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Evaluation of Risk of High Elevation Colorado Waters to the Establishment of *Myxobolus cerebralis*

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ABSTRACT. During 1992 and 1996, fish were inadvertently stocked into 226 waters in Colorado from hatcheries that were later identified as *Myxobolus cerebralis*-positive. Seventy-two high-elevation waters, previously classified as *M. cerebralis*-negative, were sampled to determine if stocking of fish from those hatcheries contributed to the spread of *M. cerebralis* in these locations. Pepsin-trypsin digest (PTD) and polymerase chain reaction (PCR) tests were used to test for *M. cerebralis* in 1,743 fish. A total of 190 fish and 23 separate waters were identified as *M. cerebralis*-positive, with PTD. PCR identified 410 fish and 42 waters as positive for the parasite. Logistic regression and Akaiki information criterion model selection was used to identify parameters contributing to the *M. cerebralis* establishment in these high elevation waters. Within-drainage distance to the nearest known *M. cerebralis*-positive water and relative abundance of *Tubifex tubifex* habitat were found to be more important contributors to the establishment of *M. cerebralis* than the accidental stocking events.

Myxobolus cerebralis, the parasite responsible for salmonid whirling disease, was first identified in the United States in the 1950s and has been detected in at least 22 states (Bartholomew and Reno, this volume). The parasite can be potentially spread by several mechanisms. Infected fish may swim upstream or downstream from a site of initial exposure, carrying the parasite throughout a given drainage. The waterborne triactinomyxon spore (the stage infective to fish) can be carried downstream from infected areas with water currents. Mature myxospores can be passed through the digestive systems of fish-eating birds, such as great blue heron (Meyers et al. 1970), kingfishers (Schaperclause 1954), black crested night heron, and mallard ducks (Taylor and Lott 1978), facilitating transport of the organism into previously unexposed habitats. Past evaluations suggest that the most common route of transmission of *M. cerebralis* to new locations is transport of infected fish (Meyers et al. 1970). In Colorado, accidental stocking of *M. cerebralis*-suspect fish raised the concern that many high-elevation waters, previously free of the organism, may have become contaminated with the parasite. A total of 226 high-elevation waters were stocked by Roaring Judy State Fish Fatchery in 1992 and the Pitkin and Durango State Fish Hatcheries in 1996, when the facilities were considered to be free of the parasite. Most of these high-elevation stockings were aerial plants of fingerling trout. Testing of the hatcheries by the pepsin-trypsin digest method (PTD; Markiw and Wolf 1975), during annual inspections later during those years, resulted in identification of *M. cerebralis*. Additional fish were inadvertently stocked from these hatcheries the following year into some of the same or additional high-elevation habitats because they were not removed from the stocking schedules for those hatcheries. Actual *M. cerebralis* status and infection severity of the fish stocked into these high elevation waters is unknown, but it is likely that the fish were exposed to the parasite before stocking. The objective of this study was to determine the extent of the spread of *M. cerebralis* into high elevation waters presumed to be free of the parasite, identify the contribution of spread due to accidental stocking events, and determine the threat of the parasite to high-elevation trout populations in Colorado.

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METHODS

During 1998 and 1999, resident rainbow trout *Oncorhynchus mykiss*, brown trout *Salmo trutta*, brook trout *Salvelinus fontinalis*, and cutthroat trout *Oncorhynchus clarki* were sampled from 72 waters classified as *M. cerebralis*-negative before the accidental stocking events. Sampling sites included, both, waters that had been stocked from the suspect hatcheries and those that had not been stocked from suspect hatcheries. This was done to distinguish between stocking- and nonstocking-related transfer of *M. cerebralis* to these high-elevation habitats. Waters with a wide range of elevations, 2,103–3,840 m (6,900–12,600 ft), and habitat types were sampled with gill nets, hook and line, and electrofishing. An attempt was made to collect a representative 60-fish sample from each body of water to detect *M. cerebralis* at a higher than 5% prevalence, with 95% confidence intervals (Ossiander and Wedemeyer 1973). In many cases, very few fish or only native cutthroat trout were found, so smaller samples were taken to preserve the existing populations. Sampling was focused on fish that were obviously a result of natural reproduction, to identify establishment of the parasite rather than presence of the organism in stocked fish. Wild brook, brown, and cutthroat trout, as well as younger age classes of fish, were preferentially sampled. Fish submitted for testing were at least 1 year old, to increase the likelihood that if mature myxospores were present, they would be detected using the PTD method. Parallel single-round polymerase chain reaction (PCR) and PTD tests (Schisler et al. 2001) were conducted on 1,743 fish, during the study. Myxospore burden was estimated for each fish (half-head) tested with PTD. PCR band-strengths were rated as one of five categories: negative (0), weak positive (1), positive (2), strong positive (3), and very strong positive (4). Prevalence and infection intensity were evaluated for fish in each of the waters tested. The waters were then categorized as *M. cerebralis*-positive or negative, based on the testing results.

