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Differences in pathogen resistance within and among cultured, conservation-dependent, and endangered populations of Atlantic salmon, *Salmo salar* L.

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Abstract We report genetic differences for resistance to the pathogen *Listonella anguillarum* within and among one cultured and two wild Canadian populations of Atlantic salmon, *Salmo salar*, using a common-garden experimental protocol. Following exposure to the causative agent for vibriosis, parr originating from the endangered Stewiacke River population experienced significantly higher mortality than cultured parr, four generations removed from the Saint John River population, and wild parr from Tusket River. Pathogen resistance differed between sexes; males consistently experienced higher survival than females. There was no evidence that maturity influenced pathogen resistance in male parr. The population and sex differences in pathogen resistance documented here have implications for risk assessments of the demographic consequences of interbreeding between wild and farmed Atlantic salmon.

Keywords Disease · *Listonella* · Mature male parr · Sex bias · Genetic

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Introduction

Genetic variation among populations for traits that directly affect survival or fecundity can provide compelling support for the hypothesis that phenotypic differences in the wild represent adaptive responses to local environments. Among various species, salmonid fish are particularly suited for studies of local adaptation because of restrictions in gene flow, attributable to migration strategies that involve homing to natal streams that have resulted in the formation of local populations across a diversity of environments (Ford 2004; Hendry et al. 2004). Among other things, such philopatry provides the opportunity for wild populations to be genetically differentiated from one another. In the wild, natural selection favours those genotypes whose life history traits generate the highest fitness, leading to adaptive genetic variability at the population level (Taylor 1991; Garcia de Leaniz et al. 2007). Similarly, within the hatchery and sea-cage environments experienced by cultured/farmed salmonids, artificial and domestication selection will favour those genotypes best suited to these milieux (Hutchings and Fraser 2008).

As a consequence of their different habitats, one might expect differential selection pressures to generate genetic differences in fitness-related traits between wild salmonids and their farmed counterparts. Pathogen resistance is one of the most significant fitness-related traits because of the considerable demographic consequences associated with disease outbreaks each of which, depending on their frequency and virulence, will affect the strength of the selection intensities resulting therefrom. Among the most compelling studies of local adaptation in salmonids are those on disease susceptibility (Chevassus and Dorson 1990; Fjålestad et al. 1996; Balfry et al. 1997; but see Fraser et al. 2008). Drawing upon one well-known example, Atlantic salmon, *Salmo salar*, inhabiting rivers flowing into the Baltic Sea are resistant to the endemic ectoparasite *Gyrodactylus salaris*, whereas those elsewhere are not (Bakke et al. 1990; Johnsen and Jensen 1991). Population differences in susceptibility of coho salmon, *Oncorhynchus kisutch*, to the myxosporean parasite *Ceratomyxa shasta* have been similarly explained as locally adaptive responses to selection (Hemmingsen et al. 1986).

From a conservation perspective, it would be instructive to know whether pathogen resistance in domesticated populations differs significantly from that of wild populations with which they might interbreed upon escape from sea cages. From an ecological perspective, one question arising is whether disease resistance differs between sexes or between stages of maturity within sexes. Such differences might be anticipated given sexual differences in energetic trade-offs, particularly as a consequence of maturation (Hutchings et al. 1999; Arndt 2000; Jonsson and Jonsson 2003).

The pathogen examined here was *Listonella anguillarum*, a Gram-negative, motile bacterium that is the causative agent for vibriosis, a systemic bacterial infection. Although *L. anguillarum* generally occurs in marine and estuarine environments, it can pose a health risk to salmonids in fresh water, causing up to 100% mortality in 5 to 8 days following an acute outbreak (Ackerman and Iwama 2001). In eastern Canada, vibriosis is one of the five main diseases affecting Atlantic salmon in the aquaculture industry (Olivier and MacKinnon 1998). Genetic variation in resistance to vibriosis has been previously documented within (Balfry et al. 1997) and among (Beacham and Evelyn 1992) populations of chinook salmon, *O. tshawytshcha*, and between two hatchery strains of coho salmon (Balfry et al. 2001). Although genetic differences in resistance to vibriosis have been reported among aquaculture strains of Atlantic salmon by Gjedrem and Aulstad (1974), their experimental protocol lacked appropriate controls and replicates.

