 31, 2012 we sampled 475 band-tailed pigeons and 28 birds from three sympatric species for trichomonad infection. Trichomonad infection was confirmed in 21.5% (102/475) of band-tailed pigeons that were live-trapped, hunter-killed, hospitalized, or involved in mortality events. Three additional birds had clinical disease but were either test-negative or not laboratory-tested. Infection was common among birds sampled at mortality events (55/57, O.R. 205.96) and admitted to rehabilitation centers (30/77, O.R. 2.70), whereas live-caught (11/268, O.R. 0.05) and hunter-killed (6/54, O.R. 0.40) birds were rarely infected. Infections were significantly associated with disease ($P < 0.001$). Among the 448 laboratory-tested band-tailed pigeons from all surveillance activities, adults were more likely to be infected than juveniles (86/314 adults vs. 15/134 juveniles, $P = 0.0002$). Prevalence of infection was similar among males (51/221, 23%) and females (40/138, 29%) in this study.

3.1. Live-caught birds

A total of 288 birds (268 band-tailed pigeons, 3 California quails, and 17 mourning doves) were live-trapped during the band-tailed pigeon breeding and nesting seasons of 2011 and 2012 at 11 locations in California (Table 1 and supplementary data Fig. S1). Ten adult band-tailed pigeons (6 males, 4 females) and one juvenile (gender undetermined) were infected with trichomonad protozoa (11/268, 4.1%). Three of these infected birds had mild to severe caseous lesions in the oral cavity. Test-positive band-tailed pigeons were identified at collection sites in Lassen (2/84, 2.3%), El Dorado (2/25, 8.0%), Contra Costa (3/39, 7.7%), Santa Barbara (1/16, 6.3%) and San Diego (3/34, 8.8%) counties (Table 1).

Sequence analysis revealed that 10 out of 11 trichomonad isolates cultured from live-caught band-tailed pigeons were identical at the ITS1/5.8S/ITS2 locus to *T. gallinae* Sequence Group A (GenBank EU215369) previously isolated in a variety of bird species in the U.S. and in finches in the United Kingdom (Anderson et al., 2009; Gerhold et al., 2008; Lawson et al., 2011a) (Table 1, Fig. 1). At the Fe-hydrogenase locus, all but two of these isolates were identical to *T. gallinae* isolated in a Madagascar turtle dove (*Streptopelia picturata*) sampled in the Republic of Seychelles (Lawson et al., 2011a) (e.g. CA012226, Table 1, Fig. 2). Recently, this genotype was named Fe-hydrogenase (FeH) subtype A2 (Chi et al., 2013). All *T. gallinae* FeH subtype A2 sequences detected in band-tailed pigeons that were live-trapped or sampled from other sources were identical. A second *T. gallinae* FeH sequence type was identified in two live-caught birds sampled in Lassen County and was identical to FeH subtype A1 (e.g. CA012245, Table 1, Fig. 2) which was previously amplified in British greenfinches during epidemic trichomonosis transmission (Fig. 2) (Lawson et al., 2011a). As for FeH subtype A2, all *T. gallinae* FeH subtype A1 sequences detected in live-caught band-tailed pigeons, as well as those sampled elsewhere, were identical. In a 927 bp alignment of the two sequence types, FeH subtype A1 differs from FeH subtype A2 by six nucleotides (position numbers refer to GenBank KC244200): C → T at position 582, T → C at position 708, A → G at position 756, T → C at position 783, and C → T at position 873. All six substitutions were third position synonymous changes.

Trichomonad isolate CA015840 was obtained from a live-caught, lesion-free band-tailed pigeon in El Dorado County and was an example of the newly-described species *T. stableri* n. sp. (Girard et al., 2014) (Figs. 1 and 2). The isolate had 98.8% pairwise similarity to *T. vaginalis* strain Tv30:GZ and 93.4% pairwise similarity to *T. gallinae* Sequence Group A at the ITS1/5.8S/ITS2 locus. At the Fe-hydrogenase locus, *T. stableri* isolate CA015840 had 94.4% pairwise similarity to *T. vaginalis* HDGL1, and only 81% pairwise similarity to *T. gallinae* FeH subtype A2. Isolate CA012229 sampled from a live, lesion-free mourning dove in San Diego County was identical to isolate MODO-22 originating from a hunter-killed mourning dove in Texas (Gerhold et al., 2008) at both the ITS1/5.8S/ITS2 and Fe-hydrogenase loci (Figs. 1 and 2). Fe-hydrogenase

Table 1
Trichomonas spp. infections in live-caught and hunter-killed Pacific Coast band-tailed pigeons and select sympatric species detected using the InPouch™ TF culture device. See supplementary data Fig. S1 for a map of collection locations.

