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[Communication]

Use of Site Occupancy Models to Estimate Prevalence of *Myxobolus cerebralis* Infection in Trout

KEVIN G. THOMPSON*

Colorado Division of Wildlife, 2300 South Townsend Avenue, Montrose, Colorado 81401, USA

Abstract.—Empirical estimates of pathogen prevalence in samples of fish may underestimate true prevalence because available detection techniques are incapable of perfect detection. Trout of several species were collected from enzootic (*Myxobolus cerebralis*, causative agent in whirling disease) habitats, and individual fish were examined for presence of the parasite two or six times by one of four methods: pepsin–trypsin digest (brown trout *Salmo trutta*), plankton centrifuge (brown trout), polymerase chain reaction (rainbow trout *Oncorhynchus mykiss*), or histopathology (brook trout *Salvelinus fontinalis*). The presence–absence data were modeled for prevalence of infection (ψ) and probability of detection (p) of the parasite via occupancy models that accounted for imperfect detection of the organism. Based on estimates from the most-supported model for comparison, two myxospore concentration methods underestimated prevalence by about 12% for whole-head results and 34% for the expected value of half-head analysis. Polymerase chain reaction and histopathology gave virtually the same prevalence estimates for whole-head results as the best models but underestimated prevalence by about 6% and 12%, respectively, for the expected value of half-head analysis. The probability of detecting the parasite in a single survey of a fish head, conditional on the parasite's presence, was 0.66 for myxospore concentration methods, 0.81 for histopathology, and 1.0 (left halves) or 0.89 (right halves) for polymerase chain reaction. The occupancy models used in this study may be extended to large-scale monitoring of *M. cerebralis* to estimate expansion or contraction of the parasite's range over time.

Whirling disease was the subject of extensive research in the 1960s through the 1980s (Halliday 1976; El-Matbouli et al. 1992) and held much interest in the USA because of its recent introduction to North America and its former status as an emergency prohibitive fish pathogen (its current status as a notifiable pathogen was agreed to by the Colorado River basin states after an emergency conference and allows individual states to exercise stricter control if desired; CRWC 1988). The malady, caused by parasitic infection of salmonids by *Myxobolus cerebralis*, was first reported to have negative impacts on wild trout populations in intermountain areas of the western USA (Nehring and Walker 1996; Vincent 1996; Nehring et al. 1998). An intense research effort continues in an effort to find effective ways to combat the parasite (Bartholomew and Wilson 2002).

Much of the work in the years since trout population impacts were first documented has dealt with species susceptibility, biological and ecological factors associated with parasite transmission and spread, and the search for trout strains that are resistant to whirling disease. Many studies included a component of estimating prevalence or severity of infection in fish (e.g., Baldwin et al. 1998; Hedrick et al. 1999a, 1999b, 2001a, 2001b; Ryce et al. 2001, 2004; Sandell et al. 2001). These estimates are obtained by such methods as polymerase chain reaction (PCR; Andree et al. 1998; Schisler et al. 2001; Cavender et al. 2004), pepsin–trypsin digest (PTD; Markiw and Wolf 1974), plankton centrifuge concentration (PC; O'Grodnick 1975), or histopathology (FHS 2005).

Half-heads (possibly pooled) may be used for presumptive diagnosis or detection of subclinical *M. cerebralis* infection by one method, saving the other half-head for confirmatory diagnosis by histopathology or PCR (Lorz and Amandi 1994; FHS 2005). An implied assumption for this practice is that both halves of a fish head harbor evidence of the presence of *M. cerebralis*, particularly if prevalence estimation is one of the desired outcomes of the testing (Williams and Moffitt 2001). If both halves do not contain *M. cerebralis*, prevalence of infection will be underestimated when using half-heads to examine fish samples by PTD, PC, PCR, or histopathology. Moreover, underestimation of parasite prevalence is possible even when all fish are parasitized because commonly used detection methods are incapable of perfect detection.

A site occupancy analysis strategy (MacKenzie et al. 2002) fits the imperfect detection of *M. cerebralis*. Site occupancy studies involve multiple visits to sites that are likely to harbor the species of interest. In the present case, each fish head is a "site" that *M. cerebralis* may occupy. Each half-head analysis or histopathology section is then a "site visit." When the result of each examination is recorded separately, the resulting pattern of failure or success in detecting the species of interest (*M. cerebralis*) at each site allows

* E-mail: kevin.thompson@state.co.us

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the estimation of site occupancy (prevalence), as well as probability of detection.

This technique was used for each of the four methods mentioned previously to investigate the degree to which treating half-head analyses or histopathology sections as empirical estimates of prevalence may underestimate actual prevalence.

