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Detecting Burrowing Owl Bloodmeals in *Pulex irritans* (Siphonaptera: Pulicidae)

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Abstract

Pulex irritans L. is a cosmopolitan flea species that infests a wide variety of hosts. In North America it generally parasitizes large wild mammals, but in the Pacific Northwest an association has emerged between *P. irritans* and the western burrowing owl (*Athene cunicularia hypugaea*). While investigators have recognized this association for decades, it has not been clear if *P. irritans* feeds on burrowing owls, or if the owls serve exclusively as phoretic hosts. Here we describe using a real-time assay that was originally developed to identify bloodmeals in Ugandan cat fleas (*Ctenocephalides felis* Bouché) to detect burrowing owl DNA in *P. irritans* collected from burrowing owls in southern Idaho. Of 50 fleas tested, 12 had no detectable vertebrate bloodmeal. The remaining 38 (76%) contained burrowing owl DNA. The assay did not detect vertebrate DNA in unfed fleas exposed to owl or mouse pelts and is therefore unlikely to detect DNA in fleas from vertebrates that have served exclusively as phoretic hosts. We conclude that *P. irritans* feeds on burrowing owls. We discuss the potential implications of this finding for burrowing owl conservation and enzootic plague dynamics.

Keywords

Pulex irritans; burrowing owl; *Athene cunicularia*; bloodmeal identification; flea

Pulex irritans L., the so-called human flea, parasitizes a wide variety of hosts, including rodents, large wild mammals, and livestock (Gratz 1999). In North America it generally parasitizes large mammals, particularly carnivores, but specific host associations vary between geographic regions (Hopla 1980, Lewis et al. 1988). In the northwestern United States and southern British Columbia, investigators have observed a surprising association between *P. irritans* and western burrowing owls (*Athene cunicularia hypugaea*; hereafter burrowing owls), small, ground-dwelling owls of western North America (Hopla 1980, Smith and Belthoff 2001, Belthoff et al. 2015). Recently, Belthoff et al. (2015) reported that *P. irritans* comprised >99% of 4,791 fleas collected from breeding burrowing owls or their

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nestlings in study sites in Idaho, Oregon, and Washington. This phenomenon appears to occur primarily in the Pacific Northwest, although *P. irritans* have also been collected from burrowing owls in western Montana and northern Utah (Hopla 1980). *Pulex irritans* was the only species of flea collected from adult burrowing owls captured from one study site in Colorado, but only a single flea was collected from a single individual. No fleas were detected on 55 adult burrowing owls captured from another study site in South Dakota (Belthoff et al. 2015). Skoruppa et al. (2006) found no fleas on burrowing owls wintering in south Texas.

While investigations have shown a relationship between burrowing owls and *P. irritans*, it has not been clear if *P. irritans* feeds on the owls, or if the owls serve exclusively as phoretic hosts. Phoretic hosts provide transport for fleas but do not provide bloodmeals. The nature of this arthropod–host relationship could have implications for burrowing owl conservation. The United States Fish and Wildlife Service lists the burrowing owl as a national bird of conservation concern, and this species is endangered in Canada and threatened in Mexico (Klute et al. 2003). Ectoparasitism of bird species in decline merits concern because fleas can decrease nestling growth and survival, affect parental feeding rates and sleeping regimens, and prompt increased grooming and nest sanitation, which can take time and energy away from caring for young (Richner et al. 1993, Christe et al. 1996, Boughton et al. 2006, Cantarero et al. 2013). Hematophagous ectoparasites deplete nutrients which could otherwise contribute to host growth, maintenance, or reproduction. Moreover, flea saliva contains potent immunogens that induce energy-demanding immune responses in the host (Møller et al. 2005, Krasnov 2008). Thus, feeding fleas could challenge declining burrowing owl populations.

Whether or not *P. irritans* feed on burrowing owls may also have implications for plague ecology. *P. irritans* is known to be a competent vector of *Yersinia pestis*, the etiologic agent of plague (Verjbitski 1908, Blanc and Baltazard 1941), and it is incriminated as a *Y. pestis* vector in Madagascar, parts of Southern and Central Africa, South America, South Asia, and the Middle East (Gratz 1999, Drancourt et al. 2006, Laudisoit et al. 2007, Ratovonjato et al. 2014). While avian species are generally resistant to infection with *Y. pestis* (Meyer 1950), some investigators have suggested that birds of prey, including the burrowing owl, may play a role in sylvatic plague dynamics by transferring fleas between susceptible rodent populations (Jellison 1939, Wheeler et al. 1941, Brown 1944, Smith and Belthoff 2001). Previous work (Eisen et al. 2006, 2007) showed that taking an uninfected bloodmeal after taking an infectious one decreased the probability that ground squirrel fleas (*Oropsylla montana* Baker) would subsequently transmit *Y. pestis* to an uninfected host. This finding appears to be generalizable, at least within *Oropsylla* spp. (Wilder et al. 2008a, b). Therefore, taking an uninfected bloodmeal after taking an infectious one may have a similar effect on *P. irritans* transmission efficiency. If burrowing owls provide bloodmeals to *P. irritans* rather than serving exclusively as phoretic hosts, it may reduce the probability that a *Y. pestis*-infected *P. irritans* will subsequently transmit the bacterium to a susceptible host.