Bird source	County	Trapping time period	BTPI InPouch™		MODO InPouch™		CAQU InPouch™		No. infected/No. collected (%)	Oral lesions	*T. gallinae*		*T. vaginalis*-like sp.
			–	+	–	+	–	+			FeH subtype A2	FeH subtype A1	
Live-caught	Lassen	July 2011; June, July, Aug. 2012	84	2	10	0	3	0	2/99 (2)	0		2 BTPI	
	Plumas	August 2011	1	0	0	0	0	0	0/1 (0)	0			
	El Dorado	May, June 2012	25	2	0	0	0	0	2/27 (7.4)	0	1 BTPI		1 BTPI[a]
	Contra Costa	Sept., Oct. 2011; March, May 2012	39	3	1	0	0	0	3/43 (7.0)	0	3 BTPI		
	Alameda	July 2011; April 2012	5	0	2	0	0	0	0/7 (0)	0			
	Santa Clara	July 2011; June 2012	46	0	0	0	0	0	0/46 (0)	0			
	Santa Cruz	June 2011	7	0	0	0	0	0	0/7 (0)	0			
	Santa Barbara	Aug., Sept. 2011	16	1	0	0	0	0	1/17 (5.9)	1 BTPI	1 BTPI		
	Riverside	July 2012	0	0	1	0	0	0	0/1 (0)	0			
	San Diego	July 2012	34	3	2	1	0	0	4/40 (10)	2 BTPI	3 BTPI		1 MODO
Total			**257**	**11**	**16**	**1**	**3**	**0**	**12/288 (4.2)**	**3 BTPI**	**8 BTPI**	**2 BTPI**	**1 BTPI; 1 MODO**
Hunter-killed	Monterey	Dec. 2011	39	6	0	0	0	0	6/45 (13.3)	0	6 BTPI		
	Shasta	Sept. 2011	9	0	0	0	0	0	0/9 (0)	0			
Total			**48**	**6**	**0**	**0**	**0**	**0**	**6/54 (11.1)**	**0**	**6 BTPI**		

MODO, mourning dove; CAQU, California quail; BTPI, Pacific Coast band-tailed pigeon; FeH, Fe-hydrogenase.
[a] Infected with *T. stableri*.

sequences from these mourning dove isolates formed a polytomy with *T. vaginalis* and *T. stableri* (Fig. 2), and had a pairwise similarity of 93.3% to *T. vaginalis* HDGL1, and 83.1% to *T. gallinae* FeH subtype A2. Meanwhile, their ITS1/5.8S/ITS2 sequences held a basal position to the clade containing *T. vaginalis*, *T. stableri* and uncharacterized trichomonad isolates from other mourning doves and white winged doves (Fig. 1).

3.2. Hunter-killed birds

Clinical signs of trichomonosis were not apparent in the 54 band-tailed pigeons examined and sampled at hunter check stations during sampling at the Monterey County or Shasta County locations in the fall and winter of 2011 (Table 1 and supplementary data Fig. S1). Sampling of the oral cavity revealed that six of the lesion-free birds from Monterey County (6/45, 13.3%) were infected with *T. gallinae* FeH subtype A2 (Table 1). All six infected birds were sampled at Chew's Ridge where the site-specific prevalence was 17.6% (6/34). No association between age or sex and infection status was observed.

3.3. Birds submitted by wildlife rehabilitation centers

Nine wildlife rehabilitation centers in California submitted samples from 98 birds (90 band-tailed pigeons, 5 mourning doves and 3 rock pigeons (Table 2 and supplementary data Fig. S2) during the study period. Band-tailed pigeons primarily died of trichomonosis ($n = 25$) and trauma ($n = 18$) ($P > 0.05$). All non-band-tailed pigeon species examined post-mortem died of trichomonosis. Clinical trichomonosis was confirmed or highly suspected in 26.7% (24/90) of band-tailed pigeons. Of the 73 laboratory-tested band-tailed pigeons, 26 (35.6%) were infected with either *T. gallinae* FeH subtype A2 ($n = 15$), FeH subtype A1 ($n = 6$, $P = 0.006$ subtype A2 vs. subtype A1), *T. stableri* ($n = 1$) or a *Tritrichomonas blagburni* n. sp.-like species ($n = 2$) and only four of these animals did not have obvious oral lesions (Table 2). A single *T. gallinae* variant in band-tailed pigeons, CA005639, had 99.2% pairwise distance to *T. gallinae* Sequence Group A in the ITS1/5.8S/ITS2 locus (Fig. 1). Infections in band-tailed pigeons were more common in adults (15/20, 75%) compared to juveniles (11/51, 21.57%, $P < 0.0001$). Where >5 band-tailed pigeons were available for testing, parasite infection prevalence was high in rural (Nevada County, 66.7%), urban (Contra Costa County, 75%; Los Angeles County, 29%) and coastal settings (Monterey 22.2% and Marin 17.6%).

Live organisms with genetic and morphologic similarity to *T. blagburni* were cultivated from the oral cavity of two band-tailed pigeons admitted to the Pasadena Humane Society and SPCA in Los Angeles County within one week of each other in May of 2011. One bird, CA011842, had oral lesions on admission, but, in lesion tissues only *T. gallinae* FeH subtype A2 DNA could be amplified. The second *T. blagburni*-like isolate was made from a healthy fledgling (CA005618) with no lesions who was treated with metronidazole and released. Isolates from both birds were identical to each other and to *T. blagburni* isolates AUTf-10 (GenBank EU569309) and AUTf-11 (EU569310) across 371 bp of the ITS1/5.8S/ITS2 region (Fig. 1). Within the ITS2 region, band-tailed pigeon tritrichomonad isolates shared a single nucleotide polymorphism with published feline tritrichomonad isolates including NCSU Tfs-1 (AF644749) and *T. blagburni* isolates AUTf 1–7 and AUTf 9–13 (EU569301–EU569312) that distinguish them from tritrichomonad isolates from other host species including bovine *T. foetus* (JN105456), *T. suis* (JN006998) and *T. mobilensis* (TMU86612) (Reinmann et al., 2012) (Fig. 1).

