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Comparison of direct and indirect techniques for evaluating endoparasite infections in wild-caught newts (*Taricha torosa* and *T. granulosa*)

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ABSTRACT: Studies of amphibian parasites have increased over the past 20 yr, in part because of their role in amphibian population declines and deformities. Such patterns underscore the importance of non-lethal methods for detecting and quantifying endoparasitic infections. The goal of this study was to compare results of indirect methods (fecal smears and fecal floats) with quantitative necropsies to detect endoparasitic infections in adult newts. In 2015, we collected fecal samples from 68 adult newts (*Taricha granulosa* and *T. torosa*) in the East Bay region of California and used fecal smears, sodium nitrate fecal flotation solution, and Sheather's sugar flotation solution to assess infection (i.e. the presence and/or abundance of a parasite). Across all methods, we detected 3 protozoans (*Eimeria tarichae*, *Tritrichomonas* sp., and *Balantidium* sp.) and 3 nematodes (*Rhabdias tarichae*, *Cosmocercoides variabilis*, and *Chabaudgolvania* sp.). Based on generalized linear mixed models, the likelihood of detection varied between hosts (with *T. torosa* showing more overall infection relative to *T. granulosa*) and by assessment method: while fecal smears were more sensitive in detecting protozoans, comprehensive necropsies were the most reliable for quantifying infections of *R. tarichae*. Nonetheless, both the likelihood of *R. tarichae* detection within fecal samples as well as the number of infectious stages observed correlated strongly with infection intensity from necropsy, highlighting the utility of non-lethal assessment methods. The overall congruence between indirect methods and gross necropsy helps to validate the use of less-invasive methods for parasite detection and abundance, especially for sensitive or protected host taxa such as amphibians.

KEY WORDS: Nematode · Protozoan · Amphibian decline · Parasite · Conservation · Disease ecology · Veterinary parasitology

1. INTRODUCTION

A key prerequisite of parasitological research is to identify approaches that reliably and accurately characterize patterns of host infection. Linking infections to pathology-related outcomes in hosts often requires comparative information on which hosts are infected, with which parasites, and at what intensity or burden (Mabbott 2018). Nonetheless, approaches for quantifying infection often incur tradeoffs among accuracy, effort, and impact on the host population (Latham & Poulin 2003, Huver et al. 2015, LaFonte et al. 2015). Accurate and precise methods, such as systematic necropsy of the host, provide perhaps the most reliable data but are labor-intensive and require sacrifice of multiple hosts per population (e.g. between 10 and 50 hosts per population; Poulin 1998, Dove & Cribb 2006, Marques & Cabral 2007, Hartson et al. 2011, Rhoden & Bolek 2012). More indirect methods, such as antibody testing in which host sera is screened for specific antigens (e.g. enzyme-linked immunosorbent assays, ELISA), can make it difficult to differentiate between current and previous infections or quantify the amount of infection (e.g. load or

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intensity) (Robertson et al. 1988, Cheesbrough 2006). Common veterinary tools such as fecal floats and fecal smears, which are minimally invasive to the host, can sometimes generate false negatives or underestimate burden (i.e. fecal egg count may not accurately reflect parasite infection intensity; Dryden et al. 2005, Levecke et al. 2011, Tully 2012). For example, eggs per gram of host feces may vary with worm age, the time of year (seasonality of infection), parasite sex ratio, coinfection, and host condition, limiting the correlation between fecal counts and actual infection prevalence or intensity (Cattadori et al. 2005, Kim et al. 2011). These tradeoffs emphasize the value of using multiple methods to assess infection and quantify their relative efficacy and uncertainty through comparative analysis (LaFonte et al. 2015).

Ongoing declines in amphibian populations and the potential contributions of parasite infections and disease have prompted efforts to characterize the parasite communities within frogs, toads, and salamanders (Densmore & Green 2007, Schotthoefer et al. 2009, Koprivnikar et al. 2012). Larval amphibians are infected by a diverse assemblage of micro- and macroparasites, including bacteria, viruses, protozoans, trematodes, nematodes, and cestodes (Sutherland 2005, Densmore & Green 2007, Koprivnikar et al. 2012). As amphibian disease research progresses, non-lethal methods to assess parasitic infections have become increasingly essential, particularly for sensitive host populations (Retallick et al. 2006, Gray et al. 2012). While direct methods (e.g. necropsy) often offer the most comprehensive assessment of the current parasite community and loads, such approaches cannot be repeated over time and are inappropriate for use on vulnerable hosts or life stages. Quantitative PCR methods using external swabbing of amphibians have been widely used to diagnose *in vivo* infection for chytridiomycosis and ranaviruses (Retallick et al. 2006, Gray et al. 2012). Non-lethal methods have similarly been used to quantify certain helminths that infect amphibians. For example, adult *Halipegus* sp. trematodes can be collected from the eustachian tubes or under the tongue of bullfrogs; after collection, the frogs can be released (Esch 2004). Environmental DNA (eDNA)-based approaches are also increasingly being harnessed to test water or sediment samples for wildlife pathogens, including those infectious to amphibians (Huver et al. 2015), although such results are unlikely to provide information about infection occurrence or load within specific hosts.