Methods

Feral fish were collected from known enzootic habitats and examined for the presence of *M. cerebralis* by PTD, PC, PCR, or histopathology. We examined 32 brown trout *Salmo trutta* from the Williams Fork River via the PTD method and 32 via the PC method; fish had been held in flow-through sentinel cages in the Colorado River below Windy Gap Reservoir (Nehring et al. 2002). We used the PCR method to examine 40 rainbow trout *Oncorhynchus mykiss* collected from the effluent channel of a hatchery, and we used histopathology to examine 24 brook trout *Salvelinus fontinalis* collected from a private pond. Each of the chosen collection sites was known from previous sampling to exhibit high infectivity for *M. cerebralis*.

The fish in each sample were euthanized with an overdose solution (>250 mg/L) of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories), and the heads were removed. For the PTD, PC, and PCR samples, each head was split along the sagittal midplane using a clean scalpel and cutting surface. Head halves were individually bagged, assigned a random number, and designated with "R" or "L" to indicate right or left half. The full set of half-heads in each sample was submitted in blind fashion to laboratories specializing in the appropriate detection method for *M. cerebralis*. A reference list was kept on file so that the halves of each fish head could later be paired.

The heads of the brook trout used for histopathology were placed whole into Bouin's solution and submitted to the laboratory, where they were halved and prepared for examination via standard histopathology techniques. Three sections were taken from each half of each head in a progression from near the midplane toward the eye. Each section was independently scored (MacConnell–Baldwin scale; Baldwin et al. 2002) and reported.

The results for each group of fish were formatted for input into Program MARK (White and Burnham 1999) as site occupancy data. The myxospore concentration methods (PTD and PC) were treated as a single data set with two groups (by method), whereas PCR and histopathology were treated as separate data sets. In every analysis, a fish head was analogous to a single site regarded as suitable habitat for *M. cerebralis*, and each separate evaluation of the presence of *M. cerebralis* equated to a site visit. Hence, each site was visited twice for the PTD, PC, and PCR methods and six times for the histopathology method.

A discrete set of a priori models were considered for each data set, each model being grounded in biological possibility. Parameters estimated were the incidence of infection or occupancy rate (ψ) and the probability of detection with a single examination (p), whether it was a half-head or a histopathology section. Some of the models allowed a right–left hemisphere effect for probability of detection. Model sets were larger for myxospore concentration because of the two analysis methods and for histopathology because fish length was available as a covariate. (In more complicated data sets, the occupancy models may be extended to incorporate other covariates, such as body condition, species, or water of origin.)

The best models were selected based upon bias-corrected Akaike's information criterion (AIC_c; Burnham and Anderson 2002). This criterion, based in information theory, estimates the relative distance between the model in question and the unknown true mechanism that generated the data. It incorporates a means for penalizing overparameterization, and the bias-corrected version is intended for small-sample data. Models with the lowest AIC_c score fit the data better than those with higher scores, and models within a few points of the lowest-scoring model are typically given consideration.

Results

The PTD and PC Methods

Generally, simpler models better explained the observed data (Table 1). The MARK model most supported by the data recognized no difference between the two myxospore concentration techniques or any left–right hemisphere effect (Table 2). Under this model, $\hat{\psi}$ was equal to 0.903 (SE = 0.0720). The estimate of the probability of detection, $\hat{p}$, in half a head was 0.658 (SE = 0.0630). Thirteen half-head pairs (40.6%) gave incongruent results by each concentration method. The expected values for prevalence from half-head analysis of the data would be 0.61 for PTD and 0.58 for PC (the means of the right- and left-half estimates for each method; Table 2). Whole-head prevalence of infection in these groups, based on the combined results of both half-heads for each fish, was 0.813 for the sample analyzed by PTD and 0.781 for the sample analyzed by PC (Table 2), both of which were lower than the prevalence estimated by the most supported model.

The estimated average numbers of myxospores per head obtained for these samples were 16,343 for PTD and 8,668 for PC. The differences between incongruent

TABLE 1.—Models run in Program MARK for each of three analysis methods used to examine for *Myxobolus cerebralis* in trout. Model ranking was based on Akaike's information criterion corrected for small sample size (AIC_c). Symbols are p = probability of detection, L = left head hemisphere, R = right head hemisphere, ψ = occupancy rate (prevalence), m = method of myxospore concentration, F = fish length, and NP = the number of parameters in each model. Asterisks = interactions; plus symbols indicate additive effects.

