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Identification of Zoonotic and Vector-borne Infectious Agents Associated with Opossums (*Didelphis virginiana)* in Residential Neighborhoods of Orange County, California

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ABSTRACT: Opossums and cat fleas have been epidemiologically linked to flea-borne rickettsial disease transmission in residential backyards of Orange County, California. In 2013, a study was initiated to better elucidate the life history of opossums and their role as vectors of disease and hosts for both internal and external parasites. The study population consisted of adult opossums collected year-round from flea-borne rickettsial disease exposure sites, and moribund opossums submitted by wildlife rehabilitators in Orange County. Carcasses were examined for ectoparasites and necropsied, which included the removal and collection of endoparasites, organ tissues, feces, and urine. Reproductive life history data suggested one brood of young per year, with an average litter size of 7 ($n = 9$, range 2-11). Average adult weight was 2.49 kg (range 1.30-4.41 kg). Cat fleas were present on each opossum with an average of 96 fleas per opossum ($n = 82$, range 2-725). Thirty of 33 cat flea pools tested PCR-positive for one of the following bacteria: *Rickettsia felis* (53%), *R. typhi* (3%), the *R. felis*-like organisms, *Candidatus* Rickettsia senegalensis (28%) and *Ca.* Rickettsia asemboensis (3%), or *Bartonella vinsonii* subsp. *arupensis* (1.5%). Sticktight fleas (*Echidnophaga gallinacea*), the only other flea detected, were present on less than 6% of opossums, and ticks were not detected on any carcasses ($n = 83$). Endoparasitic nematodes *Cruzia americana* and *Physaloptera turgida* were present in each stomach and cecum, and *Didelphostrongylus hayesior* or *Heterstongylus heterostrongylus* was noted in lung samples of opossums ($n = 83$). *Salmonella* spp. were detected in 52% of fecal samples ($n = 50$), with subsequent typing of strains indicating the presence of human pathogens in all but three of the samples ($n = 26$). Blood and spleen samples were negative for *Bartonella* spp., *Brucella* spp., and *Yersinia pestis* ($n = 33$). Sera were negative for *Leptospira*-specific antibodies and *Leptospira* DNA was not detected in urine ($n = 83$). Results from this multi-agency study show that the presence of opossums in the backyard environment put Orange County residents and their pets at risk of flea-borne bartonella and rickettsial diseases and salmonellosis.

KEY WORDS: *Bartonella*, *Candidatus* Rickettsia asemboensis, *Candidatus* Rickettsia senegalensis, cat fleas, *Ctenocephalides felis*, *Didelphis virginiana*, disease, flea-borne rickettsial disease, *Leptospirosis,* opossum, Orange County CA, public health, *Rickettsia felis*, *Rickettsia typhi*, *Salmonella*

INTRODUCTION

Understanding how landscape, host, and pathogen traits contribute to disease exposure requires systematic evaluations of pathogens within and among host species and geographic regions; the relative importance of these attributes is critical for management of wildlife and

mitigating domestic animal and human disease, particularly given rapid ecological changes, such as urbanization (Carver et al. 2016). The Virginia opossum (*Didelphis virginiana*) and its most common ectoparasite the cat flea (*Ctenocephalides felis*), have been implicated in flea-borne rickettsial disease transmission, and in many human-wildlife conflicts in residential backyards of Orange County, CA. In 2013, in order to advise residents and policy makers about infectious agents and disease risk posed by opossums, the Orange County Mosquito and Vector Control District (District) initiated a study to learn more about the biology and life history of opossums in Orange County. The study included an assessment of internal and external parasites, and the potential role of opossums in the transmission of flea-borne rickettsiae (*Rickettsia typhi* and *R. felis*) and other pathogens (*Bartonella* spp., *Salmonella* spp., *Leptospira* spp.) in the residential environment. The Virginia opossum (opossum) is not native to Southern California and was likely introduced to the region in the 1910s as a potential food source (Grinnell 1915). The first museum collection records for opossums in Southern California are from Covina and Glendale in 1917 and Downey in 1918 (University of California-Los Angeles, Los Angeles County Museum of Natural History). The earliest record linking opossums, fleas, and flea-borne typhus disease transmission occurred in 1933, when a human case of murine typhus reported exposure to opossums and fleas in an orchard in Orange County to public health officials (CDPH 1950).

The District began investigating the role of opossums and cat fleas as vectors of flea-borne rickettsial pathogens in the 1970s. Early studies from Southern California describe the differences between the urban and suburban flea-borne typhus disease cycles (Adams et al. 1970, Sorvillo et al. 1993). The urban disease cycle, "murine typhus", involves Norway rats (*Rattus norvegicus*), the Oriental rat flea (*Xenopsylla cheopis*), and the infectious agent *Rickettsia typhi*. The suburban transmission cycle is known as flea-borne spotted fever and linked to cat fleas, cats, opossums and the infectious agent *R. felis* (Sorvillo et al. 1993). Since the 1970s, Orange County experienced rapid urbanization leading to a substantial reduction of agricultural areas. With the loss of agriculture, the Norway rat population decreased in abundance and is now rarely collected. Filling the niche left behind, roof rat (*Rattus rattus*) and opossum populations increased in newly-developed urban and suburban areas. Contrary to popular belief, roof rats are not commonly infested with fleas in Orange County and are not involved in flea-borne rickettsia transmission in Southern California (Schwan et al. 1985).

Since 2006, the County has experienced a reemergence of human cases of flea-borne rickettsial disease. Prior to 2006, no human cases of flea-borne rickettsiosis had been reported in the County since 1991. From 2006 to 2015, 404 human cases of flea-borne rickettsiosis were reported to the California Department of Public Health (CDPH) as occurring in Southern California, with 136 confirmed exposures in Orange County (CDPH 2015). Although the incidence of flea-borne rickettial disease cases has increased in California, isolation of the etiologic agent in a California victim has yet to occur. Therefore, it is unknown whether the flea-borne rickettsial disease is murine typhus, flea-borne spotted fever, or both.

