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Quantifying larval trematode infections in hosts: A comparison of method validity and implications for infection success

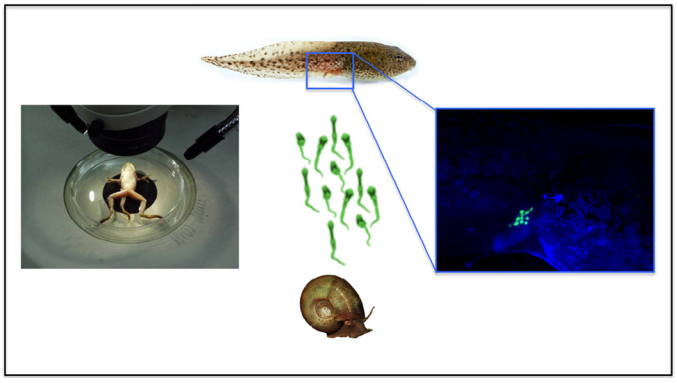
Bryan E. LaFonte [1], Thomas R. Raffel [2], Ian N. Monk [2], Pieter T.J. Johnson [1,*]

[1] Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309, USA
[2] Biological Sciences, Oakland University, Rochester, MI 48309, USA

HIGHLIGHTS

- Accurate methods to quantify trematode infections in intermediate hosts are essential.
- We contrast the efficacy of necropsy, tissue clearing, and fluorescent labeling.
- Optimized fluorescent labeling offered a persistent, accurate, and non-lethal method.
- In amphibians, infection was a linear function of exposure, with no density dependence.

GRAPHICAL ABSTRACT



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ABSTRACT

Accurately estimating parasite transmission success and subsequent infection load has important ramifications for a wide range of disease-related questions and research disciplines. Recent interest in the role of parasites in amphibian population declines and deformities, for instance, has prompted increased interest in approaches to quantify infection and pathology. Here, we introduce a novel method of fluorescently labeling trematode cercariae and optimize its application to interactions between the pathogenic trematode *Ribeiroia ondatrae* and amphibian hosts (*Lithobates sphenocephalus*). We then compare the efficacy of this method with two other approaches commonly used to assess infection in second intermediate hosts – necropsy and tissue clearing – with a focus on accuracy, precision, and bias. Dye (Invitrogen, BODIPY® FL C12) concentrations of <200 nM and DMSO solvent concentrations <0.2% offered a highly visible, long-lasting marker with no detectable effects on cercariae survival or metacercariae establishment. Among methods, the necropsy approach yielded the highest proportion of detected parasites and the lowest standard error around the mean. However, the fluorescent labeling method offered highly similar results ($r = 0.99$), with an estimated 75% of administered parasites establishing successfully. At low to moderate parasite exposure dosages, tissue clearing was comparable to the other two methods, but tended to underpredict infection at the higher exposures (i.e., 'proportional bias'), likely because individual parasites became more difficult to distinguish. Conceptually, these findings illustrate that initial infection within amphibian hosts is a consistent, linear function of exposure dosage, suggesting that parasite density dependence does not

* Corresponding author. Fax: 303 492 869.
E-mail address: pieter.johnson@colorado.edu (P.T.J. Johnson).

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regulate initial establishment. From an applied standpoint, our results offer a methodological foundation for subsequent research using fluorescently labeled parasites, which offer distinct advantages such as allowing *in vivo* parasite tracking within living hosts.

1. Introduction

Ongoing declines and deformities in amphibian populations have catalyzed increased interest in the effects of parasites and pathogens on amphibian ecology and conservation (Blaustein et al., 2012; Densmore and Green, 2007; Kilpatrick et al., 2010; Koprivnikar et al., 2012). Alongside habitat loss, climate shifts, and pollution, infectious diseases represent one of the most important threats confronting amphibians worldwide (Collins and Storfer, 2003; Lips et al., 2006; Stuart et al., 2004). Amphibians become infected with a wide range of parasites, several of which can cause significant pathology in host species. While attention has often focused on the invasive chytrid fungus *Batrachochytrium dendrobatidis*, viral infections can cause population die-offs (e.g., ranaviruses such as FV3 and ATV; Chinchar et al., 2011) and macroparasites (e.g., roundworms, flukes, tapeworms and ectoparasites) can significantly alter amphibian growth, behavior and habitat use (Koprivnikar et al., 2012). Larval stages (metacercariae) of the trematode parasite *Ribeiroia ondatrae*, for instance, can induce severe limb deformities (e.g., extra limbs, missing limbs, and misshapen limbs) and mortality in a broad range of North American amphibian species (Johnson et al., 2012).

