

# Intestinal pH profile in rainbow trout *Oncorhynchus mykiss* and microhabitat preference of the flagellate *Spironucleus salmonis* (Diplomonadida)

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**ABSTRACT:** In farmed rainbow trout *Oncorhynchus mykiss*, the flagellate *Spironucleus salmonis* (Diplomonadida) is often found in the pyloric region of the intestine. While previous *in vitro* studies report a pH of 7.5 to 8.0 as optimal for presumed *S. salmonis*, no previous *in vivo* studies have investigated the relationship between pH and microhabitat preference. Therefore, in 698 rainbow trout (75% were 5 to 6 mo old juveniles, 10 to 20 cm total length), we recorded occurrence and density of *S. salmonis*, and pH, in the pyloric, anterior, middle, and posterior intestine. There were no significant differences in total length or weight between infected and uninfected fish. *S. salmonis* preferred the pyloric region, with occurrence and density decreasing significantly from pyloric to posterior regions. In infected fish, pH in pyloric (6.8 to 7.9, mean 7.3) and posterior regions (6.5 to 8.0, mean 7.1) was significantly lower than in anterior (6.5 to 8.5, mean 7.7) and middle (6.8 to 8.2, mean 7.7) regions; in uninfected fish, the pH profile was similar. At the individual level, 90% of infected fish and 79% of uninfected fish showed this pH profile. In the pyloric region, pH was not significantly different among uninfected fish, and fish with light, moderate, or heavy infections. Our *in vivo* study suggests the optimal pH for *S. salmonis* is between 7.1 and 7.5, possibly close to 7.3 (the mean in pyloric region of infected fish). We conclude that while the presence of *S. salmonis* reflected tolerable pH, density of infection was not correlated with pH, and thus a causal relationship between microhabitat preference and pH is unlikely.

**KEY WORDS:** Diplomonad flagellate · Intestinal tract · Microhabitat preference · *Oncorhynchus mykiss* · pH profile · Rainbow trout · *Spironucleus salmonis*

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## INTRODUCTION

Diplomonad flagellates can cause intestinal and systemic infection, with significant morbidity and mortality in aquaculture (Woo & Poynton 1995). Despite their economic importance, many aspects of host-diplomonad interactions, including microhabitat preference, are not well understood. Different species of piscine diplomonads have different microhabitat preferences

(Table 1), and among the enteric species, *Spironucleus salmonis*, *S. torosa*, and *S. vortens* prefer the pyloric region, rectum, and middle intestine, respectively (Moore 1922a,b, Davis 1926, Ferguson 1979, Poynton & Morrison 1990, Poynton et al. 1995, 2004, Sterud 1998a,b). These preferences may reflect adaptations to the variations in morphology, physiology and metabolic gradients in the fish intestine (see Weinreb & Bilstad 1955, Anderson & Mitchum 1974).

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Table 1. *Spironucleus* spp. Microhabitat preferences of well-characterised piscine diplomonads. A: aquaculture; D: anadromous; F: freshwater; S: seawater; W: wild

Species	Host	Locations	Geographic locality		Source
<i>S. barkhanus</i>	Arctic char	Intestine, gall bladder	W,D	Norway	Sterud et al. (1998)
	Grayling	Intestine, gall bladder	W,F	Norway	Sterud et al. (1997)
<i>S. salmonicida</i> <sup>a</sup>	Arctic char	Systemic (intracellular)	A,S	Norway	Sterud et al. (2003)
	Atlantic salmon	Systemic	A,S	Norway	Sterud et al. (1997, 1998)
	Chinook salmon	Systemic	A,S	Canada	Kent et al. (1992)
<i>S. salmonis</i> <sup>b</sup>	Brook trout	Intestine (pyloric)	A,F	USA	Moore (1922a,b)
	Brown trout	Intestine (pyloric)	A,F	USA	Davis (1926)
	Lake trout	Intestine (pyloric)	A,F	USA	
	Rainbow trout	Intestine (pyloric)	A,F	USA	
	Rainbow trout	Intestine (pyloric)	A,F	Ireland	Ferguson (1979) Poynton et al. (2004)
<i>S. torosa</i>	Burbot	Intestine (rectum)	W,F	Norway	Sterud (1998a)
	Cod	Intestine (rectum)	W,S	Norway	Sterud (1998b)
	Cod	Intestine (rectum)	W,S	Canada	Poynton & Morrison (1990)
	Haddock	Intestine (rectum)	W,S	Canada	Poynton & Morrison (1990)
	Saithe	Intestine (rectum)	W,S	Norway	Sterud (1998b)
<i>S. vortens</i>	Angelfish	Intestine (middle), lip tumor	A,F	USA	Poynton et al. (1995)
	Angelfish	Intestine, head lesions	A,F	UK	Paull & Matthews (2001)
	Discus	Systemic	A,F	UK	Paull & Matthews (2001)