In 2012, the District entered into a Memorandum of Understanding (MOU) with the Orange County Health Care Agency (OCHCA) to enable sharing of human case exposure sites for ecological investigation (OCHCA, 2011). Ecologic investigations of human case exposure sites found that as many as 77% of cases reported owning pets and 65% reported opossums or feral cats visiting backyard environments (Cummings et al. 2014). Surveillance of opossums and fleas using molecular methods [quantitative real-time polymerase chain reaction (qPCR) assays] have shown that *R. felis* is the overwhelmingly abundant pathogen collected from fleas in flea-borne rickettsial disease-endemic areas (Eremeeva et al. 2008, Abramowicz et al. 2011, Eremeeva et al. 2012). Ongoing surveillance by the District has shown the prevalence of *R. felis* in cat fleas removed from opossums and pet cats to be around 48%, with less than 2% of fleas positive for *R. typhi* (Eremeeva et al. 2012, Cummings et al. 2014). A study of cat fleas collected from feral cats in Los Angeles County found a 39% prevalence of *R. felis* but did not detect *R. typhi* (Billeter et al. 2016).

Similar to other urban areas of the United States, the rapid urbanization of Orange County has favored the establishment of opossum populations (Wright et al. 2012). As the opossum population has become established in the County, contact between opossums, other urban wildlife, and household pets has increased. These interactions have the potential to indirectly increase contact between opossums, cat fleas, and humans, and the likelihood of pathogen transmission. The most likely route of flea-borne rickettsia transmission involves infected cat fleas moving from opossums and feral cats to household pets that frequently contact people (Eremeeva et. al. 2012).

Opossums are classified as non-game animals by the California Department of Fish and Wildlife (CDFW), and can be taken if damaging property. However, if taken, the animals must be humanely euthanized, as relocation of trapped animals is prohibited (CDFW Fish and Game Code §4152). Unfortunately, there are limited options available for homeowners to humanely euthanize large-bodied, non-game wildlife in urban environments in the County. This conflict has led to intense activism from animal rights groups to protect opossums, with backlash from pet owners and flea-borne rickettsiosis survivors demanding governmental resources to euthanize disease carrying wildlife. In 1988, the Opossum Society of the United States protested against disease surveillance and nuisance animal practices at the Orange County Animal Control Shelter (OCAC Shelter) and the District, effectively ending euthanization of nuisance opossums in Orange County by any governmental agency (Cummings et al. 2014). In 2012, a County resident, attempting to euthanize an injured opossum, was arrested for possible animal cruelty, resulting in multiple lawsuits against the city for false arrest which resulted in a substantial settlement (Orange County Register 2012). Feral cat feeding stations, which also provide food for opossums, were implicated in flea-borne rickettsial disease transmission to a County employee while at work on

County property (Orange County Grand Jury 2015). Although the County stated employee safety as a top priority, the Board of Supervisors was unable, or unwilling, to adopt a "no feeding of wildlife policy" on County lands. The situation became contentious enough to warrant a grand jury investigation of the OCAC Shelter (Orange County Grand Jury 2015). The grand jury report suggested free-ranging opossums, fed at feral cat feeding stations at the OCAC Shelter, may be responsible for transmitting *Leptospira* to a caged domestic dog under care of the Shelter (Orange County Grand Jury 2015).

As opossums have increased in prevalence in Orange County, concerned residents have contacted the District to inquire about potential zoonotic pathogens and parasites associated with the animals. The District has recommended exclusion as the main form of opossum management, and treatment of household pets with flea control products year-round for prevention of flea infestations. However, residents comment that exclusion is not possible because most residential properties are surrounded by block wall fences that act as elevated passageways between properties. A property owner who feeds animals (feral cats, pet cats, dogs) outside could attract other wildlife to the feeding station, causing flea and nuisance wildlife issues for the entire neighborhood. When flea-borne rickettsial disease reemerged in the County in 2006, residents of flea-borne rickettsiosis hotspots repeatedly demanded that feral cat feeding stations be removed from the neighborhood, with remaining wildlife dependent on the feeding station to be trapped and removed in an attempt to stop infected fleas from propagating in the neighborhood (Orange County Register 2013). As flea-borne rickettsial disease cases continued to increase in prevalence in the County, investigations by District staff revealed that opossums and feral cats could support area-wide infestations of cat fleas in neighborhoods, in addition to producing large volumes of feces (Cummings et al. 2014, Orange County Grand Jury 2015).

Although opossums have been found in the County since the late 1910s, there have been few studies systematically analyzing infectious pathogens and internal parasites associated with these animals and their fleas in urban areas. To better address concerns from Orange County residents and policy makers about infectious agents and disease risks posed by opossums and cat fleas, the District initiated this multiagency study. This paper presents findings, to date, from specimens collected in 2013 and 2014 in residential neighborhoods of Orange County, CA.

MATERIAL AND METHODS
Study Area
Orange County, California, is the third-most-populous county in the state, with an estimated population of 3,169,776 people living in a largely metropolitan area of 2,047 km^2 (790.6 mi^2); the population density is approximately 1,548 persons/km^2 (4,009 persons/ mi^2) (U.S. Census Bureau 2015). The County's climate is typically maritime Mediterranean, with mild winter temperatures and warm, dry summers moderated by easterly winds from the Pacific Ocean. The mean annual

temperature and precipitation are 18.1°C (64.6°F) and 345 mm (13.6 inches), respectively; on average, measurable rainfall occurs on only 22 days per year (OC Weather 2015).

Opossum Trapping and Euthanization
Opossums were obtained through live-trapping and by coordination with local wildlife rehabilitation centers from February 2013 throughout January 2014, covering a year-long period. Live-trapping of opossums was conducted in response to human cases of flea-borne rickettsial disease by District staff specifically trained to trap opossums, and comply with all Health Insurance Portability and Accountability Act (HIPPA) regulations set forth in the MOU with the Orange County Health Care Agency (OCHCA 2012). Opossums submitted to the study from local wildlife rehabilitation centers were primarily moribund animals that suffered trauma (i.e., hit by cars, attacked by dogs). Live-trapping of opossums was conducted using 107×30×30-cm Tomahawk traps (Tomahawk Live Trap Co., Tomahawk, WI). Traps were baited with pet food or fruit and checked before 9:00 AM the next day. All animals collected by the District were humanely trapped and euthanized in accordance with protocols established in a CDFW Scientific Collection Permit according to guidelines from the American Veterinary Medical Association (AVMA 2013).

