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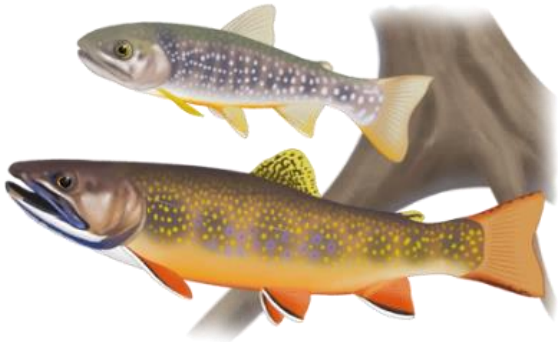
Mechanisms of sustainable introgressive hybridization between native white-spotted charr (*Salvelinus leucomaenis*) and non-native brook trout (*S. fontinalis*)



Sho Fukui

A dissertation submitted to Division of Biosphere Science of Doctor of Philosophy in Graduate School of Environmental Science, Hokkaido University, Japan

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Abstract

Human-mediated hybridization is one of the most serious threats to native taxa. Introgressive hybridization of native species with non-native species can cause the breakdown of inherent gene complexes and ecological adaptations in native populations. This breakdown can pose a major threat to the persistence of rare or endangered species, yet the fitness consequences of introgression and their determining factors have not been fully evaluated in the wild. I am investigating factors affecting the spread and/or sustainability of introgression between introduced brook trout (*Salvelinus fontinalis*) and native white-spotted charr (*S. leucomaenis*) in the upper Sorachi River system, Hokkaido, Japan. The fundamental questions of my doctoral work are: how long can non-native genes be sustained and what factors can contribute to sustain the introgression? To answer these questions, I first assessed the distributional pattern and its temporal changes of the parental species and their hybrids in the Sorachi River systems. The distributional range of introduced brook trout has decreased from 2003 to 2013, but introgression has nevertheless occurred during this period. Secondly, I quantified the reproductive success of parental species and their hybrids in a wild population, and also tested whether male's reproductive characteristics, such as elongated jaws and deeper bodies, affect hybrid fitness. These characteristics should influence male-male interactions. The results of parentage analysis suggest that the reproductive success of hybrids was lower than that of their parental species and that the male's reproductive traits influence the fitness of hybrid males. Finally, I compared other fitness components (i.e. survival and growth) and movement between the hybrids and their parental species, using 1089 tagged individuals followed over 4 years.

The survival and growth of hybrids were equivalent or even higher than those of their parental species. Also, hybrids showed high mobility similar to native charr, while brook trout showed the lowest mobility. Together, these results suggest that despite a decreasing distributional range and low mobility, non-native brook trout can pose a threat to native white-spotted charr through introgressive hybridization.

Chapter 1

INTRODUCTION TO DISSERTATION

General introduction

Introductions of non-native species are a major threat to biodiversity and can have serious economic impacts (Vitousek et al., 1997; Allendorf & Lundquist, 2003; Gozlan et al., 2010). Introduced species interact with native species in various ways, such as through competition, predation, hybridization, or spread of diseases and parasites (Mack et al., 2000; Allendorf et al., 2001; Peeler et al., 2011). These effects have contributed to the decline or extinction of many populations of plants and animals (Allan & Flecker, 1993; Rhymer & Simberloff, 1996).

Hybridization and introgression with non-native species are a serious problem to native species worldwide (Rhymer & Simberloff, 1996; Allendorf et al., 2001). The irreversible effects of introgressive hybridization between native and non-native species can cause the breakdown of inherent gene complexes and ecological adaptations in native populations (Rhymer & Simberloff, 1996). The rate of hybridization and introgression can further be exacerbated by increasing land use and global climate change (Allendorf et al., 2001; Muhlfeld et al., 2014; Muhlfeld et al., 2017). Therefore, there is a growing need to better understand the consequences of hybridization.

Effects of hybridization should, in part, depend on the sterility and fitness of hybrids (Rhymer & Simberloff, 1996; Writz, 1999; Allendorf et al., 2001). When F1

hybrids are sterile, the parental species would waste their reproductive efforts (Leary et al., 1993). On the other hand, when hybrids have high fitness, hybrid swarms can be formed in which essentially all individuals are of hybrid origin (Allendorf et al., 2001), and genetic introgression will occur and further hybridization can lead to long-term effects on native species (Rhymer & Simberloff, 1996; Allendorf et al., 2001). Thus, to measure the relative difference in fitness between hybrids and parental species under wild conditions is the most informative approach for estimating such effects of hybridization (Svedin et al., 2008).

Most studies in natural and human-mediated hybrid zones have focused on hybrid fitness during a limited portion of the life cycle. Studying selection against hybrids in this manner can be misleading because the magnitude and direction of selection can shift across the life stage. For example, Lemmon & Lemmon (2010) have experimentally demonstrated that although hybrids are disfavored in mate choice and fertilization, hybrid viability is greater than parental crosses. Therefore, it is necessary to evaluate multiple fitness components from several life-stages.

Furthermore, even though strong selection against hybrids occurs, continuous immigration of hybrids can sustain introgression. For an excellent instance focusing on the balance between natural selection and movement of wild hybrids, Kovach et al. (2015) have documented that only 17% of the fish in a stream were pure native cutthroat trout despite the fact that selection coefficients acting against hybrids between native cutthroat trout and non-native rainbow trout were as strong as 0.88. How human-mediate introgression is sustained? To answer to this question, to evaluate both multiple fitness components and dispersal potential of hybrids are necessary, but such studies in human-mediated hybrid systems are limited.

