



## The use of fluorescent fatty acid analogs as labels in trematode experimental infections

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Trematode

Fluorescent dye

Fatty acid analog

BODIPY FL C<sub>12</sub>

BODIPY 558/568 C<sub>12</sub>

### ABSTRACT

We examined the utility of fluorescent fatty acid analog dyes for labeling larval trematodes to use in experimental infections. Our goals were to identify two dyes that label larval trematodes belonging to the species *Maritrema novaezealandensis* and *Coitocaecum parvum*, determine if the dyes influence survival and infectivity of larval trematodes and/or host mortality, and if larval trematodes labeled with alternative dyes could be distinguished post-infection. The two dyes tested, BODIPY FL C<sub>12</sub> and BODIPY 558/568 C<sub>12</sub>, successfully labeled all treated larval trematodes, did not influence cercariae survival or infectivity, and did not influence host mortality in either host–parasite system. All larval parasites were fluorescent and distinguishable after 5 days in amphipod intermediate hosts. In addition, larval *Acanthoparyphium* sp. were strongly fluorescent with both dyes after 5 weeks within cockle hosts. This method should be extremely useful for experimental studies using trematode–host systems as models for addressing a range of ecological and evolutionary questions.

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### 1. Introduction

Host–parasite systems present exciting opportunities for examining the evolution of animal life history traits and behaviors. Digenetic trematodes are particularly amenable to experimental studies, and are rapidly becoming useful model systems. Trematodes typically utilize multiple hosts to complete their life cycles, including a molluscan first intermediate host in which asexual reproduction occurs producing hundreds of identical genetic clones. These clones can subsequently infect second intermediate hosts, such as crustaceans, bivalves or fish, which are consumed by definitive hosts in which the parasites complete their life cycles. Many trematodes are able to alter their intermediate host's phenotype and/or behavior to facilitate transmission to definitive hosts via predation (Helluy, 1984; Thomas and Poulin, 1998; McCurdy et al., 2000). Recent studies have found both different conspecific trematode genetic clones and multiple copies of the same genetic clone occurring within second intermediate hosts (Rauch et al., 2005; Keeney et al., 2007a,b), setting the stage for the evolution of mechanisms such as kin recognition and competitive strategies dependent on genetic relatedness.

The degree of relatedness of individuals within a parasite population or subpopulation can have important implications for the evolution of their life history traits and behaviors. Theoretical models predict that parasite characteristics such as growth rates,

virulence and host manipulation, can be influenced by the relatedness of the parasites within individual hosts (Brown, 1999; Parker et al., 2003), but few empirical studies have examined trematodes. Although field sampling can identify the degree of relatedness of parasites within hosts, rigorous tests of the influence of relatedness on parasite behavior require experimental studies to remove the confounding influence of factors such as time of infection. By experimentally infecting hosts with parasites of different genotypes, measurements can be made of the influence of a parasite's genotype on its ability to infect hosts and/or the influence of relatedness on parasites' abilities to manipulate host behavior. One method of doing this with trematodes would be to use genetic markers, such as microsatellites, to identify the relatedness of individual trematodes within snails and use appropriate combinations of genotypes for experimental infections. We are currently using this approach in our lab with several trematode species (Keeney and Lagrue, unpublished data). Because not all cercariae used in infections successfully infect the host, post-infection genotyping of metacercariae is required to accurately determine the genotype of each parasite within the host. This can be exceedingly expensive and time consuming if numerous metacercariae need to be genotyped for multiple loci. Distinguishing between different clones of the same trematode species is only one situation in which post-infection recognition of metacercariae is essential. For example, some experimental designs may involve exposure of the same host to batches of cercariae at intervals of a few days, with the need to distinguish between these batches later when the host is dissected. Or one might want to study local adaptation of trematodes,

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by exposing hosts from one locality to combinations of sympatric and allopatric cercariae, again involving the need to distinguish between them following infection. These and other experimental scenarios call for reliable discrimination between cercariae of different clones, batches or populations.