Importantly, the pathology associated with macroparasite infections is often intensity-dependent; thus, low levels of infection may have few detectable effects on a host whereas high parasite loads are associated with an increased risk of pathology and both direct and indirect effects on fitness. For this reason, the ability to accurately measure infection and its consequences are fundamentally important to infectious disease researchers. Historically, researchers have relied on one of several methods to quantify trematode infections within second intermediate hosts, with few efforts to compare efficacy and uncertainty among them (but see Lepitzki et al., 1994; McFarland et al., 2003). The first and most common of these methods is gross necropsy, which involves the careful dissection of host tissues to isolate, identify, and quantify parasites within the host (Hartson et al., 2011). The second method, commonly referred to as "clearing and staining" or "tissue clearing", involves bleaching the pigments and infusing with a liquid (e.g., glycerol) whose refractive index is similar to body tissues, thereby rendering the specimen transparent and allowing visualization of metacercariae within an intact specimen (Raffel et al., 2010). One advantage of tissue clearing over necropsy is that infections can be counted multiple times on the same specimen, even by different observers, allowing for error estimation. A major disadvantage, however, is that many parasites – particularly larval macroparasites – require molecular methods of analysis to identify taxonomically, which is often not possible in a cleared host due to DNA degradation during formalin fixation and hydrogen peroxide bleaching. A final method involves the use of acid digestion to remove the host tissue and leave only encysted parasites remaining, although this approach can adversely affect counts of incompletely formed metacercariae as well as their size measurements (see Lepitzki et al., 1994).

Both of these methods also require that hosts be destructively sampled to gather infection data. However, such 'snapshot' methods might do a poor job of capturing dynamic changes in infection within a host following exposure. Recent evidence that amphibian hosts differ in the capacity to "clear" trematode parasites post-infection emphasizes the importance of quantifying temporal changes in infection load. For instance, LaFonte and Johnson (2013) showed that the median survival time of *Ribeiroia* metacercariae within amphibians could vary from 10 hrs to >600 hrs among different frog species (LaFonte and Johnson, 2013). Such observations have prompted growing interest in sub-lethal methods for quantifying parasite infections *in vivo*, such as qPCR of skin swabs to track chytridiomycosis infection at multiple time points (Herbert et al., 2011). The use of fluorescently labeled parasites, for example, allows researchers to track both initial infection success as well as the subsequent fate of each colonizing parasite within living hosts over time, at least for parasites that are visible externally through the host tissue (Herbert et al., 2011; Keeney et al., 2008; LaFonte and Johnson, 2013). Because this technique is non-destructive, it also provides an opportunity to compare estimates of infection between different methods, thereby yielding measures of accuracy, precision and bias.

Here we had two primary objectives. The first was to further evaluate and optimize the fluorescent-labeling method for trematode parasite infections by quantifying how varying concentrations of the dye itself or its solvent (DMSO) affected both the survival of *Ribeiroia* cercariae over 24 hrs and their success in infecting a larval amphibian. While non-lethal methods of parasite tracking have some distinct advantages, their utility will depend on how the labeling process affects the behavior and infectivity of cercariae. Second, using the optimized form of the labeling method, we sought to compare the relative efficacy of standard host necropsy, tissue clearing, and fluorescent labeling in quantifying *Ribeiroia* metacercariae within amphibian hosts. We did this by exposing individual larval amphibians to one of six dosages of *Ribeiroia* cercariae and then comparing their infection estimates between methods, either at the individual host level or at the treatment level. This allowed us to assess the accuracy, precision and bias of these methods. Finally, as an applied illustration of the biological insights available through such tools, we use the collected data to examine how parasite establishment success varies with exposure dosage.

2. Materials and methods

2.1. Parasites and amphibian hosts

We obtained *Ribeiroia ondatrae* cercariae from naturally infected *Helisoma trivolvis* snails collected from wetlands in the San Francisco Bay Area of California. Infected snails were maintained in the laboratory and parasites were identified as *R. ondatrae* based on cercaria morphology (Johnson et al., 2004). Morphological identifications have previously been validated using molecular techniques (Johnson and Hoverman, 2012; Wilson et al., 2005). To obtain cercariae for experiments, we isolated infected snails in plastic centrifuge tubes filled with 40 mL of purified water and placed them in the dark. Within 3 hr, we harvested any released cercariae within the vials, pooled them among snails, and used them in experiments.

Egg masses of *Lithobates sphenocephalus* were collected near Tampa, Florida, and allowed to hatch in the laboratory at 25 °C on a 12:12 light dark cycle. Tadpoles were reared in 20 L Rubbermaid containers at a density of approximately 2–3 per liter. Complete water changes were performed three times per week or as needed. Tadpoles were fed an *ad libitum* diet of ground TetraMin flakes both prior to and during experiments. Only tadpoles at (Gosner, 1960) stage 27 were selected for use in our experiments. All procedures described in this study were reviewed and approved by the University of Colorado Institutional Animal Care and Use Committee.