<sup>a</sup>Diplomonads initially reported as *S. barkhanus*, and renamed *S. salmonicida* by Jørgensen & Sterud (2006)  
<sup>b</sup>Diplomonads initially reported as *Hexamita salmonis*, and renamed *S. salmonis* by Poynton et al. (2004)  
 Note: Uldal & Buchmann (1996) found diplomonads in intestine from rainbow trout in Denmark, preferentially in the pyloric and anterior regions and referred to them as *H. salmonis*; we presume they were *S. salmonis*

The concentration of diplomonads in the pyloric region of trout is likely to be predominantly due to physiological and metabolic factors, rather than morphology, since the one valve between the intestine and the rectum is indistinct (Ezeasor & Stokoe 1980). Both pH and bile have been suggested to play decisive roles in determining the microhabitat preference of diplomonads in trout (Buchmann & Uldal 1996, Uldal & Buchmann 1996). This suggestion is based on *in vitro* studies showing a pH tolerance of 5 to 10 (optimum 7.5 to 8.0), and that bile in low concentrations (30 to 960 mg l<sup>-1</sup>) enhanced flagellate propagation slightly. However, there are no *in vivo* studies that test this hypothesis, or any other hypothesis about factors determining microhabitat preference in piscine diplomonads.

In the present study, we therefore investigated *in vivo* the role of pH in microhabitat preference of *Spironucleus salmonis* in the intestinal tract of rainbow trout. The simple intestinal tract in trout comprises a short cranial ascending limb bearing pyloric caecae, and a longer caudal descending limb (intestine) extending to a distinct rectum with annulo-spiral septa arranged as a stack of caudally directed funnels (Anderson & Mitchum 1974, Ezeasor & Stokoe 1980, Smith 1989, Willers 1991). In our studies we divided the intestinal tract into 4 regions, consistent with previous studies by Uldal & Buchmann (1996).

We approached our investigations by posing 6 main questions: (1) what is the occurrence of infection (presence/absence) in different intestinal regions; (2) what is the density of infection in different intestinal regions, and what is the relationship between density of infection in the pyloric region and that in each of the 3 downstream intestinal regions; (3) what is the pH in different intestinal regions in uninfected fish; (4) what is the pH in different intestinal regions in infected fish; (5) what is the difference between the pH in uninfected and infected fish per intestinal region; and (6) what is the relationship between the pH and density of infection in different intestinal regions?

We also used our data set to investigate 2 further questions: (7) what is the relationship between size of fish and infection; and (8) what is the relationship between size of fish and pH?

## MATERIALS AND METHODS

**Experimental design.** New data were collected on occurrence and density of diplomonad infection in rainbow trout, along with concurrent measurement of pH conditions in the same fish in order to allow comparison of these parameters.

**Source and size of fish.** A total of 698 juvenile rainbow trout were collected during summer and winter

between 2001 and 2005 from Seltershof farm near Berlin, Germany (52.061° N, 12.884° E), where they had been fed with Skretting Classic trout food (Skretting). After transport to the laboratory, the fish were held in an indoor flow-through system, supplied with tap water at 9 to 11°C, and fed 3 times d<sup>-1</sup> with Forellenmastfutter FM 49/22 EXSP (1.8 mm) (Aquavalent).

