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Estimating parasite infrapopulation size given imperfect detection: Proof-of-concept with ectoparasitic fleas on prairie dogs

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ABSTRACT

Parasite infrapopulation size - the population of parasites affecting a single host - is a central metric in parasitology. However, parasites are small and elusive such that imperfect detection is expected. Repeated sampling of parasites during primary sampling occasions (e.g., each host capture) informs the detection process. Here, we estimate flea (Siphonaptera) infrapopulation size on black-tailed prairie dogs (*Cynomys ludovicianus*, BTPDs) as a proof-of-concept for estimating parasite infrapopulations given imperfect detection. From Jun–Aug 2011, we live-trapped 299 BTPDs for a total of 573 captures on 20 plots distributed among 13 colonies at the Vermejo Park Ranch, New Mexico, USA. During each capture, an anesthetized BTPD was combed 3 times consecutively, 15 s each, to remove and count fleas. Each flea ($n = 4846$) was linked to the BTPD from which it was collected and assigned an encounter history ('100', '010', '001'). We analyzed the encounter histories using Huggins closed captures models, setting recapture probabilities to 0, thereby accounting for flea removal from hosts. The probability of detecting an individual flea (p) increased with Julian date; field personnel may have become more efficient at combing fleas as the field season progressed. Combined p across 3 combings equaled 0.99. Estimates of flea infrapopulation size were reasonable and followed the negative binomial distribution. Our general approach may be broadly applicable to estimating infrapopulation sizes for parasites. The utility of this approach increases as p declines but, if p is very low, inference is likely limited.

1. Introduction

Parasite transmission is expected to increase with parasite abundance (May and Anderson, 1979). Consequently, parasite 'infrapopulation size' for individual hosts is a key metric in parasitology and disease ecology (Bush et al., 1997). Yet, parasites are relatively small and elusive, and imperfect detection is expected. Moreover, technological and methodological limitations are influential when estimating infrapopulation sizes for parasites (McClintock et al., 2010; Huyvaert, 2021).

Investigators have called for improved detection and enumeration of parasites (Huyvaert et al., 2018; Han and Ostfeld, 2019). In this context, repeated sampling of hosts during each primary occasion (e.g., each host capture) informs the detection process (McClintock et al., 2010; Huyvaert, 2018). Mark-recapture models might be used, but most parasites are difficult to mark. Fortunately, models are available to estimate infrapopulation size of unmarked parasites while accounting for the probability of detecting individuals (p). If model assumptions are met, or

mostly met, and p is reasonable, then the resulting estimates of parasite infrapopulation size might be useful. If p is relatively low, however, inference is limited (White et al., 1982).

Here, we estimate infrapopulation sizes of adult fleas (Siphonaptera) on black-tailed prairie dogs (*Cynomys ludovicianus*). Black-tailed prairie dogs (BTPDs) are colonial, burrowing sciurids in the grasslands of western North America. BTPDs are currently restricted to <2% of their historic range. Arguably, plague – an introduced disease caused by the flea-borne bacterium *Yersinia pestis* – is the greatest threat to BTPDs and associated species, including the endangered black-footed ferret (*Mustela nigripes*), a specialized predator of four *Cynomys* species (Eads and Biggins, 2015). Considerable effort is devoted to studies of BTPDs, fleas, and plague, and much remains to be learned (Salkeld et al., 2016; Eads et al., 2022).

Fleas are small, wingless, blood-feeding insects found parasitizing vertebrates around the world (Krasnov, 2008). Fleas have been marked by injecting hosts with iron-59 citrate (Warren-Hicks et al., 1979) or

injecting fleas with radioactive isotopes (Kharlamov, 1965). Fleas have also been marked by removing terminal segments from legs (Mead-Briggs, 1964) or painting or dying their exoskeletons (Kosminsky and Soloviova, 1959). In all cases, marking negatively affected flea survival and/or behavior, such that fleas are rarely marked for individual identification.