Elevation, stocking history, presence of *Tubifex tubifex* habitat, and proximity to known infected waters were recorded for each of the waters tested. Multiple fish species were often taken from the same body of water, so waters were not classified by species. One lake was sampled in both 1998 and 1999, so data were combined for that particular lake. Samples from two other waters were incomplete, or fish were too young for valid PTD testing and were not included in the data set. The end result was 69 waters used in the analysis. The waters sampled in the study were classified into three different categories, based on stocking history. Waters never stocked with fish from suspect or positive facilities were classified as "Stock 0" waters. Waters stocked with fish from hatcheries the year before or the year of finding infected fish at the facility (suspect fish) were classified as "Stock 1" waters. Waters stocked with fish from known-positive hatcheries (year after identification of infected fish at the facility) were classified as "Stock 2" waters.

T. tubifex can be found in pristine environments and are fairly ubiquitous throughout freshwater salmonid habitat (Granath and Gilbert 2001). However, the likelihood of finding abundant *T. tubifex* is much greater in locations with fine sediments and eutrophic conditions. Sauter and Güde (1996) found that *T. tubifex* tend to prefer silt-clay substrate. Lazim and Learner (1987) found that most tubificids occurred in areas of high organic content and silt-clay sized particles. *T. tubifex* may occur in very high densities in cases of extreme organic enrichment or habitat degradation, when many other invertebrate species are eliminated (Aston 1973; Brinkhurst 1965; Lestochova 1994). Studies in Colorado have shown that eutrophic, silty locations favorable to *T. tubifex* can acr as point sources of *M. cerebralis* infection (Allen 1999; Thompson and Nehring 2000). Waters were categorized based on the presence of obvious *T. tubifex* habitat. Very oligotrophic locations with cobble-boulder or bedrock substrates and minor amounts of available *T. tubifex* habitat were classified as "Tubifex 0." Waters with an intermediate level of sedimentation, gravel-cobble substrate, and a moderate amount of organic material deposition were classified as "Tubifex 1." Locations with silt-sand-clay substrate and eutrophic conditions, such as beaver ponds, were classified as "Tubifex 2." Classification into these types of habitats was not based on an absolute measure of total sedimentation, eutrophication, or total *T. tubifex* abundance. However, they are distinct enough for any layperson to identify the differences in these habitat types and rate them on a scale of 0–2.

Proximity to known *M. cerebralis* waters was evaluated by relying on past pathogen testing, conducted by the Colorado Division of Wildlife Aquatic Animal Health Laboratory in Brush, Col-

orado. Both straight-line distances and within-drainage distances (km) to closest known *M. cerebralis*-positive sites were estimated using measurements from Delorme 1:160,000 maps. Straight-line distance was simply the most direct distance to the nearest location that had previously tested positive for *M. cerebralis*. Within-drainage distance was the distance, following the streambed, to the nearest upstream or downstream site that had previously tested positive for *M. cerebralis*. Many sites in Colorado have not been tested for *M. cerebralis*, so the parasite is likely established in many more locations than the current data reflects, and distances recorded are based only on known positive sites. Maximum distance recorded was limited to 40.2 km (25 mi), due to the presence of some waters in dead-end drainages or in areas where limited testing had been done, which would have otherwise resulted in recording of some waters as having infinite within-drainage distances to the next known positive site.

