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Subclinical *Listonella anguillarum* infection does not impair recovery of swimming performance in rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: This study examines whether injections of the commonly used bacterial-challenge pathogen *Listonella anguillarum* (formerly *Vibrio anguillarum*) negatively impact the ability of rainbow trout *Oncorhynchus mykiss* Walbaum to perform repeat swimming trials. Fish were given intraperitoneal injections of either a sub-lethal (10^5 colony forming units; CFUs) or a lethal (10^7 CFUs) dose of *L. anguillarum*, held for 48 h, and then given 2 successive ramp critical swimming speed (U_{crit}) tests separated by 45 min. Compared with saline-injected control fish, the low-dose injection did not significantly impair swimming performance and recovery. Similarly, U_{crit} and re-performance for fish surviving the high-dose injection were comparable to control (2 of 6 fish died after injection and before testing). In contrast, a positive control test of seawater challenge did impair recovery of swimming performance. In view of these results and common use of *L. anguillarum* as a challenge pathogen for toxicological studies, it seems unlikely that the consequences of pathogenesis impact the important cardiorespiratory changes associated with exercise.

KEY WORDS: *Listonella anguillarum* · Vibriosis · Seawater · Swimming performance · Recovery · Critical swimming speed · U_{crit}

INTRODUCTION

Listonella anguillarum (basionym *Vibrio anguillarum*) is a naturally occurring bacterial pathogen and the etiological agent of vibriosis, known to cause substantial mortality in farmed and wild salmonid stocks. Vibriosis is primarily a disease of salt and brackish waters (Egidius 1987) that often occurs when cultured salmonids are relocated to saltwater (Egidius & Andersen 1979). The purpose of the present study was to test the hypothesis that subclinical *L. anguillarum* infection impairs swimming performance of rainbow trout *Oncorhynchus mykiss*.

Behaviors such as feeding, predator avoidance and migration all rely on swimming ability (Beamish 1978), so it is axiomatic that any impairment to swimming could impact other aspects of physiology and behavior. Interactions between swimming performance and immunological stress have been reported previously. For example, the swimming endurance of bull trout *Salvelinus confluentus* was ~20% decreased by *Renibacterium salmoninarum* infection (Jones & Moffitt 2004), and critical swimming speed (U_{crit}) was ~13% lower in Atlantic salmon *Salmo salar* with high numbers of sea lice *Lepeophtheirus salmonis* (Wagner et al. 2003) and ~18% lower in Delta smelt *Hypomesus transpacificus* with *Mycobacterium* sp. infections (Swanson et al. 2002). The ability of fish to recover from a U_{crit} test can also be impaired with disease. Indeed, wild, adult sockeye salmon *Oncorhynchus nerka* with incidental *Listonella* (*Vibrio*) and *Sporocytophagosis* infections were unable to recover within 1 h from a U_{crit} test and could only reach ~61% of an earlier U_{crit} on a second U_{crit} test (Jain et al. 1998). Whether the *Listonella* infection was the causative factor in decreasing swimming performance has not

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been tested. Therefore, the principle aim of this study was to examine the effect of *L. anguillarum* injections on rainbow trout swimming and recovery ability using repeated ramp-U_{crit} testing (Jain et al. 1997).

MATERIALS AND METHODS

Fish. Rainbow trout (mass 379 ± 12 g, fork length 29.8 ± 0.3 cm) were acquired from Sun Valley Trout Farm (Mission, BC) and maintained in flow-through dechlorinated municipal water (temperature 8.9 ± 0.3°C; dissolved oxygen >8 mg O_2 l^{-1}) at the Department of Fisheries and Oceans/University of British Columbia (DO/UBC) Centre for Aquaculture and Environmental Research (West Vancouver, BC). Fish were fed commercial trout chow ad libitum once daily.