The present study compared disease susceptibility of Atlantic salmon parr to *L. anguillarum* among three genetically different populations, using a common-garden experimental protocol. The study populations included a cultured population that originated from the Saint John River, New Brunswick, Canada, and has experienced four generations of artificial selection (Glebe 1998), and a wild population from Stewiacke River, Nova Scotia, assessed as endangered by COSEWIC (Committee on the Status of Endangered Wildlife in Canada), the national science advisory body responsible for assessing the status of species at risk in Canada. Following a microsatellite-DNA analysis to quantify gene flow among populations, the common-garden experimental protocol allowed for an examination of whether disease susceptibility differs genetically between cultured and wild populations, males and females, and immature and mature male parr.

Materials and methods

Study populations

Experimental fish were obtained from three populations of Atlantic salmon in November 2001: two wild populations from Nova Scotia (NS) and one cultured population from New Brunswick (NB; Fig. 1). Spawning adults from the wild populations (Stewiacke River and Tusket River, NS) were collected by staff from the Coldbrook Biodiversity Facility (Department of Fisheries and Oceans Canada [DFO], Coldbrook, NS). Cultured adult salmon were obtained from the Atlantic Salmon Broodstock Development Programme in St. Andrews, NB.

The Stewiacke River is one of a group of more than 30 rivers that flow into the Inner Bay of Fundy that has been assigned a status of Endangered under Canada's national *Species At Risk Act* (www.sararegistry.gc.ca). Since the early 1970s, Stewiacke River salmon are estimated to have declined by 99% (COSEWIC 2006). Although *L. anguillarum* has been identified in wild salmonid populations in the Grand River, part

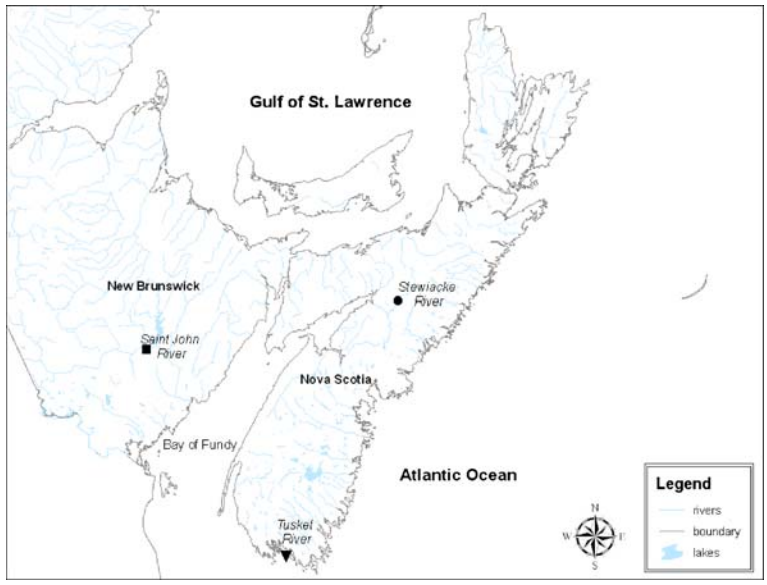

Fig. 1 Geographical locations of the study populations of Atlantic salmon in eastern Canada. The two wild populations are located in Nova Scotia; the population from which the cultured salmon were originally derived is in New Brunswick

of the Stewiacke River drainage basin (MacKinnon et al. 1998), none has been documented in wild Stewiacke Atlantic salmon.