Mourning doves admitted to rehabilitation centers were infected with *T. gallinae* FeH subtype A2 and A1, and a *T. vaginalis*-like species distinct from mourning dove CA012229 (Table 2,

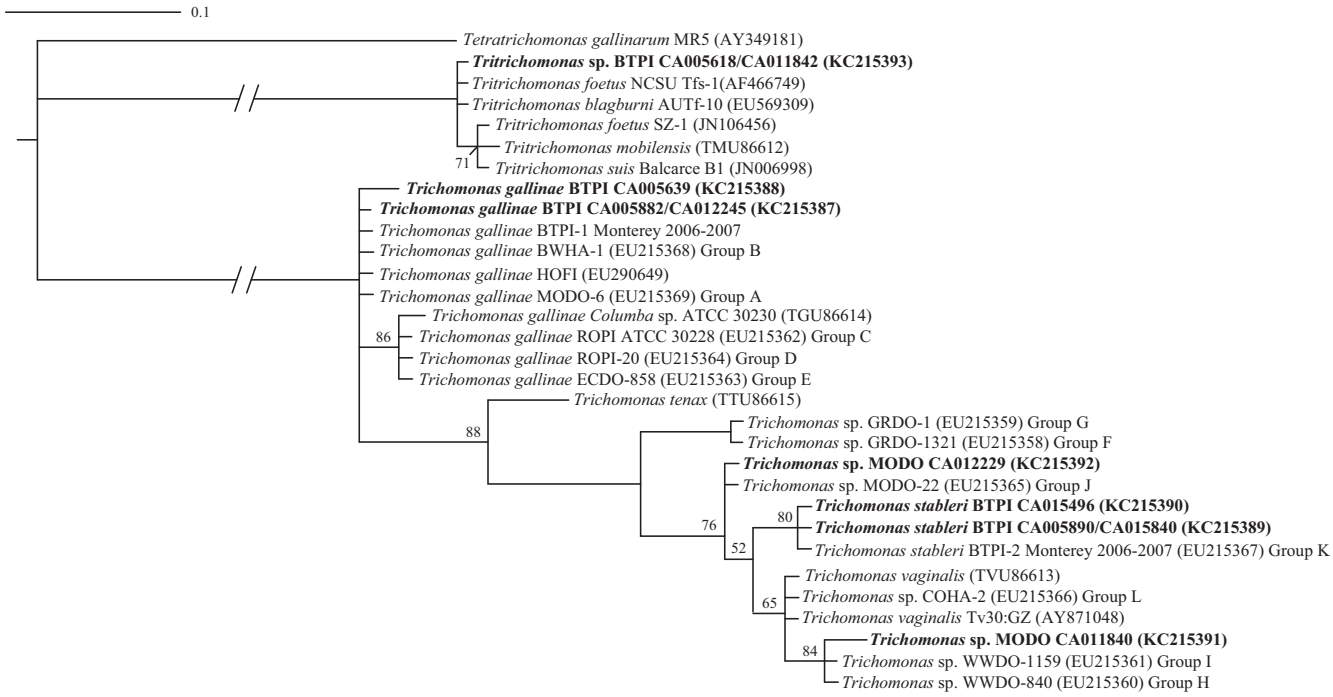

Fig. 1. Fifty percent majority-rule consensus tree of the trichomonad ITS1/5.8S/ITS2 locus (269 nt alignment) determined by Bayesian inference. Sequence Group designations are based on a previous study (Gerhold et al., 2008). Sequences in bold are from birds sampled in this study. Scale bar represents substitutions per site. Posterior probabilities <90% are given at nodes. BTPI, Pacific Coast band-tailed pigeon; BWHA, broad-winged hawk; COHA, Cooper's hawk; ECDO, Eurasian collared dove; HOFI, house finch; GRDO, common ground dove; MODO, mourning dove; ROPI, rock pigeon; WWDO, white-winged dove.