A potential alternative to genotyping metacercariae post-infection is labeling different cercariae clones (or batches used on different days, etc.) with alternative fluorescent fatty acid analog probes before infection, which may allow for identification of clonal identity post-infection. Fluorescent dyes have been used with parasites for several applications, including studying lipid utilization in *Schistosoma mansoni* (Furlong et al., 1995), tracking individual cestodes within intermediate hosts (Kurtz et al., 2002), and investigating monogenean infection behaviors (Glennon et al., 2007; Ohhashi et al., 2007). Similar methods may be useful in experimental studies with trematodes to identify genetic clones post-infection and determine the influence of relatedness on their behavior. Once the relatedness of cercariae within snails has been initially determined via genotyping, cercariae from specific snails can be stained with alternative fluorescent dyes and used for experimental infections. If the metacercariae retain the fluorescent dyes, their genetic identity can be determined after infection by examination with an epifluorescent microscope, removing the need to extract DNA from and genotype each individual metacercaria. However, for accurate measurements of the influence of relatedness on such parameters as infection success and host manipulation, any effects the dyes might have on parasites and/or hosts must be identified. For example, if one dye causes higher mortality of cercariae, it may select for more robust cercariae that will have higher rates of infection or growth within the host.

Our goal for this study is to determine the feasibility of using fluorescent fatty acid analog probes for experimental infections involving trematodes. In particular, we examine the feasibility of using multiple fluorescent dyes in trematode experimental infections, the effect the different dyes have on the survival and infectivity of larval trematodes, the effect the dyes may have on host mortality, and the ability to distinguish parasites labeled with alternative dyes post-infection.

## 2. Materials and methods

### 2.1. Effect of fluorescent dyes on cercariae mortality

This work utilized two New Zealand species of trematodes that have been used to experimentally infect second intermediate hosts in our laboratory (Fredensborg et al., 2004; Lagrue and Poulin, 2007) and represent different taxonomic families and diverse environments (marine versus freshwater). *Maritrema novaezealandensis* (Microphallidae) is a marine trematode that uses the snail *Zeacumantus subcarinatus* as a first intermediate host, several crustaceans (including the amphipod *Paracalliope novaezealandiae*) as second intermediate hosts, and shorebirds as definitive hosts (Martorelli et al., 2004). *Coitocaecum parvum* (Opecoelidae) is a freshwater trematode that uses the snail *Potamopyrgus antipodarum* as a first intermediate host, the amphipod *Paracalliope fluviatilis* as a second intermediate host, and freshwater fishes (mainly the common bully *Gobiomorphus cotidianus*) as definitive hosts (Macfarlane, 1939; Holton, 1984). Differences in our methodologies for the two species in this study reflect our current experimental protocols for them. For *M. novaezealandensis*, approximately 200 *Z. subcarinatus* were collected from Lower Portobello Bay, Otago Harbor, South Island, New Zealand in December 2006. Snails were screened for *M. novaezealandensis* infections by incubating individuals in 60 mm Petri dishes containing seawater at 25 °C for 1–6 h under constant illumination. Snails shedding *M. novaezealandensis* cercariae were identified under a stereomicroscope. For *C. parvum*,

approximately 10,000 *P. antipodarum* were collected in Lake Waihola, 40 km southwest of Dunedin, South Island, New Zealand. Snails were screened visually and infected individuals were obtained by selectively choosing individuals that displayed an altered shell shape, an indication of infection by *C. parvum* (Lagrue et al., 2007).