Atlantic salmon inhabiting Tusket River comprise one of several southern upland populations in Nova Scotia whose persistence has been negatively affected by acid rain since the 1970s (Amiro et al. 2000). The number of adult salmon returning to spawn in Tusket River was approximately 140 and 50 in 1998 and 1999, respectively, 40% to 80% less than the estimated abundance of wild adults returning to Tusket River from 1980 through 1985 (Amiro et al. 2000). The population can be characterized as being conservation-dependent because of its comparatively small population size and because of past and present negative influences of acid rain to its persistence.

The cultured population was a product of the Atlantic Salmon Broodstock Development Programme (ASBDP) which was initiated in 1974 (complete details are provided by Glebe 1998). Salmon were initially obtained from pure Saint John River stock and used to create pedigreed stocks. Selection of gamete donors for future generations was based on multiple traits, including parr length, percent yearling smolt, market size and body size of mature two-sea-winter broodfish. The cultured salmon used in the present study are estimated to have been subjected to domestication selection for four generations.

Population genetic variation

To quantify genetic variation within and among the study populations, the following five microsatellite loci were amplified by polymerase chain reaction, using Qiagen DNeasy^{TM} extraction kits, and run on acrylamide gels: *Ssa197* (O'Reilly et al. 1996), *SSsp2201*, *SSsp2213*, *SSsp2215*, and *SSsp2216* (Verspoor et al. 2002; Table 1). Ladder and microsatellite alleles were visualized using an FMBIO II® fluorescent imaging system (Hitachi Software Engineering America, Ltd., San Francisco, CA, USA). We used GENEPOP 3.4 (Raymond and Rousset 1995) to quantify observed (H_o) and expected (H_e) heterozygosities, deviations from Hardy–Weinberg equilibrium, and heterozygote excess/deficiency. Pairwise differences in microsatellite allele identity (F_{st}; Wright 1931), a metric of population differentiation, were estimated using GENEPOP 3.4.

Experimental protocol

Gametes collected from 10 adult males and 10 adult females in each population were crossed to yield 30 pure full-sib families, each of which was reared separately for 1 year. For a period of five months thereafter, 50 randomly chosen fish from each of the 10 families were combined to produce three population groups of 500 fish each. Prior to the disease challenge, random samples of fish from each

Table 1 Metrics of microsatellite DNA variability within and among the three experimental populations of Atlantic salmon (n=20 fish for each locus within each population; a=number of alleles; H_E=expected heterozygosity; H_O=observed heterozygosity)

Population	Locus					
	Ssa197	SSsp2201	SSsp2213	SSsp2215	SSsp2216	All loci
Cultured						
a	7	14	10	11	14	11.2
H_E	0.76	0.91	0.85	0.85	0.86	0.85
H_O	0.60	0.95	0.90	1.00	0.90	0.87
Stewiacke						
a	9	14	10	9	11	10.4
H_E	0.83	0.91	0.83	0.79	0.86	0.84
H_O	0.90	1.00	0.90	0.85	0.85	0.89
Tusket						
a	8	14	10	9	11	10.4
H_E	0.81	0.91	0.83	0.79	0.86	0.84
H_O	0.85	1.00	0.90	0.85	0.85	0.89

population were tested according to standard DFO and provincial disease protocols and declared disease-free.

For the challenge experiment, 475 parr were randomly selected from each of the three populations and randomly assigned to 1 of 12, 100-L flow-through tanks (N≈40 fish in each of the 12 tanks). Individuals were acclimated to the experimental environment for two weeks prior to the challenge during which they were exposed to a 12-h light:12-h dark photoperiod and fed a 1.5 mm Corey High Pro starter diet (Corey Aquafeeds, Fredericton, NB) once daily (approximately 1% body-weight). Tanks were routinely cleaned of uneaten food and faeces approximately one hour after feeding. Throughout the challenge period, water temperature ranged between 12.5°C and 13.3°C (average=12.9+ 0.1°C S.E.).