In veterinary medicine, non-invasive techniques such as fecal smears and fecal flotations are often used to detect intestinal parasites in livestock, domestic animals, and wild animals (Dryden et al. 2005, Clayton & Gore 2007, Stitt et al. 2007). Fecal smears involve microscopic examination of a sample of feces (<1 g) for motile protozoa or nematode reproductive stages (e.g. ova and larvae) (Foreyt 2001, Dryden et al. 2005, Zajac & Conboy 2006), whereas fecal flotations are more often used when the objective is to concentrate and detect reproductive stages (e.g. eggs of hookworms, roundworms, or whipworms; Zajac & Conboy 2006). The method requires solutions calibrated to a certain specific gravity that induces ova to float, facilitating detection and quantification even when infections are rare (Dryden et al. 2005). However, if the specific gravity is inappropriate for a particular parasite, or if the operculum of the ova has already opened, the method can also yield false negatives (Dryden et al. 2005). Similarly, if parasites within the hosts are not currently producing eggs (e.g. immature or males only), such methods may also yield biased results. Calibrating the application of these non-lethal techniques to minimize (or quantify) bias could allow novel opportunities to detect the presence of endoparasites or estimate parasite load (abundance of parasite individuals host^{-1}), without adversely impacting naturally occurring populations of amphibians.

In western North America, 4 species of *Taricha* (Salamandridae) have been described: rough-skinned newt *T. granulosa* (Skilton 1849), red-bellied newt *T. rivularis* (Twitty 1935), Sierra newt *T. sierra* (Twitty 1942), and California newt *T. torosa* (Rathke 1833). Within this study, we focused on 2 newt species, *T. torosa* and *T. granulosa*, which co-occur in the Bay Area region of California (Myers 1942, Jennings & Hayes 1994, Stebbins & McGinnis 2012). Knowledge of the parasite communities of *Taricha* remains incomplete, due both to the rarity of parasitological studies on these amphibian hosts and the generally depauperate parasite fauna reported (e.g. Goldberg et al. 1998, Johnson et al. 2018). Previous studies have reported intestinal parasites, including protozoans (*Eimeria tarichae*, Levine et al. 1980; and *Tritrichomonas* sp., Lehmann 1960) and several nematodes (*Cosmocercoides variabilis*, Martínez-Salazar et al. 2013; *Rhabdias tarichae*, Kuzmin et al. 2003; and *Chabaudgolvania moraveci*, Richardson & Adamson 1988). Holl (1928) first reported finding *C. variabilis* in the intestines and rectum of *T. torosa*. A less common intestinal nematode of *T. granulosa* is *C. moraveci*, which was described from Vancouver Island, British Columbia, Canada (Richardson & Adamson 1988). *R. tarichae* was first reported in the lungs of *T. torosa* from Berkeley, California (Kuzmin et al. 2003). In a recent study of adult newts (*Taricha* sp.) collected

from California, one of the most common infections observed through necropsy was *R. tarichae* (28.6% of sampled hosts) (Johnson et al. 2018), which helped motivate the current effort to develop and validate non-lethal methods for endoparasite detection.

Here, we evaluated naturally occurring newts (*T. torosa* and *T. granulosa*) collected within ponds from the Bay Area of California to address 2 primary objectives: (1) identify the endoparasite assemblages from this understudied genus of amphibians, and (2) compare the efficacy of non-lethal techniques for detecting and quantifying infections with more intensive, necropsy-based approaches, including the presence of each parasite (i.e. infection prevalence) and the number of parasites per host (i.e. infection load). Specifically, we collected multiple fecal samples from 26 *T. torosa* and 42 *T. granulosa* and compared both parasite detections and estimates of load among 3 methods of non-lethal assessments: fecal flotation with sodium nitrate, fecal flotation with Sheather's sugar solution, and direct fecal smears. These results were compared to necropsy findings, with the ultimate aim of identifying optimal methods for parasite screening in future research efforts. To the best of our knowledge, non-lethal techniques for detecting macroparasitic and protozoan infections in host feces have not been well-studied in wild populations of amphibians, highlighting an opportunity to apply veterinary techniques to an ecologically important group of vertebrates (Densmore & Green 2007).