Graham et al. (2012, 2013) previously developed a real-time polymerase chain reaction (PCR)-based assay for identifying blood-meals in cat fleas (*Ctenocephalides felis* Bouché) collected from huts in the West Nile region of Uganda. Here we sought to determine if 1)

This assay was flexible enough to identify burrowing owl blood, and 2) We could detect burrowing owl bloodmeals in *P. irritans* collected from burrowing owls in southern Idaho. To address these questions, we needed to ensure that if we did detect burrowing owl DNA in field-collected fleas it was not contaminating DNA from exposure to an owl's skin and feathers. We therefore also sought to determine if our assay would detect vertebrate DNA in unfed fleas exposed to animal pelts.

Materials and Methods

Study Area, Owl Blood Collection, and Flea Collection and Identification

During May–June 2012, we collected fleas from burrowing owls (Fig. 1) nesting in artificial burrows within the Morley Nelson Snake River Birds of Prey National Conservation Area (NCA) in southwestern Idaho. This area is described in detail elsewhere (Smith and Belthoff 2001, Belthoff and King 2002). The population of burrowing owls in the NCA has been under long-term study, and fleas are prevalent on the owls (Smith and Belthoff 2001, Belthoff et al. 2015). As part of long-term monitoring, we also obtained ~ 100 µl of blood from owls using venipuncture of a wing vein. Blood was collected in micro hematocrit tubes, immediately transferred to 1.5-ml centrifuge tubes, stored on ice in the field, and then frozen at −20°C. The burrowing owl pelt we used (see below) was from a nestling that died in a nest burrow of unknown causes prior to fledging and was stored frozen at −20°C. Field research procedures for burrowing owls were approved by Boise State University's Institutional Animal Care and Use Committee and authorized by appropriate federal and state permits issued to J. Belthoff.

We used an aspirator and forceps to collect fleas from owls at one nest for the present study. We made no attempt to remove all fleas from the owls; rather, we sought to collect a sample. Fleas were held on ice in a plastic bag in the field and subsequently stored at −20°C. Fleas, pelts, and blood were shipped on dry ice to the Centers of Disease Control and Prevention Division of Vector-Borne Diseases (Fort Collins, CO) for analysis. We identified the fleas using morphologic keys (Furman and Catts 1982).

DNA Extraction, Real-Time PCR, and Sequencing

Only fleas with a visibly intact exoskeleton were used for bloodmeal analysis. We soaked each flea in 50% bleach (3.08% sodium hypochlorite) to remove surface contaminants, rinsed and homogenized it in calcium- and magnesium-free Dulbecco's Phosphate Buffered Saline (Life Technologies, Grand Island, NY), extracted DNA as previously described (Graham et al. 2012), and eluted with 70 µl RT-PCR-grade water (Life Technologies). Each set of DNA extractions from field-collected fleas included a newly emerged (unfed) colony-reared *Xenopsylla cheopis* Rothschild (Division of Vector-Borne Infectious Diseases, Fort Collins, CO) as a negative extraction control. We stored all DNA at −80°C until PCR analysis. We extracted DNA from burrowing owl blood using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA).

All templates were amplified and analyzed using a slightly modified version of a previously described SYBR Green I-based real-time PCR assay (Graham et al. 2012). Briefly, we employed M13-tagged primers that amplify an approximately 100-nt variable region of the 12S mitochondrial RNA gene in vertebrates but do not amplify flea DNA. Each reaction included the primers at a concentration of 135 nM each, 1X iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and 10 µl template. A 5-min initial denaturation was followed by 40 amplification cycles. The final cycle was immediately followed by a melting analysis cycle. Each real-time run included at least one no template control (water) and positive control DNA isolated from *Rattus norvegicus* whole blood. The control DNA also served as an inter-run calibrator, ensuring equivalent threshold settings across runs. We ran all samples in duplicate. We purified and sequenced selected amplicons and generated a single sequence for each sample as previously described (Graham et al. 2012).