Bacterial suspension

A primary isolate of *L. anguillarum* was obtained from a moribund fish that had been previously exposed to the pathogen during a disease optimization challenge. A single bacterial colony removed from a trypticase soy agar (TSA; Difco, Sparks, MD, USA) plate supplemented with 1.5% sodium chloride (NaCl) was incubated overnight at 20°C with agitation in 3 mL of tryptic soy broth (TSB; Difco, Sparks, MD, USA). This starter culture was used to inoculate 1,200 mL of TSB supplemented with 1.5% NaCl and grown overnight with agitation at 20°C. The culture was adjusted to an absorbance of 1.0 at 600 nm, and then washed once with cold, sterile peptone–saline solution (P-S; 0.1% peptone and 0.85% NaCl) and re-suspended in a total volume of 240 mL P-S. The volume of the *L. anguillarum* suspension (40 mL) added to each challenge bath (40 L) was estimated to contain a final concentration of 1.7×10^7 colony-forming units per millilitre (cfu mL^{-1}). This concentration was selected to produce an approximate LD_{50} dose, based on an optimization challenge test. The bacterial suspensions were held on ice until the challenges were performed.

Among the experimental tanks, six (two replicates per population) were designated disease treatment tanks; the remaining six tanks (two replicates per population) were controls (Table 2). The treatment groups received the *L. anguillarum* suspension; the control groups were exposed to an equal volume of peptone saline. Tanks were randomly assigned to serve as either controls or treatments.

The actual concentration of *L. anguillarum* in the treatment groups was determined by direct colony counts obtained from serial dilutions in P-S of the OD_{600}=1.0 bacterial suspension that had been incubated in triplicate on TSA (supplemented with 1.5% NaCl) overnight at room temperature. Colonies were counted and the *L. anguillarum* dose estimated. Fish in all but one of the treatment tanks were exposed to an actual challenge concentration of 10^6 cfu mL^{-1}; fish in the remaining tank were exposed to a concentration of 10^5 cfu mL^{-1} (Table 2).

Table 2 Random assignment of the three study populations of Atlantic salmon parr to treatment and control tanks

Population	Exposure	M	MM	F	Survival (%)	SM	SMM	SF	LM	LF	WM	WF
Cultured	C	15	5	19	100	15	5	19	170.3	196.4	48.9	68.1
	C	18	1	18	100	18	1	18	202.8	198.2	67.2	62.6
	T	25	3	16	45.50	8	2	10	194	227.4	61.8	86.3
	T	12	5	22	46.20	9	2	7	217.9	195.7	60.5	63
Tusket	C	12	2	27	100	12	2	27	190.8	207.8	69.9	85
	C	16	4	19	100	16	4	19	200.2	201.7	73.5	78.4
	T	14	0	26	50.00	7	0	13	205	210.8	73.4	80.5
	T	13	2	26	46.30	6	2	11	204.3	204.2	71	76.1
Stewiacke	C	9	15	16	100	9	15	16	142.9	143.1	32.8	33.6
	C	11	9	18	100	11	9	18	163.1	164.9	39.9	40.2
	T	7	9	21	29.70	3	3	5	141	166.4	24.9	36.6
	T	9	12	19	27.50	1	5	5	140.3	152.2	25.9	32.8

Data for each tank also include exposure category (*T* treatment, *C* control), number of males (*M*), number of mature males (*MM*), number of females (*F*), percentage total survival, number of surviving males (S*M*), number of surviving mature males (*SMM*), number of surviving females (*SF*), average length (mm) of all surviving males (*LM*), average length (mm) of surviving females (*LF*), average weight (g) of all surviving males (*WM*), and average weight of surviving females (*WF*).

Disease susceptibility experiment

Fish were challenged by immersion in plastic containers filled with 40 L of tank water to which a concentration of 1.7×10^7 cfu mL^{-1} of *L. anguillarum* in peptone–saline had been added. Tank water was used to minimize any stress that might be attributed to temperature or water quality differences. The fish were exposed for 30 min with gentle aeration. Lids were placed on the containers and the fish were undisturbed during this time. Following the challenge, the fish were returned to their designated resident tanks. The control groups experienced an identical handling protocol.