2. MATERIALS AND METHODS

2.1. Field collection and newt husbandry

Between July and August 2015, a total of 26 adult *Taricha torosa* and 42 adult *T. granulosa* were collected from 14 pond ecosystems in the East Bay region of California (only Contra Costa and Alameda counties). Within this area, the 2 newt species have sympatric distributions, frequently co-occur within the same ponds, and share similar body sizes and breeding phenologies, likely exposing them to similar parasites (Tan & Wake 1995, Stebbins & McGinnis 2012, Johnson et al. 2018). Adult newts were collected using dip-net sweeps, seine hauls, and by hand from within ponds or on the surrounding shoreline (Johnson et al. 2018). After collection, newts were maintained individually in environmental chambers within a 2.25 l plastic container with a lid and the bottom covered with a damp paper towel (dampened with tap water that had been UV-sterilized and carbon-filtered; hereafter referred to as 'treated water') at a constant temperature (22°C) with a 12 h light:12 h dark photoperiod. During the holding period, the newts were not fed to ensure intestinal parasites woul not be introduced to the host.

2.2. Fecal collection

The containers were checked daily, and approximately 2 fecal samples were collected per newt. When the paper towel appeared soiled, we rinsed the content with treated water through a 500 µm filter (to eliminate large debris in feces) into a sterile 50.0 ml centrifuge tube. After a 15 min settling period, we used a 1.0 ml glass pipette to aliquot 0.5 ml of each fecal sample into 2.2 ml sterile centrifuge tubes that were assigned to 1 of 3 fecal analysis methods: direct fecal smear, Sheather's sugar flotation solution (Jorvet) or Fecal float (Phoenix), commonly known as sodium nitrate solution (hereafter referred to as $NaNO_3$). In 5 (19.0%) *T. torosa* and 14 (33.0%) *T. granulosa*, only a single fecal sample was obtained from the host; therefore subsamples were used for each of the 3 fecal treatments as described above.

2.3. Parasitological analysis

2.3.1. Direct smear

For one of the 0.5 ml subsamples from each fecal sample, we examined the material directly on a glass slide (approximately 30 µl) with a cover slip using an Olympus BX51 microscope at 400× magnification. Any detected parasites or ova were measured using an ocular micrometer and photographed using an Olympus DP imaging system. Across all methods, we identified parasite eggs, larvae, and/or protozoans using the taxonomic keys outlined in Duszynski et al. (2007), Khan (2008), and Anderson et al. (2009).

2.3.2. Fecal flotations

Two different solutions were used to perform fecal flotations following standard methods outlined by Dryden et al. (2005). Using the 0.5 ml fecal aliquots collected above, we added either 2.0 ml of $NaNO_3$ (specific gravity [SG]: 1.18–1.20 g ml^{-1}) or 2.0 ml of Sheather's sugar solution (SG: 1.27 g ml^{-1}) to the appropriate vial until a convex meniscus formed. Cover slips were placed on top of the centrifuge tubes to

remain in contact with the fluid and maintained for 15 min (see Zajac & Conboy 2006 for detailed methods). After 15 min, each cover slip was removed from the vial and placed on a clean glass slide and examined at 400× magnification on an Olympus BX51 microscope for eggs or larva using methods described in Dryden et al. (2005).

2.3.3. Necropsy

Following the fecal collection period (~1 wk), we humanely euthanized each host using a 10% solution tricaine methanesulfonate (MS-222) (Western Chemicals) and sodium bicarbonate (Fisher) for 10–15 min before identifying all infectious stages of parasites with a focus on nematode worms and protozoans. We completed gross necropsy on each individual host by systematically removing and dissecting major organs or tissues (i.e. skin, muscle, tongue, mandible, gall bladder, heart, kidneys, liver, lungs, intestines, rectum and spleen) for parasite infections (Hartson et al. 2011, LaFonte et al. 2015, Johnson et al. 2018). After a thorough examination with the aid of an Olympus SZX10 stereo dissection microscope, all organs and organelles were then individually compressed between 2 plastic gridded petri dishes to further facilitate detection of embedded parasites. For the digestive tract, a shallow cut was made from the start of the esophagus through to the rectum, and any contents were removed by both a saline flush and a gentle scraping with forceps. Contents were then viewed using the same microscope. The intestinal tract tissue was then compressed between petri dishes to look for any remaining parasites. The lungs were examined using a similar method. Here, we focused on nematodes in the intestines, rectum, and lungs, and protozoans found in the intestines and rectum. Nematodes (adults, eggs, and larvae within the host) were enumerated as observed through necropsy in the host and used in the Poisson analysis described below, whereas protozoans were documented as present or absent (binomial analysis). For nematodes only, we also quantified the number of adult female worms detected in necropsied hosts because these are the individuals responsible for the generation and release of eggs in the host feces.

