Prevalence and shedding of *Renibacterium salmoninarum* in Brook trout (*Salvelinus fontinalis*) in Michigan

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ABSTRACT: Bacterial Kidney Disease has been reported wherever susceptible salmonid populations are present. There has been a dispute about the status of the diseases among different salmonid species and their susceptibility to the infection over the past years. In this regard, a considerable number of indicated the susceptibility of brook trout to BKD infection. In order to determine the status of bacterial kidney disease (BKD) in hatchery and wild populations of brook trout (*Salvelinus fontinalis*) in Michigan, (*Renibacterium salmoninarum*) *R. salmoninarum* prevalence and intensity were determined in representative samples from adult hatchery raised and wild stocks as well as their offspring from 2001 through 2005. The hatchery raised adult Iron River brook trout presented higher BKD prevalence than the brook trout caught from Cherry Creek. Generally, the BKD prevalence and intensity in hatchery and wild brook trout strains gradually decreased throughout the period from 2001 to 2004. The critical role played by hatchery practices to control the spread and minimize the prevalence of BKD among Michigan brook trout populations was discussed. Although most of the previous studies reported unimportant role of the male in transmission of *R. salmoninarum*, yet our results clearly demonstrated that males shed more *R. salmoninarum* along with their gametes than females. [Nature and Science. 2007;5(1):8-17].

Keywords: BKD, *Renibacterium salmoninarum*, prevalence, Shedding, Brook trout, *Salvelinus fontinalis*

INTRODUCTION

   Brook trout (*Salvelinus fontinalis*) is an indigenous salmonid species in the Great Lakes (Coon 1999) that has been artificially propagated and stocked in Michigan's public waters for years (Dexter and O'Neal, 2004). Two strains of brook trout, the Assinica and Iron River strain, are being reared by Michigan Department of Natural Resources (MDNR) for stocking resident stream populations where there is a deficiency of natural recruitment (Dexter and O'Neal, 2004). Iron River Brook trout strain is considered a pure native strain which was originally from the Iron River in Michigan's Upper Peninsula. Unlike the Assinica strain, Iron River brook trout are slow to reach maturity and are characterized by a very slow growth rate because of their wild characteristics (Driver 1995., Dexter and O'Neal 2004).

        Fish health poses major challenges to development and progress of the brook trout restoration in the Great Lakes basin. Among these health challenges, bacterial kidney disease, caused by *R. salmoninarum*, is an eminent threat due to the enzootic nature of the pathogen within Great Lakes waters (Eissa 2005). Moreover, affinity of *R. salmoninarum* for the kidneys, which possesses an essential lymphoid function and its obligate intracellular nature, makes this pathogen and its soluble antigens a major threat to the host by suppressing the fish immune system (Ellis 1999; Fredriksen et al. 1997; Grayson et al. 2002).

   A considerable number of studies have been performed on brook trout in the USA which indicated the susceptibility of brook trout to BKD (Belding and Merrill 1935; Snieszko and Griffin 1955). However, most of these studies involved the use of experimental infection, whilst few discussed natural BKD infection among brook trout (Allison (1958); Bullock et al. (1971); Mitchum et al. 1979; Mitchum and Sherman 1981).

   Interestingly, the first report of BKD in the USA occurred in brook trout at a Massachusetts State fish hatchery (Belding and Merrill, 1935). During the late 1940s and early 1950s, *R. salmoninarum* infection caused mass mortalities in brook trout at the federal hatcheries in Berlin, New Hampshire, Cortland, and

New York (Snieszko and Griffin, 1955). Mitchum et al. (1979) found that the prevalence of BKD in brook trout during epizootics in southeastern Wyoming, USA were 100% and 83% among dead and live brook trout respectively.

In Michigan, the first case of BKD was discovered in 1955 in brook trout yearlings at the Oden and Marquette state hatcheries, where eggs were originally imported from a hatchery in New England in which BKD had been endemic for many years (Allison, 1958). Since the first report of the disease in 1955, none of the published studies have reported any data about the recent occurrence of BKD outbreaks, prevalence, or magnitude of the disease in brook trout in Michigan or in the Great Lakes basin.

Thus, the aim of the current research was to investigate the status and magnitude of BKD in brook trout in Michigan by assessing the *R. salmoninarum* prevalence in the hatchery raised brook trout populations in Michigan. The role-played by the male and the shedding of the bacteria along the gamete were also studied.