To determine if two alternative fluorescent dyes could be utilized and the ideal concentration of each dye to use in experimental infections with each species, we initially examined the survival of cercariae after exposure to the fluorescent dyes. We selected the fatty acid analog probes BODIPY FL C<sub>12</sub> (green dye) and BODIPY 558/568 C<sub>12</sub> (red dye) from Molecular Probes, Inc. as labels for our larval parasites based on their excitation wavelengths matching available filters. In addition, the previous study of Furlong et al. (1995) suggested that at least one of these (BODIPY FL C<sub>12</sub>) would be potentially useful with our species. Fluorescent-labeled parasites were examined with a Leica MZ FLIII fluorescence stereomicroscope equipped with a Leica DFC320 Digital Camera system and GFP2 and DiI fluorescent filter sets suitable for BODIPY FL C<sub>12</sub> and BODIPY 558/568 C<sub>12</sub>, respectively. Following purchase of the dyes, 10 mM stocks and 100 μM working stocks of both dyes were created by dissolving the dyes in DMSO. For both *M. novaezealandensis* and *C. parvum*, cercariae were exposed to four concentrations of each dye: 2 μM, 200 nM, 100 nM, and 50 nM in 1 ml of 0.22-μm filtered water (sea water for *M. novaezealandensis* and lake water for *C. parvum*) in 24-well plates (3 ml well volume). Controls were set up for each set of replicates and were manipulated in the same manner as cercariae receiving the dyes. To obtain cercariae, 200 *C. parvum* and ten *M. novaezealandensis* infected snails were incubated at 25 °C in lake and seawater, respectively, for 30 min to induce shedding of cercariae. Cercariae were pooled by species into 60 mm Petri dishes. Approximately 25 cercariae were then added to each well containing one of the dyes or control water and incubated for 45 min at 25 °C. The numbers of cercariae alive and dead were then counted under a stereomicroscope. To determine if cercariae had been successfully labeled with fluorescent dye, cercariae were subsequently examined with the epifluorescent stereomicroscope. For *M. novaezealandensis*, five replicates were set up for each treatment (5 replicates × 3 treatments × 4 concentrations × 25 cercariae = 1500 cercariae) and for *C. parvum*, two replicates were set up for each treatment (2 replicates × 3 treatments × 4 concentrations × 25 cercariae = 600 cercariae).

### 2.2. Effects of dyes on trematode infection success and host mortality

To determine if the fluorescent dyes affect cercariae infection success and host mortality, amphipod second intermediate hosts were exposed to fluorescently labeled cercariae (*P. novaezealandiae* with *M. novaezealandensis* and *P. fluviatilis* with *C. parvum*). Experiments were initially conducted with *M. novaezealandensis* as it is easier to obtain large numbers of cercariae with this species and we wanted to examine the longevity of fluorescence and influence of light on the light-sensitive dyes in the relatively transparent amphipod hosts. For *M. novaezealandensis*, four treatments were set up: fluorescent dyes in light, fluorescent dyes in dark, unlabeled (control) cercariae in light, and unlabeled (control) cercariae in dark. Light treatments were exposed to natural day–night cycles while dark treatments were kept without light for the entire duration. Forty-eight amphipods were used for each treatment. Cercariae were fluorescently labeled by placing in 10 ml filtered seawater containing the optimal concentration of dye determined during the last experiment (200 nM used for both species) for 45 min at 25 °C and then rinsed by replacing seawater twice to decrease dye carryover. Control cercariae were manipulated in the same manner with filtered seawater. Individual experimental amphipods were exposed to five cercariae from each dye (10 total

cercariae/amphipod) in 75  $\mu\text{l}$  water in 96-well plates (250  $\mu\text{l}$  well volume), and control amphipods were exposed to 10 unlabeled cercariae; all amphipods were incubated for 5 h at 25 °C. After incubation, amphipods were transferred to 1-L tanks containing sea lettuce (*Ulva lactuca*) and an airstone. One tank was used for each treatment. Two sets of experiments were set up to examine the dye longevity for light and dark conditions, a 2-day and 5-day set. After 2 days and 5 days, the number of amphipods alive in each tank was recorded, and 12 amphipods from each treatment were haphazardly selected and measured, dissected, and the number of parasites within them counted. All experimental metacercariae were examined with a Leica MZ FLIII stereomicroscope as described for cercariae.