Mortality was monitored for 15 days post-challenge. Tanks were inspected three times daily and deceased animals were immediately removed. Following removal, length, weight, sex, and state of maturity were measured for each individual. Posterior kidney swabs were also taken from each fish. The sex of each fish was determined by internal observation. Maturity of males was determined by gently squeezing the fish to check for milt at the urogenital opening prior to dissection. Kidney swabs were cultured on TCBS (thiosulfate citrate bile salt sucrose; Difco, Sparks, MD, USA) vibrio-selective medium. Mortality was considered *L. anguillarum*-related if individuals displayed clinical signs of disease and if yellow colonies were isolated when the posterior kidney was cultured on TCBS (Alsina and Blanch 1994). Post-challenge survivors were euthanized with eugenol (2 ppt concentration; Sigma-Aldrich, Oakville, ON, USA) and data obtained were those previously described for deceased fish.

Statistical analysis

Data on infected individuals were pooled for statistical analysis. *G* tests were used to test for differences among the total number of survivors at the end of the study and to determine whether the proportion of groups infected with the disease differed by sex or, for males, by state of maturity. The effects of day of experiment, population, and sex on survival were assessed using a generalized linear model (GLM) of proportion data with binomial error (Crawley 2002). A minimal adequate model was obtained for the independent variables day, population, and sex, using S-Plus (Version 6.1). Given that the data exhibited over-dispersion for the binomial parameter, model simplification was carried out using an *F*, rather than a χ^2, statistic (Crawley 2002). Statistical significance for all tests was set at $\alpha=0.05$.

Results

Genetic variation

The number of alleles per locus varied between 7 and 14 for cultured salmon, 9 and 14 for Stewiacke salmon, and 8 and 14 for Tusket salmon (Table 1). There was no

evidence of per-locus heterozygote excess or deficiency in any population. Observed levels of heterozygosity across the five loci were high, ranging from 0.82 among Stewiacke salmon to 0.87 and 0.89 among cultured and Tusket salmon, respectively. Allelic identity differed among populations, reflecting genetic differences among populations. Pairwise estimates of F_{st} were as follows: 0.061 for cultured and Stewiacke populations; 0.038 for cultured and Tusket populations; and 0.065 for Stewiacke and Tusket populations ($P<0.01$ for all pairwise estimates).

Variation in pathogen susceptibility

The pathogen *L. anguillarum* influenced the survival probability of Atlantic salmon parr. Fifteen days after exposure, fish in the treatment tanks experienced mortality rates ranging from 27% to 50%; by comparison, none of the control fish died (Table 2). Based on the results of the GLM, probability of death was significantly influenced by time post-exposure, population, and sex. Independently of population and sex, mortality depended on the time that had elapsed following exposure ($F_{1,\ 192}=225.79$, $P<0.0001$); mortality was greatest between 3 and 7 days within each population (Fig. 2).

Cumulative mortality (at 15 days post-challenge) differed significantly among populations ($F_{2,\ 192}=10.44$, $P<0.0001$); there were no tank effects, survival between replicates did not differ within treatments ($P>0.05$ for all comparisons). Comparing the extremes, survival was lowest among parr from Stewiacke River (average survival for the two replicates: 28.6+0.01% S.E.) and highest among those from Tusket River (49.4+0.02% S.E.). Survival of cultured salmon (45.8+0.01% S.E.) did not differ from that of Tusket salmon ($G_1=0.14$, $P>0.05$), but was significantly greater than that of Stewiacke salmon ($G_1=5.10$, $P<0.05$).

There was evidence of a sex bias in pathogen resistance in Atlantic salmon parr, as revealed by a significant sex × population interaction term ($F_{2,\ 192}=4.84$, $P=0.009$). Within each of the three study populations, males experienced higher survival than females (Fig. 3), although the difference was statistically significant in only the cultured population ($G_1=4.06$, $P<0.05$).