Necropsy, Tissue Preparation, Feces and Urine Collection
All juvenile and adult opossums admitted into the study were sexed, weighed, and crown-to-rump and tail length measurements were recorded. A veterinary pathologist conducted necropsies of juvenile and adult opossums under a biologic containment hood in a negative pressured room under biosafety level-2 conditions. Significant findings were recorded by organ and system. Three tissue samples each of brain, lung, heart, kidney, liver, ovaries, testes, uterus, ear, and rump muscle were harvested. Two samples of each organ were frozen and kept in a low temperature freezer at -60°C for future testing. The remaining organ tissue was stored in 10% neutral-buffered formalin until fixation and then cut and processed for future immunohistochemistry studies. Slide preparation was outsourced to a commercial laboratory (Harris Histology, Tustin, CA), and slides were saved for future analysis.

If urine was present in the bladder, the urine was collected and split into two 2-ml vials and stored at -60°C. A standard sterile swab was used to collect feces present in the colon and then stored at -60°C for *Salmonella* testing. When available, feces were collected from the cage. Fecal material was collected and stored in 10% neutral-buffered formalin at room temperature.

Internal Parasite Collection and Identification
Intestinal contents were obtained from all opossums entered in the study. Stomach, small intestine, and cecum samples were washed separately with water and the contents strained using a 500-μm sieve. The contents of the sieve were examined for parasitic worms. Worms were then stored in 10% neutral-buffered formalin based on

collection location (stomach, small intestine, cecum) from each animal. A dissecting microscope was used to identify and count worms based on published morphological criteria and scanning electron microscopy images (Crites 1956, Chabaud 1974, Matey et al. 2001). Lung tissue was removed and examined by the veterinary pathologist. If lung worms were present, they were collected and stored in 10% neutral-buffered formalin for identification. Lung worms were identified and counted using a compound microscope.

Opossum Blood Collection

Opossum blood samples were taken immediately following euthanization by cardiac puncture using a 21-gauge needle and 10-cc syringe. Opossum blood was then distributed into a 2 ml EDTA tube, a 5-ml sterile conical tube, and used to make two peripheral blood smear slides. The EDTA tubes were immediately frozen at -60°C. Blood collected in the sterile conical tube was spun down using standard methods. The blood serum was then separated from the clot and stored at -60°C.

Flea Collection, Identification, and Historical Abundance of Fleas on Opossums

After euthanization and blood collection, each opossum carcass was placed in a plastic bag, labeled with collection number, and stored in a refrigerator for 48 hours. After 48 hours, if fleas were still observed alive on the carcass, laboratory technicians applied the pesticide 565 Plus XLO® (active ingredients: 0.5% pyrethrins, 1% piperonyl butoxide, 1% n-octyl bicycloheptene dicarboximide) on the carcass and resealed the bag for an additional two hours in an attempt to knock down fleas. The carcass was then examined under a biological fume hood and fleas were collected, counted, and placed into vials by species, when possible, using gross examination. If necessary, fleas were examined under a dissecting microscope using morphological characteristics for speciation according to a standard taxonomic key (Lewis et al. 1988). Flea species counts were compiled and the monthly average abundance per opossum was analyzed using a paired *t*-test in Microsoft Excel®.

Historical flea index data from opossums collected in Orange County in 1967 and 1986 were compiled in Microsoft Excel® (OCMVCD 1967 and OCMVCD 1986).

Bartonella Testing of Individual and Pooled Fleas

Twenty fleas were collected from each host and were separated into two pools of ten for testing. One pool from each host was tested first and if positive, fleas from the second pool were individually tested. If the first pool indicated negative results, the remaining fleas were tested as a pool, not individually. DNA extraction and molecular detection of *Bartonella* and *Rickettsia* spp. were conducted by the Centers for Disease Control and Prevention (CDC), Fort Collins, CO. DNA was first extracted by placing flea pools or fleas in sterile tubes containing brain heart infusion media and five sterile glass beads. The samples were then placed in a Mixer Mill MM200 (Retsch GmbH, Haan, Germany) and homogenized. DNA was extracted using a QIAXtractor (Qiagen, Valencia, CA) tissue kit protocol following the manufacturer's instructions. For

Bartonella detection, the DNA was tested by conventional PCR (Polymerase Chain Reaction) targeting the 16S-23S intergenic spacer region (ITS) (325s: 5' CTT CAG ATG ATC CCA AGC CTT CTG GCG and 1100as: 5' GAA CCG ACG ACC CCC TGC TTG CAA AGC A) used by Diniz et al. (2007) and two regions of the citrate synthase gene (*gltA*); a 767 base pair (bp) region (443f: 5' GCT ATG TCT GCA TTC TAT CA and 1210r: 5' GAT CYT CAA TCA TTT CTT TCC A) used by Billeter et al. (2011) and a 1090 bp region (120f: 5'-TTTYACTTATGATCCTGGYTT and 1210r: 5' GAT CYT CAA TCA TTT CTT TCC A) with cycle parameters used by Kabeya et al. (2010).

To ensure validity of each PCR, nuclease-free water was used as a negative control and *B. doshiae* DNA was used as a positive control. Gel electrophoresis was used to visualize bands. Any bands of the appropriate amplicon size were purified with QIAquick PCR Purification Kit (Qiagen) and sequenced with a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were assembled and analyzed using Lasergene 11 software (DNAstar, Madison, WI). Samples were only considered positive if *Bartonella* DNA could be sequenced.