At the time of examination, the total length of fish ranged from 5.5 to 22.5 cm (mean 12.7, SD ± 3.5), and their weight ranged from 1.4 to 113.0 g (mean 26.8, SD ± 23.7); we estimate our fish were 3 to 7 mo old (since the farm frequently graded the fish, no exact age was available). Although there was a large size range of fish, most (75 %) were 10 to 20 cm total length (5 to 6 mo old juveniles), and the mean total length ranged from 11.5 to 12.7 cm in 4 of 5 data sets.

**Examination of fish, quantifying infection, and recording pH.** Only 5 % of fishes were examined the day of collection. The remaining 95 % were examined within 4 wk of collection, by which time they should have acclimated to the holding conditions in the laboratory. Similar numbers of fish were examined in each of the 4 wk after collection. The time interval between feeding and examination varied from 0.5 to 5.0 h.

The intestine was divided into 4 regions, as suggested by Uldal & Buchmann (1996), viz. pyloric region (ascending intestine bearing pyloric caecae), anterior (anterior ¼ of length from anterior descending intestine to anus, and comprising only descending intestine), middle (middle ¼ of length from anterior descending intestine to anus, comprising descending intestine and part of rectum), and posterior (posterior ¼ of length from descending intestine to anus, comprising rectum only).

For each region, occurrence (presence or absence) of diplomonads was recorded. Density was semi-quantitatively estimated as the number of flagellates under a 22 × 22 mm cover slip (484 mm<sup>2</sup>) (n = 1 to 3 per fish). We considered light infection (1+) as less than 10 flagellates/484 mm<sup>2</sup>, moderate infection (2+) as 11 to 40 flagellates/484 mm<sup>2</sup>, and heavy infection (3+) as >40 flagellates/484 mm<sup>2</sup> (Saghari Fard 2001).

The pH of the intestinal contents was recorded with an accuracy of 0.2 by using pH indicator strips (pH Indikatorstäbchen: VWR 1.09543.0001) (VWR International). After the samples of intestinal contents had been taken for determining occurrence and density of diplomonads, a pH strip was laid tangentially in each of the 4 regions (hence 4 strips per fish), and gently pressed into the remaining intestinal contents. After a few minutes (allowing for maximum colour change), the strips were removed, and the pH was determined against a reference strip.

The pH was recorded in 37 uninfected fish, in 21 infected fish with *Spironucleus salmonis* present in all

4 intestinal regions, and in 65 fish with *S. salmonis* present in only 1, 2 or 3 regions of the intestine.

**Identification of the diplomonad.** We confirmed the identification of the diplomonad as *Spironucleus salmonis* based on our previous ultrastructural studies and molecular characterisation (Fard et al. 2007).

**Statistical analysis.** Our analysis began by using the Kolmogorov-Smirnov (K-S) test to determine whether data were normally distributed. Levene's test was used to determine homogeneity of the variance.

For the 6 main questions in our study, the  $\chi^2$  test was used to test for differences in occurrence and density of infection between intestinal regions (Questions 1 and 2), (the test was performed on the number of fish, not the percentage, and for density, we compared the proportions of infections that were light, moderate or heavy pairwise between regions). The Mann-Whitney test was used to determine the relationship between density of infection in the pyloric region and that in each of the 3 downstream intestinal regions (Question 2). One-way ANOVA was used to test the significance of differences in pH between the intestinal regions in uninfected and infected fish (Questions 3 and 4, respectively), followed by Tukey's honestly significant difference (HSD) test to compare regions pairwise. The *t*-test was used for comparing the pH in uninfected and infected fish per region (Question 5). The Kruskal-Wallis test (for non-normally distributed data) was used to test for significant differences in pH in the pyloric region between uninfected, lightly, moderately, and heavily infected fish (Question 6).

For the 2 supplementary questions, the Mann-Whitney test (for non-normally distributed data) was used to test for significant differences in size (total length and weight) between uninfected and infected fish (Question 7). Pearson's correlation coefficients were used to test the relationship between size of fish (total length and weight for 37 uninfected fish) and pH in each intestinal region (Question 8).