Hybridization is more common among fish species than among other

vertebrate taxa because most fishes have external fertilization (Hubbs, 1955; Leary et al., 1995; Scribner et al., 2001). In addition, many fish species have been introduced around the world for both recreational and commercial purposes. Brook trout (*Salvelinus fontinalis*) are originated from eastern North America and the second most widely introduced salmonid fish (49 countries; Fausch, 2008). In western North America, introduced brook trout have displaced native cutthroat trout (*Oncorhynchus clarki*) populations through interspecific competition (Griffith 1988; Dunham et al. 2002; Benjamin et al. 2007). Furthermore, it is concerned that introduced brook trout is prone to hybridize with native bull trout (e.g. Leary et al. 2003: Patrick et al. 2010). In a stream of western North America, rapid displacement of pure bull trout by invasive brook trout for only eight years has been documented (Leary et al. 1993). Introduced brook trout population have been also established in Japan: Yu-kawa of the upper Tone River, Azusa stream and Ryugakubo spring pond of the upper Shinano River, Nunobe and Furebetsu stream of the upper Sorachi River, and the upper Nishibetsu River (Kitano, 2004; Kitano, 2018). In the upper Sorachi River, hybridization between introduced brook trout and native white-spotted charr has been confirmed (Kitano et al. 2014). As previous experimental studies have indicated that F1 and the later-generation hybrids between these species have some fertility (Suzuki & Fukuda, 1971, 1973, 1974), introgression could occur in the wild, threatening pure white-spotted charr.

In this thesis, I investigated the patterns and mechanisms of introgressive hybridization between introduced brook trout and native white-spotted charr in the upper Sorachi River. First, I assessed the distributional pattern and its temporal changes of the parental species and their hybrids. Because it is difficult to visually distinguish parental species and their hybrids, especially post-F1 hybrids, I employed molecular analysis to

species identification. Second, I quantified the reproductive success of parental species and their hybrids in a wild population, with a specific question whether male reproductive characteristics, such as elongated jaws and deeper bodies, affect hybrid fitness. Third, I compared survival, growth, body size, and movement between the hybrids and their parental species, using 1087 tagged individuals followed over 4 years. Finally, I discussed how introgression can persist and/or spread by integrating all the findings. Based on this, management/control of the nonnative was also discussed. This thesis is one of a few studies that evaluate the multiple fitness components and dispersal of hybrids in human-induced hybrid area.

Chapter 2

TEMPORAL CHANGE IN THE DISTRIBUTION AND COMPOSITION OF NATIVE, NON-NATIVE, AND HYBRID CHARRS IN NORTHERN JAPAN

Abstract

Introductions of non-native species have caused various negative impacts on native species and their ecosystems. Hybridization is particularly prevalent among closely related species, and can result in displacement, hybrid swarms, or the disruption of a locally adapted gene complex. Although hybridization between native and non-native species is widespread, long-term monitoring is generally lacking. In this study, I compared the distribution and composition of native white-spotted charr (*Salvelinus leucomaenis*), introduced brook trout (*Salvelinus fontinalis*), and their hybrids in the upper Sorachi River, Hokkaido, Japan in 2003 and 2013, especially focusing on (1) if genetic introgression or hybrid swarm has occurred and (2) if white-spotted charr have declined, since a previous study indicated a potentially harmful asymmetric hybridization with the mothers of hybrids being all white-spotted charr. I found no evidence of decline in native white-spotted charr; rather, the distribution and abundance of introduced brook trout had decreased. Of 142 charr (i.e., genus *Salvelinus*) collected, 18 individuals (13%) were hybrids but no unidirectional hybridization was observed. However, most of the hybrids were post-F1 individuals with biased mating with white-spotted charr. The effects of long-term

introgression on native white-spotted charr should be further examined.

Introduction

Introductions of non-native species are a major threat to biodiversity and can have serious economic impacts (Vitousek et al., 1997; Allendorf & Lundquist, 2003; Gozlan et al., 2010). Introduced species interact with native species in various ways, such as through competition, predation, hybridization, or spread of diseases and parasites (Mack et al., 2000; Allendorf et al., 2001; Peeler et al., 2011). These effects have contributed to the decline or extinction of many populations of plants and animals (Allan & Flecker, 1993; Rhymer & Simberloff, 1996).

Hybridization is more common among fish species than among other vertebrate taxa because most fishes have external fertilization (Hubbs, 1955; Leary et al., 1995; Scribner et al., 2001). In addition, many fish species have been introduced around the world for both recreational and commercial purposes. This can exacerbate non-native and native species interactions through invasive hybridization which may become more serious with increases in land use and global climate change (Allendorf et al., 2001; Muhlfeld et al., 2014). Hybridization and introgression of native with non-native species can cause the breakdown of inherent gene complexes and ecological adaptations in native populations, which can threaten the persistence of rare or endangered taxa
(Rhymer & Simberloff, 1996).

Hybridization can result in three major consequences. First, if hybrids are fertile and have relatively high fitness, hybrid swarms can form in which the majority or all of the individuals in a population are of hybrid origin resulting in genomic extinction of parental

species (Epifanio & Philipp, 2000; Allendorf et al., 2001). A famous example of a hybrid swarm is the one between native cutthroat *Oncorhynchus clarki* and introduced rainbow trout *O. mykiss* (Allendorf & Leary, 1988). Alternatively, if hybrids are sterile or have very low fitness, there will be two outcomes depending on the direction of hybridization. If the mating is unidirectional, the maternal species may decline or even be displaced by the paternal species because the production of eggs has higher energetic costs than sperm and is often limited as a resource for population growth. For example, this unidirectional hybridization might have significantly reduced native bull trout *Salvelinus confluentus* populations in cases where brook trout *S. fontinalis* have been introduced (Leary et al., 1993). Finally, if hybrids have low fitness and the direction of mating is more or less random, two interbreeding species might be able to coexist, as in the many cases of natural hybrid zones (Taylor, 2004).