To verify that the assay detected burrowing owl DNA, we used DNA isolated from burrowing owl blood. By running 10-fold dilutions, five replicates per dilution, we determined that the assay amplified *A. cunicularia hypugaea* DNA and had a linear range of detection (LRD) of at least 1 pg–10 ng (R^2 =0.999, efficiency =93.9%). The limit of detection (1 pg burrowing owl DNA) corresponded to a threshold cycle (Ct) value of 35.81. We selected the highest whole cycle value that fell within the LRD, 35, as our Ct cutoff for this study. We considered a sample positive if the collective Ct value (combined replicates) was ≤35, neither replicate had a Ct value >37, and both replicates yielded similar melting curves with single peaks. We repeated any sample that yielded dissimilar replicates or melting curves with multiple peaks.

To establish an *A. cunicularia hypugaea* target sequence, we amplified the target from 10 replicates of 10 ng burrowing owl blood DNA, sequenced each amplicon, and constructed a consensus sequence.

Testing Unfed Fleas Exposed to Animal Pelts

To simulate a scenario in which fleas infest a vertebrate host but do not feed on it, we exposed 27 newly emerged (unfed), colony-reared *X. cheopis* to a colony-reared mouse (AG129) pelt and 27 to a piece of burrowing owl pelt. Both pelts had been removed from frozen carcasses and stored at −20°C. They were allowed to thaw at ambient temperature immediately before the experiment. We attached a capsule to each pelt by cutting off the top portion of a 50-ml conical tube and securing it with a mixture of hot wax and resin. The area inside the capsule remained covered with fur or feathers. We then placed the fleas in the capsules and secured the conical tube lids so that the fleas were contained on the pelts (Fig. 2). After a 1-h exposure, we collected all the fleas and stored them at −20°C. We also collected pieces of skin from inside each capsule and stored these samples at −20°C. The fleas were later rinsed with DPBS and examined via light microscopy to verify that the exoskeleton was intact. They were then preserved at −20°C pending DNA isolation.

We isolated and amplified DNA from each set of fleas, sequenced the amplicons, and analyzed the sequences using the same protocol we used for the field-collected fleas. As a positive control, each batch of extractions included a colony-reared *X. cheopis* that had been frozen immediately after feeding on a colony-reared SKH1 mouse. We also amplified and sequenced DNA extracted from the mouse and owl skin tissue using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Animal procedures were approved by the Centers for

Disease Control and Prevention Division of Vector-Borne Infectious Diseases Institutional Animal Care and Use Committee.

Results

Flea Identification

Of 81 fleas collected, 6 were too damaged to identify to genus. All of the remaining 75 fleas were identified as *Pulex*: 36 males and 39 females. Four of the males were too damaged to identify to species. We identified the remaining males (*n* =32) as *P. irritans.* We could not distinguish between *P. irritans* and *Pulex simulans* Baker females based on morphology, but because all identified males were *P. irritans*, all females were also presumed to be *P. irritans.*

Burrowing Owl Target Sequence

Using DNA extracted from burrowing owl whole blood, we established a 102-nt target sequence for this species. This consensus sequence matched the only *A. cunicularia* 12S sequence in the NCBI database (AF231330.1:27–119), except that our sequence included a 9-nucleotide insertion (CGAGCACTA) between nucleotides 69 and 70. Basic Local Alignment Search Tool (BLAST) analysis indicated that our 102-nt *A. cunicularia* sequence did not match any other sequence in the NCBI nucleotide collection; homologous sequences from other species differed by at least 9 nucleotides. We therefore considered samples yielding amplicons with this sequence positive for burrowing owl DNA.

Bloodmeal Identification

Of the 75 fleas identified as *Pulex irritans* or *Pulex* sp., 25 were too damaged to test using our bloodmeal assay. Of the 50 fleas we tested, 12 had no detectable vertebrate bloodmeal. The remaining 38 (76%) contained burrowing owl DNA.

Testing Unfed Fleas Exposed to Animal Pelts

All of the mouse-fed control flea samples and both the mouse and owl pelt samples to which the fleas had been exposed tested positive for vertebrate DNA. BLAST analysis indicated that the mouse skin amplicon sequence was consistent with the corresponding sequence for *Mus musculus* (KC663621.1:516-616). The owl skin amplicon sequence was identical to our consensus sequence from burrowing owl whole blood DNA. We did not detect vertebrate DNA in any of the unfed, pelt-exposed fleas (*n* =54).