MATERIALS AND METHODS

Fish. To investigate the prevalence and intensity of *R. salmoninarum* infection among brook trout (BKT) populations in Michigan waters, a total of 628 adult brook trout were collected from the hatchery raceways of Marquette State fish hatchery (MSFH) and the Cherry Creek water stream outside the hatchery in Marquette, Michigan, from 2001-2004. MSFH is the primary facility for brook trout broodstock that are used for the production of fingerlings to be stocked in both inland and Great Lakes waters.

A total of 567 hatchery-raised brook trout broodstocks (529 Iron River strain (IR-BKT) sample, 38 Assinica strain (AS-BKT) sample) and 61 adult Cherry Creek wild brook trout (CC-BKT) (Table1) were used for BKD prevalence analysis. Following approximately 18 months of egg incubation and fish rearing in the Marquette State Fish Hatchery, MDNR releases fingerlings in the spring of each year. From 2002-2005, a total of 420 pre-stocking fingerlings were collected to determine the prevalence of *R. salmoninarum* prior to release into the basins of Lakes Michigan (Table 1).

The fish were euthanized by exposure to an overdose of MS-222 (tricaine methane sulfonate, Finquel-Argent Chemical Laboratories, Redmond, WA). During collection of the broodstock fish samples, following gamete collection, the abdominal cavity was cut open and all internal organs were examined for signs of BKD. About one gram of tissues were sampled from anterior, posterior and middle kidney sections using separate sterile dissecting tools.

Fingerlings were collected, euthanized and whole kidney tissue was collected individually using separate sterile dissecting tools.

To test the shedding of *R. salmoninarum* antigens with the gametes, a total of 200 coelomic/ovarian fluid samples were collected from females using sterile transfer pipette during egg collection, transferred to 5 ml sterile polypropylene tubes, and kept on ice until processed at the laboratory at Michigan State University, East Lansing, MI. Additionally, a total of 200 semen samples were collected from males by squirting the middle stream of semen directly into sterile 5 ml polypropylene tubes.

Clinical examination. Fish were euthanized using an overdose of MS 222 and examined externally for the presence of any lesions, parasites, or abnormal growths on the skin or gills. Fish were dissected and examined internally for any lesions, swelling, or color changes in the kidneys other internal organs and viscera.

Sample processing. Kidney samples representing the anterior, posterior and middle sections of the kidney were transferred in sterile 7.5 cm x 18.5 cm Whirl Pak® bags (Nasco, Forte Atkinson, and WI), kept on ice, and were softened as much as possible through multiple cycles of physical pressure. The homogenized kidney tissues were diluted in 1:4 (w/v) Hank's Balanced Salt Solution (HBSS, Sigma Chemical Co, St. Louis, MO) then stomached for 2 minutes at high-speed in a Biomaster Stomacher-80 (Wolf Laboratories Limited, Pocklington, York, UK). In the case of coelomic/ovarian fluid or milt samples, 1 ml from fluid sample was diluted 1:2 (v/v) in HBSS for Q-ELISA testing.

Measurements of *Renibacterium salmoninarum* antigen using the Quantitative Enzyme-linked Immunosorbent Assay (Q-ELISA). Aliquots of 250 µl volume of each processed samples were transferred into 1.5 ml safe-lock microfuge tube to which an equal volume of 0.01 M phosphate buffered saline-Tween 20 (0.05 %) (PBS-T20) (Sigma) with 5 % natural goat serum (Sigma) (Olea et al., 1993) and 50 µl CitriSolv solution (Fisher Chemicals, Fairlawn, New Jersey) (Gudmunsdottir et al., 1993) were added.

The mixture were thoroughly mixed by vortexing then incubated at 100 °C on heat blocks with rotary shaker for 15 minutes and followed by 2 hours of incubation at 4 ºC. After incubation, the mixture was centrifuged at 6000g for 15 minutes at 4 ºC. The aqueous supernatant of each sample was carefully collected and then transferred to a 1.5 ml microfuge tube for Q-ELISA testing. The Q-ELISA method used in this study is that described in details by Pascho and Mulcahy (1987) and Alcorn and Pascho (2000). The positive negative threshold absorbance is calculated according to the method described by Meyers et al. (1993) .The positive–negative cutoff absorbance for the kidney homogenate was 0.10. The tested positive samples were assigned the following antigen level categories: low (0.10 to 0.19), medium (0.20-0.99) and high (1.000 or more) (Pascho et al., 1998). Intensity of infection among certain group of fish is expressed by percent of fish with high titers of *R. salmoninarum* soluble antigens using Q-ELISA.