Although hybridization between native and introduced species is globally widespread (reviewed in Rhymer & Simberloff, 1996), long-term monitoring is generally lacking (but see Muhlfeld et al., 2014). Displacement of native by non-native species can be rapid (e.g., < 10 years) especially when unidirectional hybridization occurs and the fitness of hybrids is very low (Leary et al., 1993; Konishi & Takata, 2004). To assess the impacts of introduced species appropriately and develop management schemes, long-term monitoring is necessary.

In Japan, hybridization between native white-spotted charr (*Salvelinus leucomaenis*) and non-native brook trout (brook charr, *S. fontinalis*) has been documented in the upper Sorachi River of Hokkaido (Kitano et al., 2014). In addition, mitochondrial DNA (mtDNA) of all F1 hybrids (N = 7) were identical to that of white-spotted charr, indicating unidirectional hybridization (Kitano et al., 2014). Suzuki (1974) indicated in a

breeding experiment that the fertility of F1 hybrids was lower than that of parental species, although the fitness in the wild is unknown. Therefore, I can predict either ongoing genetic introgression, which might result in hybrid swarm, or decline of native white-spotted charr through the seemingly unidirectional hybridization in the upper Sorachi River. However, populations of native white-spotted charr have not been monitored since the initial survey in 2003 (Kitano et al., 2014). In addition, because the previous study only used three microsatellite loci with a relatively small number of individuals for genetic analysis (N = 63), a more detailed survey is required.

In the present study, the current status of the native and introduced charr was evaluated to determine (1) whether genetic introgression or hybrid swarm has occurred and (2) whether decline of native white-spotted char has occurred via unidirectional hybridization. A follow-up survey in the same 22 study sites as the previous study (Kitano et al., 2014) was conducted, and using a greater number of microsatellite markers and hybrid individuals, I compared the distributions of parental species and their hybrids between the two sampling times separated by a 10-year interval.

Materials and methods

Study area and field surveys

The study area was located in three major tributaries of the upper Sorachi River, central Hokkaido, Japan (Fig. 1). The same 22 sites were sampled as in the previous survey (Kitano et al., 2014) in order to directly compare the distribution and abundance of individuals between 2003 and 2013. Reach lengths of each site were set at least 100 m, the

same length, or longer than the previous survey (Kitano et al., 2014) for more accurate sampling. Brook trout, white-spotted charr, their hybrids, rainbow trout, Dolly Varden (*Salvelinus malma*), freshwater sculpin (*Cottus nozawae*), Siberian stone loach (*Nemacheilus barbatulus toni*), Japanese dace (*Tribolodon hakonensis*), and brook lamprey (*Lethenteron reissneri*) inhabit this study area.

Brook trout are coldwater-adapted species originally distributed in northeastern North America. In Japan, brook trout have been recorded in 14 prefectures of Honshu and Hokkaido but self-reproducing populations have been reported in only five freshwater systems of spring-fed streams or cold mountain lakes (Kitano, 2004; Kitano 2018). Although the current distribution is limited, introduced populations could affect native salmonids via hybridization, redd superimposition, and possibly competition (Kitano, 2004). Brook trout were most likely introduced into the study area during the 1950s to 1990s for aquaculture purposes (Kitano et al., 2014).

A one-pass backpack electrofishing survey (200–300 V) was conducted during July, 2013 at the 22 study sites. The fish collected were anesthetized with clove oil, identified to species using phenotypic characteristics, and measured to the nearest 1 mm (fork length for salmonids and total body length for other species) (Nakabo, 2000). Hybrid individuals often showed intermediate physical characteristics and coloration between white-spotted charr and brook trout, especially with regard to the dorsal fins. Individuals with ambiguous wavy lines on the dorsal fin were marked as putative hybrids according to Kitano et al. (2014). Adipose fin tissues were collected from all charr and preserved in 99% ethanol for subsequent DNA analysis to verify species identification.

DNA analysis

Total genomic DNA was extracted from fin tissues with a PureGene DNA isolation kit (Applied Biosystems) following manufacturer's instructions. Eight microsatellite loci were used (Sfo12, Ssa197, Sco200, Ots101, Sle6, u-85, Sco211, Sle5), of which Sfo12, Ssa197, and MST-85 were expected to be diagnostic markers to identify brook trout, white-spotted charr, and their hybrids (Kitano et al., 2014). Allele sizes of other loci were partially overlapped but significantly differentiated between white-spotted charr and brook trout (F_{ST} > 0.4 for all loci combined), resulting in high resolution for species identification (e.g., Vähä & Primmer, 2006). PCR reactions were performed in 10 ul volumes using a thermal cycler (Takara; Thermal cycler TP650). The reaction mixture contained 0.5 U Master Mix (GoTaq, Promega), 0.2 µM of each primer, 0.2 mM dNTP, 50 mM KCL, 15 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, and approximately 50–100 ng/µl of genomic DNA as a template. PCR was carried out for 2 min at 95ºC followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 56ºC for 30 s, and extension at 72ºC for 30 s. The amplified products were analyzed on the genetic analyzer ABI 3130 (Applied BioSystems) and allele sizes were scored by GeneMapper (GeneMapper v.4.0; Applied BioSystems).