The above protocol was followed with *C. parvum* but slightly modified due to the difficulty in obtaining large numbers of cercariae. Only 5-day dark treatments were conducted. Cercariae were fluorescently labeled by placing in 10 ml filtered lake water containing 200 nM dye for 45 min at 25 °C and then rinsed twice. Control cercariae were manipulated in the same manner with filtered lake water. Individual experimental amphipods were exposed to 2 cercariae from each dye (4 total cercariae/amphipod) in 3  $\mu\text{l}$  water in 500  $\mu\text{l}$  Eppendorf tubes, and control amphipods were exposed to four unlabeled cercariae; amphipods were incubated for 5 h at room temperature. After incubation, amphipods were transferred to 1-L tanks containing macrophytes (*Elodea canadensis*) from Lake Waihola. Eight treatment and eight control tanks were set up and ten amphipods were transferred to each tank. After 5 days in the dark, the number of amphipods alive in each tank was recorded, all amphipods were measured and dissected, and the number of parasites within each amphipod was counted. All experimental metacercariae were examined for fluorescence.

### 2.3. Statistical analyses

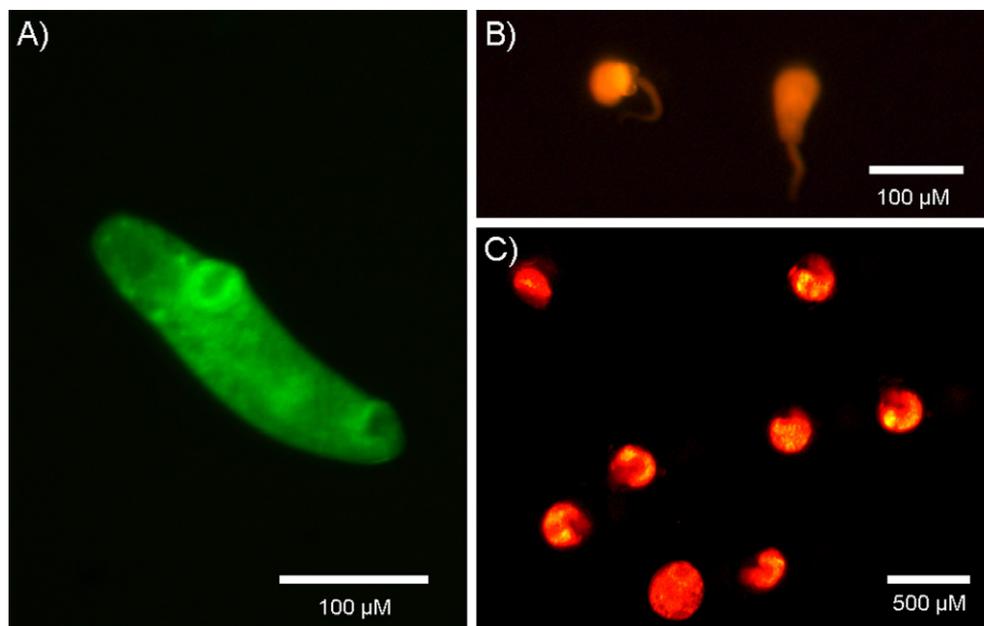
All statistical analyses were performed with SPSS 13.0 for Windows for both species. Nonparametric tests were used for variables deviating from the assumptions of normality. Kruskal–Wallis tests (K–W) were performed among treatments (green, red, and control) for each concentration as well as among concentrations for each

treatment to examine if the dyes had an effect on cercariae survival. When a significant value was found, post-hoc Mann–Whitney *U* tests (M–W) were performed between each treatment. Pairwise M–W tests were performed between each treatment and control to examine if the dyes had an influence on the mean number of cercariae successfully infecting the amphipods. For each treatment, M–W tests were also used to determine if the dyes had a differential effect on trematode infectivity by examining if there was a difference in the mean number of metacercariae stained with either dye within amphipods. For *C. parvum*, a *t*-test was performed to examine if there was a difference in amphipod survival between those infected with dyed versus control cercariae.