All statistical tests were done using SPSS (version 9), and the results were considered significant if  $p < 0.05$ .

## RESULTS

### Overview

Both occurrence and density of *Spironucleus salmonis* differed significantly along the length of the intestine, being highest in the pyloric region and then decreasing posteriorly. The pH profile in uninfected and infected fish was characterized by more neutral conditions in the pyloric and posterior regions, and significantly more alkaline conditions in the anterior and

middle regions of the intestine. In the pyloric region, the pH was not significantly different in uninfected and lightly, moderately and heavily infected fish.

Among the 698 fish examined, 168 were infected (prevalence of infection 24%). There was no significant difference in size of infected and uninfected fish. Furthermore, there was no significant correlation between size of fish and pH in any of the 4 intestinal regions.

### Occurrence of infection and region

The occurrence of *Spironucleus salmonis* was significantly higher in the pyloric region (24% of fish were infected in this site) than in each of the other 3 regions (anterior 15%, middle 13% and posterior 10%) ( $\chi^2$ ,  $p < 0.001$ ), and the occurrence in the anterior region was also significantly higher than in the posterior region ( $\chi^2$ ,  $p < 0.005$ ) (Fig. 1).

### Density of infection and region

The density of *Spironucleus salmonis* in the pyloric region was significantly higher than in the anterior, middle and posterior regions ( $\chi^2$ ,  $p < 0.0001$ ), and density in the anterior region of the intestine was also significantly higher than in the middle and posterior regions ( $\chi^2$ ,  $p < 0.05$ ,  $p < 0.005$  respectively). Results for 3 categories of density (upon which the  $\chi^2$  tests were based) per intestinal region are shown in Fig. 1.

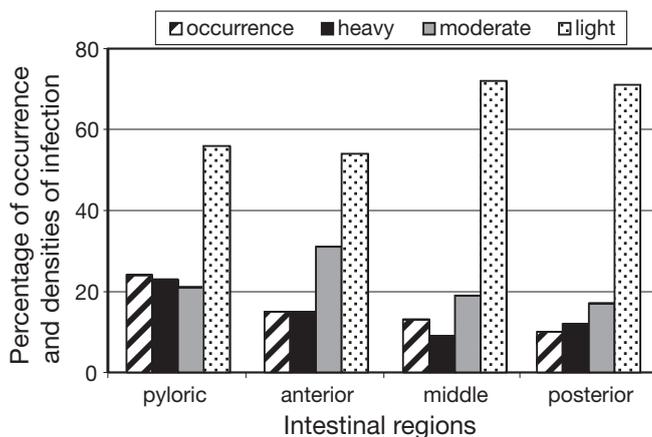


Fig. 1. *Spironucleus salmonis* infecting *Oncorhynchus mykiss*. Occurrence and density of the diplomonads in each of 4 regions of the intestinal tract of juvenile rainbow trout. Occurrence expressed as percentage of infected fish per region ( $n = 698$  fish for each region). Densities expressed as percentage of infected fish with each density per region ( $n = 168$  fish for pyloric region,  $n = 105$  fish for anterior region,  $n = 89$  fish for middle region, and  $n = 69$  fish for posterior region)

In 38 fish heavily infected in the pyloric region, the density of infection in each of the 3 downstream intestinal regions was significantly higher than that in the 94 fish lightly infected in the pyloric region (Mann-Whitney test,  $p < 0.0001$ ). Furthermore, in 100% of fish heavily infected in the pyloric region, all 3 downstream regions were infected, whereas there were no fish infected in all 3 downstream regions when the pyloric region was lightly infected.

### pH and region in uninfected fish

Along the length of the intestine of 37 uninfected fish, pH ranged from 6.7 to 8.2 ( $n = 37$ ) (Table 2). The pH was significantly lower in the pyloric and posterior regions than in the anterior and middle regions (ANOVA,  $F_{(3,144)} = 15.97$ ,  $p < 0.0001$ ) (Table 2) (Fig. 2).