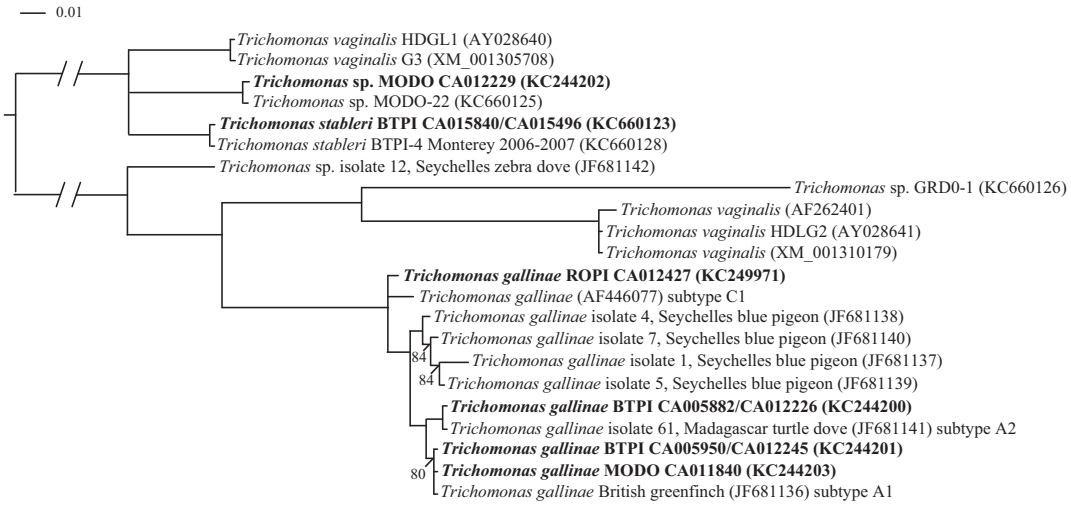

Fig. 2. Fifty percent majority-rule consensus tree of the trichomonad Fe-hydrogenase locus, (763 nt alignment) determined by Bayesian inference. Fe-hydrogenase subtype designations are based on a previous study (Chi et al., 2013). Sequences in bold are from birds sampled in this study. Scale bar represents substitutions per site. Posterior probabilities <90% are given at nodes. BTPI, Pacific Coast band-tailed pigeon; MODO, mourning dove; ROPI, rock pigeon.

Fig. 1). Mourning dove CA011840 was co-infected with *T. gallinae* FeH subtype A1 (Fig. 2) and a *T. vaginalis*-like sp. that had 99.2% pairwise similarity to *Trichomonas* sp. Sequence Groups I and H isolated from hunter-killed white-winged doves (*Zenaida asiatica*) in Texas (Fig. 1). Rock pigeons were infected with either *T. gallinae* FeH subtype A1 or a third subtype (CA012427) with sequence similarity to FeH subtype C1 (AF446077, Fig. 2).

3.4. Mortality events

Whereas no band-tailed pigeon mortality events were identified in the 2010–2011 winter and spring seasons, eight events were detected the following year between December 2011 and March 2012 (Table 3, Fig. 3a and b). Time periods of each die-off overlapped considerably, particularly in January and February, towards the end of winter and the beginning of spring migration (Fig. 3b). Trichomonosis was confirmed in 96.5% (55/57) of birds examined at seven mortality events (Table 3). Over 98% (56/57) of carcasses were from adult age birds.

The largest outbreak involving ~600 birds occurred in Carmel Valley, Monterey County between January 11 and March 18, 2012 (Table 3). Out of 28 carcasses recovered from this outbreak, 27 had caseonecrotic lesions in the oral cavity or upper digestive tract. Twenty-one laboratory-tested birds (21/25, 84%) were

Table 2
Trichomonad parasite infections in Pacific Coast band-tailed pigeons, mourning doves (MODO) and rock pigeons (ROPI) submitted to wildlife rehabilitation centers in California. See supplementary data Fig. S2 for a map of collection locations.

Rehabilitation center (county)[a]	Band-tailed pigeons						Other bird species				
	No. examined	No. infected/No. tested	*T. gallinae*			Other	No. infected/No. tested		*T. gallinae*		Other
			FeH subtype A2	FeH subtype A1	Un-typed		MODO	ROPI	FeH subtype A2	FeH subtype A1	
Bird Rescue Center (Sonoma)	1	1/1	1								
Lindsey Wildlife Museum (Contra Costa)	13	6/8 (75%)		3	2	1 *T. stableri*					
Pasadena Humane Society & SPCA (Los Angeles)	38	9/31 (29%), 3 w/o lesions	6	1		1 *Tritrich.*; 1 *Tritrich.*/FeH subtype A2	1/2				
Project Wildlife (San Diego)	0	0					2/2	1/1	1 MODO	1 MODO; 1 ROPI	1 MODO: *Trichomonas* sp./FeH subtype A1
Rose Wolf Rehab Center (Tuolumne)	0	0									
SPCA for Monterey County	11	2/9 (22.2%)	2					1/1			1 ROPI: *T. gallinae* FeH subtype C1-like
Tri County Wildlife (Amador)	1	1/1	1								
Wild Care (Marin)	19	3/17 (17.6%), 1 w/o lesions	2	1			0/1	0/1			
Wildlife Rehabilitation & Release (Nevada)	7	4/6 (66.7%)	2	1		1 unknown					
Total	**90**	**26/73 (35.6%)**	**14**	**6**	**2**	**4**	**3/5 (60%)**	**2/3 (66.7%)**	**1**	**2**	**2**

SPCA, Society for the prevention of cruelty to animals; *Tritrich.*, *Tritrichomonas* sp.; FeH, Fe-hydrogenase.
[a] Birds examined did not always originate in the same county as the rehabilitation center.

Table 3
Trichomonas spp. infecting Pacific Coast band-tailed pigeons during mortality events in California, 2011–2012. See Fig. 3 for a map of outbreak locations.