Model	AIC_c	Delta AIC_c	AIC_c weight	Model likelihood	NP
Pepsin–trypsin digest and plankton centrifuge					
$[p(L = R)\psi]$	175.524	0.00	0.619	1.000	2
$[p(L = R)\psi(m)]$	177.631	2.11	0.216	0.349	3
$\{p[m*(L = R)]\psi(m)\}$	179.888	4.36	0.070	0.113	4
$\{p[m*(L \neq R)]\psi(m)\}$	180.009	4.48	0.066	0.106	6
$\{p[m*(L + R)]\psi(m)\}$	181.627	6.10	0.029	0.047	5
Polymerase chain reaction					
$[p(L \neq R)\psi]$	53.325	0.00	0.832	1.000	3
$[p(L = R)\psi]$	56.528	3.20	0.168	0.202	2
Histopathology					
$[p(L = R)\psi]$	127.778	0.00	0.483	1.000	2
$[p(L \neq R)\psi]$	129.305	1.53	0.225	0.466	2
$[p(L = R)\psi(F)]$	129.470	1.69	0.207	0.429	3
$[p(L \neq R)\psi(F)]$	131.274	3.50	0.084	0.174	4

head halves were substantial, in some cases amounting to greater than 5,000 myxospores in the positive half (four cases for PTD, three cases for PC). For each method, the maximum estimated myxospore concentration in a positive half-head that had a corresponding negative half-head was greater than 33,000.

The PCR Method

Four half-head pairs (10.0%) gave incongruent results by the PCR technique. All four incongruent half-head pairs were negative in the right hemisphere and positive in the left hemisphere. Because of this circumstance, the model best supported by the data predicted different probabilities of detection for the right and left hemispheres. However, this model predicts a consistent bilateral asymmetry in the distribution of *M. cerebralis*, an unlikely scenario that did not rank as best in any other data set. The PCR technique proved to have such a high probability of detection that the estimate obtained from the highest-ranked model ($\hat{\psi} = 0.925$; SE = 0.0416) was the same as the empirical estimate derived from combining half-head results (0.925; Table 2). The $\hat{p}$-values given by the model were 1.0 (SE = 0.0000) for left halves and 0.892 (SE = 0.0510) for right halves. The expected value for a prevalence estimate from half-head PCR analysis for these data would be 0.875. The average score for the PCR data were positive to strongly positive on the scale used by Schisler et al. (2001).

Histopathology

Four of the 24 half-head pairs (16.7%) were incongruent when analyzed by histopathology. Histology scores from the positive head halves were uniformly 1 in one case, 2 in two cases, and 3 in one case. Three sections were examined from each head half, so incongruence between head halves was only realized when all individual sections were incongruent with the matched (distance from midline) sections from the opposite head half.

When the sections were considered by pairs based upon distance from the midline, those arising closest to

TABLE 2.—Modeled and empirical prevalence estimates ($\hat{\psi}$) of *Myxobolus cerebralis* infection in brown trout based on pepsin–trypsin digest (PTD) and plankton centrifuge concentration (PC) techniques, in rainbow trout based on polymerase chain reaction (PCR), and in brook trout based on histopathology. Whole-head empirical data are from the combined right and left halves of each fish head. Estimates of prevalence from the MARK program are those generated from the model having the most support from the selection criterion (i.e., Akaike's information criterion corrected for small sample size).

Data set	N	MARK estimate	MARK 95% confidence interval	Empirical estimate by head portion		
				Whole	Right	Left
PTD	32	0.903	(0.650, 0.979)	0.813	0.719	0.500
PC	32	0.903	(0.650, 0.979)	0.781	0.531	0.625
Histopathology	24	0.667	(0.461, 0.824)	0.667	0.542	0.625
Polymerase chain reaction	40	0.925	(0.792, 0.976)	0.925	0.825	0.925

the midline of the skull exhibited the greatest amount of incongruence (eight pairs, 33.3%). The middle and outer section pairs were each incongruent in only five instances (20.8%). The average severity of infection score from the histology analysis was 0.93, based on the MacConnell–Baldwin rating system (Baldwin et al. 2000; Andree et al. 2002).

The MARK model most supported by the data for the histopathology analysis gave a value of $\hat{\psi}$ equal to 0.667 (SE = 0.0962), the same as the empirical estimate (16 of 24 heads). The model assumed no differences in detection probability between halves of a head and yielded a $\hat{p}$ of 0.812 (SE = 0.0399). The expected value for a prevalence estimate from half-head histology analysis for these data would be 0.584.