Rickettsia Testing of Pooled Fleas

To screen flea specimens for the presence of rickettsial DNA, a genus specific qPCR assay (Rick17b) that amplifies and detects a 115-bp segment of the 17-kDa antigen gene (Jiang et al. 2012) was used by the Viral and Rickettsial Disease Department at the Navy Medical Research Center. Subsequently, the flea DNA preparations were further tested using a group-specific qPCR assay (RFelB) that detects *R. felis* and *Candidatus* Rickettsia senegalensis (Odhiambo et al. 2014) and two species-specific assays targeting the *ompB* gene of *Ca.* R. asemboensis-specific assay (Rasem) (Jiang et al. 2013) and the *R. typhi* (Rtyph) (Henry et al. 2007). The rickettsia positive samples were confirmed by PCR amplification and sequencing of the *gltA* and/or the *ompB* gene for all the flea pools positive for *Rickettsia*. Additional sequencing was performed on one flea pool positive with Rasem qPCR assay for *rrs, ompB, ompA and sca4* genes as previously described (Jiang et al. 2013). Sequencing reactions were performed utilizing both DNA strands using Big Dye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Life Technologies, Foster City, CA), according to the manufacturer's instructions on an ABI 3500 genetic analyzer (Applied Biosystems). Sequences were assembled using CodonCode Aligner version 5.0.1 (CodonCode Corp., Centerville, MA) and exported to MEGA version 6 software (CEMI, Tempe, AZ) where they were compared with other strains available in GenBank.

Detection of *Bartonella* spp. in Opossum Blood by Culture

Blood was tested for *Bartonella* spp. by culture methods published elsewhere (Bai et al. 2009). Briefly, samples were diluted 1:4 with brain heart infusion supplemented by 5% with amphotericin B and then plated on brain heart infusion agar supplemented with 10% rabbit blood. Plates were incubated at 35°C and kept for 4 weeks.

They were inspected once a week for growth of *Bartonella*-like colonies.

Molecular Detection of *Bartonella* spp. in Opossum Blood

DNA was extracted from blood samples using a QIAamp DNA Mini Kit (Qiagen) following the manufacture's protocol. The DNA was tested for *Bartonella* spp. by conventional PCR targeting the RNA polymerase beta-subunit gene (*rpoB*) (1400f: CGCATTGGCTTACTTCGTATG and 2300r: TGTAGACTGATTAGAACGCTG) following cycle conditions described by Renesto et al. (2001), the cell division protein gene (*ftsZ*) (BFP1: AGA GTT TGA TCC TGG CTC AG and BFP2: GGT TAC CTT GTT ACG ACT T) described by Zeaiter et al. (2002), and the primers described above that were used to screen fleas: ITS (325f, 1100r), *gltA* (443f, 1210r), and *gltA* (120f, 1210r).

Opossum Spleen Testing to Detect Species of *Bartonella, Brucella,* and *Yersinia* Bacteria

Spleen samples were homogenized with a Bullet Blender® (Next Advance, Inc., NY) and DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) following manufacturer's instructions. Samples were tested for *Bartonella* spp., *Yersinia* spp., and *Brucella* spp. in a real-time multiplex PCR assay, targeting the transfer-messenger RNA (ssrA), the peptidoglycan-associated lipoprotein (Pal), and *Brucella* specific insertion (IS711), respectively. The primers and probes used for each genes were: ssrA-F: 5'-GCT ATG GTA ATA AAT GGA CAA TGA AAT AA-3', ssrA-R: 5'-GCT TCT GTT GCC AG GTG -3'and ssrAprobe:5'-HEX: ACC CCG CTT AAA CCT GCG ACG -3'); IS711-F: 5'-GCT TGA AGC TTG CGG ACA GT-3', IS711-R: 5'-GGC CTA CCG CTG CGA AT-3', and IS711 probe: 5'-FAM AAG CCA ACA CCC GGC CAT TAT GGT-3'; PAL-F: 5'-CGC AAA TAA TGA CCA ATC TGG-3', PAL-R: 5'-CGT GGC CTT CAA CAA CAA C-3', and Pal probe (5'-Quasar 670 CGG TTC TGA CTT CGC TCA AAT GCT GG-3'). Cycle conditions were 1 min at 60°C, 10 min at 95°C, followed by 39 cycles of 15 sec at 95°C and 1 min at 60°C, and a final step of 1 min at 60°C. A negative control and positive controls for each gene target were used and samples considered negative if the Critical threshold (Ct) value was > 40. Samples with a Ct value ≤ 40 were further tested by PCR of 16S ribosomal RNA using primers 63-F (5' -CAG GCC TAA CAC ATG CAA GTC -3') and 1387-R (5'-GGG CGG WGT GTA CAA GGC -3') used by Marchesi et al. (1998).

Leptospirosis Testing of Sera and Urine

Blood sera from 38 opossums were sent to the California Health and Animal Food Safety Laboratory (CHAFSL) to test for the presence of antibodies to 6 *Leptospira* serovars (*L. Bratislava*, *L. canicola*, *L. grippotyphosa*, *L. hardjo*, *L. icterohemorrhagiae*, *and L. pomona*) using the microscopic agglutination test (MAT). Urine from opossums was collected and stored at -60°C prior to sending to a commercial veterinary pathology laboratory (Research Associates Laboratory, Dallas, TX) for molecular detection of *Leptospira* DNA by real time

PCR (rt-PCR). The primers used for the test are proprietary and not provided for publication, but would amplify DNA from the six serovars.

Salmonella Detection in Feces

Fecal swabs from 50 opossums were collected at the time of necropsy, frozen at -60°C, and sent to the CAHFSL for molecular detection of *Salmonella* by qPCR and culture confirmation of strain. For each specimen, the sample was first tested via PCR to detect the presence of *Salmonella* and then cultured to determine the strain(s) of *Salmonella* present in the sample.

RESULTS
Opossum Collections

From February 2013-February 2014, 162 opossums from across Orange County were submitted to the District for inclusion in the study (Figure 1). Of the 162 opossums, 63 were pouch young, 16 juveniles, and 83 adults (39 male and 44 female). As animal age can be an indicator of pathogen exposure time, the 63 pouch young were not necropsied for pathogen testing. A total of 101 adult and juvenile opossums were necropsied by a veterinary pathologist. Of the 101 opossums, 36 were submitted by wildlife rehabilitation groups and animal care shelters, and 65 were collected by District staff in response to human cases of flea-borne rickettsiosis. Life history data indicated the presence of pregnant females and pouch young from May-August, with an average liter size of seven ($n = 9$, range 2-11). Juvenile opossums were collected April-August, and adult opossums were collected year-round.