Little is known about the probability of detecting individual fleas on hosts while combing them. Existing knowledge from unadjusted indices of flea combing indicates that p may vary widely (e.g., indexed $p = 88\%$ in Mize [2009] vs. 21% in Beaumont et al., [2019]). In our study system, transmission of *Yersinia pestis*, the bacterial agent of plague, is known to generally increase with flea abundance (Lorange et al., 2005) and accurate estimation of flea infrapopulation size is an important objective (Eisen et al., 2020). If p is low or highly variable under a given flea detection method, then surveillance, research, and disease management efforts may be inefficient or even ineffective. Here, we estimate flea infrapopulation sizes on BTPDs while accounting for variation in p, and we evaluate potential effects of observer experience with flea combing over time on detection of individual fleas.

A variety of sampling methods and statistical models are available for estimating animal abundance from unmarked populations like those of fleas. We implemented removal sampling (Seber and Whale, 1970) and Huggins closed captures models (Huggins, 1989, 1991) to estimate flea infrapopulation size. We hypothesized the following: (1) within each trapping occasion, p would decline with repeat combings as fleas awoke from anesthesia and were better able to resist removal via combing and (2) p would increase with Julian date, as field personnel became more efficient at combing.

2. Materials and methods

Data from this study are available from Eads (2022). We studied BTPDs and their fleas in the short-grass prairie of Vermejo Park Ranch, New Mexico, USA (36°32′N, 104°45′W) from "Jun 3" to match "Aug 30" 2011. We live-trapped and sampled BTPDs on 20 plots at 13 colonies. Local terrain and habitat determined plot size (1.54 or 2.25 ha). Each plot contained 16 live-traps ha^{-1}. We distributed trapping effort as evenly as possible among plots (Eads et al., 2016). Upon capture, we anesthetized each BTPD and its fleas with isoflurane and combed the BTPD as thoroughly as possible for ectoparasites. DAE, who started studying BTPDs and their fleas in 2005, trained field technicians on the combing procedures, which are described below; DAE demonstrated the procedures several times firsthand, and then monitored and guided each technician's learning over 5 consecutive days (3 Jun through 7 Jun). We marked each BTPD's ears with tags (Monel size 1; National Band and Tag Co., Newport, Kentucky) for permanent identification and released it at the point of capture upon recovery from anesthesia.

During each capture event, BTPDs were anesthetized in an induction chamber, then held vertically by the nape, and combed thoroughly, 3 consecutive times, 15 s each, over 3 separate water-lined tubs to remove and trap fleas. Thus, each processing event was a 'primary occasion' comprising three 15 s 'secondary occasions' (details in Eads et al., 2013, 2015). Individual BTPDs were trapped 1 to 6 times ($\bar{x} = 1.92$ times, *SD* = 1.21) over the course of the field season. Herein, each primary occasion for an individual BTPD, separated by 5 or more days, was considered independent of other primary occasions. Biologically, primary occasions are likely to be independent for a variety of reasons (Krasnov et al., 2006; Eads et al., 2016); for example, between primary occasions, BTPDs can acquire or dispel new fleas when traversing the burrows they excavate and use as refuge and shelter and when interacting with conspecifics (Biggins and Eads, 2019).

One or more fleas sometimes fell from a BTPD into the anesthesia chamber, before combing. Thus, our analyses estimate the number of fleas on a BTPD after anesthesia, during standardized combing. Considering the 263 primary occasions (49% of primary occasions) in which at least 1 flea fell into and was detected in the anesthesia

chamber, numbers of fleas in the chamber (x) were positively correlated with total numbers of fleas (y) combed from BTPDs ($r^2 = 0.559$, $y = 1.509x + 4.138$). In only 10 cases were flea(s) (1–3) found in the induction chamber and no fleas combed from the associated BTPD.

BTPD hosts might be envisioned as "islands" with varying numbers of fleas. In this context, Huggins models assume:

1. Individual fleas were not recounted: Each flea combed from a BTPD was pulled from a water-lined tub, counted, and placed in a vial which was linked to the BTPD from which the flea was collected. Thus, duplicate counts were eliminated.
2. Misclassification errors were eliminated: Misclassifications (i.e., classifying another ectoparasite as a flea when it was not) were eliminated by distinguishing fleas from other collected ectoparasites under light microscopy in a laboratory.
3. Flea infrapopulations were "closed": Births, herein the emergence of adult fleas from pupal cocoons on BTPD bodies, were presumably absent. Fleas are thought to develop as pupae within BTPD burrows, not on BTPDs (Krasnov, 2008), and we have not detected flea pupae on BTPDs. Flea deaths during sampling would facilitate removal and collection. Processing of BTPDs by hand, away from burrows and other BTPDs, should eliminate flea immigration. Emigration was reduced but not eliminated by anesthesia; if fleas awoke from anesthesia, they dove deeper into BTPD fur, which should have reduced emigration (while potentially affecting p).
4. Individual fleas were detected independently: Sometimes, a female and a male flea were collected attached for breeding, but this event was rare (<0.01% of flea identifications). Otherwise, fleas were detected independently.
5. All fleas were equally detectable: This assumption was probably not met fully, given interspecific and intraspecific variation in flea body size, anatomy, and behavior; presumably, larger fleas are easier to detect than smaller fleas (Eads et al., 2015). Nevertheless, as described below, overall p was considered "high", indicating most fleas were detectable (i.e., if they were "available" for detection, a topic considered in the Discussion; Kellner et al., 2022).

We implemented Huggins closed captures models in Program MARK (White and Burnham, 1999). Individual fleas, the sampling unit, were removed from BTPDs, so we set recapture probabilities to 0. Individual fleas were "grouped" by individual BTPD host. Flea encounter histories were '100' if combed into the 1st water-lined tub, '010' if combed into the 2nd tub, and '001' if combed into the 3rd tub. We evaluated both constant p and potential variation in p among secondary occasions. We also considered all possible time variations. Regarding full time variation (i.e., different estimates of p for each secondary occasion), the last p is not identifiable unless a constraint is imposed; we imposed a constraint by considering a linear variable from 1st to 3rd combing (1,2, 3). In addition, we considered a potential linear effect of Julian date of combing on p; detection of individual fleas might have increased over time, as field personnel became more familiar with, and skilled at, combing fleas from BTPDs over the course of the field season. The models estimated flea infrapopulation size ($\hat{N}$) on individual BTPDs as a derived parameter (i.e., conditioned out of the likelihood).

We used an information theoretic approach for model selection based on Akaike's Information Criterion for small sample sizes (AICc) (Burnham and Anderson, 2002). We fit all possible models and calculated AICc differences (ΔAICc) and Akaike weights (w_i = the probability model i is the "best" model given the data and model set). We interpreted the best supported model and all models within ≤ 2 AICc units of the top model.

3. Results

We live-trapped and sampled 299 individual BTPDs a combined total

of 573 times. Two flea species, both known vectors of *Y. pestis*, were most prevalent (*Oropsylla hirsuta* and *Pulex simulans*; Eads, 2014). The Huggins closed captures models 'condition' on primary occasions with at least 1 flea detected. We detected 4846 fleas during 476 primary occasions of standardized combing; the effective sample size of fleas was 14,538 (i.e., 4846 fleas × 3 secondary occasions per primary occasion). Raw flea counts ranged from 1 to 100 per primary occasion and followed the negative binomial distribution characteristic of flea parasitism (Fig. 1; variance-to-mean ratio [VMR] = 150:10).

The quasi-likelihood parameter ($\hat{c}$) for the most general Huggins model was 1.28 (~1.00), indicating no need to correct for over-dispersion (Burnham and Anderson, 2002). Two models were well supported (Table 1); no other model was within 7 AICc units of these models. We interpreted estimates of *p* from both top models, the relationship between Julian date and *p* from the best supported model, and $\hat{N}$ derived from the top model.

The most supported model indicated *p* declined over 3 secondary occasions, from the 1st 15 s combing to the 2nd (the latter of which was equal to the 3rd; Table 1). The second competing model indicated *p* declined from the 1st combing to the 2nd but rebounded in the 3rd (=1st). In both models, *p* was 0.94 after 30 s combing and 0.99 after 45 s combing. Both models included an effect of Julian date on *p*. Flea detection increased by about 10–14% from 3 Jun through 30 Aug (Fig. 1).

Derived estimates of flea infrapopulation size $\hat{N}$ from the most supported model were reasonable, ranging from 1 to 103. Like raw flea counts from BTPDs, estimates of $\hat{N}$ from the most supported model followed the negative binomial distribution (Fig. 1; VMR = 162:11). On average across all BTPD combings, lower and upper 95% confidence limits were 0.36 and 2.95 away from $\hat{N}$.