A correlation matrix produced by PROC CORR, a procedure in SAS system software, was used to determine if significant collinearity existed between factors. Elevation was correlated with our measure of *T. tubifex* habitat ($R^2 = 0.2628$, $P < 0.0001$). Eutrophication and sedimentation leading to abundant *T. tubifex* habitat can occur at any elevation given the correct circumstances. We felt that relative amount of *T. tubifex* habitat was more likely to have a direct effect on establishment of *M. cerebralis* than elevation, and because these factors were correlated, we removed elevation as a factor in the modeling procedure. Straight-line distance was correlated with within-drainage distance to other *M. cerebralis* positive sites ($R^2 = 0.4578$, $P < 0.0001$). Because both of these parameters represent essentially the same factor and were correlated, we removed straight-line distance as a factor.

Apparent effects from individual factors may be confounded due to interactions, and considering each factor alone in highly complex natural systems can be misleading.

Therefore, logistic regression analysis (PROC GENMOD) in SAS system software was used, to test all factors simultaneously and to determine which (if any) of the parameters recorded contributed to *M. cerebralis* status of the waters tested. The major factors recorded for each water and included in the analysis as independent variables were stocking history, *T. tubifex* habitat, within drainage distance to the nearest known positive water, and all interaction terms. Stocking was used as a categorical variable, with each of the three different stocking classifications as a different category. *T. tubifex* habitat was treated as a categorical variable in one set of models and as a continuous variable in another set of models. *T. tubifex* habitat was used as a categorical variable in some models to reflect the three distinct habitat types, as they were classified during sampling as minimal, moderate, or abundant habitat. *T. tubifex* habitat was used in other models as a continuous variable to describe a range of habitats from low to high *T. tubifex* habitat abundance. Treatment of the variable in this manner is useful because all waters may not always fit perfectly into one of the three categories described.

For all of the models, the sampling unit used was individual waters, and the dependent variable was the presence or absence of *M. cerebralis*. All of the different combinations of variables resulted in 36 different models to be evaluated. Second order Akaiki information criterion (AICc) (Burnham and Anderson 1998) was used as the model selection procedure to identify the model that best described the data without over-parameterization. AICc is much like AIC (Lebreton et al. 1992), except a small-sample bias adjustment term is added to the formula and is described as follows:

$$AICc = -2\log(L(\hat{0})) + 2K\left(\frac{N}{N-K-1}\right)$$

where
N = number of samples, and
K = number of parameters.

AICc values were calculated for each of the alternative logistic regression models generated. The models were then ranked based on these values, with small AICc values representing the best models and large AICc values representing poor models of the information in the data. The AICc values were then rescaled as follows to give the model with the minimum AICc a value of 0 and to allow easily comparable, rankable models:

$$\Delta_i = AICc_i - \min AICc$$

Akaiki weights (Burnham and Anderson 2001) were then calculated for each of the models, to provide "weight of evidence" for the strongest models when compared with other models evaluated. The Akaiki weight for each model is interpreted as approximately the probability that the model

is the best in the set evaluated. The weights are calculated as follows:

$$w = \frac{\exp(-\Delta_i / 2)}{\sum_{r=1}^{R} \exp(-\Delta_r / 2)}$$

This information was used to identify the best models for describing the data from the 69 high-elevation waters sampled. The logistic regression and subsequent model selection allowed us to not only identify the best models but also to quantify relative importance and effects of the factors used in the models.

RESULTS

Raw data suggested lower mean spore burdens, and PCR band strengths occurred at higher elevation waters. Mean myxospore burdens (half-heads) increased from 1,386 per fish, at elevations greater than 3,500 m, to 3,302 per fish, at elevations of 2,501–3,500 m. At elevations below 2,500 m, mean myxospore burden increased to 5,727 per fish. Corresponding mean PCR band strengths had a similar pattern of stronger strength with lower elevation. Prevalence of infected fish with increasing elevation was fairly pronounced (Table 1). There were some obvious exceptions, including waters above 3,500 m that still produced high proportions of infected fish. Some low-elevation waters also produced low proportions of infected fish. This indicates that other factors influenced infection prevalence besides simply elevation.