Discussion

We found that a molecular assay developed to identify vertebrate bloodmeals in Ugandan cat fleas was flexible enough to detect burrowing owl DNA in North American *P. irritans.* We further demonstrated that the assay did not detect vertebrate DNA in fleas that had not taken a vertebrate bloodmeal but had been exposed to mouse or owl pelts. The assay is therefore unlikely to detect DNA in fleas from vertebrates that have served exclusively as phoretic hosts. Using this assay, we detected burrowing owl DNA in 38 *P. irritans* collected from burrowing owls in southern Idaho. We conclude that *P. irritans* feeds on burrowing owls.

Our finding raises questions about the potential effects of ecto-parasitism on owls. The few available studies of fleas on burrowing owls indicate that 1) Juveniles infested with fleas disperse from natal areas significantly later than those treated with insecticide, but only in some years (V. Garcia and C. Conway, unpublished data), 2) Nests with higher flea loads have lower productivity (J. Welty and J.R.B., unpublished data), and 3) Nest-site reuse does not appear to be affected by the presence or absence of fleas (Riding and Belthoff 2015). Other important aspects of the host–parasite relationship between burrowing owls and *P. irritans* are poorly understood, e.g., if and how this relationship alters owl physiology, immunology, parental care, or juvenile survival after leaving nests, and if it has any long-term reproductive consequences for the owls (e.g., Richner and Tripet 1999). Given that blood feeding depletes nutrients and can induce energy-demanding immune responses in the host (Møller et al. 2005, Krasnov 2008), our finding suggests that additional studies are merited to determine the extent to which *P. irritans* impact declining burrowing owl populations.

The finding that burrowing owls serve as a bloodmeal source for *P. irritans* also has potential implications for enzootic plague dynamics. Avian hosts are generally considered resistant to *Y. pestis* infection (Meyer 1950), although the wheatear (*Oenanthe spp.*), has been found to be infected with *Y. pestis* in the former Soviet Union and Mongolia (Shevchenko et al. 1980, Galdan et al. 2010). A vertebrate host must develop a high bacteremia ($\geq 10^6$ colony forming units/ml) to reliably infect feeding fleas (Engelthaler et al. 2000, Lorange et al. 2005). If an infected *P. irritans* feeds on a burrowing owl, it is unlikely that the burrowing owl will develop a sufficient bacteremia to subsequently infect other fleas. Also, taking an uninfected bloodmeal can reduce the probability that an infected flea transmits *Y. pestis* the next time it feeds (Eisen et al. 2006, 2007; Wilder et al. 2008a, b). Thus, if *P. irritans* serves as a *Y. pestis* vector within a landscape shared by burrowing owls and *Y. pestis*-susceptible mammals, *P. irritans* feeding on burrowing owls may actually reduce the force of infection within that ecosystem.

It is important to note, however, that in the Pacific Northwest, the geographic region where *P. irritans* most commonly parasitize burrowing owls, the owls are unlikely to have contact with mammals carrying infected *P. irritans* because the other primary *P. irritans* hosts are various Canidae (Hopla 1980). Canids, while susceptible to plague, rarely succumb to infection and are considered unlikely to provide a source of infection for feeding fleas (Gage et al. 1994). Burrowing owls in Idaho, Oregon, and Washington often nest in burrows dug by American badgers (*Taxidea taxus*) (Belthoff et al. 2015). Serologic surveys in this region indicate that the American badger has exposure to *Y. pestis*, but it develops only a transient infection (Messick et al. 1983). Thus, though there may be interaction between owls and some carnivores that are carrying fleas, those carnivores are unlikely to infect fleas and thus are unlikely to serve as a source of infected *P. irritans*. In this region, ground squirrels are the mammals most likely to develop a transmissible bacteremia. These species almost never host *P. irritans* (Hopla 1980, Barnes 1982).

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Fig. 1.

A nesting pair of adult burrowing owls occupying an artificial burrow nest site within the Morley Nelson Snake River Birds of Prey National Conservation Area in southwestern Idaho. The tunnel entrance to the subterranean nest chamber is visible.

Fig. 2.

Fleas inside a capsule on a pelt from a burrowing owl nestling. To simulate a scenario in which fleas infest a vertebrate host but do not feed on it, unfed *Xenopsylla cheopis* were secured on owl or mouse pelts in capsules with screw-top lids (not pictured) for a 1-h exposure period. Fleas were subsequently tested using our bloodmeal assay to determine if the assay would detect vertebrate DNA.