Outbreak city (county)	Estimated event date range (mm/dd/yyyy)	Estimated mortality[a]	No. birds examined	Confirmed cases[b]	*T. gallinae*			*T. stableri*	FeH subtype A2/*T. stableri* co-infection
					FeH subtype A2	FeH subtype A1	Un-typed		
Santa Margarita (San Luis Obispo)	12/10/2011–1/31/2012	400	0						
Coarsegold (Madera)	1/10/2012–3/9/2012	400	21	21	19	0	0	0	1
Carmel Valley (Monterey)	1/11/2012–3/18/2012	600	28	26	14	0	7	3	1
Santa Barbara (Santa Barbara)	1/23/2012–2/23/2012	30	1	1	1	0	0	0	0
Amador City (Amador)	2/1/2012–2/20/2012	10	1	1	1	0	0	0	0
Grass Valley (Nevada)	2/4/2012–2/10/2012	30	4	4	3	0	1	0	0
Corralitos (Santa Cruz)	2/10/2012–2/22/2012	20	1	1	1	0	0	0	0
Feather Falls (Butte)	2/20/2012–2/27/2012	20	1	1	1	0	0	0	0
Total		**1510**	**57**	**55**	**40**	**0**	**8**	**3**	**2**

[a] Estimated mortality was calculated based on the number of pigeons found sick, freshly dead, and scavenged during site visit and regular monitoring by local personnel during the duration of the event after considering available habitat and scavenger activity.
[b] Trichomonosis cases were confirmed by parasite cultivation, immunohistochemistry, and/or PCR amplification of trichomonad DNA in tissue or culture.

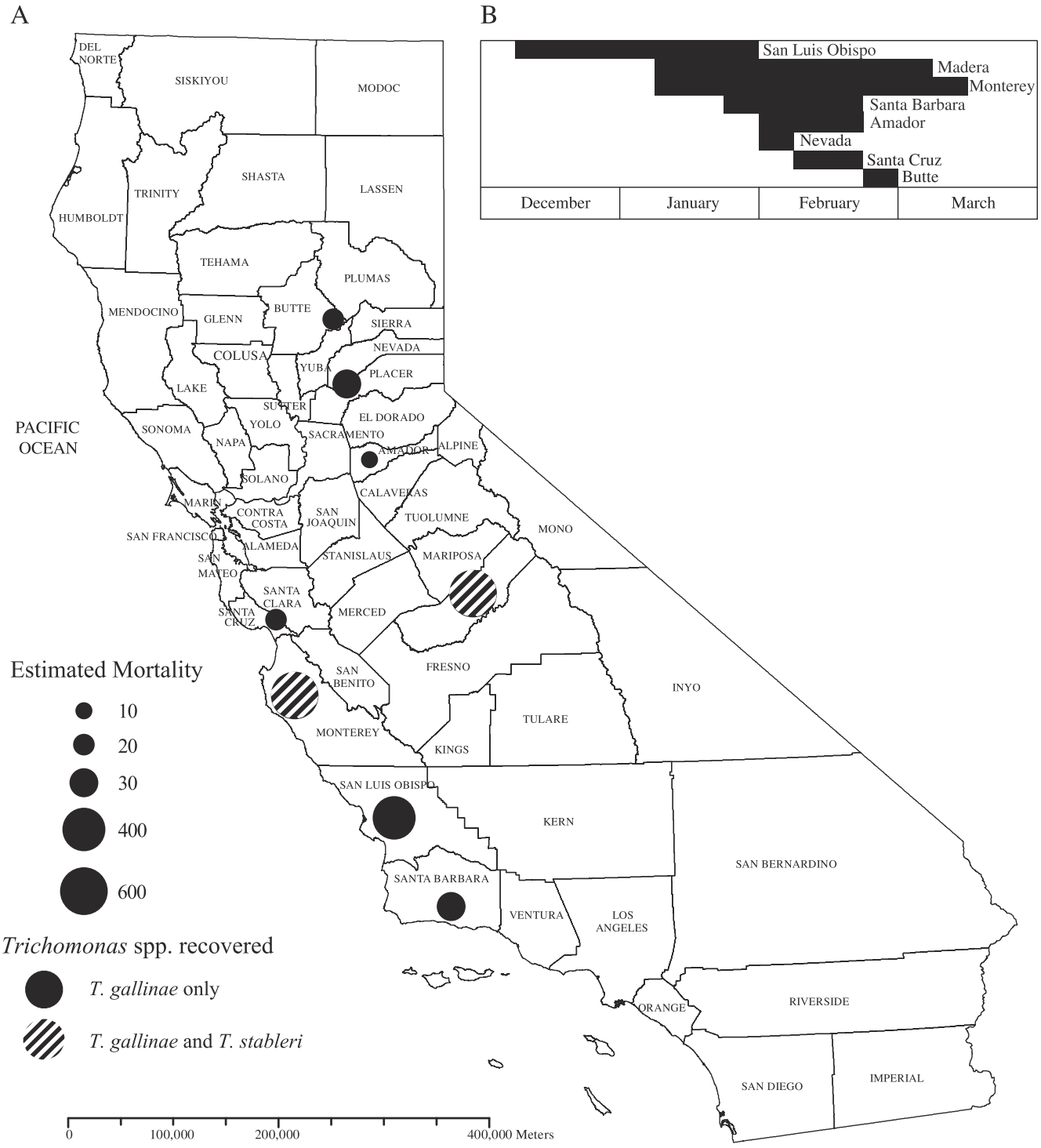

Fig. 3. Temporal and geographic distribution of trichomonosis mortality events in Pacific Coast band-tailed pigeons in California, USA. (a) Geographic locations and estimated mortality at the eight outbreaks detected between December 2011 and March 2012 (b) temporal overlap of mortality events by California county. Figure legends indicate estimated number of birds that died at each event (top) and trichomonad parasite species recovered (bottom).