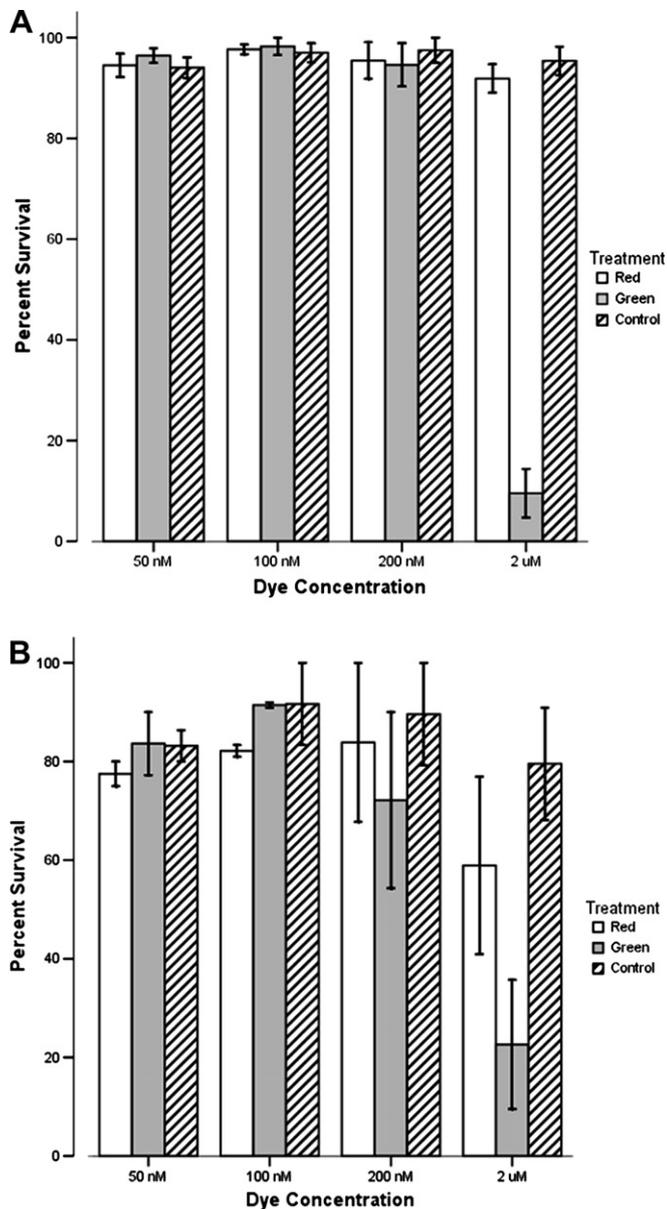
### 3. Results

All dead and live cercariae were fluorescent when viewed under UV light after initial dye labeling for all dye concentrations. Cercariae labeled with 50 nM dyes were noticeably less intensely bright than other concentrations. *C. parvum* cercariae were brighter than *M. novaezealandensis* with both dyes and cercariae of both species were fluorescent throughout their entire body (Fig. 1). After 2–5 days inside amphipod hosts, all dye-labeled metacercariae remained fluorescent throughout. *M. novaezealandensis* within amphipods kept in the dark appeared similar to those recovered from amphipods experiencing normal day–light cycles after 5 days, with the possible exception of several green metacercariae appearing slightly fainter in the light samples. The larger *C. parvum* remained brighter than *M. novaezealandensis* after 5 days. Parasites could not be consistently identified within amphipod hosts prior to dissection as the hosts' cuticles glowed faintly under epifluorescent conditions, and dissections were therefore required to obtain accurate parasite counts.

Mean *M. novaezealandensis* cercariae survival ranged from 9.5% to 98.3% for the green dye, from 91.9% to 97.7% for the red dye, and from 94.1% to 97.5% for the controls (Fig. 2). The 2  $\mu\text{M}$  concentration was the only group in which cercariae survival varied significantly among the three treatments (K–W,  $P = 0.006$ ; all other concentrations  $P > 0.681$ ). This was caused by cercariae in the green dye having significantly lower survival than those in red