At the individual level, among 37 uninfected fish, 79% adhered to this pH profile (defined as more neutral, more alkaline, more alkaline, more neutral, for pyloric, anterior, middle, and posterior regions, respectively).

### pH and region in infected fish

Along the length of the intestine of 21 fish that were infected in all regions of the intestinal tract, pH ranged from 6.5 to 8.5 (Table 2). The pH was significantly lower in the pyloric and posterior regions than in the anterior and middle regions (ANOVA,  $F_{(3,80)} = 3.711$ ,  $p < 0.05$ ) (Table 2) (Fig. 2).

At the individual level, among 21 infected fish, 90% adhered to this pH profile (more neutral, more alkaline, more alkaline, more neutral).

### pH in uninfected and infected fish per region

There were no significant differences in pH between 37 uninfected and 21 infected fish in the pyloric, anterior and middle regions; however, in the posterior region, the pH was significantly more alkaline in uninfected fish than in infected fish ( $t$ -test,  $t(56) = -2.58$ ,  $p < 0.05$ ) (Table 2).

### pH and density of infection in different regions

In the pyloric region, there were no significant differences in pH between the 37 uninfected fish, 22 lightly, 22 moderately, and 21 heavily infected fish (Table 2). The low number of fish with moderate and heavy infections in anterior, middle and posterior regions precluded statistical analysis (Table 2).

Table 2. *Oncorhynchus mykiss*. pH in 4 different intestinal regions of uninfected and *Spiroucleus salmonis*-infected juvenile rainbow trout. –: mean and SD not available, as n = 1

Intestinal regions	Uninfected fish <sup>a</sup>	Infected fish <sup>b</sup>	Infected fish <sup>c</sup> (density)		
			Light	Moderate	Heavy
<b>Pyloric</b>					
Range	6.7 – 7.8	6.8 – 7.9	6.8 – 8.0	6.8 – 7.9	6.5 – 8.0
Mean ± SD	7.2 ± 0.3	7.3 ± 0.3	7.4 ± 0.4	7.3 ± 0.3	7.3 ± 0.5
No. of fish	37	21	22	22	21
<b>Anterior</b>					
Range	7.1 – 8.1	6.5 – 8.5	7.0 – 8.6	6.5 – 8.5	6.7 – 8.0
Mean ± SD	7.7 ± 0.3	7.7 ± 0.5	7.7 ± 0.4	7.7 ± 0.5	7.4 ± 0.5
No. of fish	37	21	23	18	7
<b>Middle</b>					
Range	7.1 – 8.2	6.8 – 8.2	6.7 – 8.5	6.8 – 8.2	8.0 – 8.1
Mean ± SD	7.7 ± 0.3	7.7 ± 0.4	7.7 ± 0.4	7.7 ± 0.4	8.1 ± 0.0
No. of fish	37	21	33	8	2
<b>Posterior</b>					
Range	6.8 – 8.1	6.5 – 8.0	6.8 – 8.2	6.5 – 8.0	7.0
Mean ± SD	7.4 ± 0.4	7.1 ± 0.5	7.3 ± 0.4	7.1 ± 0.5	–
No. of fish	37	21	20	6	1

<sup>a</sup>Fish which were uninfected in all 4 regions  
<sup>b</sup>Fish which were infected in all 4 regions  
<sup>c</sup>Fish which were infected in only 1, 2 or 3 regions; total numbers were 65 (pyloric region), 48 (anterior), 43 (middle) and 27 (posterior)

### Size of fish and infection

Among the 698 fish, there was no significant difference in total length or weight between 530 uninfected fish and 168 infected fish.

### Size of fish and pH

In 37 uninfected fish, there were no significant correlations between size of the fish (total length or weight) and pH, in any of the 4 intestinal regions.