infected with *T. gallinae* Sequence Group A, and 14 infections could be further subtyped as *T. gallinae* FeH subtype A2 (e.g. CA005882, Table 3, Figs. 1 and 2). *Trichomonas stableri* DNA was amplified in lesion tissue sampled from three of the remaining four birds (CA015496, CA015499, and CA015500), while band-tailed pigeon CA015497 was apparently co-infected: *T. stableri* protozoa were cultivated from the oral cavity, and *T. gallinae* FeH subtype A2 DNA was amplified from lesion tissue (Figs. 1 and 2, Table 3). In Coarsegold, Madera County, an estimated 400 birds died between January 10 and March 9, 2012 (Table 3). Trichomonosis was confirmed as the cause of mortality in all 21 birds examined post-mortem. *Trichomonas gallinae* FeH subtype A2 DNA was isolated from 20 birds, one of whom (CA005890) was co-infected with *T. stableri* (Table 3). *Trichomonas gallinae* FeH subtype A2 was the only organism amplified in tissue obtained from band-tailed pigeons found dead at outbreaks in Amador, Nevada, Santa Cruz, Santa Barbara, and Butte counties, although very few birds were sampled at these events (Table 3).

3.5. Pathogenesis of trichomonosis in band-tailed pigeons

Birds diagnosed with trichomonosis typically presented with caseonecrotic lesions in the oropharynx, esophagus, crop, or at the esophageal/proventricular junction (Fig. 4a). Caseonecrotic lesions were accompanied by intralesional protozoa and bacteria. Lesions were occasionally observed obstructing the opening of the larynx or the proximal trachea, or extending into the choanal slit. Lesions frequently extended through the mucosa, or into the deep mucosa through the submucosa and muscularis. In more than one case, necrotic lesions in the distal esophagus/proventriculus region extended through the esophagus into the trachea and adjacent lung. Evidence of aspiration of protozoa along with necrotic debris from the oropharygeal region was not uncommon in the trachea and lungs, especially in mortality event birds. The presence of protozoa in the lungs was previously confirmed by immunohistochemistry (Girard et al., 2014). Necrotizing and pleocellular pneumonia and bronchopneumonia were likely related to the aspiration of necrotic material and bacteria. Birds examined from the Madera County mortality event had either mild or moderate portal pleocellular hepatitis, interstitial nephritis, pancreatitis, tracheitis, bursal or splenic lymphoid depletion and focal necrotizing coelomitis. As previously noted, no obvious differences between the pathogenesis of infections with *T. gallinae* vs. *T. stableri* were observed (Girard et al., 2014).