2.4. Statistical analysis

To evaluate the efficacy of the alternative methods in detecting infection by different protozoan and nematode infections, we compared the capacity of each assay to predict the presence of a given parasite within a newt host. The presence of the parasite was treated as a binomially distributed response variable within generalized linear mixed models (GLMMs) with a logit link (implemented using the 'glmer' function in 'lme4' package; Bates et al. 2015). As predictors, we included the assay method (fecal smear, sugar flotation, $NaNO_3$, or necropsy), host species (*T. torosa* or *T. granulosa*), and body size (snout–vent length [SVL], in mm). Individual host identity was included as a random intercept term to account for the repeated, non-independent observations on each individual (i.e. across the 4 different methods). This analysis was repeated separately for each parasite taxon. Our goal was to determine whether specific methods were significantly more effective at detecting infection, while accounting for the fact that observations on the same host were not independent. We used the 'multcomp' package to perform multiple pairwise comparisons between the methods using Tukey's all pair-comparisons (Hothorn et al. 2017). All analyses were conducted in the R statistical environment (R Development Core Team 2016).

For the most common nematode, *Rhabdias tarichae*, we further examined how infection intensity (i.e. the number of female worms detected during necropsy, $\log_{10}[x + 1]$ transformed), influenced the likelihood of detection among the 3 non-lethal assays, using the same analytical approach. This transformation was only applied to *R. tarichae* because it was the only nematode with sufficiently abundant infections to analyze statistically. Thus, in this case, we examined the relationship between the observed number of female nematodes in a host (as assessed at necropsy) and nematode detection (yes or no) by each of the non-lethal methods. Among the subset of hosts determined to be infected at necropsy, we also tested the degree to which the number of female worms predicted the number of infectious stages (eggs or larva) observed in the non-lethal assay methods. We modeled the number of infectious stages as an overdispersed Poisson distribution with a log-link function and included fixed effects for the number of female worms, assay method, and host species, with random intercept terms for host identity and each individual observation (Johnson et al. 2018). This approach is similar to using a negative binomial distribution to account for the overdispersion that is common to count data. Numeric predictor variables were scaled and centered prior to inclusion.

3. RESULTS

In total, we collected 52 fecal samples from 26 *Taricha torosa* hosts and 84 samples from 42 *T. granulosa* (average of 1.7 fecal samples host^{-1}). Across all examination techniques, a total of 6 parasite taxa were encountered: 3 nematodes, representing both direct and indirect life cycles, and 3 protozoans, all of which have direct life cycles. Of those infections, the non-lethal methods detected 97.5% of protozoan infections determined at necropsy. One of the protozoans, *Tritrichomonas* sp., was found in the rectum of both newt species, while *Eimeria tarichae* was only detected in the intestines of *T. torosa* and *Balantidium* sp. was only in the rectum of *T. granulosa*. Infection prevalence — as determined at necropsy — varied among species: *Tritrichomonas* sp. (30.8% of *T. torosa* and 21.4% of *T. granulosa*), *E. tarichae* (26.9% in *T. torosa* only), and *Balantidium* sp. (45.2% in *T. granulosa* only). Protozoans were only detected through fecal examination or necropsy, not through either flotation technique (see Section 3.1.1).

For nematodes, non-lethal methods detected 75% of nematode infections found in the host during necropsy. We detected adult females (i.e. those bearing eggs) of *Rhabdias tarichae* in the lungs of both newt species, adult *Cosmocercoides variabilis* in the small and large intestines, and adult *Chabaudgolvania* sp. in the intestines. Interestingly, both the ova and larvae of *R. tarichae* were detected in the intestines of infected newts (Kuzmin et al. 2003). With the aid of an Olympus BX51 microscope at 400× magnification, we used relative characteristics (elongated body, rounded head, and pointed tail) to help identify *R. tarichae* larvae (Kuzmin et al. 2003), and also confirmed morphological identifications with genetic approaches (D. M. Calhoun et al. unpubl. data). Despite sampling more *T. granulosa* than *T. torosa* (42 and 26, respectively), we found strikingly little nematode infection among *T. granulosa*, regardless of the method (binomial GLMM: host species [*T. torosa*] coefficient ± SE: 6.09 ± 1.51, $p < 0.0001$; n = 816 observations on 68 hosts). Infection prevalence, as determined by necropsy, consisted of *R. tarichae* (42.3% of *T. torosa* and 2.4% of *T. granulosa*), *C. variabilis* (7.7% in *T. torosa* only), and *Chabaudgolvania* sp. (3.8% in *T. torosa* only) (Table 1). Among *T. torosa* hosts with infection at necropsy, the number of female worms per host averaged (±1 SE) 4.3 ± 0.51 for *R. tarichae* (range: 1–14; n = 11); only 2 hosts were infected with *C. variabilis*; for *Chabaudgolvania* sp. only a single *T. torosa* was infected with 2 adult females. One *T. granulosa* was infected with a single adult female *R. tarichae*.