Opossum Weight

The average weight of opossums submitted by wildlife rehabilitators was 2.3 kg for females ($n = 22$) and 2.9 kg for males ($n = 14$). The average weight of opossums from flea-borne rickettsial disease exposure sites was 2.0 kg pounds for females ($n = 21$) and 2.5 kg for males ($n = 17$). The difference in average body weight between opossums submitted by wildlife rehabilitators and those collected from flea-borne rickettsial disease exposure sites was significantly different as determined by paired student *t*-test ($p < 0.02$). An opossum was considered a juvenile if the weight was below 0.9 kg. The largest opossum collected was a male weighing 4.4 kg (it was not submitted by a rehabilitator). For adult opossums, the average head-to-rump measure was 38.8 cm for females and 40.8 cm for males, and the average tail length measurement was 28.25 cm for females and 30.74 cm for males.

Internal Parasites

This study identified four species of nematode parasites present in opossums collected from residential properties in Orange County, CA. The internal parasite *Physaloptera turgida* (Nematoda: Spiuridae: Physalopteridae), was present in 94% of stomachs and small intestines examined ($n = 78/83$). The average *P. turgida* burden in stomach and small intestine of opossums, by month, is presented in Figure 2. Upon examination, 98% of cecums ($n = 82/83$) contained abundant *Cruzia americana* (Nematoda: Ascaridida: Kathlaniidae) worms. Approximately 30% of cecums sampled ($n = 100$) contained over 500 worms, with
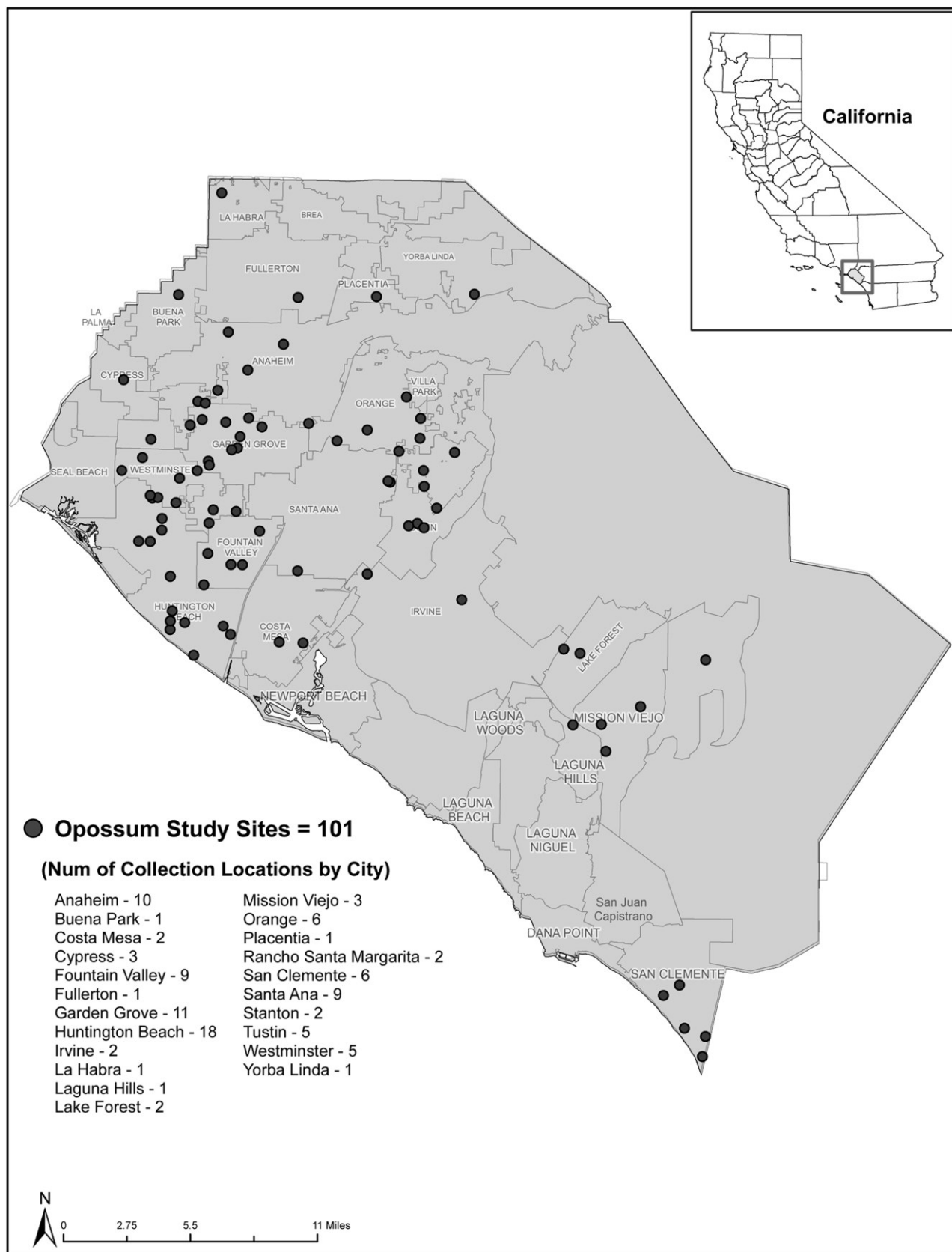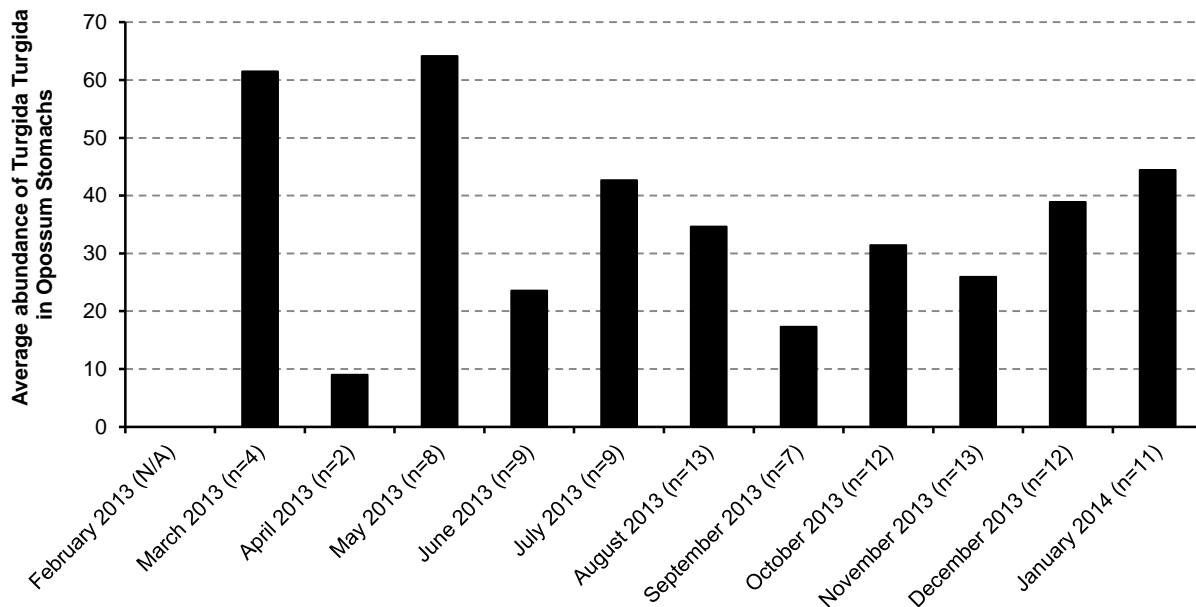
272