Swimming performance protocol. Swimming measurements were performed in a 215 l, mobile Brett-type respirometer described in Farrell et al. (2003). Individual trout were taken to stage 3 anesthesia (Summerfelt & Smith 1990) with 0.2 mg l^{-1} MS222 (Tricaine methanesulfonate, Syndel Laboratories) in buffered freshwater ($NaHCO_3$ 0.2 mg l^{-1}) for mass and length measurements and to facilitate placement into the swim chamber. Fish recovered for 45 min at a low water speed (0.3 body lengths [BL] s^{-1}), which allowed fish to remain stationary on the bottom of the chamber without swimming. After the recovery period, trout were given a conditioning swim that consisted of an abbreviated step velocity test (described in Jain et al. 1997). For the conditioning swim, water speed was increased in regular increments of 0.15 BL s^{-1} every 2 min until the fish was unwilling to swim faster and rested on the electric grid at the rear of the swim chamber. The conditioning swim was intended to eliminate the potential training effect of repeat swimming (Farlinger & Beamish 1978, Jain et al. 1997) and also provide an index of individual performance to set the ramp velocities. Following acclimation and beginning at approximately the same time of day (11:00 to 12:00 h), fish were given a ramp-U_{crit} test as described by Jain et al. (1997, 1998). For ramp-U_{crit} testing, each fish was brought up to ~75% of the maximum speed achieved in the conditioning swim using 5 min increments of 0.15 BL s^{-1}. Following this, 20 min increments of 0.15 BL s^{-1} were given until the fish reached failure. Failure was judged to have occurred when the fish impinged on the rear electrified grid for a period of at least 20 s. U_{crit} was calculated as in Brett (1964), where $U_{crit} = U_{fi} + (t_f / t_i)\ U_i$, and U_{fi} is the water velocity of the last fully completed increment, t_f is the time spent on the last step, t_i is 20 min, and U_i is the increment of 0.15 BL s^{-1}. After a 45 min recovery period at acclimation water speed (0.3 BL s^{-1}), a second identical ramp-U_{crit} test was given to determine U_{crit2}. The repeat U_{crit} protocol was chosen since healthy salmonids are capable of repeating exhaustive swimming after as little as a 40 min recovery (Farrell et al. 1998, 2003, Jain et al. 1998, Lee et al. 2003), whereas stressed or ill salmonids are not (Farrell et al. 1998, Jain et al. 1998, Tierney et al. 2004).

Validation of experimental approach. Traditional U_{crit} testing includes a lengthy overnight acclimation period (e.g. Brett 1964, Nikl & Farrell 1993), which in a flow-through situation may produce much wastewater and potential for *Listonella anguillarum* release. However, Peake et al. (1997) demonstrated that hatchery trout reached the same U_{crit} regardless of acclimation length. Therefore we shortened the acclimation period as a means of reducing wastewater production and the risk of pathogen release. To confirm these earlier results, we first verified that reducing the overnight (12 to 14 h) acclimation to only 2 h had no significant effect on U_{crit} and its re-performance. Fish tested after a conventional overnight acclimation period had a U_{crit1} (first U_{crit} test) of 1.62 ± 0.06 BL s^{-1} (n = 6), and repeated that performance after a 40 min recovery period, reaching an identical speed (U_{crit2} = 1.64 ± 0.07 BL s^{-1}). With a 2 h acclimation, U_{crit} and re-performance were identical ($p = 0.540$; $\beta = 0.811$) (U_{crit1} = 1.67 ± 0.02 BL s^{-1}, and U_{crit2} = 1.67 ± 0.05 BL s^{-1}; n = 6), confirming the work of Peake et al. (1997). Consequently, we were confident that our main experiments could be conducted using a short acclimation protocol.