4. Discussion

As hypothesized, flea detection increased with Julian date. Within primary occasions, *p* declined from the 1st combing to the 2nd and 3rd combing, or *p* declined from the 1st combing to the 2nd but rebounded in the 3rd. Both of these top models make biological sense. Detection may decline from the 1st to 2nd and 3rd combing because many fleas awake from anesthesia by the 2nd combing and dive deeper into BTPD fur. Fleas harbor spines and setae that catch in BTPD fur, helping the fleas to avoid removal (Krasnov, 2008). As flea combing continues from the 2nd to 3rd combing, *p* may remain lower as fleas escape detection, or *p* might increase as fleas become exhausted due to repeated combing and

disturbance. Additional factors could influence detection of ectoparasites on hosts. For instance, some locations on host bodies may be prime locations for escaping host grooming, and perhaps even combing by humans. If there is competition for such locations, dominant ectoparasite individuals might be harder to detect, producing heterogeneity in detection among individual ectoparasites on the same host.

Huggins closed captures models produced reasonable and precise estimates of flea infrapopulation size in our study system. In some studies, abundance estimates from unmarked populations are summed across sampling units to estimate superpopulation size (i.e., all individuals at a particular place and time; Bush et al., 1997). In our case, estimates of $\hat{N}$ from individual BTPDs might be summed to index flea superpopulation sizes on sampling plots, for instance. However, we suggest flea superpopulation size should not be calculated using our approach because we did not sample all BTPDs occupying a plot during a given Julian date and infrapopulation size varies widely among BTPDs. Further, Huggins models assume population closure, which seems reasonable with flea infrapopulations on hosts sampled in hand, but unreasonable for flea superpopulations distributed among BTPDs on plots.

As a proof-of-concept, we analyzed data from the second year (2011) of a 3-year study (Eads, 2014). This particular year, with severe drought in northeastern New Mexico (Eads et al., 2016), proved useful for estimating flea infrapopulation sizes, because we detected at least 1 flea during 83% of primary sampling occasions and the closed captures models conditioned on primary occasions with at least 1 flea detected. Our general approach will be less useful in cases when parasite prevalence or the detection of at least 1 parasite is very low, or when most parasite counts are zero or one. In such cases, prevalence may be a more appropriate measure of parasitism (e.g., Eads et al., 2020), and occupancy (prevalence) models can be used for estimation while accounting for imperfect detection (such models are receiving increased use in parasitology; e.g., Lachish et al., 2012; Eads et al., 2013, 2015; Elmore et al., 2014; Peron et al., 2016; Zanet et al., 2017; Rodriguez et al., 2021; Infante et al., 2022).

Previously, we analyzed these 2011 data to investigate flea occupancy (Eads et al., 2013). In the occupancy framework, *p* is defined as the probability of detecting at least 1 flea on a BTPD carrying at least 1 flea; *p* was 0.99 after three 15 s combings (Eads et al., 2013) – identical to the overall estimate of *p* in this study. Still, estimates of *p* from occupancy and abundance models may differ. With occupancy models, *p* of at least 1 flea is presumably reduced at lower flea abundance (Royle and Nichols, 2003) and flea abundance declines with successive combings as fleas are removed from hosts (Eads et al., 2013). With abundance

Fig. 1. Left: Frequency histogram of raw (field) flea count indices from prairie dogs. Middle and right: Huggins closed captures model estimates for fleas combed from prairie dogs, including a histogram of estimated flea counts (infrapopulation size = $\hat{N}$) and a positive correlation between Julian date and individual flea detection probability (here, *p* from the first combing occasion within primary trapping occasions). In the histograms, counts of 0 fleas (gray bars) are presented for illustration; those data were not analyzed herein, because the Huggins closed captures models 'condition' on primary occasions with at least 1 flea being detected (black bars). Model output is from the top model in Table 1. On the right, dotted lines are 95% confidence intervals.