To evaluate the direction of hybridization between brook trout and white-spotted charr, maternally inherited mtDNA was amplified using the primers HN20 (5'-GTG TTA TGC TTT AGT TAA GC3') and Tpro2 (5'-ACC CTT AAC TCC CAA AGC3'), which are located in the proline and phenylalanine tRNAs, respectively (Brunner et al., 2001). The PCR condition was 2 min at 94ºC followed by 40 cycles of denaturation at 93ºC for 60 s, annealing at 47ºC for 60 s, and extension at 72ºC for 60 s. The PCR products were purified by PEG precipitation. Purified products were cycle sequenced using Big dye terminator v.3.1 (Applied BioSystems) and run on the ABI 3130 (Applied BioSystems). The base

sequences were analyzed with the software MEGA ver.6.0 (Tamura et al., 2013). The mother species was determined by comparing the mtDNA sequences with known sequences of brook trout (AF154850) and white-spotted charr (KF513161) from DDBJ (DNA Data Bank of Japan) database.

Data analysis

To evaluate the changes of salmonid compositions, proportions of captured salmonids were compared between 2003 and 2013 with a χ^2 test. Following Kitano et al. (2014), data were summarized to four regions due to small sample sizes in some of the 22 sites (Fig. 1). I also compared the proportion of salmonids in site FB5 where most hybrids were collected in 2003. Parental species and hybrids were genetically determined by the software NewHybrids (Anderson & Thompson, 2002). I assigned each individual to one of six genotypic classes based on the posterior probabilities: two parental (P0, P1), first-generation hybrids (F1), second-generation hybrids (F2), backcrosses of F1 with the first parental (B0), and backcrosses of F1 with the second parental (B1). Software parameters were set as follows: without individual or allele frequency prior information and the ‘‘Jeffreys-like’’ priors for both mixing proportions and allele frequencies. Posterior distributions were evaluated after discarding an initial ‘‘burnin’’ of 25,000 sweeps and 10^5 iterations of the Monte Carlo Markov Chain. Individuals were assigned to the class with the highest posterior probability. Since NewHybrids detects only F1 and second hybrid generations, I also performed a Bayesian clustering method to infer potential later generation hybrids by the software STRUCTURE (Pritchard et al., 2000). Although STRUCTURE cannot determine the generations of hybrid classes, I assumed the presence

of introgressive hybridization when different levels of genetic admixture were observed among hybrid individuals. I ran the program for a user-defined number of clusters *k* (1–5) under the following conditions: 10^6 replicates after a burn-in of 10^5, admixture model, correlated allele frequency, and no prior population information. When all charr samples (i.e., white-spotted charr, brook trout, and their hybrid) were analyzed, the most likely number of genetic clusters was $k = 2$, representing white-spotted charr and brook trout (see Results). I determined F1 hybrids when the probability of assignments of either one of the parental species was larger than 40% and smaller than 60% (theoretically 50%), whereas I assumed post-F1 hybrids when it was 5–40% or 60–95%.

Results

Seven fish species and putative hybrids were caught in the distribution survey, of which a total of 188 individuals were salmonids (Supplementary Table 1). The results from NewHybrids and STRUCTURE were largely consistent: the former indicated 18 hybrids and the latter indicated 18 hybrids (Fig. 2; Table 1). Both analyses suggested that only 1–2 hybrids were F1 and the rests were post-F1, with backcrosses between F1 and white-spotted charr being dominant. Hereafter, I will only report the result from STRUCTURE due to the consistency with NewHybrids. Of the 22 sites surveyed, occurrence sites of white-spotted charr, brook trout, and their hybrids were 16 (73%), 4 (18%), and 5 sites (23%), respectively.

Contrary to the prediction, white-spotted charr have not declined: rather, opposite trends were observed. The occurrence site of white-spotted charr increased from 13 sites in 2003 to 16 sites in 2013, whereas that of brook trout decreased from 9 to 4 sites.

The occurrence of hybrids increased from 2 sites in 2003 to 5 sites in 2013. In site FB5, where unidirectional hybridization had been observed in 2003, pure brook trout were not detected, however pure white-spotted charr and hybrids were observed in the current survey (Fig. 3). Additional sampling in and around the site was conducted several times, but pure brook trout were not captured. The composition of salmonid species at the Shimonosawa stream was significantly different between 2003 and 2013 ($\chi^2 = 22.469$. $P < 0.01$) with the proportion of brook trout having decreased (Fig. 3). In sites NS2–NS4, which are above an erosion control dam, few salmonids were collected, even during additional intensive sampling in and around the site. Compositions of salmonids were relatively stable in the other three regions (Furebetsu $\chi^2 = 6.488$, $P = 0.09$; Nunobe main $\chi^2 = 1.915$, $P = 0.59$; Nishitappu $\chi^2 = 1.561$, $P = 0.46$).

In total, 98 (69%) white-spotted charr, 25 (18%) brook trout, and 18 (13%) hybrids were collected from the present survey. Contrary to the previous study, no evidence for sex-specific unidirectional hybridization was observed (Table 1). In site FB5 half of the hybrids had white-spotted charr and brook trout mtDNA, respectively. In site NM10, all the six hybrids had brook trout mtDNA. However, direction of mating was biased after post-F1: most were either backcrosses between F1 and white-spotted charr or later generations mating with white-spotted charr. The varying degrees of genetic admixture shown in STRUCTURE suggest ongoing introgression.