Discussion

The data examined for this study demonstrate that *M. cerebralis* is not equally distributed between hemispheres of infected fish heads. However, for the fish samples analyzed herein, the PCR and histopathology techniques performed admirably, giving prevalence estimates that were virtually the same as the modeled prevalence when using whole-head data and only slightly lower (5–12%) when using the expected value of a half-head analysis. Using the myxospore concentration methods, prevalence of *M. cerebralis* infection in fish samples was substantially underestimated (mean = 34.1%) by examination of half-heads compared with the model estimates because these techniques exhibited lower detection probability. The mean underestimation was 11.8% when comparing the model estimate to whole-head empirical results. It should be noted that the fish samples used in this study exhibited high prevalence and moderate (histopathology, PTD, PC) to high (PCR) intensity of infection. At lower prevalence and intensity, this disparity between empirical estimates and occupancy model estimates may be different than those presented here.

This underestimation is an important consideration for investigators conducting studies in which prevalence of infection is a metric of evaluation, particularly if numbers of myxospores are also of interest and experimental subjects are limited in number. In those situations, prevalence would be best adjusted by performing a PCR test on the whole-head product of the chosen myxospore concentration technique (Baldwin and Myklebust 2002); the PCR assay could serve as the confirmatory test, if needed (FHS 2005). Such a strategy would allow the investigator to get the best myxospore concentration estimates and still allow a confirmatory diagnosis of the parasite. Alternatively, one could independently survey each head two or more times by examining multiple hemacytometers of PTD or PC product and recording the results separately. This method will probably yield a higher $\hat{p}$ than those observed on the half-heads used in this study because it will eliminate the possibility of encountering cases where the parasite is truly absent in one hemisphere of the head. As a result, agreement between modeled estimates of prevalence and the empirical values observed would probably be greater. Additional occupancy studies are being performed with whole-head PTD product to address this hypothesis. If prevalence of infection is of interest but myxospore concentrations are not, the analysis techniques advocated for pooled samples by Williams and Moffitt (2001, 2005) may also be appropriate. Either technique will provide more robust estimates of prevalence than empirical estimates based on half-head prevalence.

This study supports the asymmetry of *M. cerebralis* infection and myxospore distribution suggested by Schisler et al. (2001) as an explanation for disparate results between PTD and PCR methods. In that study, 12.6% of free-ranging trout that were found positive by PTD tested negative by PCR on the other half-head. Among hatchery-reared fish, the same result occurred in 8.1% of fish. Disparities between PCR and histopathology or PTD have been previously noted (Andree et al. 1998, 2002). Andree et al. (1998) tested 20 fish (half-heads) by nested PCR and PTD at 5 months postexposure to 200 triactinomyxons/fish and confirmed the presence of *M. cerebralis* in seven fish by PCR but in only one fish by PTD. The authors did not note whether the half-heads were paired or whether the PTD-positive half-head was from a fish that also tested positive by PCR on the other half-head. Although PCR is known to be a far more sensitive test (Andree et al. 1998; Schisler et al. 2001), the greater sensitivity of the PCR technique is not the only factor contributing to the disparate results. This is apparent from the degree of disparity observed in some of the incongruent head-half pairs analyzed by PTD, PC, and histopathology.

Several models that had good support based on AIC_c in this study showed a right–left effect for $\hat{p}$, but without a consistent handedness. Probability of detection was higher in the left hemispheres for PC and PCR, but higher in the right hemispheres for PTD. The histopathology data were reported without information on the right and left hemispheres, so although differences were suggested in that data set, it is not possible to determine which hemisphere exhibited the higher $\hat{p}$.

MacKenzie et al. (2006) discussed both the simple, single-season model used in this paper and an extension of it that would allow this method to be used as a wide-ranging survey technique suitable for

monitoring expansion or contraction of the range of *M. cerebralis*. In the latter case, each site would be a physical location, and each fish collected would be a site visit to that location or portion of the fish population. To model expansion or contraction of the parasite's range, collections would be required over several years and from locations randomly chosen from among the wider population of locations to which the investigator wishes to make inference (MacKenzie 2005a, 2005b; MacKenzie et al. 2006). MacKenzie and Royle (2005) considered a probability of detection greater than 0.5 in a given survey to be high. All of the methods used to detect *M. cerebralis* in the fish host in the present study exhibited probabilities of detection considerably greater that 0.5. However, all of the $\hat{p}$-values in this study are estimates of the probability of detection given that the parasite is present in the individual fish. In the monitoring model, $\hat{p}$ would be the estimate of the probability of detecting the parasite in a single fish given *M. cerebralis* presence in the fish population from which the fish was collected. Preliminary data collected in high-elevation streams in Colorado indicates that $\hat{p}$ may be 0.5 or more for PCR on age-0 fish, a level that MacKenzie and Royle (2005) suggested will allow researchers to minimize surveys of individual sites and still gain reliable knowledge of a parasite's range.

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