The raw data suggested that stocking history had an effect on *M. cerebralis* status of the waters tested (Table 2). A higher percentage of "Stock 2" waters were identified as *M. cerebralis*-positive than "Stock 1" or "Stock 0" waters. Only 25.0% of "Stock 0" and only 25.7% of Stock 1 waters were identified as *M. cerebralis*-positive, with PTD. PCR testing identified 66.7% of "Stock 0" and 51.4% of "Stock 1" waters as *M. cerebralis*-positive. Lower-elevation waters adjacent to known infected waters made up the bulk of the *M. cerebralis*-positive "Stock 0" waters. By contrast, 80.0% of the "Stock 2" waters were identified as *M. cerebralis*-positive, by both PCR and PTD.

Tubifex tubifex habitat was generally reduced at higher elevations, due to the lower sediment load and organic content of most of the higher-elevation waters. Presence of obvious *T. tubifex* habitat influ-

Table 1. Mean PTD myxospore counts, mean PCR band strength ratings (half-head), and infection prevalence for fish sampled at elevations ranging from 2,103–3,840 m (6,900–12,600 ft).

Elevation	Test	Fish	Infected (%)	Mean	St. Dev	Range
< 2,500	PTD	221	25.8	5,727	17,528	0–175,000
	PCR	221	62.0	1.71	1.57	0–4.00
2,501–3,500	PTD	965	11.4	3,302	19,538	0–291,644
	PCR	965	23.8	0.56	1.14	0–4.00
> 3,500	PTD	557	4.1	1,386	14,072	0–278,133
	PCR	557	7.7	0.17	0.67	0–4.00

Table 2. Infection prevalence of fish and percent of waters identified as *M. cerebralis* positive by PTD and PCR from waters classified by stocking history. "Stock 0" waters were never stocked with fish from suspect or known-positive facilities. "Stock 1" waters were stocked with fish from hatcheries the year before or the year of finding infected fish at the facility (suspect fish). "Stock 2" waters were stocked with fish after confirmation of *M. cerebralis* at the facility.

	Fish			Waters		
Stocking	N	%PTD+	%PCR +	N	%PTD+	%PCR+
Stock 0	751	8.3	17.6	24	25.0	66.7
Stock 1	676	5.6	14.8	35	25.7	51.4
Stock 2	316	28.5	56.3	10	80.0	80.0

enced M. *cerebralis* status of the waters tested (Table 3). PTD testing resulted in classifying 9.1%, 23.8%, and 61.5% of the populations in "Tubifex 0," "Tubifex 1," and "Tubifex 2" waters as infected with M. *cerebralis*. PCR testing identified a higher proportion of the populations as infected, but the pattern remained the same, with 45.5%, 52.4%, and 80.4% of the populations in "Tubifex 0," "Tubifex 1," and "Tubifex 2" waters identified as infected.

Average within-drainage distance to the nearest infected site was 11.2 km (N = 23; SD = 14.3) for waters found to be M. *cerebralis*-positive with PTD testing, and 16.7 km (N = 42; SD = 14.7) with PCR testing. Average within-drainage distances to the nearest infected sites were 24.9 km (N = 46; SD = 13.1) for waters found to be M. *cerebralis* negative with PTD testing, and 26.1 km (N = 27; SD = 13.6) for PCR testing.

Model Selection

The PCR data were best described using a model including *T. tubifex* habitat as a continuous variable and within-drainage distance (T_1, D). The second-choice model included *T. tubifex* habitat as a categorical variable and within-drainage distance (T_2, D). Other models with interaction terms or fewer parameters had larger AICc values and low Akaiki weights (Table 4). The PTD data were best

Table 3. Infection prevalence of fish and percent of waters identified as M. cerebralis positive by PTD and PCR for three categories of T. tubifex habitat.

	Fish			Waters		
Tubifex habitat	N	%PTD+	%PCR+	N	%PTD+	%PCR+
Minimal habitat	553	4.0	7.5	22	9.1	45.5
Moderate habitat	543	4.4	15.7	21	23.8	52.4
Abundant habitat	647	22.3	43.7	26	61.5	80.4

Table 4. Best-fitting alternative models for predicting presence of *M. cerebralis* in high elevation Colorado waters with PCR and PTD testing. N = 69 for each model.