Discussion

I found no evidence of a decrease in native white-spotted charr. Rather, the distribution and abundance of introduced brook trout has decreased in the past 10 years. This is especially

the case for site FB5 where in 2003 pure brook trout and hybrids had been observed, yet presently pure brook trout individuals have disappeared. Moreover, in the Shimonosawa stream, brook trout are now rare when in 2003 they dominated. Post-F1 hybrids were detected in several sites, indicating ongoing introgressive hybridization, although no hybrid swarm was observed in any site. All the hybrids found 10 years ago had white-spotted charr mtDNA, but hybrids found in this study were produced from both mothers of the native and non-native charr. Taken together, these results suggest that hybridization levels between native and introduced charr fluctuate substantially even within a relatively short time span of 10 years, which highlights the need for long-term monitoring of introduced populations.

Most hybrids were post-F1 individuals with varying degrees of genetic admixtures from the two parental species. This indicates that hybrids of white-spotted charr and brook trout are fertile even after F1 generations (e.g., Suzuki, 1974) but their fitness is not so high as to cause a hybrid swarm. Alternatively, 20–60 years (i.e., since the introduction of brook trout, Kitano et al., 2014) may not be enough time for a hybrid swarm to form. Few studies have examined direct fitness of hybrids between native and non-native salmonids so far, but Muhlfeld et al. (2009) suggest that even lowered reproductive success of hybrids can cause long-term introgression. I should continue to monitor the introgression and also investigate the ecological consequences of the introgression, such as losses in locally adapted gene complexes (Rhymer & Simberloff, 1996).

My data also indicate that patterns of hybridization change both spatially and temporally. For example, all the hybrids in the upper Furebetsu stream (FB3–FB5) in 2003 were produced by female white-spotted charr and male brook trout, but the hybrids in 2013 were produced from both mothers of the native and non-native charr. In addition, all the hybrids found in MN10 had brook trout mtDNA. Interestingly, most hybrids collected in

this study were post-F1 hybrids with higher proportions of genetic admixture from white-spotted charr. This is probably because the native white-spotted charr have been dominating the upper Sorachi River, leading to the higher probability of mating between hybrids and white-spotted charr. It is known that population sizes should affect the frequency and direction of inter-specific hybridization (Wirtz, 1999). Hybridization is often unidirectional in salmonids (Redenbach & Taylor, 2003; Baumsteiger et al., 2005; Kozfkay et al., 2007), but spatial and temporal variations in the patterns of hybridization have also been reported (Kanda et al., 2002; Rubidge & Taylor, 2004; Gunnell et al., 2008; DeHaan et al., 2010). It seems that many different factors affect the frequency and directionality of hybridization, such as population size (Kanda et al., 2002; Rubidge & Taylor, 2004; DeHaan et al., 2010), sneaking mating behavior (Kitano et al., 1994), and breeding periods. In my system, relative abundance may be one of the important factors, but a more detailed survey is certainly required.

Populations of non-native brook trout have been declining in the last 10 year period, which may be partly due to hybridization. In the upper Furebetsu stream (FB3–FB5) where a relatively large number of hybrids were observed in 2003, no pure brook trout were collected in this survey, whereas post-F1 hybrids were still observed. White-spotted charr have dominated in this stream and pure brook trout might have had lower chances to mate with conspecifics, resulting in the near disappearance. However, many other ecological factors could affect the decline of brook trout other than hybridization. For example, the compositions of non-native rainbow trout increased in some tributaries, which may be replacing brook trout because rainbow trout are much larger (e.g., 15-25 cm in brook trout compared to 20–50 cm in rainbow trout) and fecund (e.g., Clark & Rose, 1997). Also, rainbow trout could be a potential threat to native white-spotted charr (Morita et al.,

2004). In the upper Shimonosawa stream (NS2–NS4) a local population of brook trout have almost collapsed in the past 10 years. Interestingly, rainbow trout have also significantly declined in this stream (I. Koizumi, personal observation), whereas freshwater sculpin have increased. Some portions of the Shimonosawa stream flow in pristine natural forests and conditions should be favorable for salmonids. Therefore, it is difficult to imagine why certain salmonids declined dramatically. One possible factor for near local extinction could be loss of genetic diversity (Saccheri et al., 1998). Local population sizes of brook trout and rainbow trout would have been small because the populations had been isolated by an erosion control dam (ca. 3 m in height, no fish passage) in the middle of the stream. Strong genetic drift, as well as founder effects, would have lowered the genetic diversity of the introduced species.

Climate change might also have mediated the distributions and species compositions, including hybrids. In this region, mean annual air temperature has been increased by one degree Celsius over the past 30 years (Pearson's correlation, $r = 0.529$, $P = 0.001$, data source: the Japan Meteorological Agency, the Rokugo station, Fig. 1). A similar trend was observed during 2001–2013 (Pearson's correlation, $r = 0.739$, $P = 0.003$) and the mean summer temperature (June–August) differed by 2.17 ºC between the years 2001–2003 and 2011–2013 (considering the years affecting the dominant year classes, i.e., age-0+, 1+, and 2+, during the study periods). Increase in water temperature alone, or in conjunction with temperature-dependent competition (Taniguchi & Nakano, 2000) might have influenced populations of white-spotted charr, brook trout, rainbow trout, as well as hybrids in the last decade. More detailed surveying, as well as long-term monitoring, will be required.

Figure Captions

Fig. 1 Study location in central Hokkaido, Japan showing the location of 22 study sites in the upper Sorachi River where distributions of brook trout, white-spotted charr and hybrids were compared with that of 10 year ago. Site numbers correspond to Supplementary Table 1. The location of the Rokugo meteorological station from the Japan Meteorological Agency is indicated by a star. Dark and pale oranges show the distribution of non-native pure brook trout and hybrids with non-native genes, respectively.