2.2. Effect of fluorescent dye concentrations on cercariae survival

We assessed how eight concentrations of a green fluorescent dye (manufactured by Invitrogen [Carlsbad, California] and purchased through Life Technologies, BODIPY® 558/568 C12) affected the survival of *R. ondatrae* cercariae over a 24 hr period. Trematode cercariae are typically short-lived (<24 hr); however, biotic or abiotic factors that reduce their survival have the potential to inhibit infection success and transmission. Following the method described by Reed and Muench (1938), Keeney et al. (2008), and LaFonte and Johnson (2013), we exposed *R. ondatrae* cercariae to one of eight different concentrations of fluorescent dye (0 nM [control], 100 nM, 200 nM, 400 nM, 800 nM, 1600 nM, 3200 nM, and 6400 nM). Cercariae were initially pooled in a Petri dish and haphazardly transferred to a 50 mL centrifuge tube containing one of the eight dye concentrations for approximately 45 minutes to absorb the dye. We then transferred individual cercaria through a water rinse and into an individual well within a standard 96 well-plate that contained 250 μL of purified water. Each treatment was replicated 12 times for a total of 96 cercariae used in the experiment. Using a stereomicroscope, we classified the status of each cercaria (alive vs. dead) after 12 hours. Cercariae were considered effectively dead if they were not moving within the well and failed to respond to a gentle prod from a Pasteur pipette. The researcher conducting the survival observations was blind to treatment.

2.3. Effects of fluorescent dye and solvent concentrations on cercariae survival time

We performed a follow-up experiment in which we tested how cercariae survival was affected by water (control), the solvent used to dissolve the fluorescent fatty acid analog, dimethyl-sulfoxide (DMSO) (2 concentrations), and two combinations of the green fluorescent dye and DMSO. A polar aprotic solvent is necessary to dissolve the fluorescent fatty acid analog in water, and pilot studies showed that DMSO was superior to acetone for this purpose. Cercariae were collected as described above, pooled among multiple snails, and randomly assigned to one of five treatment groups (purified water [control], 0.1% DMSO, 0.2% DMSO, 100 nM dye with 0.1% DMSO, or 200 nM dye with 0.2% DMSO). After cercariae were given adequate time to absorb their treatment solution (~45 min, conducted within 50 ml centrifuge tubes on batches of parasites, as above), we transferred each cercariae through a water rinse and then into an individual well within a standard 96 well-plate that contained 250 μL of purified water. Each treatment was replicated 19 times for a total of 95 cercariae. Following the methods described above, observations were conducted immediately following the addition of parasites to the wells and every two hours thereafter. In no cases did cercariae classified as dead during one observation show signs of life at subsequent time points.

2.4. Effects of fluorescent dye on cercariae infectivity

We further evaluated whether the use of a fluorescent label affected the ability of *Ribeiroia* cercariae to successfully infect amphibian hosts, as measured 48 hrs after exposure. We randomly assigned cercariae to one of three treatment groups: control (no fluorescent label), sham (no fluorescent label but administered to tadpoles after a time delay comparable to the labeling process), or fluorescently labeled. In each case, we added 30 cercariae to an 800 mL container with one larval amphibian host (*L. sphenocephalus*). We included 10 replicate tadpoles per treatment. Cercariae in the control group were exposed only to purified water and administered to tadpoles within 3 hrs of emergence from the snail host. Cercariae in the fluorescent treatment group were labeled with the 200 nM dye solution following the same procedure outlined in section 2.2. Because the labeling process generally requires 1.5 to 2 additional hours, these cercariae were slightly older when they are added to the tadpole containers. We therefore chose to include the sham treatment to determine whether any effects on cercariae infectivity were due to cercarial age or the application of the fluorescent label. Cercariae in the sham treatment were treated exactly as though they were being labeled, except they were not exposed to the fluorescent dye (i.e., maintained in water only). Thirty-six hours post-exposure, tadpoles were necropsied to quantify the number of encysted metacercariae, which was divided by the number administered to estimate infection success. Investigators were blind to cercarial treatment while quantifying infections.

2.5. Comparison of methods for quantifying metacercariae

Finally, we sought to compare among different methods used to quantify subcutaneously forming metacercariae in amphibian hosts, including necropsy, tissue clearing, and fluorescent labeling. All cercariae used in this experiment were fluorescently labeled using the 200 nM dye solution following the same procedure outlined in section 2.2. To help assess how the methods differed in their estimates of parasite infection, we selected six doses of *R. ondatrae* cercariae (10, 20, 30, 40, 50, and 60) and exposed six *L. sphenocephalus* tadpoles to each dose. We chose these doses because they represent a range of common experimental exposure doses over which we wanted to compare how well these methods agree. Thirty-six hours post-exposure, we euthanized tadpoles using MS-222 and used the fluorescence method to quantify *R. ondatrae* metacercariae in all hosts. After imaging the animals with a fluorescent microscope, we preserved tadpoles in 10% neutral-buffered formalin. Because the necropsy and the tissue clearing techniques cannot both be performed on a single host, we randomly selected half of the hosts from each exposure dose for each method of quantification (see below). Investigators were blind to exposure dose while quantifying infections, regardless of technique.

2.5.1. Necropsy technique

Necropsies were conducted with the aid of a stereoscope (Olympus SZX-10) and precision dissection tools (micro-scissors, fine forceps). In brief, this involved careful removal of the host skin (which is a common site of infection for *R. ondatrae*), laying it out flat on a Petri dish, and examining it and variable magnifications for encysted metacercariae. Subsequently, the musculature of the tail, hindlimb buds, and oral disc were also examined for parasites. The host's gills were removed and flattened between two Petri dishes before examination. Finally, all internal organs were isolated and carefully inspected for any parasites. Any observed metacercariae were isolated from the host and viewed under high magnification (100–400×) to confirm their identity and number. Infections in all of the tadpoles from section 2.3 (n = 30) and in half of the tadpoles from section 2.4 (n = 18) were quantified by standard necropsy technique.