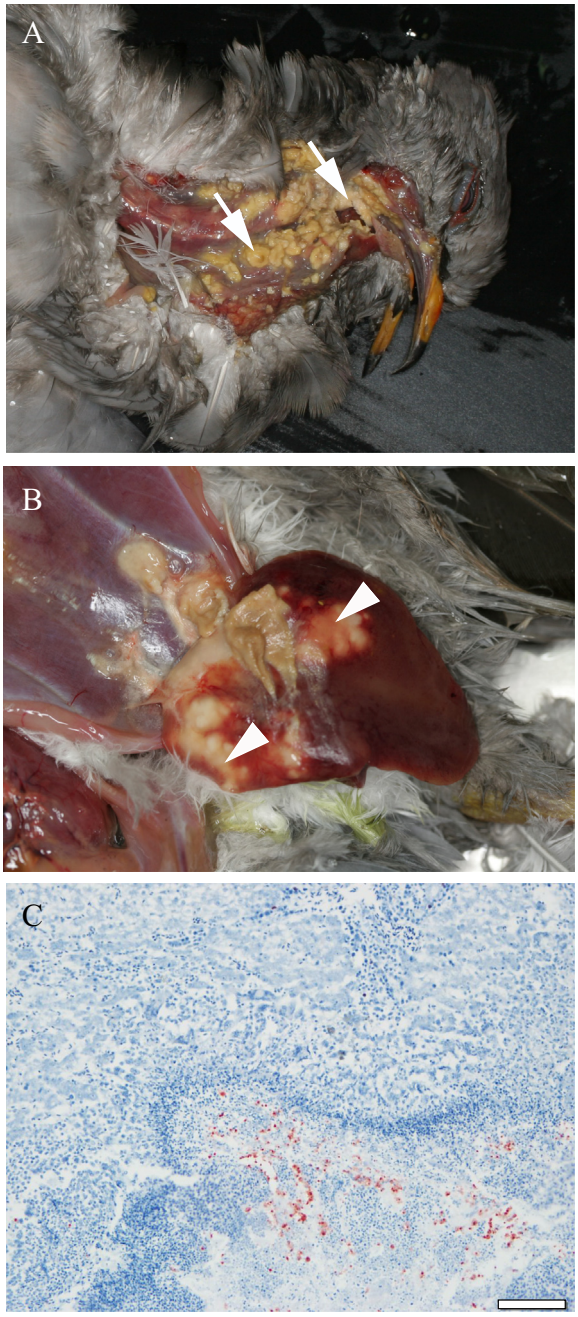

Fig. 4. (a) Caseonecrotic lesions in the oral cavity and upper digestive tract (white arrows) of a band-tailed pigeon collected during a mortality event in Madera County, California on January 17, 2012. (b) Caseonecrotic lesion of the liver (white arrowheads) in band-tailed pigeon CA015192 submitted by the SPCA for Monterey County on March 25, 2012. (c) Immunohistochemical staining of trichomonad antigen (red) in the liver of band-tailed pigeon CA015192. Scale bar is 50 μm.

Systemic trichomonosis was observed during necropsy of a juvenile female band-tailed pigeon (CA015192) admitted to the SPCA for Monterey County on March 25, 2012 due to injuries. Based on the physical and temporal proximity of this bird to the Carmel Valley, Monterey County mortality event, we hypothesize that it was infected during epidemic transmission in the region. Despite the bird having no lesions in the alimentary tract, *T. gallinae* FeH subtype A2 was cultivated from the bird's oral cavity. Associated caseonecrotic lesions were observed in the liver (Fig. 4b), air sacs and skeletal muscle. Immunohistochemistry and PCR confirmed the presence of trichomonad protozoa in the liver (Fig. 4c).

3.6. Trichomonad species and disease

Trichomonad infections were laboratory-confirmed in 21.5% (102/475) of band-tailed pigeons sampled in the study from all source categories (Table 4). Of those, 82.4% (84/102) represented confirmed cases of trichomonosis, while the remaining birds had no visible signs of disease. *Trichomonas gallinae* was identified as single infections in 88.2% (90/102) of infected birds, only 17.8% (16/90) of which were asymptomatic (Table 4). The second most common species infecting band-tailed pigeons was *T. stableri*, which was identified in 6.9% (7/102) of infected animals. Trichomonosis was diagnosed in six of these birds, two of which were co-infected with *T. gallinae* (Girard et al., 2014) (Table 4). Among band-tailed pigeons infected with a genetically-characterized parasite, the odds of being diagnosed with trichomonosis were greater for birds infected with *T. gallinae* (O.R. = 1.32) than for birds infected with *T. stableri* (O.R. = 0.88) (Table 4). Among *T. gallinae* infections, there was no association between FeH subtype and disease; however, FeH subtype A2 infected a far greater number of birds ($n = 73$) compared to FeH subtype A1 ($n = 10$) ($P < 0.001$) (Table 4).

4. Discussion

In this report, we describe the prevalence, genetic diversity and pathogenesis of trichomonad infections in Pacific Coast band-tailed pigeons during a temporally and spatially extensive sampling effort that led to the examination of over 500 birds throughout the species range in California. Infections and associated disease were detected year-round, although the prevalence varied by season, geographic location, and sampling source. *Trichomonas gallinae* FeH subtype A2 was the dominant pathogenic genotype infecting band-tailed pigeons throughout the year including during epidemics. The only previous reports of this genotype were in a live-caught disease-free Madagascar turtle dove sampled in Mahé, Seychelles and in a captive Budgarigar in Scotland that had trichomonosis lesions (Chi et al., 2013; Lawson et al., 2011a). The less common *T. gallinae*

Table 4
Relationship between disease and genetic background of the infecting trichomonad protozoa in Pacific Coast band-tailed pigeons.

Trichomonad species group	Infecting trichomonad species/Fe-hydrogenase genotype	Trichomonosis		Total	Odds ratio
		–	+		
Trichomonas gallinae	*T. gallinae* FeH subtype A2	14	56		
	T. gallinae FeH subtype A1	2	8	90	1.32
	T. gallinae (FeH subtype not determined)	0	10		
Trichomonas stableri	*T. stableri*	1	4	7	0.88
	T. stableri/*T. gallinae* FeH subtype A2 co-infection	0	2		
Tritrichomonas sp.	*T. blagburni*-like	1	0	2	nd
	T. blagburni-like/*T. gallinae* FeH subtype A2 co-infection	0	1		
Unknown spp.	Infected, but trichomonad species was unable to be determined	0	3	3	nd
Not identified	InPouch™TF-negative	352	1	373	nd
	Not tested	18	2		
Total		**388**	**87**	**475**	

nd, not determined.

Fe-hydrogenase genotype in band-tailed pigeons, FeH subtype A1, is the etiologic agent of epidemic finch trichomonosis in Britain, where infections have lead to significant declines in greenfinch and chaffinch populations (Lawson et al., 2011a, 2012; Robinson et al., 2010). The same genotype has also been found in non-passerine bird species in the U.K. including birds of prey and columbids (Chi et al., 2013). While regional studies have established that these *T. gallinae* genotypes are detrimental to more local bird populations, these and other *T. gallinae* strains have yet to be characterized with regard to their global geographic distribution, host range, genetic background, and evolutionary history.

