8. Validation of a rapid counting method for assessing treatment efficacy against *Lepidotrema bidyana* infecting silver perch (*Bidyanus bidyanus*)

James M. Forwood, James O. Harris, Matt Landos and Marty R. Deveney.

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8.1 Abstract

A published sub-sampling method was used for estimating the abundance of the monogenean *Lepidotrema bidyana*, a gill parasite of silver perch (*Bidyanus bidyanus*) and determined that it also accurately predicts parasite abundance post-treatment. Post-treatment parasite abundance estimates based on the number of parasites on the first left posterior hemibranch were compared to actual counts on fish after bath and oral treatment trials with PZQ and fenbendazole (FBZ). Post-treatment parasite abundance estimates were significantly correlated to real counts of all individual hemibranchs, accurately predicting the parasite abundance on an individual host. There was no significant difference in the post-treatment parasite abundance between individual hemibranchs, however, indicating that the treatment affected *L. bidyana* abundance on each hemibranch unequally. Use of this method predicts the remaining parasite abundance accurately, aiding evaluation of treatment efficacy, while reducing post-treatment sampling time or facilitating larger sample sizes.

8.2 Introduction

Lepidotrema bidyana is a gill dwelling monogenean that infects the Australian freshwater fish, silver perch *Bidyanus bidyanus*. Heavy infections can result in substantial economic loss in aquaculture (Rowland et al., 2007). *Lepidotrema bidyana* is oviparous, and has a direct lifecycle featuring a free swimming ciliated larva, the oncomiracidium, which finds and attaches to a host (Whittington et al., 1999). Once attached, monogeneans often migrate to a preferred specific habitat, including parts of gill arches (reviewed by Rohde, 1993).

Parasite management in aquaculture often relies on reactive chemotherapy to reduce infection levels and minimise economic loss. Potential drug candidates must go through a series of testing protocols before receiving regulatory approvals, including assessment of efficacy. Several methods using both prevalence and intensity as indicators have been used to assess the number of gill monogeneans remaining post-treatment. Methods include: sub sampling of a section of gill tissue (Stephens et al., 2003; Katharios et al., 2006; Rowland et al., 2006); bathing the gills (Anshary et al., 2001; Kimura et al., 2006) or the whole host (Williams et al., 2007) in a known effective treatment and filtering the bath solution then counting the parasites from the filtrate, and (2) manually counting all parasites (Sharp et al., 2004; Forwood et al., 2013). These methods can be time consuming and require the observer to count large numbers of individual worms. Observer fatigue can lead to inaccurate counts, and estimates based on unvalidated sub sampling can be inaccurate (Forwood et al., 2012). A method was developed for determining *L. bidyana* intensity *in situ* by counting the number of individual *L. bidyana* on the first left posterior hemibranch (L1p) and dividing the number by 0.13, which is the Average Proportional Contribution (APC) (Forwood et al., 2012).

This study was designed to examine the distribution of *L. bidyana* on the hemibranchs and to evaluate the accuracy of the counting method of Forwood et al. (2012) in fish treated both orally and topically with PZQ and fenbendazole (FBZ).

8.3 Materials and methods

8.3.1 Source of fish and parasites

660 silver perch were obtained from Pioneer Fish Farm (Gloucester, NSW) and maintained in a 10 000 L fibre glass recirculation tank at Flinders University, South Australia. Fish were infected with *L. bidyana* prior to transport and the infection was maintained by cohabitation of infected fish (Hirazawa et al., 2004). PZQ and FBZ were purchased from Sigma-Aldrich Co. Ltd (lot no. P4668 and F5396, respectively). Terminology for parasite infections follows Bush et al. (1997).