2.5.2. Tissue clearing technique

The other half of the formalin preserved tadpoles from section 2.4 (n = 18) were quantified using a tissue clearing technique adapted from Hanken and Wassersug (1981). First, the intestines were removed from each tadpole to prevent intestinal contents from interfering with subsequent efforts aimed at parasite visualization. Next, tadpoles were soaked for 48 hr in deionized water to remove any formalin and then transferred to a 0.3% aqueous NaOH solution, to which we added 1 drop of 3% H_2O_2 per 10 mL of solution. This basic peroxide solution was replaced every 2 days until tadpole tissues were fully de-melanized (<7 days for tadpoles in this study). Once de-melanized, tadpoles were rinsed briefly in deionized water, transferred to a 1:1 solution of glycerol and water for 12 hr, and finally transferred to 100% glycerol. Fully cleared tadpoles were

placed between glass plates for examination with a compound microscope.

2.6. Analysis

To determine the dosage of the fluorescent dye at which 50% of the parasites died (i.e., LD-50), we analyzed the log-transformed dye concentrations using a generalized linear model with a binomially distributed response (alive vs. dead after 12 hrs) and used this model to interpolate the LD50 value (using the dose.p function in the MASS package of the statistical program R). Following this analysis, we evaluated cercariae survival as a function of treatment group (control, 0.1% DMSO, 0.2% DMSO, 100 nM dye, 200 nM dye) using Kaplan–Meier survival analysis with a Log-Rank between groups test. We tested whether fluorescent labeling altered infectivity by analyzing infection success (i.e., the number of administered cercariae that were detected as metacercariae within the host 36 hrs post-exposure) as a function of treatment group (control, age-sham, fluorescent dye) using a linear model.

To compare among the three methods used to assess trematode abundance, we calculated the slope of the relationship between exposure dosage and observed infection as an estimate of relative accuracy (which assumes that false positive detections were rare), and the standard error around the slope as an estimate of precision. We then analyzed the effect of exposure dosage, detection method, and their interaction on the number of *R. ondatrae* metacercariae observed per tadpole. Host size (snout-vent length, mm) was included as a covariate in all analyses. Because all tadpoles had infection quantified using two different methods (fluorescence and either necropsy or clearing), we used a linear mixed effects (LME) in which host identity was a random effect, thus allowing for multiple observations on the same tadpole (note: we also ran these analyses with the response variable following a negative binomial or Poisson distribution with strongly similar results but higher AIC values, leading us to focus on LME rather than GLMM). While we expected exposure dosage to be a strong, positive predictor of observed infection, we were interested in whether these estimates varied by method overall (main effect) or over particular ranges of dosages (method-by-dose interaction term). When interactions were detected, we followed up by performing pairwise comparisons between methods either with LME (if methods were used on the same individual hosts) or standard linear models (LM, in the case of necropsy vs. tissue clearing, which could not be performed on the same individual). Finally, we constructed Bland–Altman difference plots (Bland and Altman, 1986) for each pairwise combination of methods, which are commonly used to detect systematic differences between any two methods, and tested for both 'fixed bias' (i.e., consistent differences between methods) and 'proportional bias' (i.e., differences that increasingly manifest as sample values get larger or smaller).

3. Results and discussion

3.1. Effects of fluorescent labeling on cercarial quality

Based on exposures of *R. ondatrae* cercariae to progressively higher dosages of the fluorescent dye, we determined that the 12 hr LD50 (lethal dose in which 50% of parasites died) was 1082 nM (Fig. 1A). The two lowest dye concentrations, 100 nM to 200 nM, incurred relatively little (<5%) mortality at 12 hrs, making them ideal for further comparisons into the effects of both the dye and its solvent (DMSO) on cercariae longevity and infectivity. Survival trials using individual cercariae exposed to different concentrations of the dye (100 and 200 nM) and solvent (0.1 and 0.2% DMSO) indicated that parasite survival was unaffected by any of the treatment combinations used, with no significant differences detected among the