3.1. Comparison of parasite detection techniques

3.1.1. Protozoans

The flotation techniques rarely identified any of the protozoan species (3 out of 408 possible cases). We therefore focused our comparisons between direct fecal examinations and host necropsy. Interestingly, the direct fecal method was significantly more likely to detect the occurrence of 2 of the protozoans relative to necropsy (Fig. 1) (binomial GLMM: *Tritrichomonas* necropsy coefficient ± SE: −14.76 ± 2.49, $p < 0.00001$; *E. tarichae* necropsy coefficient: −14.81 ± 4.11, $p < 0.0005$; n = 136 observations on 68 hosts). For *Balantidium* sp., there was no effect of method on detection (necropsy coefficient: 0.61 ± 0.79, $p = 0.45$; n = 136 observations on 68 hosts). Overall, fecal examinations detected 17 cases of *Tritrichomonas* sp., 7 cases of *E. tarichae*, and 5 cases of *Balantidium* sp. not found by necropsy, compared with no cases in which infection was detected only based on necropsy. There was no effect of host body size (SVL) on protozoan parasite detections.

Table 1. Detections of nematode species in newts as determined through gross necropsy. For each nematode species, data are the number of examined newts (and percentage) that were positive for infection through observation of nematode eggs, larvae, adult worms, and in total (through any nematode life stage). *Tato*: *Taricha torosa* (n = 26); *Tagr*: *Taricha granulosa* (n = 42)

Parasite	Life stage of nematodes detected in newt host							
	Eggs		Larvae		Adults		Total individuals	
	Tato	*Tagr*	*Tato*	*Tagr*	*Tato*	*Tagr*	*Tato*	*Tagr*
Chabaudgolvania sp.	0	0	0	0	1 (3.8%)	0	1 (3.8%)	0
Cosmocercoides variabilis	0	0	1 (3.8%)	0	2 (7.7%)	0	2 (7.7%)	0
Rhabdias tarichae	10 (38.5%)	1 (2.4%)	9 (34.6%)	1 (2.4%)	11 (42.3%)	1 (2.4%)	11 (42.3%)	1 (2.4%)

3.1.2. Nematodes

Because infections by *Chabaudgolvania* sp. and *C. variabilis* were relatively rare in the data set, we focused the statistical analysis on *R. tarichae* in *T. torosa*. Detection likelihood was similar across all methods, with 34.6, 38.4, 38.4, and 42.3% hosts showing evidence for positive infection based on direct fecal, sugar flotation, $NaNO_3$, and necropsy, respectively (Fig. 2). Based on the GLMM analysis, there was no effect of assay method (likelihood ratio test, $\chi^2 = 2.98$, df = 2, p = 0.39; n = 272 observations on 68 hosts), nor were there effects of host species or host body size (both p > 0.18). Necropsy had the greatest number of total detections (12 of 26 total *T. torosa*), compared with 11 of 26 for any of the non-lethal methods (Table 2). However, 2 hosts showed evidence of infection based on the non-lethal assays that were not detected at necropsy; for one, larvae of *R. tarichae* were detected by all 3 indirect methods which may be indicative of host clearance (see Section 4).

For *R. tarichae*, the number of female worms per host at necropsy positively predicted the likelihood that the non-lethal assays would detect the infection (binomial GLMM: scale [$\log_{10}${females + 1}] coefficient: 5.94 ± 2.31, p = 0.01; n = 204 observations on 68 hosts; Fig. 3). There was no effect of method (p > 0.75) or host body size (p > 0.40). Host infection intensity also correlated positively with the number of nematode larvae detected using indirect approaches. Thus, among hosts that were positive for infection at necropsy, the number of female worms positively predicted the abundance of infectious stages detected by non-lethal methods (overdispersed Poisson GLMM: scale [females] coefficient: 1.32 ± 0.44, p = 0.003; n = 36; no effect of method, likelihood ratio test $\chi^2 = 0.67$, df = 2, p = 0.72; no effect of host body size, p = 0.13). This suggests that, for each one unit increase in female worms, the number of infectious stages increased by 3.73-fold.