Fig. 2 Distruct plots for STRUCTURE runs of white-spotted charr, brook trout, and hybrids collected in the upper Sorachi River. Each fish is represented by a vertical bar that denotes membership fractions (K = 2). Red bars represent pure white-spotted charr, and green bars represent pure brook trout. Bars which have both red and green indicate hybrid individuals

Fig. 3 Relative compositions of salmonid species in 2003 and 2013. Abbreviations of species names are as follows *WSC* white-spotted charr, *BT* brook trout, *HYB* hybrid between white-spotted charr and brook trout, *RT* rainbow trout. One Dolly Varden charr caught in NM12 in 2003 was not included.

Table 1 Number of hybrids collected and their mtDNA haplotypes. The number in the Extra represents hybrids caught in extra surveys.

Sites	No. of hybrids				mtDNA haplotype	
	F1	F1 × WSC	F1 × BT	F2	WSC	BT
FB3	–	1	–	–	–	1
FB4	–	-	–	1	1	–
FB5	–	6	–	–	3	3
NS5	–	1	–	–	1	–
NM10	–	6	–	–	–	6
Extra	1	2	–	–	2	1

Fig. 1

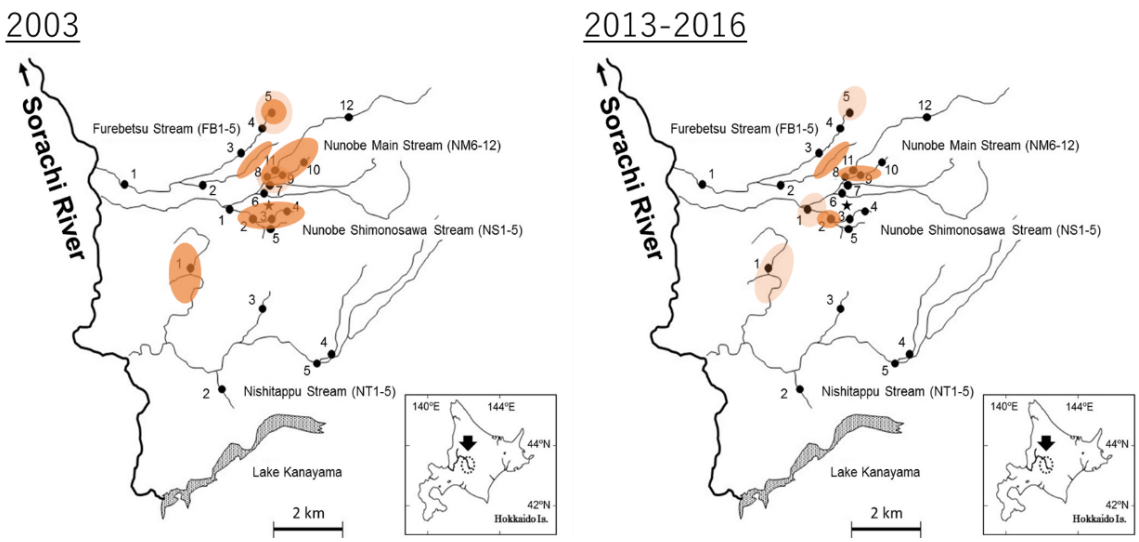

2003
2013-2016
Sorachi River
Furebetsu Stream (FB1-5)
Nunobe Main Stream (NM6-12)
Nunobe Shimonosawa Stream (NS1-5)
Nishitappu Stream (NT1-5)
Lake Kanayama
2 km
140°E
144°E
44°N
42°N
Hokkaido Is.

Fig. 2

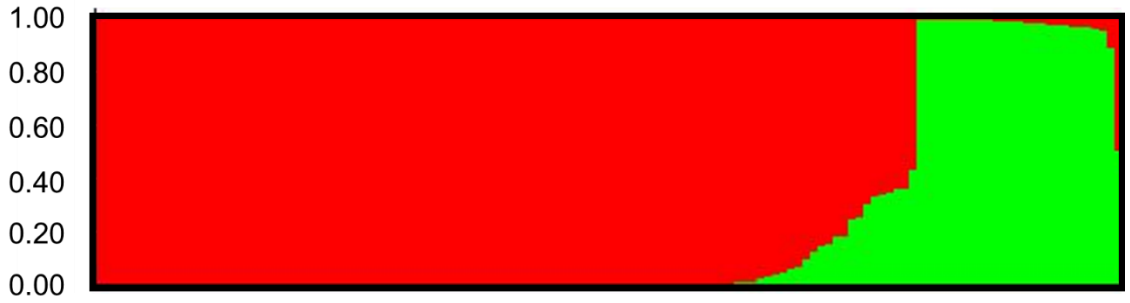
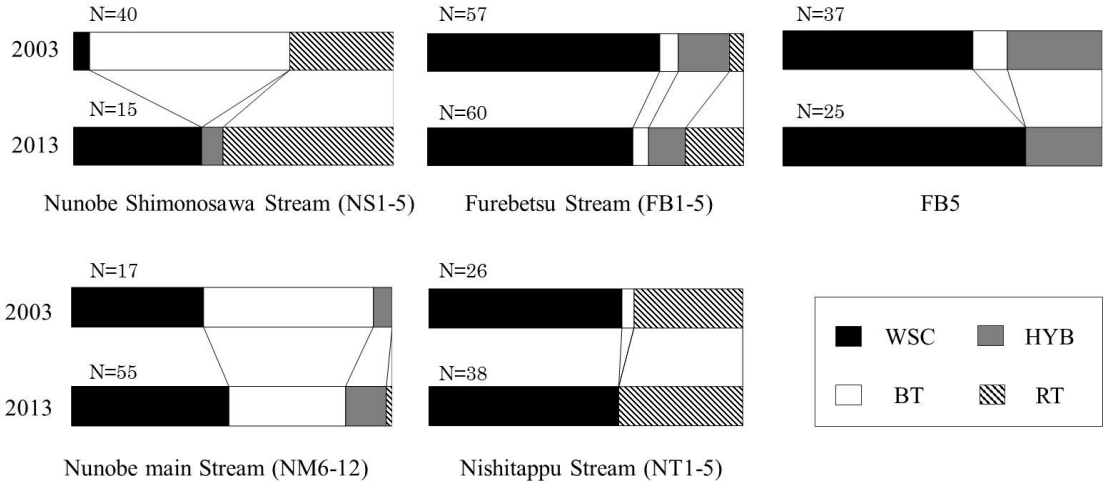