Opossum Study Sites = 101

(Num of Collection Locations by City)

Anaheim - 10
Buena Park - 1
Costa Mesa - 2
Cypress - 3
Fountain Valley - 9
Fullerton - 1
Garden Grove - 11
Huntington Beach - 18
Irvine - 2
La Habra - 1
Laguna Hills - 1
Lake Forest - 2

Mission Viejo - 3
Orange - 6
Placentia - 1
Rancho Santa Margarita - 2
San Clemente - 6
Santa Ana - 9
Stanton - 2
Tustin - 5
Westminster - 5
Yorba Linda - 1

Figure 1. Location of opossum collections, February 2013-January 2014, Orange County, CA.

Figure 2. Monthly average abundance of *Turgida turgida* removed from stomachs of *Didelphis virginiana* summarized monthly, February 2013-January 2014.

one opossum cecum containing 1,359 *C. americana* worms. Two lung worm species *Didelphostrongylus hayesi* and *Heterostrongylus* (Nematoda: Strongilidae: Angiostrongylidae) were found infesting lung tissue of 15 adult (*n* = 83) opossums examined in the study. No dual infections of lung worms were observed.

External Parasite Collection and Average Abundance of Fleas per Opossum

Fleas were the only ectoparasites removed from opossums during the study period. No ticks, lice, or mites were directly observed on the pelage of any of the 101 opossums during examination. No opossums displayed any obvious signs of mange. The flea index is defined as the number of fleas per host animal at the time of necropsy. In total, 9,687 fleas were removed from 101 opossums, with the average of fleas per individual opossum of 96 fleas/opossum (range 1-726). Of the fleas removed from opossums, more than 99% (9,660/9,687) were identified as *C. felis* and 27 as *Echidnophaga gallinacea*. The average of the total count of fleas per each individual opossum varied seasonally with the peak of 204 fleas/opossum sampled (*n* = 8) occurring in May of 2014 (Table 1). Unpublished historical reports from the District indicate that the presence of fleas on opossums has increased dramatically, as compared to previous studies conducted in Orange County by the District in 1967 and 1986 (Table 2.)

Bartonella Flea Pool Test Results

Fleas were negative for the presence of *Bartonella* DNA with *gltA* (443f, 1210r) and *gltA* (120f, 1210r) PCR assays. *Bartonella* DNA was detected in one of 33 flea pools using ITS. This positive flea pool was tested multiple times with two of three runs producing a positive sequence for *Bartonella vinsonii* subsp. *arupensis*. An additional flea pool produced a gel electrophoresis band from an ITS PCR amplicon, however, sequencing could

not confirm *Bartonella* DNA. As a result, the remaining fleas (*n* = 18) from both hosts were individually sequenced using gene fragments produced by PCR using the following targets: *gltA* (443f, 1210r), *gltA* (120f, 1210r) and ITS. All DNA from individual fleas were negative for *Bartonella* species. The remaining group of pooled fleas (*n* = 31) from each host were also negative for *Bartonella* DNA.

Table 1. Opossum by month and average number of fleas on opossums by month, Orange County, CA, 2013-2014.

Month	Number of Opossums	Average Number of Fleas on Opossums
February 2013	1	99
March 2013	4	50
April 2013	2	5
May 2013	8	204
June 2013	9	168
July 2013	9	130
August 2013	13	50
September 2013	7	80
October 2013	12	52
November 2013	13	50
December 2013	12	94
January 2014	11	132
Total	**101**	**96**

Table 2. Average cat flea (*Ctenocephalides felis*) abundance on opossums in Orange County, CA 1967-1968, 1987-1988, and 2013-2014

Study Period	Number of Opossums	Average Number of *C. felis* on Opossums
1967-1968	45	22
1987-1988	96	28
2013-2014	101	96

Table 3. Molecular detection of *Rickettsia* in pooled *Ctenocephalides felis* samples collected from opossums, Orange County, CA.

Molecular Test	Gene	Number of positive flea pools / Number of pools tested[1] (%)	Number of positive flea pools / Number of pools tested[1] (%)
		Pool 1	Pool 2
Rick17b pan rickettsia qPCR assay	*17kD* gene	31/33 (93.94)	27/31 (87.10)
Rasem qPCR (*Ca.* R. asemboensis)	*ompB*	1/33 (3.03)	1/31 (3.23)
RfelB qPCR (*R. felis* and *Ca.* R. senegalensis)	*ompB*	29/33 (87.88)	28/30 (93.33)
Rtyphi qPCR	*ompB*	1/33 (3.03)	1/31 (3.23)

[1] Two pools of 10 *C. felis* (Pool 1, Pool 2) were removed from each *Didelphis virginiana* (*n*=33) unless otherwise noted.