8.3.2 Experimental design

Silver perch (n = 360) were removed from the source population and underwent bath and oral treatment trials (Forwood et al., 2013). During bath trials 180 fish were randomly divided into twelve 500 L tanks: four tanks were treated using 10 mg/L PZQ, four tanks were treated using 10 mg/L FBZ, and four tanks received no treatment. During oral trials 180 fish were randomly divided into twelve 500 L tanks: four tanks were treated with medicated pellets using 75 mg/kg body weight (BW) PZQ, four tanks were treated with medicated pellets using 75 mg/kg BW FBZ, and four tanks received pellets with no treatment. At the completion of the treatment period fish were euthanized with an overdose (a 40 mL / 1000 L bath) of Aqui-S® and the gill baskets were removed, separated and examined under a dissecting microscope.

8.3.3 Validation of the counting methods

The total number of *L. bidyana* on each individual hemibranch was recorded for 218 silver perch that received and survived the bath and oral treatments. Remaining parasite abundance estimations were made based on dividing the number of *L. bidyana* on L1p by the APC of 0.13 (Forwood et al., 2012) and compared to the actual remaining parasite abundance. Estimates of prevalence, based on the presence of *L. bidyana* on L1p for each treatment replicate were generated and compared to the actual prevalence of each replicate.

8.3.4 Statistical analysis

Predicted and actual prevalence of each replicate, within each treatment group, was compared using a Mann-Whitney U Test. To achieve normality the predicted and actual remaining parasite abundance data were $\log_{10}$ transformed prior to analysis by linear regression. Overall differences in remaining parasite abundance between hemibranchs were analysed using a non-parametric Kruskal-Wallis test. The statistical software SPSS version 19.0 (IBM, USA) was used for all analyses. Significance for all tests was judged at $P < 0.05$.

8.4 Results

Prevalence of *L. bidyana* on silver perch receiving no treatment was 100% and the mean intensity of remaining parasites per fish was 272.3 ± 30.5 (SEM) (Range 41 to 1116) to 537.3 ± 82.6 (18 to 2449) (Table 8.1). After bath and oral treatments, prevalence was 100% and the mean intensity of remaining parasites per fish was 18.1 ± 1.8 (2 to 64) to 191.5 ± 28.8 (23 to 1045) (Table 8.1). *Lepidotrema bidyana* had a predicted prevalence of 98% in the groups receiving no treatment and the predicted mean parasite abundance per fish ranged from 250.1 ± 38.9 (0 to 1531) to 337.7 ±

56.6 (0 to 1515) (Table 8.1). After all treatments the predicted prevalence ranged from 57 to 98% and the predicted remaining mean parasite abundance per fish ranged from 6.4 ± 0.9 (0 to 31) to 113.3 ± 17.1 (0 to 600) (Table 8.1).

Table 8.1: Actual and predicted prevalence; and actual intensity and predicted mean abundance of *Lepidotrema bidyana* infecting silver perch (*Bidyanus bidyanus*) (*n* = 218) after oral and bath treatment with praziquantel (PZQ) and fenbendazole (FBZ). CI = Confidence Interval and NT = No Treatment.

Treatment	Prevalence % (95% CI)		Mean intensity ± SEM (Range)	
	Oral	Bath	Oral	Bath
NT	100 (91 to 100)	100 (95 to 100)	537.3 ± 82.6 (18 to 2449)	272.3 ± 30.5 (41 to 1116)
PZQ	100 (93 to 100)	100 (93 to 100)	191.5 ± 28.8 (23 to 1045)	18.1 ± 1.8 (2 to 64)
FBZ	100 (94 to 100)	100 (92 to 100)	90.5 ± 13.5 (9 to 543)	60.9 ± 6.2 (6 to 273)
	Predicted prevalence % (95% CI)		Predicted mean abundance ± SEM (Range)	
	Oral	Bath	Oral	Bath
NT	98 (87 to 100)	98 (90 to 100)	337.7 ± 56.6 (0 to 1515)	250.1 ± 38.9 (0 to 1531)
PZQ	98 (90 to 100)	57 (43 to 71)	113.3 ± 17.1 (0 to 600)	6.4 ± 0.9 (0 to 31)
FBZ	92 (80 to 98)	95 (86 to 99)	58.8 ± 11.7 (0 to 462)	32.2 ± 3.6 (0 to 146)

There was no significant difference in the predicted and actual prevalence between replicates of fish treated with FBZ orally and by bath (Mann-Whitney, $P = 0.343$), or between replicates of fish treated with PZQ orally (Mann-Whitney, $P = 0.686$). There was, however, a significant difference between replicates of fish treated with PZQ by bath (Mann-Whitney, $P = 0.029$).