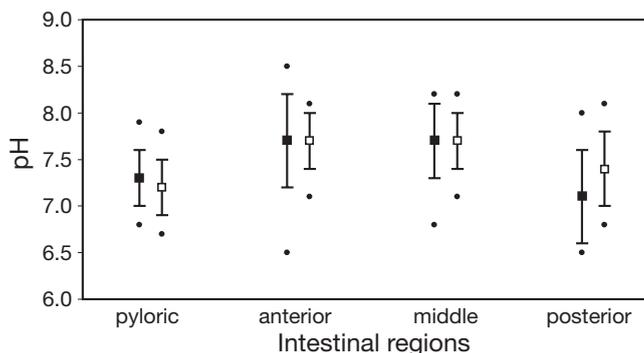


Fig. 2. *Oncorhynchus mykiss*. Mean pH ± SD in each of 4 regions of the intestinal tract of 37 uninfected (□), and 21 infected (■) rainbow trout with infection in all 4 regions of the intestine. ●: pH range

### DISCUSSION

#### Microhabitat preference

*Spiroucleus salmonis*, though found throughout the intestinal tract of juvenile rainbow trout, was concentrated in the pyloric region, with both occurrence and density significantly decreasing posteriorly. This distribution was consistent with previous studies by Davis (1926), Ferguson (1979), and Moore (1922a,b), and contrasted in part with the study by Uldal & Buchmann (1996), which did not report significant differences in occurrence and density between the pyloric and anterior regions. These differences may be due to the sizes of fish, viz. juveniles in our study (mean length 12.7 cm) and fry in the study by Uldal & Buchmann (op. cit.) (mean length 4.1 cm).

The microhabitat preference in *Spiroucleus salmonis* can initially help diagnosis of the flagellate, since the 4 other well characterized *Spiroucleus* species have other site preferences (Table 1).

#### pH profile in uninfected fish

The pH in the intestinal tract of uninfected trout in the present study, 6.7 to 8.2, partly overlapped the range of 7.0 to 9.0 previously reported for salmonids

(Steffens 1989). Along the intestinal tract, we observed a distinct variation in pH (hereafter referred to as the pH profile), with means of 7.2, 7.7, 7.7 and 7.4 in the pyloric, anterior, middle and posterior regions, respectively. Longitudinal pH profiles have been reported for other fish with stomachs, including other salmonids, channel catfish *Ictalurus punctatus*, Mozambique tilapia *Oreochromis mossambicus*, and sea bream *Sparus aurata* (Page et al. 1976, Maier & Tullis 1984).

The longitudinal pH profile in the juvenile rainbow trout reflects the diverse digestive processes and dietary physiology in the different regions. In the pyloric region, the acidic chyme from the stomach is mixed with bile and pancreatic secretions including zymogens and bicarbonate buffering compounds, and the pH becomes more neutral. The anterior and middle intestine are part of the descending intestine, having similar anatomy, physiology, and pH. Here conditions become more alkaline as buffering decreases. In the posterior intestine, conditions become more neutral.

The wide variations in pH within regions of the intestinal tract of the rainbow trout (Table 2) might be explained by differences in the time interval between feeding and examination, since pH varies as a function of feeding time (Maier & Tullis 1984, Deguara et al. 2003).

#### Possible factors affecting microhabitat preference

The range of pH along the intestinal tract of uninfected fish, 6.7 to 8.2, lay well within the rather broad pH tolerance of 5.5 to 9.0 previously reported for putative *Spironucleus salmonis* in culture (Buchmann & Uldal 1996). Thus, it is not surprising that the trophozoites were present in all 4 regions of the intestinal tract. We also note that the pH conditions in infected fish, 6.5 to 8.5, were close to the pH conditions of 6.5 to 7.0 and 7.5 to 8.0 (the latter being most suitable) that supported population growth of putative *S. salmonis* in culture (Buchmann & Uldal 1996). However, the pH in the preferred pyloric region (mean  $\pm$  SD: 7.2  $\pm$  0.3 in uninfected fish, 7.3  $\pm$  0.3 in infected fish) was lower than the reported optimum for putative *S. salmonis* in culture (7.5 to 8.0) (see discussion below on optimum pH for *S. salmonis*).