There was no significant influence of maturity on male parr survival following exposure to *L. anguillarum* ($G_1=0.06$, $P<0.75$). However, it should be noted that small numbers of mature male parr (possibly under-represented because of uncertainty in the degree to which maturity can be reliably identified by the expression of milt) rendered the power to detect such an effect negligible in the cultured ($N=8$) and Tusket ($N=2$) populations. Among male Stewiacke River parr, the survival of mature parr (38.1%, $N=21$) was marginally higher than that of their immature counterparts (32.4%, $N=37$).

Discussion

Several conclusions can be drawn from this study of population differences in resistance by juvenile Atlantic salmon to the pathogen *L. anguillarum*, the causative agent for vibriosis. Firstly, there are genetic differences

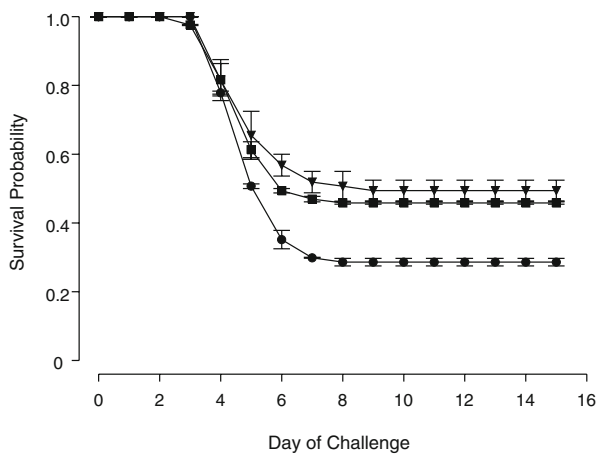

Fig. 2 Survival (means of two replicates +1 S.E.) throughout the experimental period, following exposure to *Listonella anguillarum*, of Atlantic salmon parr of cultured (*squares*) and wild origin (Stewiacke River: *circles*; Tusket River: *inverted triangles*)

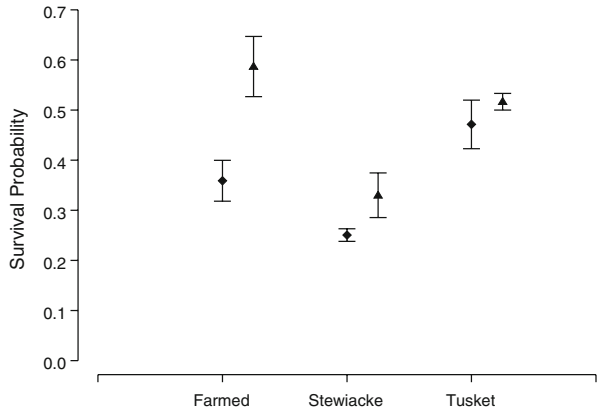

Fig. 3 Mean cumulative survival probabilities (means of two replicates +1 S.E.), following exposure to *Listonella anguillarum*, of Atlantic salmon parr of cultured and wild (Stewiacke River, Tusket River) origin. Data for females are indicated by *diamonds*, data for males by *triangles*

in resistance to vibriosis among wild and cultured populations of Atlantic salmon in Canada. Secondly, pathogen resistance in cultured salmon can differ markedly from that of wild salmon. Thirdly, to varying degrees, males appear to have greater resistance to infection by *L. anguillarum* than females. Fourthly, based on relatively limited data for the pathogen under study, disease susceptibility of mature male parr does not appear to differ from that of their immature counterparts.

The population differences documented here are consistent with previous demonstrations of a genetic basis to vibriosis susceptibility (Fjålestad et al. 1996) in aquaculture strains of Atlantic salmon. There is reason to believe that this variation can be additive, with heritability for resistance to vibriosis estimated to be as high as 0.32 (SGRP 1985). Thus, upon sufficient exposure, resistance to vibriosis can be expected to respond to selection.

It is unlikely that the differences in pathogen susceptibility between Stewiacke and Tusket River salmon can be attributed to unduly low levels of genetic variation. Heterozygosity estimates reported here are similar to those documented for wild Atlantic salmon populations in North America (McConnell et al. 1997; King et al. 2001). It also seems unlikely that tank effects could have biased the results, given that the tanks containing the 30 full-sib families from which our experimental animals were drawn were randomly distributed throughout Dalhousie University's salmon hatchery prior to the initiation of the experiment.