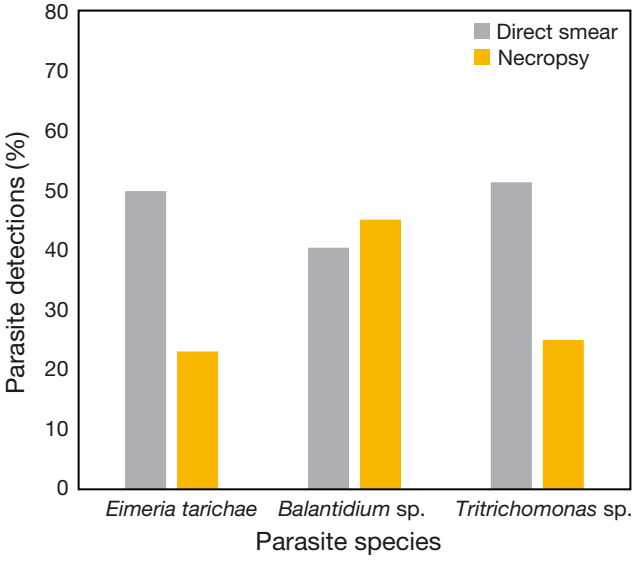

Fig. 1. Detections of the protozoans *Eimeria tarichae*, *Balantidium* sp., and *Tritrichomonas* sp. among newt hosts using direct fecal smears versus gross necropsy. For *Tritrichomonas* sp., which occurred in both *Taricha torosa* and *T. granulosa*, results represent the pooled prevalence across examined newts of both species (n = 68). In contrast, *E. tarichae* was only detected in *T. torosa* (n = 26 examined) and *Balantidium* sp. only in *T. granulosa* (n = 42 examined), such that these prevalence values were not pooled across newt species

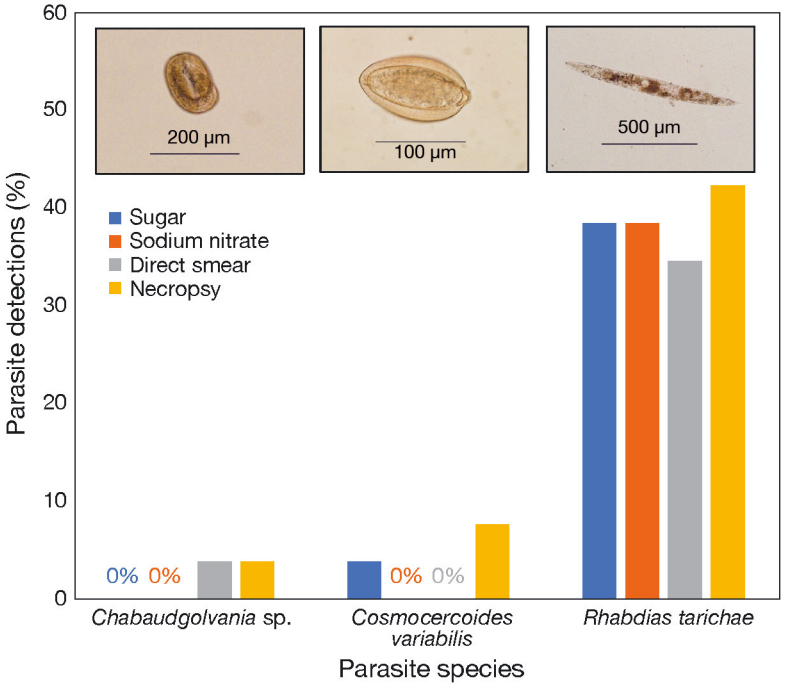

Fig. 2. Detections of the nematodes *Chabaudgolvania* sp., *Cosmocercoides variabilis*, and *Rhabdias tarichae* from California newts (*Taricha torosa*, n = 26 examined) as a function of sampling method: 3 non-lethal methods using fecal samples (Sheather's sugar solution, sodium nitrate fecal solution, and direct smears) and gross necropsy. Images were captured using an Olympus DP imaging system

4. DISCUSSION

The use of non-lethal methods to detect amphibian pathogens has applied as well as logistical advantages. Although less-invasive approaches using

Table 2. Detections of each parasite taxon as a function of newt host species and detection method. Listed is the number (and percentage) of positive detections from each method relative to the total number of hosts examined. This includes the 3 non-lethal methods (fecal floats and smears) as well as direct methods (necropsy with enumeration of adult nematodes, presence/absence for protozoans) of the same hosts. *Tato*: *Taricha torosa* (n = 26); *Tagr*: *Taricha granulosa* (n = 42)