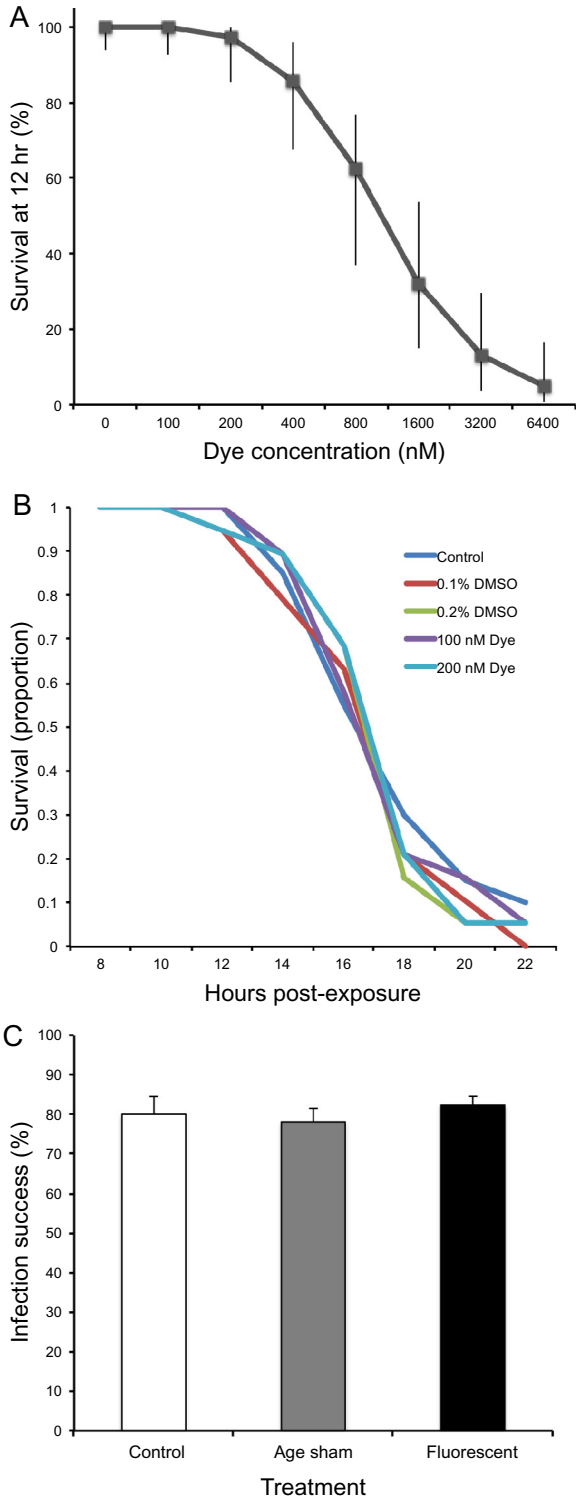

Fig. 1. Effects of fluorescent dye and solvent on the survival and infectivity of trematode (*Ribeiroia ondatrae*) cercariae. (A) LC50 curve for *R. ondatrae* cercariae exposed to increasing concentrations of the fluorescent dye. Concentrations of or below 200 nM elicited <5% mortality after 12 hrs. Error bars represent the 95% binomial confidence intervals. (B) 24 hr survival of *R. ondatrae* cercariae exposed to water (control) or varying concentrations of the dye solvent (DMSO) or the dye plus the solvent (100 vs. 200 nM). There were no significant differences in survival as a function of treatment. (C) Effects of the fluorescent dye on infectivity, or the capacity of *R. ondatrae* cercariae to infect larval amphibians (*L. sphenocephalus*) and establish as metacercariae. Approximately 80% of cercariae were successful in finding an encysting within an amphibian host after 36 hr, regardless of whether they were exposed to water (control), a sham exposure involving ageing the parasites an equivalent amount of time as the dyeing process required (age sham), or the dye method itself (fluorescent).

control, the solvent, or the solvent + dye conditions ($\chi^2 = 0.37$, $df = 4$, $p = 0.98$) (Fig. 1B). After 22 hrs (which is about the standard longevity for *R. ondatrae* cercariae), 5 of the 96 parasites were still alive, at which point we terminated the experiment and censored the data for these 5 cercariae. Based on these results, we elected to use the 200 nM dye concentration for subsequent experiments because it caused minimal mortality yet produced noticeably more intense fluorescence. Importantly, we also found no evidence that the use of this optimized version of the fluorescent labeling method affected the ability of cercariae to find and infect amphibian hosts ($F_{2, 33} = 0.17$, $p = 0.85$). Cercariae infection success after 36 hrs – quantified as the number of metacercariae detected by necropsy relative to the total number of administered cercariae – was 80.0%, 78.0%, and 82.3% in the control, age-sham, and fluorescent treatment groups, respectively (Fig. 1C). The 'missing' parasites represent both the fraction of cercariae that were unable to penetrate the host as well as any parasites that penetrated but failed to establish as metacercariae.

3.2. Comparison of parasite quantification methods

We examined the specific form of the relationship between exposure dosage and parasites detected for each of the three methods, evaluating the magnitude of the slope, the standard error around the slope, and whether the relationship was linear or non-linear (as indicated by a significant quadratic term). When the intercept was constrained to zero, the slope between exposure dosage and parasites detected with necropsy was the greatest (0.78), followed by fluorescence (0.75), and finally by tissue clearing (0.70) (Fig. 2). Biologically, this indicates that between 70 and 78% of administered parasites were detected as metacercariae, depending on the method used. Standard errors around the slope estimate were comparable for necropsy (0.013) and fluorescence (0.015) and increased only slightly when using tissue clearing (0.02), suggesting a similar amount of precision. All relationships were linear, with the exception of tissue clearing (Fig. 2C), for which a quadratic term significantly improved the fit between exposure dosage and parasites detected (dose = 1.021 ± 0.1822, $t = 5.602$, $p < 0.0001$); dose^2 = –0.005714 ± 0.00255, $t = -2.242$, $p = 0.0405$). Thus, at high exposure dosages, tissue clearing tended to yield lower estimates of observed infection relative to the other methods (Fig. 2).