Although *L. anguillarum* has been documented in wild salmonid populations in Nova Scotia and New Brunswick (MacKinnon et al. 1998), the data are insufficient to quantify differences in exposure to this pathogen among Atlantic salmon populations. In this regard, it would be useful to undertake experiments similar to those by Hemmingsen et al. (1986) on coho salmon. These authors found susceptibility to *C. shasta* to be considerably lower for salmon native to the Columbia River basin, where *C. shasta* was known to occur, than among populations where the parasite had not been previously documented. Consistent with the hypothesis that this genetic variation for resistance among coho populations was additive, the susceptibility of the crossbred progeny was almost always intermediate to that of fish from the parental populations (Hemmingsen et al. 1986).

Differential selection responses for disease resistance may render some populations more vulnerable to epizootics than others. Data presented here suggest that some wild populations may respond differently or similarly to an epizootic relative to cultured populations. All else being equal, cultured salmon might be predicted to have greater pathogen resistance than wild salmon because of the greater probability of disease transmission in the aquaculture environment. Pathogens of the genus *Listonella*, for example, are the most common among cultured salmonid populations in southern New Brunswick (MacKinnon et al. 1998), where most of the aquaculture sites in eastern Canada are located. If true, one might conclude that interbreeding between wild and cultured salmon could be advantageous to wild salmon, if it resulted in enhanced disease resistance (Hutchings and Fraser 2008). But such a prediction might not hold true if, for example, there are phenotypic or genetic costs (physiological, metabolic, immunological, energetic) to pathogen resistance. Given the ubiquity of such

trade-offs among other traits closely related to fitness (Roff 2002), it would be surprising if such costs did not exist for traits affecting disease susceptibility.

Population differences in pathogen resistance between Tusket and Stewiacke River populations raise questions from a conservation perspective. The latter has been designated Endangered by COSEWIC because of a 100-fold reduction in abundance over the past several decades (COSEWIC 2006). There is a growing concern that endangered species may have reduced variability for genes that influence host resistance to a wide variety of pathogens (Lafferty and Gerber 2002), including those in the major histocompatibility complex (MHC). Studies to date on fish have been equivocal in this regard. There is evidence, for example, that chinook salmon heterozygous for the class II MHC gene are more resistant to IHNV (infectious hematopoietic necrosis virus) than salmon homozygous for this gene, and that inbred Chinook salmon may have lower resistance to *L. anguillarum* than outbred individuals (Arkush et al. 2002). In contrast, Giese and Hedrick (2003) found no relationship between resistance to *L. anguillarum* and heterozygosity at the MHC class II locus in the endangered Gila topminnow, *Poeciliopsis occidentalis*.

The present study detected an effect of sex on individual susceptibility to *L. anguillarum*. To different degrees, males experienced greater resistance than females in each of the three study populations; among cultured salmon parr, the survival probability of males was 60% higher than that of females. Although we are unaware of a sex bias in pathogen resistance having been previously reported for salmonids, limited evidence does suggest that such a bias can exist in fish. In accordance with the sex bias documented here, Lawhavinit et al. (2002) reported that female guppies *Poecilia reticulata* are more vulnerable to infection with *Tetrahymena corlissi* than males. Female dab *Limanda limanda* appear to be more susceptible to the viral disease lymphocystis than males (Vethaak et al. 1992). In contrast, in another pleuronectiform, female flounder *Platichthys flesus* have been reported to have lower incidence of skin ulcer disease and fin rot than males (Lang et al. 1999). If disease resistance does differ between sexes, it would seem prudent to include such an effect when evaluating the consequences of interbreeding between wild and cultured salmonids, particularly given the sex biases that have been documented for other variables that can influence probability of interbreeding, such as spawning behaviour (Fleming 1996) and dispersal (Hutchings and Gerber 2002; Fraser et al. 2004).

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