		PCR testing			
Model	Parameters	−2log(L(0))	AICc	Δ_i	Akaiki weights[a]
T_1, D	3	80.5109	86.8801	0.0000	0.1904
T_2, D	4	78.4234	87.0484	0.1683	0.1750
T_1, D, S, T_1*S	7	72.3160	88.1521	1.2720	0.1077
T_1, D, T_1*D	4	80.3440	88.9690	2.0889	0.0669
T_1, D, S	5	78.5539	89.5063	2.6262	0.0512
		PTD testing			
Model	Parameters	−2log(L(0))	AICc	Δ_i	Akaiki weights[a]
T_1, D	3	60.3051	66.6743	0.0000	0.2561
T_1, D, S	5	56.6441	67.5965	0.9222	0.1615
T_2, D	4	59.2178	67.8428	1.1685	0.1428
T_1, D, T_1*D	4	59.9436	68.5686	1.8943	0.0993
T_2, D, S	6	56.3812	69.7360	3.0617	0.0554
T_1, D, S,T_1*D	6	56.4847	69.8395	3.1652	0.0526

[a]Akaiki weights for all other models were < 0.05.
T_1 = T. tubifex habitat as a continuous variable.
T_1 = T. tubifex habitat as a categorical variable.
D = Within-drainage distance to nearest infected water.
S = Stocking history as a categorical variable.
* = Interaction term.

fit with a model containing *T. tubifex* habitat as a continuous variable and within-drainage distance (T_1, D). A model with both of these variables and stocking history (T_1, D, S) was the second-best model fit. As with the PCR data, other models with interaction terms or fewer parameters had larger AICc values and low Akaiki weights.

These results indicate that a model containing within-drainage distance and *T. tubifex* habitat as a continuous variable (T_1, D) was the best overall fit for both the PTD and PCR data. Parameter estimates for models that included stocking history indicate that a within-drainage distance and *T. tubifex* habitat were the most important factors (Table 5), with stocking having a lesser influence (i.e., T_1, D, S). The best fitting model (T_1, D) provides similar results for both the PCR and PTD data, except the likelihood of finding *M. cerebralis* is greater with the PCR data (Figures 1 and 2). This is to be expected, considering the greater sensitivity of the PCR test. Increasing distance to the nearest known-infected site in the models results in lower probability of establishment of the parasite, while an increase in *T. tubifex* habitat results in greater probability of establishment.

Although the model T_1, D, S was the second-choice model for PTD testing, as identified by the AICc and Akaiki weight results, it provides some insight into the effects of stocking on *M. cerebralis* establishment in the waters in this study. Waters in the model not stocked with infected or suspect fish have the lowest overall probability of establish-

Table 5. Parameter estimates and significance levels for the two best-fitting alternative models for predicting presence of *M. cerebralis* in high elevation Colorado waters as tested by PCR and PTD.

			PCR results	
Model = T_1, D				
Parameter		df	Estimate	Standard error
Intercept		1	0.6689	0.6061
T. tubifex		1	0.7100	0.3286
Distance		1	−0.0432	0.0195
Model = T_2, D				
Parameter		df	Estimate	Standard error
Intercept		1	2.5891	0.7806
T. tubifex	0	1	−1.4971	0.6897
T. tubifex	1	1	−1.6064	0.7804
T. tubifex	2	0	0.0000	0.0000
Distance		1	−0.0505	0.0212
			PTD results	
Model = T_1, D				
Parameter		df	Estimate	Standard error
Intercept		1	−1.2710	0.7678
T. tubifex		1	1.4944	0.4739
Distance		1	−0.0685	0.0228
Model = T_1, D, S				
Parameter		df	Estimate	Standard error
Intercept		1	5.5415	1.2434
T. tubifex		1	1.2610	0.4848
Distance		1	−0.0711	0.0245
Stocking	0	1	−1.8409	1.0676
Stocking	1	1	−1.6867	1.0089
Stocking	2	0	0.0000	0.0000