Fig. 3


N=40
2003
N=15
2013
Nunobe Shimonosawa Stream (NS1-5)
N=57
N=60
Furebetsu Stream (FB1-5)
N=37
N=25
FB5
N=17
2003
N=55
2013
Nunobe main Stream (NM6-12)
N=26
N=38
Nishitappu Stream (NT1-5)
WSC
HYB
BT
RT

Chapter 3

MALADAPTIVE SECONDARY SEXUAL CHARACTERISTICS REDUCE THE REPRODUCTIVE SUCCESS OF HYBRIDS BETWEEN NATIVE AND NON-NATIVE CHARRS

Abstract

Human-mediated hybridization between introduced and native species is one of the most serious threats to native taxa. Although field studies have attempted to quantify the relative fitness or reproductive success of parental species and their hybrids**,** only a few studies have unraveled the factors determining the fitness of hybrids. Here I hypothesized that maladaptive secondary sexual characteristics may reduce fitness of hybrids between two fish species. To test this, I evaluated the reproductive success of introduced brook trout (BT: *Salvelinus fontinalis*), native white-spotted charr (WSC: *S. leucomaenis*) and their hybrids in a natural stream in Hokkaido, Japan, where the two parental species show remarkably different male secondary sexual characteristics, such as elongated jaws and deeper bodies. I predicted that introgression from WSC is maladaptive for BT males because the BT male has more prominent secondary sexual characteristics. My results suggest that both sexual selection and outbreeding depression in males and females significantly influence an individual's reproductive success. My results also suggest that asymmetric introgression may increase the risks to persistence in the recipient species.

Introduction

Human-mediated hybridization has contributed to the decline and extinction of many populations and species of plants and animals (Rhymer & Simberloff, 1996; Allendorf et al., 2001). Introgressive hybridization of native species with non-native species can cause the breakdown of inherent gene complexes and ecological adaptations in native populations, which can increase threats to the persistence of rare or endangered species (Rhymer & Simberloff, 1996). The most informative approach for estimating such effects of hybridization is to measure the relative difference in fitness between hybrids and parental species under natural conditions (Svedin et al., 2008). As the extent of hybridization and introgression can further be exacerbated by changes in land use and global climate change (Allendorf et al., 2001; Muhlfeld et al., 2014), there is a growing need for the suitable conservation planning of native species to better characterize how ecological factors involved in hybridization interact to influence the spread or existence of non-native genes within and among native populations.

Compared to extensive studies on natural hybrid zones (Lowry et al., 2008; Lemmon & Lemmon, 2010; Hochkirch & Lemke, 2011), ecological factors affecting hybrid fitness have been much less well investigated in human-mediated hybridization (but see, While et al., 2015). Since it is generally difficult to track hybrid individuals simultaneously with their parental species in the wild while measuring fitness component(s), several previous studies have illustrated the patterns or consequences of hybrid fitness in the wild (Muhlfeld et al., 2009; Taylor et al., 2012), but less frequently have the ecological mechanisms driving such patterns or consequences been studied. As an outstanding example, flycatcher birds from a natural hybrid zone have been well investigated for factors

such as hybrid inviability (Alatalo et al., 1982), hybrid infertility (Alund et al., 2013), ecological inviability (Tegelstrom & Gelter, 1990; Veen et al., 2001) and sexual selection against hybrids (Svedin et al., 2008). Understanding the factors influencing hybrid fitness is critical for forecasting the impacts of human-mediated hybridization between native and non-native (Ellstrand et al., 1999; Allendorf et al., 2001; Fitzpatrick et al., 2010).

Sexual selection can be a key factor influencing hybrid fitness (Svedin et al., 2008; Lemmon & Lemmon, 2010; While et al., 2015), but has been studied much less than other postzygotic factors, such as genetic incompatibilities (Noor, 1997; Howard et al., 1998; Brown & Eady, 2001), or hybrids performing poorly in parental niches (Grant & Grant, 1997; Hatfield & Schluter, 1999; Naisbit et al., 2001). A study by Svedin et al. (2008) estimated the fitness components of collared (*Ficedula albicollis*) and pied (*Ficedula hypoleuca*) flycatchers and their hybrids and found that sexual selection (i.e. female choice) against the intermediate phenotype (i.e., intermediate plumage characters) of hybrid males accounted for approximately one-third of the total postzygotic isolation between the two species, and approximately 75% of this selection was attributable to sexual selection. The few past studies have focused female choice (e.g. Svedin et al., 2008; Lemmon & Lemmon, 2010) probably because it is generally considered that females are the key drivers of hybridization, such us, asymmetric hybridization (Wirtz, 1999) or reproductive interference (Gröning & Hochkirch, 2008). Male-male competition, however, can be also potential driver of introgression (While et al., 2015), since dominant males often monopolize female resources at least within species (Lüpold et al., 2014).