Nested PCR. A DNeasy tissue extraction kit (Qiagen-Valencia, CA, USA) was used for the extraction of DNA from 100µl aliquots of kidney tissue homogenates. The DNA was extracted according to the manufacturer's instructions, with a few minor modifications from the method described by Pascho et al. (1998). The tissue pellets were obtained by centrifugation at 6000 g for 20 minutes at 4 °C and the pellets were incubated with lysozyme buffer consisting of 180 µl of 20 mg lysozyme (Sigma), 20mM Tris-HCl, pH 8.0; 2 mM EDTA (Sigma) and 1.2 % (v/v) Triton X 100 (Sigma) at 37 °C for 1 hour. The nPCR method and primers recommended by Pascho et al. (1998) were employed with slight modifications to the volume of DNA (5 µl for first round and 2 µl for second round PCR reaction), water, and master mixes (45 µl for first round and 48 µl for second round nPCR reaction) .The controls were composed of a PCR mixture containing no DNA template reagent (negative control), positive *R. salmoninarum* and positive tissue control. A volume of 10 µl of the nPCR product and controls were mixed with 2 µl of 6X loading dye (Sigma) and used on a 2 % agarose gel (Invitrogen Life Technologies, Carlsbad, CA). Each electrophoresis gel included a 1kbp DNA ladder with 100 bp increments (Invitrogen). Gels were run in 1 X Tris Acetate Buffer (1 X TAE) gel buffer (Sigma). Gels were visualized under the KODAK EDAS Camera System and UV Trans-illuminator. Samples were considered positive when a 320 bp band was detected. Molecular confirmation of the purified bacterial isolates was also conducted using nPCR according to the method described by Chase and Pascho (1998).

Statistical Analysis. Because of the nature of data collected in this study required basic statistical description, data analysis was primarily relied on descriptive statistics. For year to year and brook trout strains comparisons, the data was tested for normality and then student t test (parametric) or Mann-Whitney Rank Sum Test was used (with an alpha level = 0.05).

RESULTS
A. Prevalence of *R. salmoninarum* infection in captive and wild brook trout stocks. *Renibacterium salmoninarum* antigens were detected in the kidneys of Marquette State Fish Hatchery captive broodstock and fingerlings as well as Cherry Creek brook trout. The prevalence of *R. salmoninarum* in the Iron River broodstock exhibited a steady decline from 87% in 2001, to 80% in 2002, to 60% in 2003, and to 43% in 2004. Similarly, the intensity of *R. salmoninarum* in the Iron River brook trout (expressed by the percent of fish showing high titer of *R. salmoninarum* antigen) exhibited a comparable decline. The percent of fish with high *R. salmoninarum* antigen levels decreased from 17% in 2001 to 7.5% in 2004 (Figure 1).
Assinica brook trout Broodstock (BS AS-BKT) were tested only in 2003 and 2004. The results demonstrated a decrease in prevalence from 80 % in 2003 to 25% in 2004 (Table 2). However, the intensity of the *R. salmoninarum* infection in the BS AS-BKT did not exhibit a similar decline.
In the case of Cherry Creek brook trout (CC-BKT), samples were collected in the falls of 2001-2004. Prevalence of *R. salmoninarum* in CC-BKT decreased from 80% in 2001 to 67% in 2003. There was no consistent pattern in the intensity of *R. salmoninarum* (Table 2).
The prevalence of *R. salmoninarum* in IR-BKT fingerlings between 2003 and 2005 were comparable to the parents and consistent with the high prevalence in the gamete donor broodstock, although 2004 showed a significantly low prevalence. However, the intensity of *R. salmoninarum* antigens in BKT fingerlings was higher than those detected in their parents during 2003 (48% vs 17% in their parents) before sharply declining in subsequent years (table 2).
The prevalence of *R. salmoninarum* in 2005-AS-BKT fingerlings was lower (28%) than those of their parent BS AS-BKT-2003. Similarly, the prevalence of *R. salmoninarum* in 2005-AS-BKT fingerlings was

much lower than that of the 2005-IR-BKT fingerlings (28% vs 45% in IR-BKT fingerlings). The intensity of the *R. salmoninarum* infection in the AS-BKT fingerlings showed a decline, which was similar to the prevalence through the time of testing.