Estimated remaining parasite abundance and the actual remaining parasite intensity were strongly correlated in fish bathed in FBZ ($R^2 = 0.659$, 1-way ANOVA, $P < 0.001$) (Fig 8.1A) and in fish fed pellets medicated with FBZ ($R^2 = 0.444$, 1-way ANOVA, $P < 0.001$) (Fig 1B) or PZQ ($R^2 = 0.77$, 1-way ANOVA, $P < 0.001$) (Fig 8.1D) and significantly correlated in fish bathed in PZQ ($R^2 = 0.08$, 1-way ANOVA, $P = 0.037$) (Fig 8.1C).

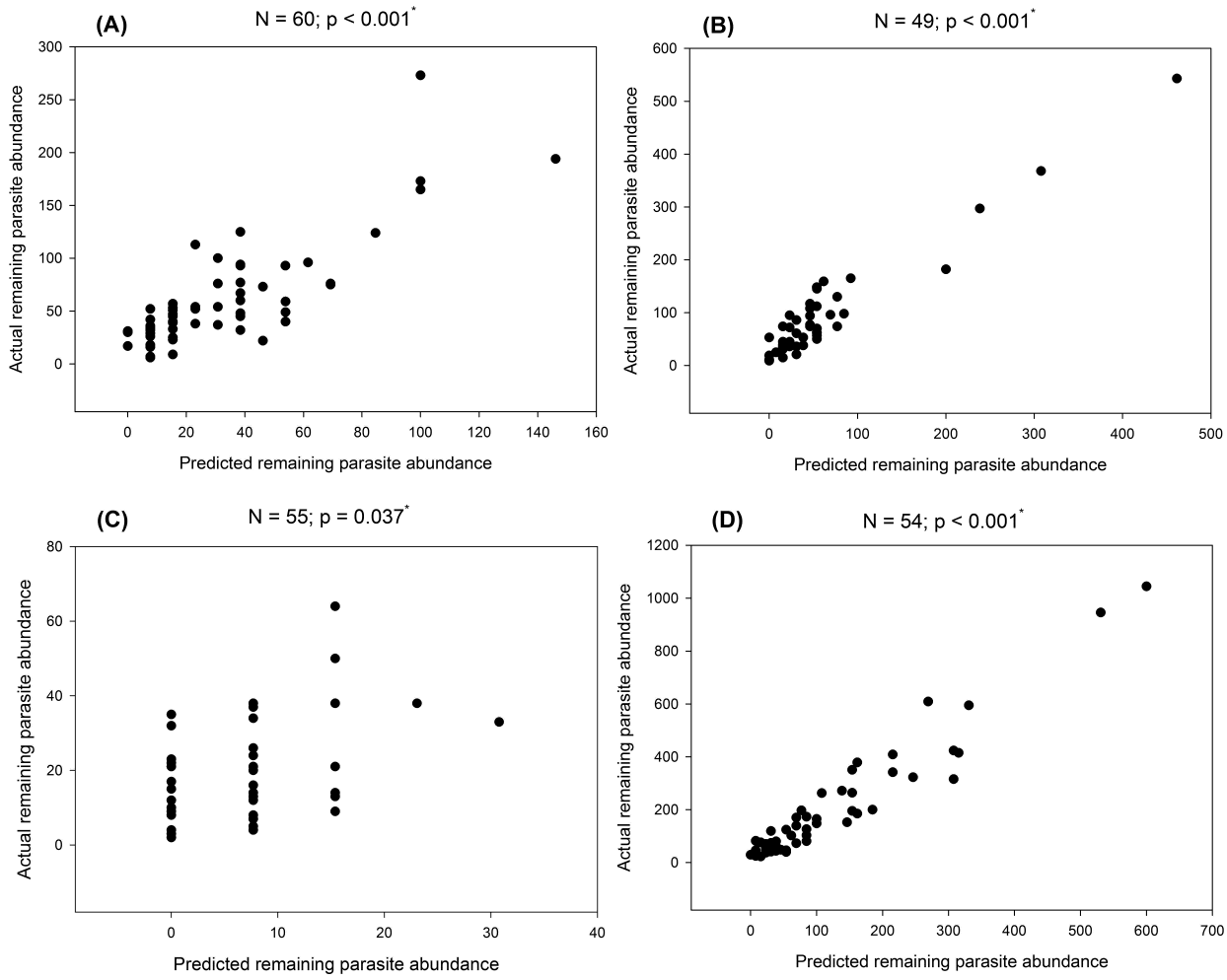

Figure 8.1: Relationship between the estimated remaining *Lepidotrema bidyana* abundance and the actual remaining *L. bidyana* abundance in silver perch (*Bidyanus bidyanus*) after treatment. *Significant relationships ($P < 0.05$) assessed by linear regression. (A) Bath treatment with fenbendazole (FBZ). (B) Oral treatment with FBZ. (C) Bath treatment with praziquantel (PZQ). (D) Oral treatment with PZQ.

The abundance of *L. bidyana* between individual hemibranchs was significantly different in the non-treatment bath trial group (Kruskal-Wallis, $P < 0.001$) and in the non-treatment oral trial group (Kruskal-Wallis, $P = 0.008$) groups. There was no significant difference in *L. bidyana* abundance between individual hemibranchs after treatment with FBZ administered both orally (Kruskal-Wallis, $P = 0.702$) and by bath

(Kruskal-Wallis, $P = 0.851$) or after treatment with PZQ administered orally (Kruskal-Wallis, $P = 0.994$) or by bath (Kruskal-Wallis, $P = 0.659$).

8.5 Discussion

Evaluation of a potential treatment for a parasite infection requires a rapid counting method specific to the target species, to determine parasite abundance so that treatment efficacy can be assessed (Grant, 1983). The method of Forwood et al. (2012) provided accurate post-treatment abundance