We further validated our repeated U_{crit} protocol with a positive control by testing swimming performance after a 48 h seawater challenge (SWC). Brauner et al. (1994) earlier demonstrated that a 24 h SWC impaired recovery from a U_{crit} test, with both wild and hatchery rainbow trout reaching <85% of U_{crit1} on a second swim about 4 h later. We replaced the freshwater supply with seawater supplied at a similar temperature (~10°C) for 48 h, and following a 24 h recovery period, 1 fish was tested daily on 4 consecutive days (n = 4). U_{crit1} (1.41 ± 0.1 BL s^{-1}) was significantly reduced from control fish above ($p = 0.012$; $\beta = 1.00$), and was independent of the time post exposure (2 to 6 d). Most of the seawater challenge fish did not recover from U_{crit1}: 3 of 4 fish died during the 45 min recovery period. The only surviving fish (tested on Day 5) reached ~25% of the initial swim speed (0.44 vs. 1.57 BL s^{-1}). Clearly, repeat swimming performance testing can resolve physiological impairment against a control and demonstrates that U_{crit} re-performance is a meaningful measure. Although Brauner et al. (1994) did not find mortality following seawater exposure, mortality following exhaustive swimming is not uncommon; it has been recorded for a variety of species (Black 1958), including similarly sized rainbow trout (Wood et al.

1983). However, mortality has not been observed in any previous repeat swimming work (e.g. Jain et al. 1997, Farrell et al. 1998, 2003, Lee et al. 2003).

Power analysis. For the main treatment (injected) groups of the disease experiments, power analysis was used to determine sample size. Since acclimation length had no impact on U_{crit} or its recovery, short and long acclimated fish were pooled (n = 12), and based on the resulting U_{crit1} and its standard deviation (mean = 1.65, SD = 0.10) and the magnitude of changes observed in other studies of disease and swimming (e.g. 13% Jain et al. 1998, and 18% Swanson et al. 2002), significant differences in treatment fish would be detectable at n = 5 while power would remain >0.80.

Disease experiments. Primary isolates of *Listonella anguillarum* (Pacific Biological Station, Nanaimo, BC isolate no. R20, serotype 01) were obtained from coho salmon *Oncorhynchus kisutch* that had died from being previously inoculated with the pathogen. *L. anguillarum* cells were cultured on tryptic soy agar (TSA supplemented with 1.5% NaCl) plates for 18 h, harvested, and transferred to sterile chilled saline (0.85% NaCl). The concentration of *L. anguillarum* was estimated from absorbance measurements made at 540 nm (540 optical density [OD] estimated to contain ~110 cells ml^{-1}). The actual concentration of colony forming units (CFUs) was subsequently determined for each inoculum by drop plating (25 µl) serial dilutions onto TSA plates. The plates were then incubated overnight and the number of colonies was counted to calculate the number of cells ml^{-1} of inoculum. Fish were intraperitoneally injected with a 0.1 ml *L. anguillarum* saline mixture. Five fish were given the low dose ($1.67 \times 10^5 \pm 1.53 \times 10^4$ CFUs), and 6 fish were given the 100-fold higher dose ($2.36 \times 10^7 \pm 4.00 \times 10^6$ CFUs). The low dose was chosen because it had proven efficacious at inducing vibriosis in rainbow trout in earlier work (Ackerman et al. 1999, Ackerman & Iwama 2001). To control for any effects of the injection or treatment system, 5 additional fish were injected with 0.1 ml sterile saline alone (0.85% NaCl). After injection, individual fish were placed into 200 l aerated tanks kept at acclimation temperature (~10°C). Water was changed once after 24 h. All fish were held for 48 h prior to measuring swimming performance, as a similar amount of pathogen had previously been shown to cause physiologic changes within this timeframe (Ackerman & Iwama 2001).

Post-swim evaluation. Following swimming experiments, fish were sacrificed using cervical dislocation. For injected fish, liver, spleen, and kidney tissue samples were taken aseptically, and streaked onto TSA plates. Fish were assumed to have vibriosis if Gram-negative, motile, curved rod-shaped bacteria were isolated from non-pigment producing, circular, cream-colored colonies, isolated on the TSA plates (DFO 1984). Leucocrit (Lct) and hematocrit (Hct) measurements were taken for saline and low-dose-injected fish. Blood was collected into 40 µl heparinized capillary tubes (VWR Canlab) and centrifuged at $10\,000 \times g$ for 2.5 min using an IEC Microcentrifuge (International Equipment Company). Hct and Lct measurements were made with the assistance of precision calipers under magnification (as described by McLeay & Gordon 1977).