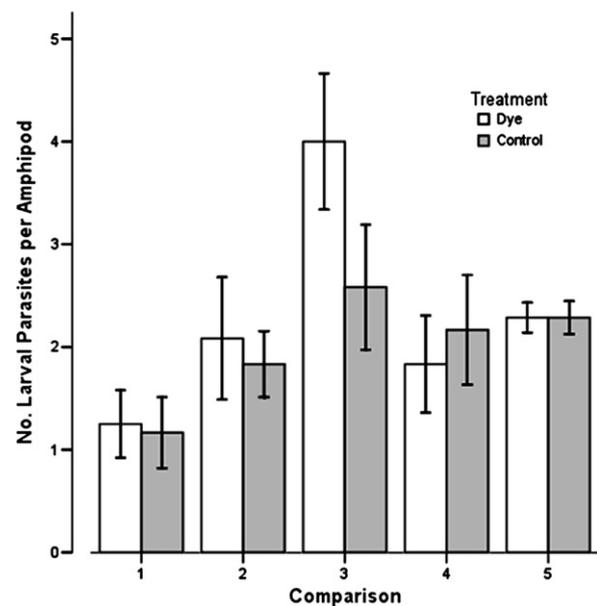
**Fig. 1.** Fluorescent-labeled larval trematodes. (A) *Coitocaecum parvum* cercaria labeled with BODIPY FL C<sub>12</sub>, (B) *Maritrema novaezealandensis* cercariae labeled with BODIPY 558/568 C<sub>12</sub>, and (C) *Acanthoparyphium* sp. metacercariae labeled with BODIPY 558/568 C<sub>12</sub> after five weeks within *Austrovenus stutchburyi* cockle hosts.



**Fig. 2.** Survival of (A) *Maritrema novaezealandensis* and (B) *Coitocaeum parvum* cercariae. Bars represent mean percentage of cercariae surviving  $\pm$  SE for each dye concentration and for the control group.

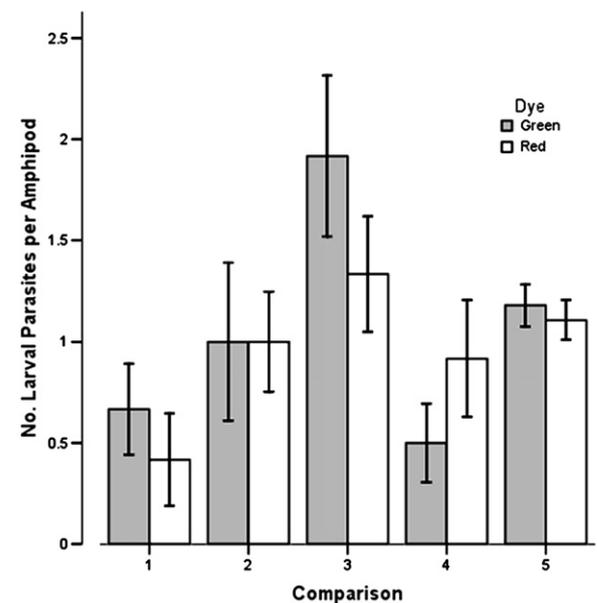
dye (M–W,  $P = 0.009$ ) and control cercariae (M–W,  $P = 0.008$ ). The red dye did not differ significantly from the control group at this concentration (M–W,  $P = 0.310$ ). Mean *C. parvum* cercariae survival ranged from 22.6% to 91.5% for the green dye, from 58.9% to 83.9% for the red dye, and from 79.6% to 91.7% for the controls (Fig. 2). *C. parvum* cercariae survival did not vary significantly among the three treatments for any concentration (K–W  $P \geq 0.156$ ). However, the lowest survival was obtained at the 2  $\mu$ M concentration for both dyes (Fig. 2).

Based on the optimal combination of fluorescence intensity and cercariae mortality, cercariae for all experimental infections were labeled with 200 nM dye. Labeling with fluorescent dyes did not have a significant effect on the ability of larval parasites to infect amphipods for any comparison with *M. novaezealandensis* or *C. parvum* (*M. novaezealandensis* 5-day light trial M–W  $P = 0.113$ ; all other comparisons  $P \geq 0.680$ ; Fig. 3). In addition, no detectable difference in infection success was observed between the two dyes with either species (M–W  $P \geq 0.281$ ; Fig. 4).