To compare the prevalence and intensity of *R. salmoninarum* in males and females BKT, two hundred pairs of IR-BKT broodstock were tested in 2004. Results indicated that the prevalence of *R. salmoninarum* infection was higher in the kidney tissue of the females than males (48.5% in females vs 37.5% in males). Similarly, the intensity of *R. salmoninarum* infection was clearly higher in the females (11% in female vs. 4 % in male). The shedding of the bacterial antigen along with the gametes was tested in broodstock IR-BKT in 2004. Data shown in Figure (2) illustrated that *R. salmoninarum* antigen shed with the gametes in both males and females IR-BKT. The prevalence of individuals that shed the bacterial antigen along with the gametes is lower in males than in females (10% in milt vs 15% in ovarian fluid). Also, the intensity of samples with high *R. salmoninarum* antigen was much higher in females (2.5%) than in males (0.5%). The majority of the females (20 out of 30) that shed the antigen in their ovarian fluids exhibited similar levels of *R. salmoninarum* antigens in their kidneys. However, 9 females shed the *R. salmoninarum* antigen along with the ovarian fluid without detecting *R. salmoninarum* antigen in the kidneys and one female shed the antigen at a low level in the ovarian fluid and tested negative for the kidney tissue. Likewise, the majority of male shedders (13 out of 20) had similar levels of *R. salmoninarum* in their kidneys with only 7 fish shed the antigen without detected titer of *R. salmoninarum* antigen in the kidney.

Table 1. Details of samples collected from brook trout broodstocks throughout the period from 2001-2004 and pre-stocking 18-month-old fingerlings collected throughout the period from 2002-2005.

Brook trout strain and life stage	Date Collected	Number of fish tested
Iron River broodstocks	October, 2001	54
	October, 2002	45
	October, 2003	30
	October, 2004	400
Assinica broodstocks	October, 2003	30
	October, 2004	8
Iron River 18 month old pre-stocking fingerlings	March, 2002	60
	March, 2003	60
	March, 2004	60
	March, 2005	60
Assinica 18 month old pre-stocking fingerlings	March, 2003	60
	March, 2004	60
	March, 2005	60

Table 2. *Renibacterium salmoninarum* **infection prevalence and intensity among brook trout broodstocks and their corresponding 18 months pre-stocking fingerlings throughout the period from 2001 - 2005.** Data in this table was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥ 1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