Overall, we found strong correspondence between the fluorescence method and both necropsy and tissue clearing (necropsy vs. fluorescence: $r = 0.997$, $t = 58.4$, $p < 0.001$, Fig. 3A; tissue clearing vs. fluorescence: $r = 0.970$, $t = 16.01$, $p < 0.001$, Fig. 3B). Based on the linear mixed effects model with tadpole as a random effect, the number of detected parasites per host was strongly related to the exposure dose ($t = 16.67$, $p < 0.0001$; Fig. 2), as expected; however, exposure dose also interacted with the method of detection (dose*fluorescence, $p = 0.0003$; dose*necropsy, $p = 0.007$). Using pairwise analyses to compare the effects of exposure dose and method, we found that necropsy yielded significantly more detected parasites than fluorescence (LME: necropsy = 0.416 ± 0.189, $t = 2.198$, $p = 0.0383$) or clearing (LM: necropsy = 2.146 ± 0.875, $t = 2.451$, $p = 0.0188$). Host body size (snout-vent length) was not a significant covariate and was removed from the final analyses.

Following the methods of Bland and Altman (1986), we tested for presence of fixed bias between each pairwise combination of methods. Relative to necropsy, the fluorescence technique had a slight yet significant fixed bias as it tended to yield an average of 0.44 fewer parasites ($t = 2.40$, $df = 24$, $p < 0.05$, 95% CI [0.06, 0.82]) (Fig. 3C). After a bias adjustment of +0.44 parasites, the fluorescence and necropsy techniques agree within ±1.80 parasites (95% limits of agreement). Parasite counts obtained using the tissue clearing and necropsy techniques did not exhibit a significant fixed bias ($t = 1.29$, $df = 5$, $p = 0.25$, 95% CI [–2.09, 6.26]), nor did comparisons between tissue clearance and fluorescence ($t = -0.53$, $df = 7$, $p = 0.61$, 95% CI [–2.23, 1.34]) (Fig. 3D). However, in both of these latter cases, the observed confidence intervals were much wider, and deviations became more evident at higher parasite loads. Correspondingly, a test of 'proportional bias', in which the difference between two methods is regressed on their average estimate, indicated that tissue clearing yielded lower infection values at higher loads (difference = –1.88 ± 0.71, $t = -2.67$, $p = 0.0168$). A similar analysis comparing tissue clearing and necropsy was not performed because these measurements were collected on different animals.

4. Conclusions

These results provide valuable insight into the optimal use and efficacy of an *in vivo* method of parasite quantification, namely the use of fluorescent labeling, and how it compares to other commonly used techniques to enumerate larval trematode infection. Using a low concentration of fluorescent label (100 to 200 nM) offered a long-lasting marker (up to several months, LaFonte unpublished) and had no adverse effects on cercariae survival or infectivity to amphibian hosts under laboratory conditions. Similarly, neither the extra time required to label parasites nor the solvent in which the label was dissolved noticeably influenced parasite viability. While all three methods yielded strongly similar estimates of observed infection within hosts, some differences did emerge. Host necropsy by an experienced professional tended to provide the highest infection estimates, suggesting that approximately 78% of administered parasites were detectable within amphibian hosts 36 hrs after exposure, regardless of dosage (i.e., the relationship was linear). The high R^2 value (0.95) and the low standard error around the slope (0.013) also suggest high precision associated with this method. The "missing" parasites likely represent the fraction of cercariae that either failed to penetrate the host or failed to establish a metacercaria successfully following penetration (see also Johnson et al., 2012), which biologically can be related to the behavioral and physiological "resistance" of this host species. Quantifying parasites through fluorescently labeling offered remarkably similar results, with 75% of parasites detectable after 36 hr in a linear manner ($R^2 = 0.91$). For tissue clearing, the results at low and intermediate exposure dosages were similar to other two methods; at high dosages, however, parasite counts derived from cleared animals tended to be slightly lower, leading to a non-linear (quadratic) relationship with exposure dose (Figs. 2 and 3). This likely reflects challenges associated with counting each individual parasite as the infection intensity becomes greater, which we also expect will become more challenging with the fluorescence method at higher dosages.

Our use of a comparative approach helps to identify the utility and best conditions in which to use the different methods outlined here. Each of the methods emphasized the importance of exposure dose in determining observed infection in a (largely) linear way (Fig. 2). Thus, at the exposure dosages utilized here, we saw very little evidence of density-dependent infection success, although it is possible such effects would manifest at higher exposures as reported for other parasites (Ashworth et al., 1996; Ebert et al., 2000; Michael and Bundy, 1989). These results parallel previous experimental work with *R. ondatrae* and amphibians, which indicated that the proportion of cercariae successful in forming metacercariae was consistent across dosages, although this proportion did differ markedly among host species (Johnson et al., 2012). Assuming false positive parasite detections are unlikely, necropsy provided the most accurate (largest slope) and most precise (lowest error around the slope) method to quantify infection. The primary downsides to this approach, however, are that it generally requires extensive previous experience and it is destructive to the sample being examined. Tissue clearing allows the specimen to be