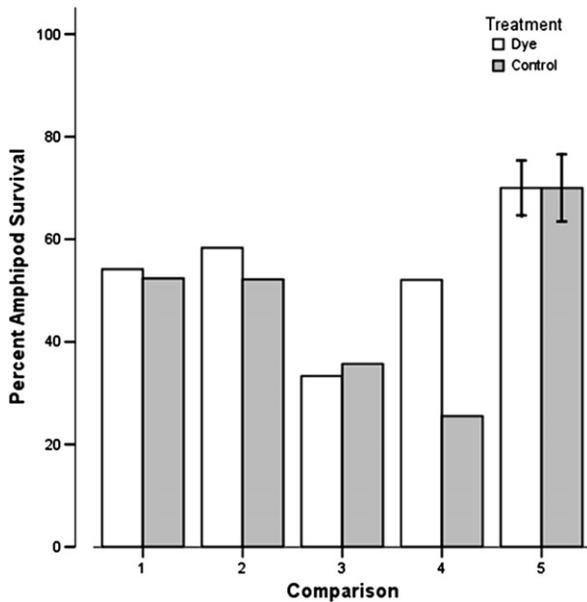


**Fig. 3.** Number of fluorescent-labeled and control larval parasites successfully infecting amphipods. Bars represent mean number of larval parasites per amphipod  $\pm$  SE. Comparisons are as follows: (1) *Maritrema novaezealandensis* 2-day light trial, (2) *M. novaezealandensis* 2-day dark trial, (3) *M. novaezealandensis* 5-day light trial, (4) *M. novaezealandensis* 5-day dark trial, and (5) *Coitocaeum parvum* 5-day dark trial.

Although treatments with *M. novaezealandensis* were not replicated, amphipod survival did not appear to be associated with the presence or absence of fluorescent dyes in the cercariae infecting them (Fig. 5). No difference in survival was detected between amphipods infected with fluorescent (means =  $70.0 \pm 15.1\%$ ) versus control (means =  $70.0 \pm 18.5\%$ ) *C. parvum* ( $t = 0.00$ ,  $df = 14$ ,  $P = 1.00$ ).



**Fig. 4.** Number of larval parasites labeled with each fluorescent dye successfully infecting amphipods. Bars represent mean number of larval parasites per amphipod  $\pm$  SE. Comparisons are as follows: (1) *Maritrema novaezealandensis* 2-day light trial, (2) *M. novaezealandensis* 2-day dark trial, (3) *M. novaezealandensis* 5-day light trial, (4) *M. novaezealandensis* 5-day dark trial, and (5) *Coitocaeum parvum* 5-day dark trial.



**Fig. 5.** Amphipod survival. For comparisons 1–4 bars represent percentage of amphipods alive per treatment and for comparison five bars represent mean percentage of amphipods alive per treatment  $\pm$  SE. Comparisons are as follows: (1) *Maritrema novaezealandensis* 2-day light trial, (2) *M. novaezealandensis* 2-day dark trial, (3) *M. novaezealandensis* 5-day light trial, (4) *M. novaezealandensis* 5-day dark trial, and (5) *Coitocaecum parvum* 5-day dark trial.

#### 4. Discussion

Our results indicate that fluorescent labeling of trematode cercariae is a promising method of identifying conspecific larval trematodes post-infection and will facilitate studying the behaviors of a range of trematode species. All cercariae labeled with either dye were strongly fluorescent and a detectable increase in cercariae mortality was only observed with the green dye at the highest concentration. Larval *C. parvum* cercariae were brighter with both dyes at all stages, likely resulting from their larger size (cercarial body  $\approx$ 270  $\mu$ m long, metacercarial body  $\approx$ 795  $\mu$ m long) and therefore larger amount of dye per parasite versus *M. novaezealandensis* (cercarial body  $\approx$ 86  $\mu$ m long, fully-grown metacercarial body  $\approx$ 367  $\mu$ m long) (Fig. 1). Therefore, the dyes successfully labeled cercariae without adversely affecting their survival in both species at a range of concentrations. Previously, Furlong et al. (1995) had used one of these fluorescent fatty acid analogs to label larval *S. mansoni* to study the uptake of the fatty acid analog. However, to use the dye in a range of experimental procedures, we needed to investigate if the dye could be successfully distinguished from a second dye in multiple trematode species as well as determine any effects the use of either dye may have on the host–parasite systems.