The highest numbers of *Spironucleus salmonis* flagellates were seen in the near neutral conditions of the pyloric region (mean pH 7.3), and the lowest numbers were seen in the near neutral conditions of the posterior region (mean pH 7.1). This distribution might be explained by the more alkaline pH of the anterior and middle regions of the intestine (mean pH 7.7 for both), being less favourable for the diplomonads, reducing occurrence and density in these regions, and allowing few diplomonads to pass to the posterior region. How-

ever, data from an *in vitro* study of putative *S. salmonis* (Buchmann & Uldal 1996) showed that pH conditions of 7.5 to 8.0 were optimum for population increase. Thus it is most unlikely that the pH of 7.7 in the anterior and middle regions were the reason for the reduced numbers of *S. salmonis*.

We are unable to confirm from our *in vivo* studies whether the preference of *Spironucleus salmonis* for the pyloric region is related to pH. As noted above, we observed a pH range of 6.8 to 7.9 (mean 7.3) in infected fish, 6.7 to 7.8 (mean 7.2) in uninfected fish, and previous *in vitro* studies of putative *S. salmonis* give 7.5 to 8.0 as the optimum for population growth (Buchmann & Uldal 1996) (see discussion below on optimum pH for *S. salmonis*). It is indeed very likely that other factors, such as bile, bacterial fauna, and nutrient content of the digesta play a key role in determining the microhabitat preference of *S. salmonis*. These other factors may be dominant, or may act in concert with pH.

Bile enters the pyloric region via the bile duct that passes among the pyloric caecae (Willers 1991), and its concentration is expected to decrease posteriorly. *In vitro* studies show that bile in low concentrations (30 to 960 mg l<sup>-1</sup>) slightly enhanced propagation of putative *Spironucleus salmonis* (Buchmann & Uldal 1996); however, statistical tests were not performed, so we do not know if the enhanced propagation was statistically significant. Thus, it may be expected that, *in vivo*, bile in the pyloric region also enhances propagation, possibly mediated by the gallic acids that emulsify fat (Steffens 1989), and/or the bile salts (Denton et al. 1974).

A further anterior-posterior gradient occurs in the bacterial fauna. There is a progressive decline in numbers of aerobic bacteria posteriorly, and anaerobes are generally restricted to the upper intestine (Austin & Al-Zahrani 1988). We suggest that the bacterial milieu of the pyloric region may be more favourable for *Spironucleus salmonis* than that of more posterior regions. Although the way in which bacteria may affect the trophozoites is not known, we suggest the mechanism is indirect (perhaps via microbial digestion), since bacteria have not been reported to attach to or lie within the cytoplasm of *S. salmonis* (Poynton et al. 2004, Fard et al. 2007).

The nutrient content of the digesta decreases posteriorly, a further factor correlated with distribution of *Spironucleus salmonis*. In the most posterior parts of the intestinal tract, digestive and absorptive functions are diminished (Smith 1989); thus, there may not be adequate nutrients for the diplomonads.

In *Spironucleus salmonis*, we have shown that while the presence of infection reflects tolerable pH, density of infection is not closely related to pH, since there was no correlation between density and pH along the length of the intestine, and thus a causal relationship is unlikely.

### Optimum pH for *Spiroucleus salmonis*

Our *in vivo* studies indicate that the optimum pH for *Spiroucleus salmonis* may be 7.3, since this was the mean pH in the pyloric region where the diplomonads were concentrated. Our determination of pH was accurate to within 0.2. Therefore, we can state that, while our *in vivo* data suggests an optimum pH for *S. salmonis* of 7.3, the optimum may lie within the range of 7.1 to 7.5.

Our *in vivo* result contrasts with the results from *in vitro* studies for putative *Spiroucleus salmonis* by Buchmann & Uldal (1996), who reported a pH optimum of 7.5 to 8.0, based on highest population density. If we assume that the same species of diplomonad was investigated in our *in vivo* study and in the previous *in vitro* study (Buchmann & Uldal 1996), how might these apparently different optima be explained? In principle, it is likely that the pH recorded *in vivo* is closer to the optimum than that determined from culture, since *in vitro* conditions represent a compromised environment for trophozoites. It is also possible that the parasites we studied from rainbow trout in Germany are a different sub-species or strain from that studied by Buchmann & Uldal (1996) from rainbow trout in Denmark. Do different sub-species or strains, if they exist, have different pH optima?