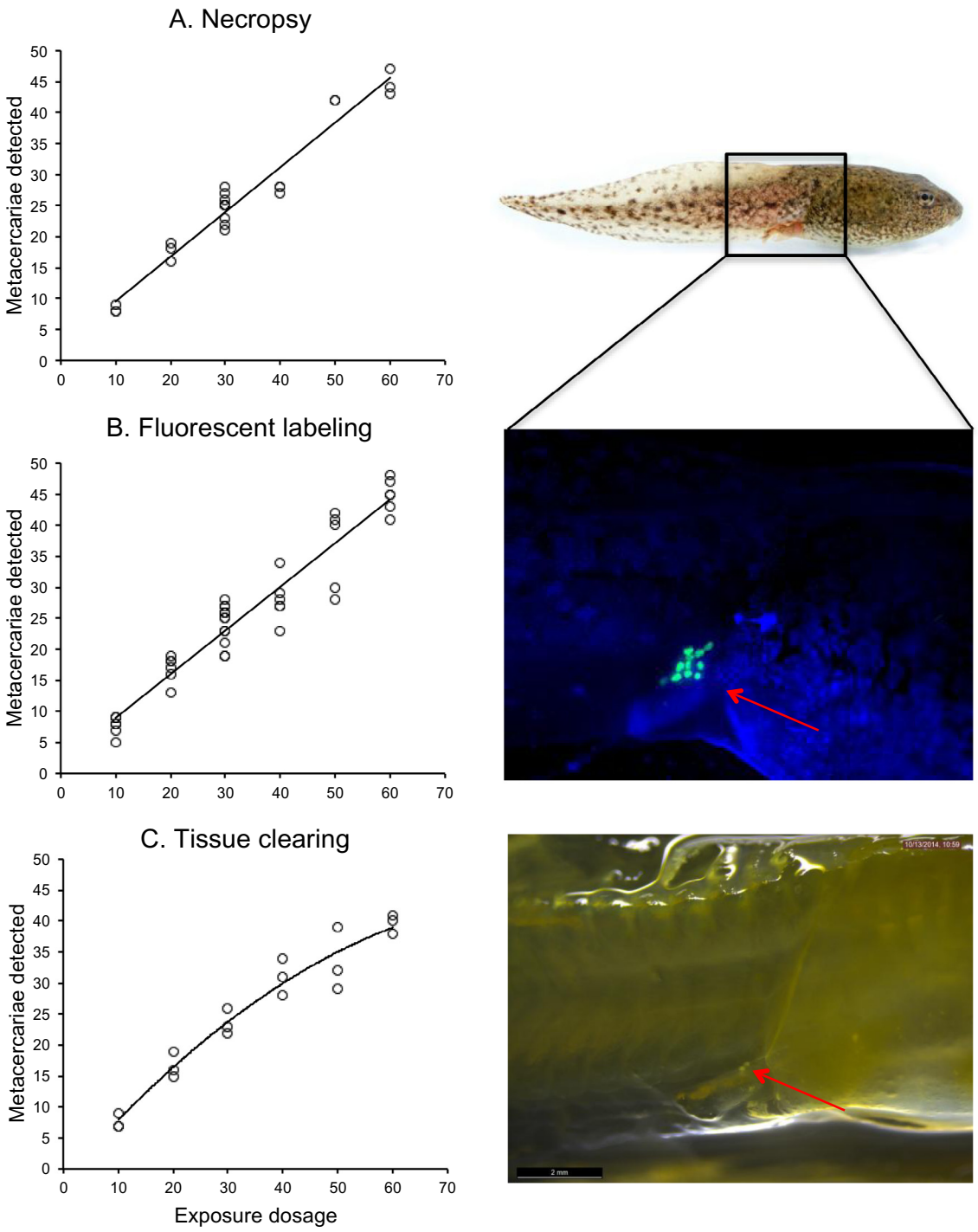

Fig. 2. Relationships between the number of administered cercariae (exposure dosage) and the number of metacercariae detected within larval amphibians (*L. sphenocephalus*) after 36 hr using different assessment techniques. In general, all methods indicate that parasite detection within amphibians is a linear function of exposure dosage, although the model for tissue clearing included a quadratic term. (A) Necropsy (observed infection = dosage * 0.78 ± 0.013, slope $t = 56.02$, $p < 0.0001$); (B) Fluorescent labeling (observed infection = dosage * 0.75 ± 0.015, slope $t = 51.54$, $p < 0.0001$); (C) Tissue clearing (observed infection = dosage * 0.70 ± 0.02 + (dosage^2)*(–0.006 ± 0.002, slope $t = 34.74$, $p < 0.0001$, quadratic $t = -2.24$, $p = 0.04$). Images to the right of each panel illustrate the techniques, including the area of primary infection by *R. ondatrae* within larval amphibians (i.e., the developing hind limbs, see rectangle superimposed upon tadpole); the red arrow in the lower two images indicates an example area where metacercariae are visible.