Since the dyes are light-sensitive, we initially examined post-infection fluorescence under both light and dark conditions with *M. novaezealandensis* as it is possible that light is able to penetrate the relatively thin cuticle of the amphipod *P. novaezealandiae*. After 5 days post-infection, all metacercariae were still fluorescent. Several 5-day light treated *M. novaezealandensis* metacercariae appeared to be slightly less fluorescent than 5-day dark treatments, indicating the possibility that there may be a detectable influence of light exposure on fluorescence in this species when kept under normal light-dark conditions. *C. parvum* 5-day dark treatments were strongly fluorescent compared to *M. novaezealandensis* 5-day dark treatments as the larger size of the parasites facilitated

detection of fluorescence. Therefore, the duration that the dyes can be distinguished may be a function of parasite size and possibly light exposure. In many cases, exposure to light will likely outweigh any decreased longevity of fluorescence as it reflects natural conditions. However, many species of trematodes utilize intermediate hosts that are practically impermeable to light and dye-labeled trematodes within them may remain fluorescent for long periods. For example, we carried out preliminary tests with the trematode *Acanthoparyphium* sp. (Echinostomatidae), which utilizes the cockle *Austrovenus stutchburyi* as a second intermediate host. Larval parasites of this species were strongly fluorescent with both dyes after 5 weeks post-infection (see Fig. 1), when labeled with 200 nM dyes as for the other two trematodes (Leung, unpublished observation). Again, larval stages of *Acanthoparyphium* sp. are large (cercarial body  $\approx$ 280  $\mu$ m long, metacercarial body  $\approx$ 400  $\mu$ m long) compared to *M. novaezealandensis* and were strikingly more fluorescent after five weeks than *M. novaezealandensis* were after initial staining.

For both species, a decrease in infection success did not occur with dye-treated cercariae versus control cercariae, indicating that the dyes do not interfere with the parasites' ability to successfully infect second intermediate hosts. In addition, the use of dyes was not associated with increased amphipod mortality in either host–parasite system. These results demonstrate that labeling of trematodes with these dyes will not bias experimental results on the behaviors or interactions of parasites labeled with either dye. Therefore, multiple dyes may be used in concert within hosts to identify specific trematode genotypes, or groups of cercariae from different waves of exposure, or from different localities, etc.

The complex multi-host life cycles, presence of multiple conspecifics of varying genetic relatedness at multiple trophic levels (Jarne and Théron, 2001; Rauch et al., 2005; Criscione and Blouin, 2006; Keeney et al., 2007a), host manipulation (Helluy, 1984; Thomas and Poulin, 1998; McCurdy et al., 2000), intraspecific competition (Davies et al., 2002; Gower and Webster, 2005) and coevolution (Dybdahl and Lively, 1996; Koskella and Lively, 2007) characterizing many trematode–host systems make them excellent models for investigating diverse evolutionary and ecological questions. Questions concerning topics such as how genetic relatedness influences parasite interactions or their ability to manipulate hosts can be addressed by experimentally infecting hosts with different trematode genotypes. Because our study did not detect effects on the trematodes resulting from the dyes, there are many potential applications for these techniques in trematode–host systems. For example, the ability of parasites to manipulate their hosts is predicted to increase with the relatedness of the parasites sharing the same host (Brown, 1999) as cheating is less advantageous among closely related individuals. With trematodes, identical genetic clones can coexist within hosts (Rauch et al., 2005; Keeney et al., 2007a,b), representing an extreme scenario of close genetic relatedness. Using our system, it is possible to label specific trematode cercarial clones being shed by snails with alternate dyes and use them in different experimental combinations to observe abilities to manipulate hosts. Post-infection, the number of each type of clone can easily be counted. As pointed out in the introduction, several other experimental designs requiring post-infection identification of conspecific cercariae can also be envisaged, and the labeling method outlined here can become a useful tool for parasitologists.

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