The suitability of pH 7.3 for *Spiroucleus salmonis* has not previously been determined. The results of an *in vitro* investigation of putative *S. salmonis* by Buchmann & Uldal (1996) appeared to exclude pH conditions between 7.0 and 7.5. Moreover, it is not known whether pH 7.5 or 8.0 was more favourable for growth, since pooled data was presented. Additional support for a true optimum pH being lower than 7.5 to 8.0 comes from our colleague's *in vitro* studies, where cultures at pH 7.0 to 7.5 were more successful than those at pH 7.5 to 8.0 (Cheng 2006). However, caution should be exercised when comparing results of our *in vitro* cultures with those of Buchmann & Uldal (1996), since as mentioned, they may have contained different sub-species or strains of *S. salmonis*, and furthermore, the culture conditions were not identical. Since our *in vitro* studies were targeted at yielding stock cultures for immunology experiments, we did not determine optimum pH.

### Size of fish and infection

Our finding that there were no significant differences in total length or weight of uninfected and infected juvenile rainbow trout (mean total length 12.7 cm) contrasts with the significant weight reduction reported for infected rainbow trout fry (mean total length 5.5 cm) by Uldal & Buchmann (1996).

As discussed earlier, we have also noted that in our juvenile fish, occurrence and density were significantly higher in the pyloric region than elsewhere, whereas in fry there were no significant differences between pyloric and anterior regions (Uldal & Buchmann 1996). These differences demonstrate that the nature of *Oncorhynchus mykiss* – *Spiroucleus salmonis* relationship is dependant upon the size of the fish. We suggest that key factors are development of immunity (affecting the consequences of the infection, with acquired immunity developing in the juvenile fish and allowing them to cope more successfully with the infection), and development of the gut (affecting the distribution of the flagellates).

### Recommendations

A number of questions about the factors determining microhabitat preference of *Spiroucleus salmonis* remain unresolved, and we now offer recommendations for further studies to refine and extend the approach that we have presented. Refinements should include (1) standardizing the intervals between collection from the farm and examination, and between feeding and examination, thus minimizing effects of stress and changes in pH respectively; (2) recording density quantitatively (actual numbers of flagellates), by slowing or stopping movement of the active flagellates, via photography or adding formalin to the preparation; and (3) using microelectrodes to measure luminal pH (Brune & Kühl 1996), and placing them precisely in the posterior region in order to measure pH in the rectum only (posterior to the annulo-spiral septa). Since age can be expected to have a marked impact on development of the gut, and hence the environment for the diplomonads, we also recommend that future studies should extend our present work on juvenile rainbow trout by comparing pH and microhabitat preference of *S. salmonis* in both fingerlings and sub-adults.

Extension of the present approach should include (4) investigating the pH preference of *Spiroucleus salmonis* by exposing organisms in culture to pH gradients, and documenting their speed and direction of movement, and (5) ascertaining the importance of bile for growth of *S. salmonis*, which can be addressed by measurement of bile concentration *in vivo*, and additional *in vitro* studies with statistical treatment of data. Successful determination of optimum pH and bile requirements will enhance the efficiency of routine *in vitro* culture of *S. salmonis*.

In a broad sense, knowledge of *Spiroucleus salmonis* microhabitat preference and determining factors may play a key role in predicting the likely impact of

changes in diet on density of diplomonads. In recent years, there have been increasing efforts made to develop diets for carnivorous fish, such as trout, that have increasing amounts of plant protein, since fish meal production is stagnant or decreasing (Cheng et al. 2003). It may be expected that such changes in diet will bring about changes in the intestinal milieu within which the diplomonads live. Improved understanding of the environmental requirements of *S. salmonis* will help predict whether changes in diet may result in increased densities of the diplomonads, which may be pathogenic in young fish.

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