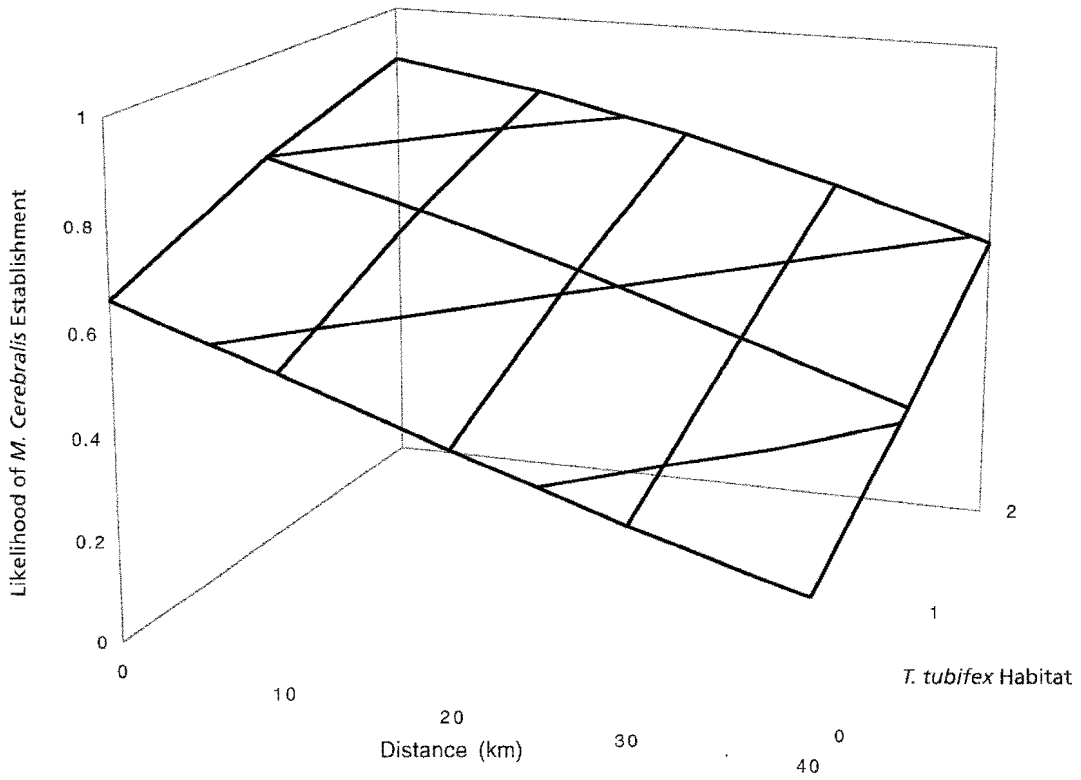

Figure 1. PCR-model results (T_1, D) for effects of *T. tubifex* habitat and distance to nearest known-positive, within-drainage site on *M. cerebralis* establishment.

ment of the parasite, followed by waters stocked with suspect fish and waters stocked from hatcheries after they were known to be contaminated with M. *cerebralis*. However, the best-fitting models indicated that the accidental stocking events had less of an effect than the other two factors.

DISCUSSION

The raw data indicated that stocking history, elevation, presence of *T. tubifex* habitat, and distance to known positive waters all contributed in some way to the M. *cerebralis* status of the waters tested. The best-fitting models identified presence of *T. tubifex* habitat and within-drainage distance to known infected waters as the most important variables in the establishment of M. *cerebralis* in high elevation waters. The importance of stocking history was low, compared with the other factors in this study. This was partially due to the small number of "Stocked 2" waters in the data set, which were the most heavily influenced by the stocking. The contribution to M. *cerebralis* establishment was not strongly influenced by the accidental stocking that occurred in the "Stocked 1" waters. The high-elevation waters in this study were stocked only one or two times with infected or suspect fish, and lower-elevation waters with long histories of stocking of infected fish are more likely to be affected by stocking history.

Available *T. tubifex* habitat has long been known to be a contributor to the intensity and prevalence of M. *cerebralis* infection. This was the case in this study as well, with low prevalence of infection and low proportion of infected waters where little *T. tubifex* habitat was available. Infection levels may have been too low to detect in some locations with little or no obvious *T. tubifex* habitat, which would give the false impression that establishment had not occurred. However, the end result is still a reduced risk from the parasite in these locations.