Statistics. To determine if there were any treatment effects on repeat swimming ability, U_{crit2} was regressed on U_{crit1} (in cm s^{-1}) using length as a covariate. As there were no treatment effects on U_{crit} repeatability, average swim speed was determined and regressed against treatment. Comparisons were then made on the residuals. Length was not a significant covariate, and so to facilitate comparisons to other studies (e.g. Jain et al. 1997, Claireaux et al. 2005), swim speed was subsequently reported standardized to length (BL s^{-1}). Hct and Lct data were arcsine transformed and compared using ANOVA. Statistics and graphing were carried out using JMP 5.0 (SAS), SigmaStat 3.0 and SigmaPlot 8.0 (SPSS Scientific). Unless stated otherwise, reported values are mean ± standard error of the mean (SEM).

RESULTS

Swimming performance

Saline-injected and uninjected hatchery rainbow trout performed the same (U_{crit1} 48.4 ± 0.92 and 49.6 ± 1.27 cm s^{-1} or 1.65 ± 0.03 1.62 ± 0.04 BL s^{-1}, respectively). Furthermore, both groups recovered this initial performance, so U_{crit1} was a good predictor of U_{crit2} (Fig. 1a). Accordingly, the injection protocol used herein had no effect on swimming or recovery performance.

Vibriosis was confirmed during a post-mortem examination for all the low- and high-dose-injected fish but none of the saline-injected fish. No external or internal clinical signs of disease were evident in any fish. Hct was high and did not differ between saline-injected fish (42 ± 2%) and low-dose *Listonella anguillarum*-injected fish (45 ± 2%). Lct also did not differ between saline-injected and *L. anguillarum*-injected fish ($p = 0.058$), but statistical power was low ($\beta = 0.40$) because Lct was undetectable in all 5 of the low-dose fish (Lct = 0%), and 2 of the 5 saline-injected fish (Lct = 0.86, 0.00, 1.09, 0.49, 0.00).

Low-dose injection did not negatively impact swimming performance or its recovery. (Fig. 1a). However, fish that received a low-dose injection (10^5 CFUs) 48 h earlier had higher U_{crit} values, as evidenced by signifi-