maintained and for parasites to be counted multiple times or by multiple observers, offering an estimate of measurement bias. As a result, this method can also be utilized by individuals with less prior experience. At high exposure dosages, our data suggest that this method could lose some of its accuracy, however. The accuracy and precision associated with parasite quantification through fluorescent labeling were generally similar to those of necropsy. While requiring more time and resources (fluorescent label, solvent, fluorescent microscope and imaging system), the advantage of this method is that it can be conducted without sacrificing the host, at least for parasites that are visible through host tissue, thus providing valuable opportunities to both track infection dynamics over time in specific

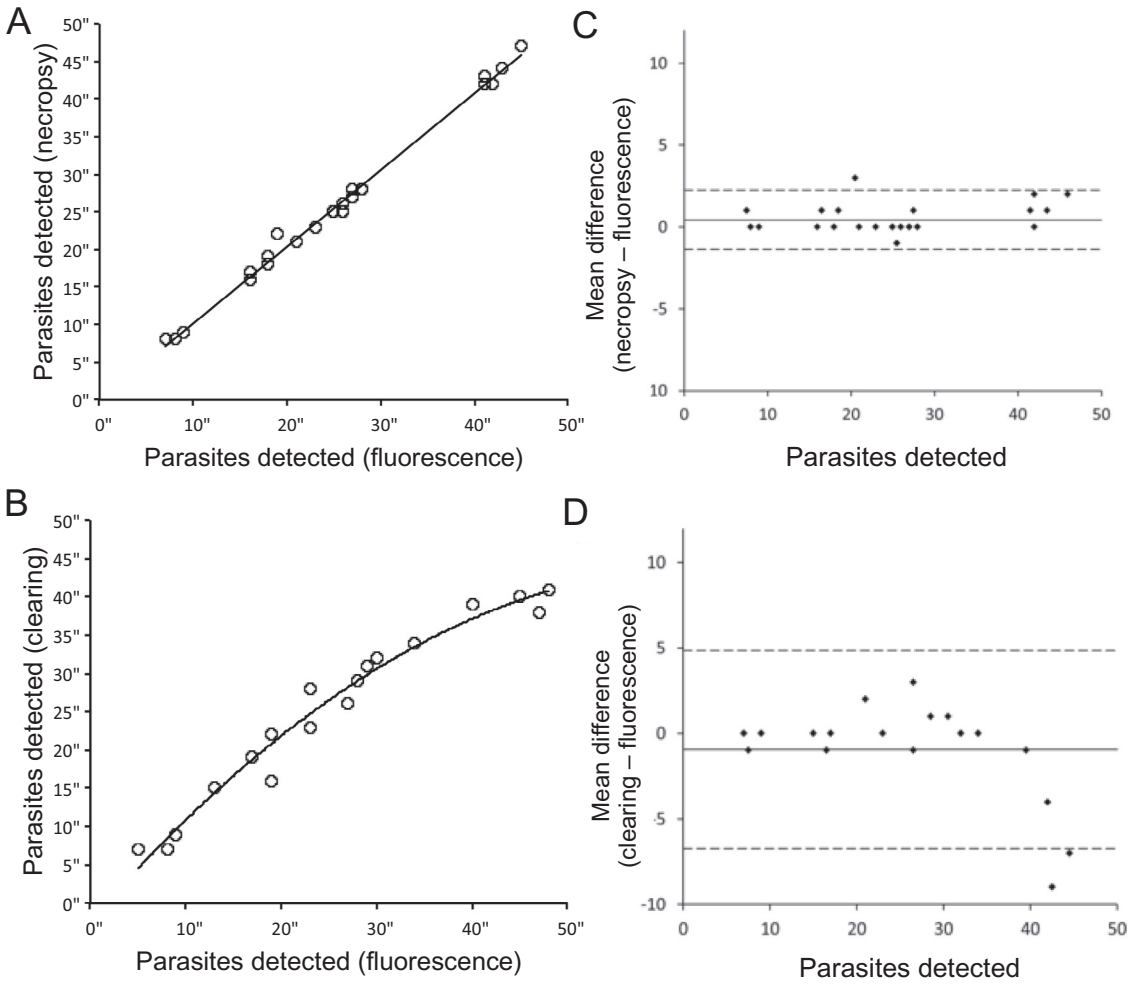

Fig. 3. Comparison of parasite quantification techniques as a function of exposure dosage. Number of parasites detected using fluorescence vs. (A) necropsy and (B) tissue clearing; in each case, counts were performed on the same hosts, first using fluorescence and subsequently by one of the other methods. For plots (C) and (D), presented is the difference in the number of parasites detected by each method; the black bar indicates the mean difference between the methods and the dashed lines show the 95% confidence intervals. (C) Necropsy vs. fluorescence; (D) Tissue clearing vs. fluorescence.

hosts (e.g., clearance) and to reduce the number of animals used in experiments relative to when destructive sampling is required. While focused here on amphibian intermediate hosts, these same approaches could easily be extended to other intermediate hosts infected by metacercariae (e.g., fishes, macroinvertebrates, zooplankton) or other parasites provided the infections are visible through the tissue.

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