Table 1

Huggins model structures and selection metrics, including Akaike's Information Criterion for small sample sizes (AICc), AICc differences (ΔAICc), and Akaike weights (w_i). No other model was within 7 AICc units of these models, which examined flea detection probabilities (p) and flea infrapopulation size ($\hat{N}$) for prairie dog hosts. Models were evaluated with detection probabilities varying or constant among 3 secondary combing occasions (15 s duration each) within each primary occasion (45 s total combing). $\hat{N}$ is conditioned out of the Huggins likelihood as a derived parameter. Estimated detection probabilities include 95% confidence intervals (CIs).

Model structure	AICc	ΔAICc	w_i	Estimated detection probability (p, 95% CI)		
				1st combing	2nd combing	3rd combing
$[p_{comb1} \neq (p_{comb2} = p_{comb3})]$ + Julian date$_{comb1-3}$	6203.79	0	0.59	0.76 (0.74–0.77)	0.59 (0.54–0.65)	0.59 (0.54–0.65)
$[p_{comb2} \neq (p_{comb1} = p_{comb3})]$ + Julian date$_{comb1-3}$	6204.63	0.84	0.39	0.78 (0.76–0.79)	0.66 (0.63–0.69)	0.78 (0.76–0.79)

models, the individual flea is of interest, and the abundance of fleas may have little influence on p of individual fleas.

Multiple studies have implemented single, standardized 30 s combings of anesthetized BTPDs with no secondary occasions (e.g., Eads and Biggins, 2019 and citations therein). Our results indicate a single 30 s combing event is useful, and relative comparisons of indexed flea abundance in prior studies were appropriate; herein, p from 30 s combing = 0.94, which is considered "high" by some investigators (e.g., Couturier et al., 2013).

Another flea sampling approach may entail combing each BTPD until no more fleas are detected. Under this approach, if few or no fleas are detected during the initial stages of combing, field personnel stop quickly, sometimes after <15 s. Conversely, if many fleas are collected, personnel may continue combing for several minutes, thinking that particular host may harbor even more fleas. When relative differences among individuals, plots, or experimental treatments, for example, are of interest, this approach could inflate relative differences in flea abundance between groups with few compared to many fleas.

Small degrees of variation in p may be important. In a study of simulated animal count data with 2 treatments, the same number of sites per treatment, and the same mean number of animals per site, there was a 50–90% risk of erroneously declaring that animal counts differed between the 2 treatments when p differed by only 4–8% (Archaux et al., 2012). In the absence of standardized flea combing protocols, p might easily vary by that magnitude or more. In such cases, findings of treatment or environmental effects are potentially spurious and might lead to inappropriate decisions for flea control and plague mitigation. The potential influence of small variation in p highlights the importance of effective training of technicians; we conducted targeted training of technicians in the field and accounted for their experience by including an influence of Julian date on p in closed captures models.

Although our approach was useful in estimating the abundance of fleas on individual BTPDs, we caution that fleas sometimes remained on BTPDs even after 45 s of combing. Namely, after combing BTPDs that harbored many fleas, we still sometimes observed fleas on the BTPD, escaping into the host's fur. We suspect our approach may underestimate the true abundance of fleas on BTPDs harboring many fleas, in particular. Put simply, 45 s of combined combing may not be enough to detect every flea on a BTPD carrying many fleas. However, given the negative binomial distribution of flea abundance, the hosts with large flea-burdens are likely to comprise small proportions of BTPD populations (e.g., Fig. 1) – but they and their fleas are likely to play important roles in the dynamics of *Y. pestis* transmission on BTPD colonies (Biggins and Eads, 2019).

In conclusion, we used Huggins closed captures models to estimate flea infrapopulation size on individual BTPDs while accounting for variation in detection probabilities. Detection of individual fleas was high but imperfect, which encourages standardization of combing methods. When sampling anesthetized BTPDs, combing for 30 or 45 s has proven effective. Our results indicate that cumulative p = 0.94 and 0.99 for 30 and 45 s combing, respectively, though we acknowledge that fleas are sometimes missed, especially from BTPDs harboring many fleas. Generally speaking, our methods might facilitate studies of parasite infrapopulations, thereby increasing scientific understanding of host-parasite relationships and dynamics, with One Health implications for disease mitigation among wildlife and humans.

Declaration of competing interest

Declaration of interest none.

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