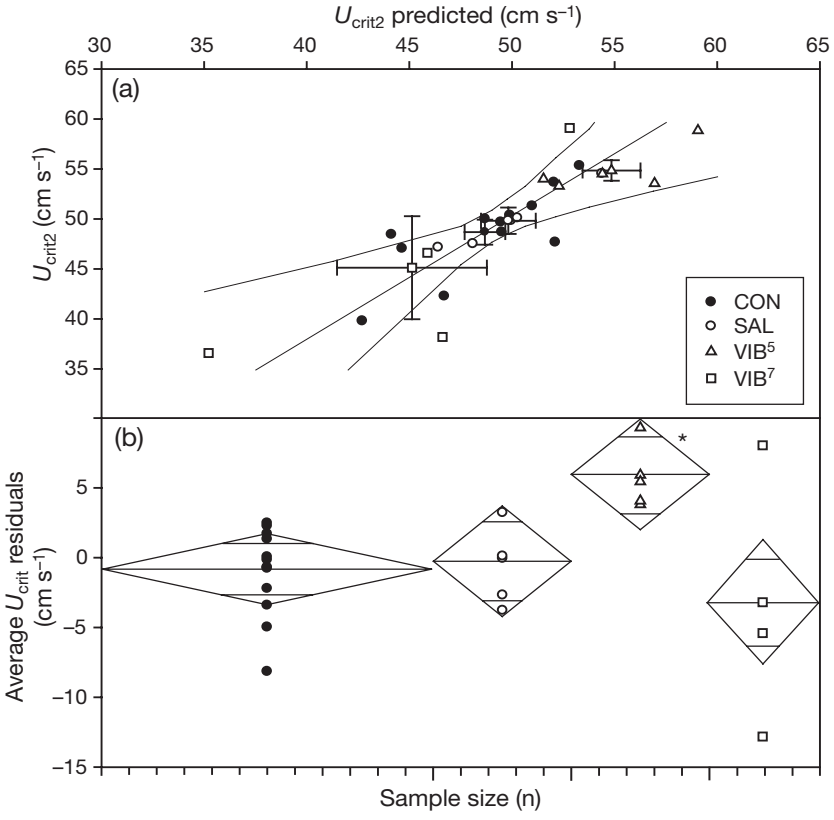

Fig. 1. *Oncorhynchus mykiss*. (a) The second critical swimming speed (U_{crit2}) versus the predicted U_{crit2} (based on U_{crit1}, determined earlier) for control (CON) and saline (SAL), low- (10^5) or high- (10^7) dose *Listonella anguillarum*- (VIB^5 and VIB^7, respectively) injected adult hatchery rainbow trout (ANCOVA, length as a covariate). Lines are for CON and SAL regression ±95 % CIs; mean ± SEM are plotted for each group. As there were no differences in successive swimming abilities, (b) residuals of an average swim speed versus treatment regression were determined and plotted. Diamond width is equal to n; middle line is mean and outer line is 95% CI. Difference from control is denoted by an asterisk

cantly greater positive residuals (Fig. 1b). Differences remain apparent when the data are considered standardized for length, with low-dose-injected fish reaching a 16.6% higher U_{crit} (in BL s^{-1}; $p < 0.05$, $\beta = 1.00$) than the saline-injected fish (U_{crit1} = 1.90 ± 0.03 BL s^{-1}, and U_{crit2} = 1.88 ± 0.03 BL s^{-1}) (Fig. 2). The higher U_{crit} value for the low-dose fish appeared due to an increase in their willingness to use burst-and-coast swimming during the later stages of the trials. In saline-injected fish, swimming cessation coincided with a transition from a steady to an unsteady swimming gait.

Only 4 of 6 fish survived injection with the higher dose of *Listonella anguillarum* (10^7 CFUs), indicating that the high dose was near a 48 h LD_{50} (lethal dose). Regardless, the surviving high-dose fish swam (U_{crit1} = 1.62 ± 0.15 BL s^{-1}) and recovered (U_{crit2} = 1.55 ± 0.20 BL s^{-1}) since their performance was not significantly different ($p = 0.830$, $\beta = 1.00$) compared with saline-injected controls (Fig. 2).

DISCUSSION

The *Listonella anguillarum* injection protocol used herein successfully caused a subclinical vibriosis at injection levels similar to those of other studies (i.e. 10^5 CFU; Ackerman et al. 1999, Ackerman & Iwama 2001). In a natural setting, fish encounter this pathogen in the water column, and although it is uncertain whether this may result in more chronic infections, a stock can suffer complete mortality in as little as 5 d (Ackerman & Iwama 2001). Our high-dose protocol achieved 33.3% mortality in 2 d. Even at these near-lethal levels of *L. anguillarum* neither swimming performance nor its recovery were compromised. In fact, the overall performance of the injected fish remained comparable with control fish and with several other studies for similar-sized hatchery rainbow trout (1.6 to 1.7 BL s^{-1}, this study; 0.53 to 0.94 BL s^{-1}, Taylor et al. 1996; 1.7 BL s^{-1}, Duthie 1987; 2.0 BL s^{-1}, Gallaugher et al. 1992). Thus, it appears that in the short term, the demands made on the immune system by *L. anguillarum* do not interact negatively with those of swimming.