Table 4. Results from sequencing of *Rickettsia* present in pooled *Ctenocephalides felis* samples collected from opossums, Orange County, CA.

Rickettsia spp. Detections	Number of positive flea pools/number of pools tested[1] (%)	Number of positive flea pools/number of pools tested[1] (%)
	Pool 1	Pool 2
R. typhi	1/33 (3.03)	1/31 (3.23)
R. felis	18/33 (54.55)	16/31 (51.61)
R. asemboensis-like (RF2125)	1/33 (3.03)	1/31 (3.23)
Rickettsia sp. PU01-02-like	10/33 (30.30)	8/31 (25.81)
Negative	3/33 (9.09)	5/31 (16.13)

[1] Two pools of 10 *C. felis* (Pool 1, Pool 2) were removed from each *Didelphis virginiana* (*n*=33) unless otherwise noted.

Molecular Detection of *Rickettsia* in Fleas

The summary of flea pool results from genus and group specific qPCR testing are summarized in Table 3 and Table 4. For arthropod vectors, any sample that had no Ct value or had a Ct value ≥35 was declared negative unless it produced an amplicon with PCR. The *gltA* and/or *ompB* gene sequence of the flea pools that were positive with RfelB qPCR assay revealed that approximately 64% and 36% were *R. felis* and *Ca.* R. senegalensis, respectively. For the one flea pool that was positive with Rasem qPCR, sequencing showed that this agent shared 99.9%, 99.86%, 100%, 100% and 99.76% homology with *Ca.* R. asemboensis for the *rrs*, *ompB*, *Sca4*, *gltA*, and *ompA* gene, respectively. The ompB sequence was also 100% identical to that of *R.* sp. DS-006 (KP398499) detected in cat fleas from California. The sequences for the *Rickettsia* sp. CF26B/US obtained from *C. felis* have been deposited in the GenBank with accession numbers KU597065, KU597066, KU597067, KU597068, and KU597069 for *rrs*, *ompB*, *sca4*, *gltA*, and *ompA*, respectively.

Detection of *Bartonella, Yersinia*, and *Brucella* in Opossum Spleens

Spleen samples from opossums (*n* = 33) were negative for *Bartonella* spp., *Yersinia* spp., and *Brucella* spp. by PCR.

Molecular Detection of *Bartonella* and *Rickettsia* in Opossum Blood

Whole blood samples (*n* = 33) were negative for *Bartonella* by culture methods. Additionally, DNA extracted from blood specimens were negative by PCR using molecular targets from *Bartonella* and *Rickettsia*: *gltA* (443, 1210), *gltA* (120, 1210), ITS, rpoB, ftsZ.

Leptospirosis Detection in Opossum Sera and Urine

All opossum sera (*n* = 38) showed no antibody detection at titers of 1:100 for exposure to six *Leptospira* strains. Leptospirosis DNA was not detected by qPCR in opossum urine samples (*n* = 64).

Detection of *Salmonella* in Opossum Feces

Of the 50 fecal samples submitted for *Salmonella* detection and culture, 24 tested culture-positive for a *Salmonella* serovar (Table 5). Detection of co-infection with two *Salmonella* serovars was found in feces from three animals (*S.* Manhattan and *S.* Montevideo; *S.* typhimurium and *S.* infantis; and *S.* Heidelberg and *S.* Newport).

Table 5. *Salmonella* strains cultured from *opossum* feces collected in Orange County, CA.

Salmonella Strain	# of Detections
S. Newport[1]	12
S. Give Group E[1]	3
S. Bredeney[1]	3
S. Montevideo[1]	3
S. Typhimurium[1]	2
S. Manhattan[1]	2
Rough "H"[2]	1
S. Heidelberg[1]	1
S. Litchfield[1]	1
S. Infantis[1]	1
S. Type undetermined[3]	1
No *Salmonella* detected in sample	26

[1] Human pathogen

[2] Not known if strain is a human pathogen

[3] Unknown strain

DISCUSSION

During the study period, opossums collected from Orange County appeared to have a single litter of young per year, unlike records from Central California of opossums rearing two litters per season (Reynolds 1952). The average litter size of seven is similar to other studies and confirms that opossums have a high reproductive rate in urban areas (Reynolds 1952). Opossums collected from wildlife rehabilitator groups had a greater average body weight than those collected from disease surveillance; this could be because wildlife rehabilitator groups cage animals during recovery, resulting in heavier-bodied opossums. In general, the size of opossums in urban Orange County is similar to opossums collected from other urban areas and larger than those collected from rural areas (Wright et al. 2012). This information can be used in the future if opossum flea control products are developed to control focal outbreaks of flea-borne rickettsiosis.

In general, the average number of cat fleas per opossum was 96: the average number of fleas removed from opossums can vary seasonally, with flea counts peaking in the summer and winter; however, in this study the variation was not statistically significant. As Orange County has a mild Mediterranean climate, flea reproduction occurs year-round, indicating the need for residents to be diligent about administering flea control products to pets year-round. As compared to prior studies conducted by the District in 1967 and 1988, the abundance of fleas on opossums has increased substantially (OCMVCD 1967 and OCMVCD 1988). This could be due to an increase in host animals available to cat fleas in the urban environment (other wildlife, household pets). The increase in cat fleas on opossums could explain the increase in flea-borne typhus disease cases as opossums share fleas with pet cats and dogs. Interestingly, no ticks were removed from opossums in this study. The brown dog tick, *Rhipicephalus sanguineus*, is found frequently in the County, and ticks have been collected from opossums (Durden and Richardson 2013).

The four nematode parasites identified from opossums in this study (*P. turgida*, *C. americana*, *D. hayesi*, and *H. heterostrongylus*) are known to cause morbidity and mortality in opossums when present in large numbers, but they are not known to parasitize humans or pets. As in this study, previous studies have shown that adult opossums are significantly more likely than juveniles to be parasitized by all three nematodes (Nichelason et al. 2008). Of the 101 opossums sampled in the study, 54% of stomachs contained at least 21 *P. turgida* worms with at least one opossum stomach containing 250 worms, a higher prevalence than observed in Central California (30% of opossums contained 21-100 worms) (Nichelason et al. 2008). However, *C. americana* adult worm burden in opossum cecae was much higher in our study than reported previously. Considering the heavy worm burden in many opossums, overall body status of opossums as scored by the pathologist at necropsy indicated that worm burden was not indicative of emaciation or poor body condition. This study did not detect *Baylisacaris procyonis* and *Parastrongylus* spp. nematodes in opossums, both of which can cause significant morbidity in humans (Roussere et al. 2003, Miller et al. 2006).