Parasite	Detection method							
	Sheather's sugar		Sodium nitrate		Direct smear		Necropsy	
	Tato	*Tagr*	*Tato*	*Tagr*	*Tato*	*Tagr*	*Tato*	*Tagr*
Nematodes								
Chabaudgolvania sp.	0	0	0	0	1 (4%)	0	1 (4%)	0
Cosmocercoides variabilis	1 (4%)	0	0		0	0	2 (8%)	0
Rhabdias tarichae	10 (38%)	0	10 (38%)	0	9 (35%)	0	11 (42%)	1 (2%)
Protozoans								
Balantidium sp.	0	1 (2%)	0	0	0	17 (40%)	0	19 (45%)
Eimeria tarichae	2 (8%)	0	0	0	13 (50%)	0	6 (23%)	0
Tritrichomonas sp.	0	0	0	0	14 (54%)	21 (50%)	8 (31%)	9 (21%)

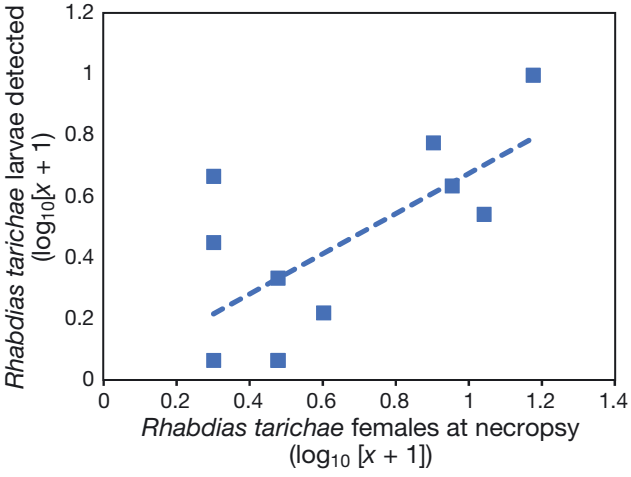

Fig. 3. Relationship between the number of female *Rhabdias tarichae* worms per newt (*T. torosa* only) at necropsy and the average abundance of nematode infectious stages (L3) detected across the non-lethal assays (Sheather's sugar solution, sodium nitrate fecal solution, and direct smears). There were no statistical differences among the non-lethal methods, and for clarity the results are combined here (averaged across methods). Both variables are $\log_{10}(x + 1)$ transformed

toe- or tail-clips, skin or buccal swabs, and eDNA have been successfully used to screen wild-caught amphibians for fungal and viral infections (Price 2013, Umile et al. 2014), relatively few such surveys have been used to quantify endoparasites in wild amphibians. Our use of a comparative approach to assess non-lethal methods for detecting amphibian endoparasites provided an opportunity to expand on widely used veterinary techniques for detecting infectious stages through fecal samples. For both protist and nematode infections, our results indicated that indirect assay methods were capable of detecting the same range of protist and nematode infections discovered during necropsy, albeit with varying degrees of sensitivity. Detection probabilities for each parasite infection varied between host species and assessment method. Among 26 *Taricha torosa* and 42 *T. granulosa*, 76.9 and 61.9% were infected with at least one parasite (nematode or protozoan). Direct fecal smears were especially effective at detecting protozoan infections (Fig. 1). For instance, *Tritrichomonas* sp. and *Eimeria tarichae* were detected in every host when the host was positive by necropsy, whereas *Balantidium* sp. was detected by direct smears in only 53.6% of cases where newts were positive upon dissection. Interestingly, direct smears also detected protozoan infections in an additional 26% of hosts found to be negative at necropsy. Fecal flotations were not effective in detecting protozoans, as expected.

For nematodes, infections were significantly more likely to be detected using necropsy compared to any of the non-lethal methods. Relative to necropsy, fecal flotation approaches yielded 5.7% fewer nematode detections, on average, in both species of *Taricha*. Because the overall prevalence of *Cosmocercoides variabilis* and *Chabaudgolvania* sp. were both low (7.7% and 3.8% in *T. torosa*, respectively), we focused our statistical analyses on *Rhabdias tarichae*, which was the most commonly detected nematode (*T. granulosa* overall prevalence = 61.9%; *T. torosa* overall prevalence = 76.9%). Overall, fecal smears and flotations detected 91.7% of *R. tarichae* infections found during necropsy, with only 2 observations of nematode larvae from the indirect methods that were not also seen during dissection. There were few differences among the flotation solutions in their estimated detection likelihood for *R. tarichae* (Fig. 2), although fecal smears were generally less sensitive.

Importantly, the number of adult female *R. tarichae* detected during necropsy was also a significant, positive predictor of both the likelihood that nematode larvae would be detected using non-lethal methods as well as the number of larvae within fecal samples (Fig. 3). Thus, while non-lethal methods were somewhat less sensitive in detecting *R. tarichae* relative to necropsy, they nonetheless offered a reasonable approximation as to whether a host was infected, as well as the parasite burden (i.e. infection intensity).