We noted a small but significant increase in U_{crit} with the low-dose injection. Jain & Farrell (2003) proposed that fish may opt to swim to different U_{crit} levels depending on either the water temperature or a resulting physiological condition. They found that warm-acclimated (14.9°C) rainbow trout were able to achieve greater U_{crit1} values than cold-acclimated (8.4°C) fish through increased use of anaerobic swimming. However, the increased U_{crit} for the warm-acclimated fish came at the cost of impaired re-performance (i.e. U_{crit2} was significantly lower than U_{crit1}), a greater plasma ionic imbalance and a higher plasma cortisol level. In the present study, sublethal infection resulted in a 16.6% higher U_{crit1}, a level that was maintained for U_{crit2}. This response was unexpected and could have a behavioral or physiological basis, neither of which were explored here. For example, the elevated plasma cortisol levels previously shown to be associated with vibriosis (e.g. Ackerman et al. 1999 noted cortisol was elevated in rainbow trout at our low-dose [10^5 CFUs] 24 h following injection, and Ackerman & Iwama 2001 noted cortisol was elevated in rainbow trout 5 d following injection) may have stimulated swimming effort or altered swimming behavior.

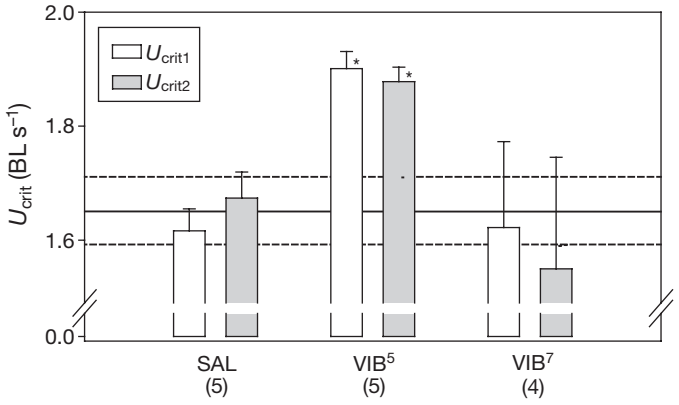

Fig. 2. *Oncorhynchus mykiss*. Ability of rainbow trout to perform 2 U_{crit} tests separated by 45 min, 48 h following saline (SAL) or a low- (10^5) or high- (10^7) dose *Listonella anguillarum* (VIB^5 and VIB^7, respectively) injection. U_{crit} values are standardized to body length (BL) and bars are mean + standard error of the mean (SEM); n is given below groups in parentheses; values significantly different from control are shown with asterisks (2-way repeated measures [RM] ANOVA, Holm-Sidak, $p < 0.05$). U_{crit} ± 95 CI for (n = 12) uninjected rainbow trout of the same stock are also shown with solid and dashed lines

Sublethal vibriosis had no effect on Hct or Lct compared with saline-injected fish. In fact, Hct was within the normal range for rainbow trout (Gallaugher et al. 1992). Vibriosis has been associated with anemia; for example, Harbell et al. (1979) noted Hct dropped from 37 to 15% as coho salmon *Oncorhynchus kisutch* became moribund 48 h following *Listonella anguillarum* exposure. Conversely, and in agreement with our findings, Ackerman & Iwama (2001), who used an injection protocol similar to the one we used, found no change in Hct up to 7 d post-injection. Lct was more variable in saline-injected control fish, with normal Lct in 3 of the 5 (Wedemeyer et al. 1983), but a negligible Lct in 2 other fish. Since Lct was not detected after *L. anguillarum* injection, the injection may have caused a leucopoenia, but statistical power was weak. Ransom et al. (1984) reported earlier that Chinook salmon *O. tshawytscha* with vibriosis experienced an 80 to 95% decrease in circulating leucocytes. Since cortisol is known to be lymphocytolytic in salmonids (McLeay 1973), the elevated cortisol levels observed by others with *L. anguillarum* injection may be leucopenic. As McLeay (1973) pointed out, the destruction of lymphocytes may free up protein and enable gluconeogenesis that can in turn be used to fuel swimming.

This study is the first to demonstrate that *Listonella anguillarum* injection does not negatively affect U_{crit} and its re-performance in rainbow trout, even at near lethal levels. The mechanism by which a sublethal vibriosis produced a modest increase in U_{crit} 48 h post-injection will require further study.

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