Strain	Parent stocks (Gamete donors)		18 months old pre-stocking fingerlings	
	Year	*Renibacterium salmoninarum* prevalence	Year	*Renibacterium salmoninarum* prevalence
Iron River	2001	87.0 % Total 16.7% high 50.0 % medium 20.4% low	2003	98.0 % Total 48.0 % high 35.4 % medium 14.6 % low
Iron River	2002	80.0 % Total 15.55 % high 13.3 % medium 51.1 % low	2004	20.0 % Total 0.0 % high 10.0 % medium 10.0 % low
Iron River	2003	60.0 % Total 10.0 % high 0.0 % medium 50.0 % low	2005	45.0 % Total 0.0 % high 12.5 % medium 32.5 % low
Assinica	2003	80.0 % Total 0.0 % high 10.0 % medium 70.0 % low	2005	27.5 % Total 0.0 % high 2.5 % medium 25.0 % low
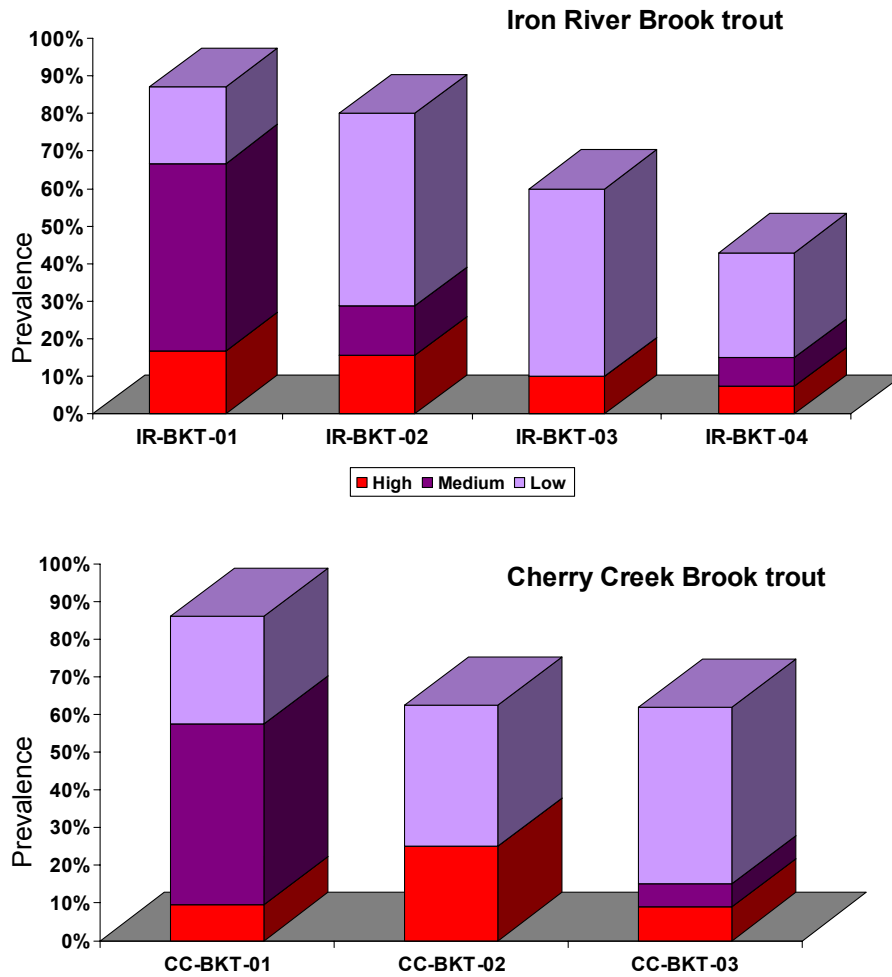
Figure 1. Prevalence and intensity of *Renibacterium salmoninarum* among the adult brook trout collected through 2001-2003。 Data in this Figure was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19. IR-BKT: Iron River Brook trout CC-BKT: Cherry Creek Brook trout
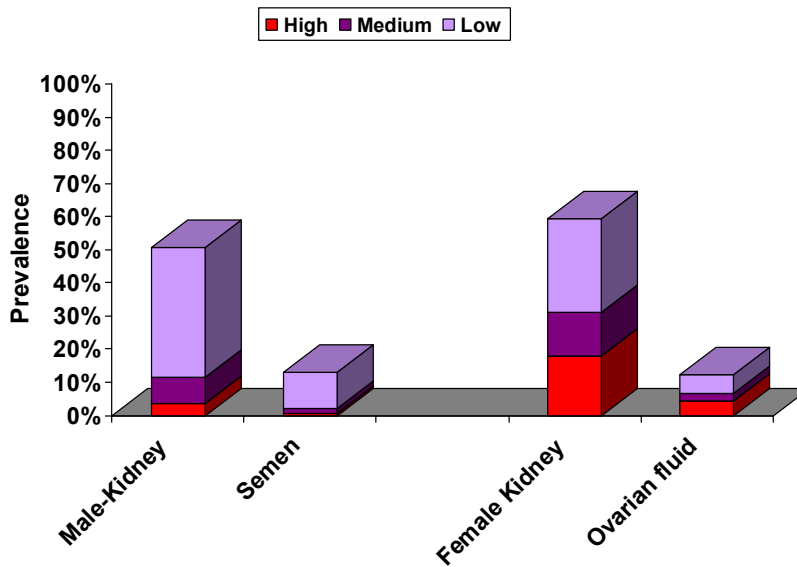
Figure 2. *Renibacterium salmoninarum* **Prevalence and intensity in kidneys and gametes of the Iron River brook trout broodstock in from Marquette State Fish Hatchery. Samples were collected during the fall spawning season of 2004.** Data in this Figure was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

DISCUSSION

For decades, brook trout has been known for its high susceptibility to *R. salmoninarum* infection (Snieszko and Griffin 1955; Mitchum and Sherman 1981). Brook trout infected with *R. salmoninarum* either naturally or experimentally, suffer from high mortalities (Belding and Merrill 1935; Snieszko and Griffin 1955; Mitchum et al. 1979).