An interesting observation was the detection of infections using indirect assay methods that were not subsequently detected by necropsy. Specifically, 66% of hosts that were negative for protozoans based on necropsy were positive for protozoans by direct fecal smear. This outcome could have at least 2 non-mutually exclusive explanations. First, the assay result could be a false positive, for instance resulting from contamination of the fecal sample. Second, the infection status of the host could have changed between fecal collection and subsequent dissection. Although the risk of contamination was limited by maintaining hosts individually, using treated water, and sterilizing containers before re-use, we cannot completely rule out this explanation as some parasitic or commensal protozoans are opportunistic, persisting outside of the host or under unfavorable conditions (Schlichting & Sides 1969, Corliss & Esser 1974). During asexual reproduction, some protozoans create cysts that allow them to establish on hosts, plants, in wind, or in water and survive desiccation, extreme temperatures, and even ultraviolet irradiation (Schlichting 1961, Schlichting & Sides 1969). These dormant cysts are able to remain intact and continue their lifecycle when favorable conditions resume (Corliss & Esser 1974).

By virtue of the study design, hosts were maintained in the laboratory for a period of between 1 and 21 d, which could have also afforded an opportunity for clearance or the loss of infections over time. Thus, changes in host infection status over the course of captivity is plausible; for instance, if protozoans or nematodes died or were cleared from hosts between collection of the fecal samples and subsequent necropsy, this could lead to higher detection probabilities in the non-lethal methods — as was observed especially for protozoans. Further study is needed to understand how both infection status and infection load change over time within infected hosts, including whether the parasites in this study can be effectively cleared through host immunity.

Our results also offer insights into infection patterns of protozoan and nematode infections in naturally occurring newt populations. Few surveys have characterized parasites found in species of *Taricha*, and those that have described their communities as 'depauperate' (Ingles 1936, Goldberg et al. 1998, Johnson et al. 2018). While most of the parasites detected here appeared to be relative generalists, occurring in both newt species (as well as some other anurans; Kuzmin et al. 2003, Martínez-Salazar et al. 2013, Johnson et al. 2018), the protozoans *E. tarichae* and *Balantidium* sp. are apparent specialists, occurring only in *T. torosa* and *T. granulosa*, respectively. Consistent with Johnson et al. (2018), we found fewer nematode infections in *T. granulosa* (overall prevalence = 2.4%) relative to *T. torosa* (overall prevalence = 46.2%), despite these species often occurring in the same ponds as well as a larger sample size for *T. granulosa* (Table 1). One potential explanation for these differences is variation in toxicity: rough-skinned newts (*T. granulosa*) often contain higher concentrations of the potent neurotoxin tetrodotoxin (TTX) relative to *T. torosa* (Johnson et al. 2018). Indeed, Johnson et al. (2018) found there may be different immunological responses between the 2 species of *Taricha* relative to the amount of TTX present and suggested parasite burdens may differ as a result. Gall et al. (2011) found that TTX levels in *Taricha* spp. decrease as development progresses from eggs to metamorphosis, after which it increases for the adult stage. Macy (1960) also reported a low fecundity of the trematode *Megalodiscus microphagus* when it occurred in *T. granulosa* relative to other amphibian species, despite presumably lower concentrations of TTX within the rectum where this parasite typically occurs. Additional study is thus needed to understand what drives the interspecific differences in infection between these congeneric and often sympatric species.

In conclusion, the non-lethal methods employed here provide a sensitive method for detecting protozoan infections as well as a reasonable approximation of nematode infection presence and load. With limited studies of indirect techniques to detect or quantify amphibian endoparasites, this study offers a valuable first step toward providing an easy, mobile, and cost-effective suite of methods for parasite assessment in field collections. Fundamentally, the effectiveness of indirect methods based on fecal collections to qualitatively estimate parasite populations depends on the relative costs to sensitive amphibian populations traded against the efficacy of such approaches to reliably detect endoparasites in aquatic systems. The development of effective, non-lethal detection methods for parasites in amphibians would

reduce the need to euthanize a large number of hosts in sensitive populations. Such methods would be especially valuable for endangered or threatened amphibian populations while also providing tools for surveillance and management programs among amphibian communities. For example, pond ecosystems in California, USA, may include threatened species such as the California red-legged frog *Rana draytonii* and the California tiger salamander *Ambystoma californiense*, for which relatively little is known regarding their endoparasitic infections. The coupling of non-lethal parasite assessment methods with mark–recapture studies could allow development of population-dynamic models to assess both long-term changes in infection or potential fitness consequences associated with parasitism.

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