Trichomonas gallinae infected all three bird species sampled in this study, while *T. vaginalis*-like strains in band-tailed pigeons, mourning doves and rock pigeons were generally restricted to those species. Gerhold and colleagues (2008) reported *T. gallinae* Sequence Group A infection in a wide range of raptors, finches, and dove and pigeon species sampled across the U.S., while *T. vaginalis*-like isolates (ITS1/5.8S/ITS2 Sequence Groups H-L) had some host specificity. The generalist behavior of *T. gallinae* is highly optimal for parasite persistence and transmission success, while the restricted host range of avian *T. vaginalis*-like spp. suggests that these organisms may have evolved more recently in birds. But whether this narrow host range has a genetic and/or functional basis, or is simply due to ecological circumstances that have limited host shift opportunity requires further analysis. We will gain a better understanding of cross-species parasite transmission and host associations of genetically diverse avian trichomonosis pathogens through studies that combine disease surveillance with analysis of bird movement (e.g. using radio telemetry) and targeted sampling at artificial food and water sources that tend to attract a variety of bird species.

The relationship between the *Tritrichomonas* sp. isolated in band-tailed pigeons and tritrichomonads infecting cattle and cats requires further analysis, particularly given recent findings that distinguish the feline agent of large-bowel diarrhea, *T. blagburni*, from the bovine agent of abortion in cattle, *T. foetus* (Walden et al., 2013). Our preliminary analysis of the ITS1/5.8S/ITS2 region indicates a feline origin of tritrichomonads isolated in band-tailed pigeons. However, based on the fact that only *T. gallinae* DNA could be amplified from lesion tissue of the diseased bird, and the second bird showed no signs of trichomonosis, we hypothesize that the *Tritrichomonas* sp. organisms were simply colonizing the oral cavity and were not invasive or pathogenic to band-tailed pigeons. Oral exposure of birds to cat fecal material, for example, could have easily occurred in the urban setting of Pasadena, California where birds and cats share habitats including back yards with bird feeders.

The sporadic nature of trichomonosis epidemics in band-tailed pigeons and mourning doves is well-documented (Cole, 1999; Stromberg et al., 2008; USGS, 2013), however, previous to this study, little was known about the genetic background of the etiologic agent, or the host and environmental drivers of these intermittent outbreaks. Although diverse protozoa were detected in columbids sampled throughout the study including three *T. gallinae* genotypes, a *Tritrichomonas* sp., and three *T. vaginalis*-like strains, only *T. gallinae* FeH subtype A2 and *T. stableri* were isolated at outbreaks. Both *T. gallinae* subtype A2 and *T. stableri* were isolated previously from band-tailed pigeon carcasses sampled during a large trichomonosis outbreak in Monterey County during the winter of 2006–2007 in which over 43,000 birds are estimated to have died (Gerhold et al., 2008; Girard et al., 2014; Stromberg et al., 2008). Considering their high pathogenicity and involvement in multiple, recent band-tailed pigeon epidemics in California, continued monitoring of the prevalence and spatial distribution of both *T. stableri* and *T. gallinae* subtype A2 is important for Pacific Coast band-tailed pigeon population health. Surveillance activities should also continue to examine the distribution of the British finch epidemic strain, FeH subtype A1, a potential pathogen for North American passerine species.

Unlike in Britain, where epidemic mortality events due to trichomonosis in breeding populations of finches occur in the late summer (Lawson et al., 2012; Robinson et al., 2010), transmission in California band-tailed pigeons followed a pattern similar to that of wood pigeons in Spain where *T. gallinae* infection prevalence is higher in adults (72.7%) than juveniles (20% or 34.6%, depending on location) (Villanùa et al., 2006) and outbreaks occur in winter and spring (Höfle et al., 2004). The high prevalence of infection in adults and the timing of mortality events in California band-tailed pigeons have strong implications for adult survival and recruitment potential. The largest numbers of Pacific Coast band-tailed pigeons are present in California during winter and spring migration, suggesting the majority of the population is at risk of infection and subsequent mortality due to trichomonosis. These factors indicate that disease may be contributing to the population decline observed in this species. Given the strong seasonal trends in band-tailed pigeon infection prevalence and the age structure of the population during peak mortality, increased transmission and pathogenesis in adults appears to be driven by environmental, physiological and behavioral changes that accompany shifts in the birds' annual cycle. As observed in other avian-pathogen systems, the energetic burden and stress of migration, or even pre-migration activity, is linked to impaired host immunity, and increased susceptibility to infections (Altizer et al., 2011; Lloyd, 1995). In the case of band-tailed pigeons, disease transmission in

the migration season may be additionally heightened during congregation of exceptionally large flocks (Stromberg et al., 2008) particularly in years of low precipitation (Bunbury et al., 2007) or limited food availability (Lloyd, 1995). Research activities exploring the relationship between climate, precipitation, habitat quality, food availability and the role of human development on disease transmission dynamics in the band-tailed pigeon population are needed, and may reveal ways to reduce individual- and population-level impacts of trichomonosis in band-tailed pigeons and other susceptible avian species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.03.002.

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