Data obtained in this study demonstrated a high prevalence and intensity of *R. salmoninarum* infection in both hatchery raised and wild stocks. This concurs with previous reports. For example, Mitchum and Sherman, 1981, recorded a relatively high prevalence and severity of *R. salmoninarum* infection in wild and hatchery raised brook trout populations (58 %, 45 % respectively).

A general trend of declining prevalence and intensity of *R. salmoninarum* in IR-BKT broodstock was observed over the period of this study. This trend might be explained by the improvement of hatchery hygienic practices. Among these practices are the prophylactic erythromycin phosphate administration and hardening of eggs in erythromycin containing water. Evelyn et al. 1986 and Lee and Evelyn (1989) found that intramuscular injection of broodstock with erythromycin phosphate dramatically minimized the vertical transmission of *R. salmoninarum*. Also, Rinsing of broodstock in iodophores solution before collecting gametes could minimize the *R. salmoninarum* on the eggshell (Ross and Smith 1972). Maule et al. (1996) described similar observations that lead to remarkable decrease of *R. salmoninarum* prevalence among other salmonid species.

Although the Assinica strain demonstrated inconsistent *R. salmoninarum* infection prevalence when compared to the Iron River, yet Iron River strain showed higher intensity than Assinica strain. The Assinica strain is known for its superior survival and growth (Gowing 1986), and these characteristics could play a vital role in the general defense of the fish against severe infections with *R. salmoninarum*. In addition, variable susceptibility of fish strains to different diseases is not unusual. For example, some strains of steelhead showed variable susceptibility to *R. salmoninarum* (Winter et al., 1980).

Wild populations of BKT (CC-BKT) showed a comparable prevalence to that of the hatchery reared BKT. The fact that Cherry Creek supplies the hatchery with water and that BKT exists in this water may explain the similarity in infection levels.

Analysis of the data of *R. salmoninarum* prevalence and intensity of infection among the Iron River brook trout pre-stocking fingerlings indicated that fingerlings from 2003 exhibited the highest prevalence and infection intensity (approaching 100 %). The 2003 pre-stocking fingerlings are the offspring of the 2001 Iron River brook trout parent stocks that also exhibited high BKD prevalence (83 %). Vertical transmission have probably played a major role in this high incidence of infection, particularly that erythromycin prophylactic administration was not implemented in 2001. On the contrary, the 2004-2005 Iron River and Assinica brook trout offspring showed relatively lower *R. salmoninarum* prevalence and intensity, although they originally hatched from fertilized eggs collected from 2002-2003 parents with relatively high *R. salmoninarum* prevalence and intensity. This could only be explained by the strict hygienic measures adopted by the hatchery starting from 2002.

Data obtained from the Q-ELISA testing of gametes indicated that the *R. salmoninarum* was shed with the gametes in both males and females (10% in males versus 15% in females), with a higher intensity in females than males. These results suggest a contribution of the male in the vertical transmission of *R. salmoninarum* to the offspring, in addition to the role played by females. Allison (1958) was the first to report vertical transmission in the brook trout, albeit with circumstantial evidence that gametes from infected adults resulted in infected offspring. Our data agreed with Wiens and Kaattari (1989), which were able to detect the *R. salmoninarum* antigens in the milt of infected males. However, the studies of Klontz (1983) and Evelyn, et al. (1986) demonstrated that males play an insignificant role in the vertical transmission of *R. salmoninarum*. However, the data may be complicated by discrepancies between levels of *R. salmoninarum* in the ovarian fluid and their levels in the kidney of corresponding individual fish.

In conclusion, this study supports the previous reports, which emphasized that brook trout are highly susceptible to *R. salmoninarum* infection. In addition, this study shed the light on the possible contribution of a number of factors to development of BKD epizootics in Michigan hatcheries, such as the seasonal changes and the presence of external parasites. Further, the possible role of males and females in shedding the bacterium and its soluble antigens was fully discussed.

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