The effect of distance to known positive waters on M. *cerebralis* status of the waters tested in this study suggests that transport by anglers, migrating fish, or other wildlife may be a contributing factor in the spread of the pathogen in

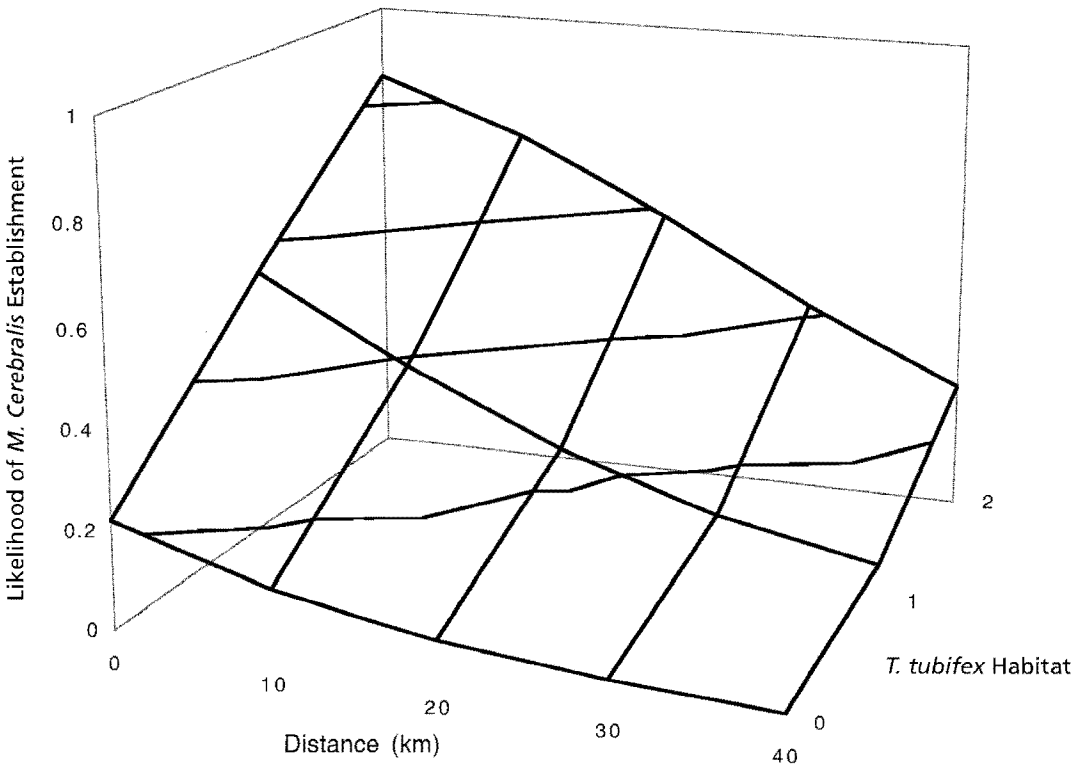

Figure 2. PTD-model results (T_1, D) for effects of *T. tubifex* habitat and distance to nearest known-positive, within-drainage site on *M. cerebralis* establishment.

Colorado. It is intuitive that waters in close proximity to other known-positive waters have a greater probability of becoming contaminated with the parasite by these means.

The models selected provide some guidelines for identifying waters at the highest risk for establishment of M. *cerebralis* and could be used to set priorities for testing or regulations. Other parameters not examined in this study may very well have an effect on presence or absence of M. *cerebralis*, and the results of this study may be an over-simplification due to exclusion of these unknown variables. The possibility exists that, with time, the range of M. *cerebralis* will expand into even the most remote locations in Colorado.

The results indicate that the accidental stocking events, occurring the year of identifying the hatcheries as M. *cerebralis*-positive, did not contribute substantially to the spread of parasite in the high-elevation waters. However, M. *cerebralis* was found in a high percentage of the few warers that were stocked the year after the hatcheries were identified as positive. Continued stocking would surely contribute more to the likelihood of M. *cerebralis* establishment. Colorado Division of Wildlife regulations will prohibit stocking of M. *cerebralis*-exposed fish into salmonid habitat by 2003. Many trout populations in Colorado are somewhat protected from M. *cerebralis*, due to their high elevations, long distances to other infected waters, lack of *T. tubifex* habitat, and preclusion of further stocking of infected fish into high-elevation waters. Populations with abundant *T. tubifex* habitat, and close proximity to known M. *cerebralis*-contaminated waters, are at high risk for establishment of M. *cerebralis* and should be closely monitored.

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