estimates when compared to real counts over a wide range of post-treatment parasite abundances. There was a significant difference in the abundance of *L. bidyana* between individual hemibranchs in the control groups, which is normal for monogenean populations (Rohde, 1993). There was, however, no significant difference between individual hemibranchs post-treatment, indicating that parasite mortality is dissimilar across hemibranchs, but the accuracy of predicted abundances indicate that this variable was insufficiently different to affect abundance predictions.

Abundance estimates based on the number of worms in five fields of view (Rowland et al., 2006) are inaccurate (Forwood et al., 2012). Manual counting of all worms on each hemibranch is accurate (Forwood et al., 2013) but time consuming and if sampling is prolonged, parasite recruitment can occur post-treatment, resulting in inaccurate efficacy estimations (Williams, 2010). Extended counting of large numbers of parasites also requires multiple counters, further increasing potential variability between individuals who have different counting training or experience (Heuch et al., 2011).

Changes in prevalence have been used as indicators of treatment efficacy (Katharios et al., 2006; Rowland et al., 2006). We therefore evaluated estimates of prevalence using the counting method, but zero counts on L1p significantly affect the accuracy of the prediction, resulting in an under estimation of prevalence, particularly when post-treatment abundances are low.

Using the counting method described by Forwood et al. (2012) provides a rapid and accurate estimate of *L. bidyana* abundance that can be used when evaluating the efficacy of treatments, but it does not accurately estimate post-treatment prevalence.