Bartonella vinsonii subsp. *arupensis* was detected from a single pool (1/33) of fleas using a PCR protocol targeting the 16S-23S intergenic spacer region (ITS) region. When screened using primers specific for *gltA*, the sample was negative suggesting that the ITS PCR assay is a more sensitive tool (Nasereddin et al. 2014, Rizzo et al. 2015). It is possible that additional fleas harbored bartonellae but negative results were obtained due to a DNA dilution effect from pooling of flea samples. Regardless, the finding of *B. vinsonii* subsp *arupensis* in pooled fleas from a single opossum does not indicate a viable infection. It is more likely that an opossum was exposed to a rodent-borne *Bartonella* species, possibly through contact with other rodents or their fleas. *B. vinsonii* subsp. *arupensis* has been associated with deer mice (*Peromyscus* spp.) and wood rats (*Neotoma* spp.) in the United States (Morway et al. 2008, Bai et al. 2011) and has been isolated from bacteremic humans in the United States and Thailand (Welch et al. 1999, Bai et al. 2012).

The lack of *Bartonella henselae* DNA in *C. felis* from opossums was surprising due to the high prevalence of this bacterium detected previously in cat fleas in California (34%, 38/112 fleas) (Chomel et al. 1995). Opossums, feral cats, and domestic pets serve as hosts for cat fleas and share the same niche in the urban and suburban environment; however, the results from this study may suggest that host switching occurs infrequently in the investigated area. Cats do serve as reservoirs for *B. henselae* and antibodies to *B. henselae* are frequently found in California felids (Carver et al. 2016). A recent study testing felids from Southern California found the seroprevalance of *Bartonella* to be >60% for domestic cats (Carver et al. 2016). Other *Bartonella* species present in urban areas of Southern California include *Bartonella rochalimae*-like DNA and *B. tribocorum*-like DNA detected from Oriental rat fleas (*X. cheopis*) removed from Norway rats (Billeter et al. 2011). Additionally, cat fleas recovered from opossums in South Carolina were found to harbor *B. clarridgeiae* (single specimens) and *B. henselae* DNA (Reeves et al. 2005, Nelder et al. 2009). The reasons why *B. henselae* or other *Bartonella* species commonly found associated with cat fleas, cats, and rodents in urban areas was not detected in opossums and their cat fleas in this study should be explored further in future studies to better understand transmission dynamics.

The prevalence of *R. typhi* (3%) and *R. felis* (53%) in pooled cat fleas is similar to other published accounts in Southern California (Karpathy et al. 2009, Abramowicz et al. 2011, Eremeeva et al. 2012, Cummings et al. 2014, Billeter et al. 2016). Interestingly, this study detected 2 *Rickettsia felis*-like organisms in pooled cat fleas, namely *Ca.* R. senegalensis strain PU01-02 (28%) and *Ca.* R. asemboensis (3%). This report confirms existence of *Ca.* R. senegalensis and *Ca.* R. asemboensis (or very closely related agents) in Orange County. The pathogenicity of the 2 agents is currently unknown.

The study demonstrated that one of the species-specific qPCR assays (Odhiambo et al. 2014) for detection of *R. felis* (RfelB) is no longer specific to *R. felis* alone, as it detects *Ca*. R. senegalensis Str. PU01-02 as well. For this reason, the results of the RfelB qPCR assay includes positive results from both *R. felis* and *Ca*. R. senegalensis.

The detection of pathogenic *Salmonella* serogroups in

over 50% of opossums sampled is similar to that found in other areas of the United States, suggesting that opossums can serve as reservoirs for these pathogenic serogroups in urban environments (Runkel et al. 1991). The District will continue to advise residents to exclude opossums from residential properties and agriculture fields, as a measure to reduce exposure of people and pets to pathogenic *Salmonella* serogroups.

The findings that all opossum sera tested negative for antibodies to six serovars of *L. interrogans*, and testing of opossum urine did not detect DNA for *Leptospira* spp., are somewhat surprising, as opossums are known to serve as reservoirs for *Leptospira* serovars throughout the world and in the United States (Bharti et al. 2003). Annually in Southern California, domestic dogs are diagnosed with leptospirosis (Los Angeles County Public Health 2016 and Orange County Grand Jury Report 2015), and these findings cause us to speculate a rodent reservoir may be responsible for disease transmission.

Opossums in Orange County can harbor significant numbers of cat fleas that may be infected with the causative agents of flea-borne rickettsial diseases and bartonellosis. The feces from opossums have been shown to contain strains of pathogenic *Salmonella*. The risk of disease transmission from cat fleas or opossum feces to humans is dependent on a variety of epizootiological factors such as prevalence of the disease organisms in the opossum and cat flea populations, density and susceptibility of the opossum and cat flea populations, and the amount of contact between cat fleas, opossums, and people. Properties where children and immunocompromised individuals frequent, such as parks, schools, and hospitals, should enforce "no feeding of wildlife" policies in an attempt to reduce the risk of transmission of these pathogens to vulnerable populations. The District advises pet owners to maintain diligence in administering flea control products to pets year-round, feed pets indoors, and exclude opossums from residential properties in an effort to prevent flea-borne rickettsial diseases, bartonellosis, and salmonellosis. If opossums are removed from a property, or are found dead on a property, a licensed pest control operator should be hired to control larval fleas that may be developing in opossum harborage areas outdoors. The best way to prevent opossum associated diseases is to limit contact (direct and indirect) between opossums, household pets, and residential environments. The District will continue to advise residents to exclude opossums and other wildlife from backyards in an effort to prevent flea infestations and contamination of properties with *Salmonella*.

ACKNOWLEDGEMENTS

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