In this study, I explored the effects of sexual selection against hybrids between native and non-native salmonid species (i.e., charr: *Salvelinus* genus). I focused on secondary sexual characteristics of hybrid males because male-male competition often

results in remarkable sexual dimorphisms in salmonid fishes (Fleming & Gross, 1994; Quinn & Foote, 1994; Fleming, 1996). While female choice has been found in salmonids, strong male-male competition often masks the effects of female choice (Petersson et al., 1999). During the breeding season, males develop elongated jaws, develop an angular tip or kype on the lower jaw, and large canine-like teeth emerge from the hooked snout which appear to function as specialized weapons in fighting competitors. In addition, the enlargement of the dorsal hump appears to act as a shield against attack from competitors and block access to ovipositing females by competing males. Males also show brilliant nuptial coloration, which may attract females. Such developed secondary sexual characteristics can contribute to reproductive success through male-male competition and female choice (Fleming & Gross, 1994; Quinn & Foote, 1994). Hybrids between introduced brook trout (BT), *Salvelinus fontinalis*, with native white-spotted charr (WSC), *S. leucomaenis*, occur in a boreal stream of Japan (Kitano et al., 2014; Chapter 2). Here, the male secondary sexual characteristics between these species express obviously distinct features; BT have more intense breeding coloration, deeper bodies, and larger jaws (Fig. 1, see Results). These observations allowed me to test whether the male reproductive characters can be one of the factors/mechanisms determining the hybrid fitness in the wild. For a similar and well-investigated example, introgression between non-native rainbow trout (*Oncorhynchus mykiss*) and native westslope cutthroat trout (*O. clarkii lewisi*) has spread across watersheds in North America (Hitt et al., 2003; Boyer et al., 2008; Yau & Taylor, 2014). Muhlfeld et al. (2009) found that the reproductive success in post F1 hybrids declined rapidly with an increasing genetic proportion of rainbow trout ancestry, although the mechanisms reducing the reproductive success of hybrids remained unknown. Here, I conducted an intensive field survey with parentage analysis in a tributary of the upper

Sorachi River, Hokkaido, Japan, to (i) quantify the reproductive success of parental species and their hybrids in a wild population, (ii) evaluate whether introgression causes outbreeding depression in the life-stages from juvenile to maturing, and (iii) test whether male reproductive characters affects hybrid fitness. For (ii) and (iii) I focused on BT and its hybrid because sample size was limited for pure WSC. I specifically predicted that transfer of the less well developed secondary sexual characteristics of native WSC would reduce the male reproductive success of introduced BT.

Materials and methods

Study area and data collection from the field

My field study was conducted in the Higashi-Furebetsu stream, a tributary of the upper Nunobe stream, in central Hokkaido, Japan (Fig. S1). Based on species distribution data from a previous survey (Chapter 2), I considered that the tributary was suitable to investigate the interactions between WSC and BT because both species and their hybrids were consistently observed in the tributary and the tributary size was relatively small (2–4 m in stream width). Fourteen contiguous sections from a 2,615 m length stream reach were used as permanent study sites with a length of 100–425 m (mean = 186.8 m) in each section. Other species collected during the field survey included freshwater sculpin (*Cottus nozawae*) and Siberian stone loach (*Barbatula barbatula*).

The field survey in the Higashi-Furebetsu stream was conducted in August, October and December 2013, May, June, July and October 2014, and July 2015. The survey was mainly concentrated on collecting adult individuals in 2013, age 0+ juveniles in 2014

and age 1+ juveniles in 2015 to conduct a subsequent parentage analysis for reproductive success. This sampling design evaluated detailed reproductive success of 2013-year adult fish by collecting their offspring (i.e., 2014-year cohort). I passed through each section two to four times using backpack electrofishing (200–300 V). Significant effort was made to collect all of the potential parents and their offspring inhabiting this study area to estimate reproductive success. Fish captured were anesthetized and measured for fork length (FL: nearest 1 mm), as an indicator of body size. The mean fork length (± SD, range, sample size) of mature BT, WSC, and hybrid (HYB) males successfully genotyped were 181.9 ± 34.4 mm (134-290 mm, n = 74), 213.6 ± 25.6 mm (162-265 mm, n = 16), and 189.2 ± 50.4 mm (129-323 mm, n = 22), respectively. Additionally, individuals captured in 2013 that exceeded 45 mm in FL were implanted with 8 or 12 mm passive integrated transponder (PIT) tags for individual identification. I implanted 1087 individuals with PIT tags in 2013 and 2014. Fin clips of all candidate parental and offspring fish were preserved in 99 % ethanol for subsequent DNA analysis. Fin tissues were stored at –20 ºC until DNA extraction.

Photographs were taken for all captured age–1+ and older fishes to measure the external characteristics of all candidate parental fish using an acrylic water tank (40cm×15cm×10cm) and digital camera (Panasonic; DMC-FT3-S). Nine morphological characters (Fig. 1) were measured from the photographic data using the software Image J (version 1.48v; US National Institutes of Health, Bethesda, Maryland) corrected by the fork length measured during the field sampling (i.e., calculated by relative lengths). Morphological differences between the three male groups (i.e., BT, HYB, and WSC) were examined by principal component analysis (PCA). All the nine characters measured were ln-transformed and size-adjusted using their FL prior to the PCA. Since the first principal

component (PC1) included secondary sexual characters such as snout length or hump height, which are known as sexually selected variables (Fleming & Gross, 1994; Quinn & Foote, 1994), and showed a significant difference between WSC and BT (see Results), PC1 was used as a value of secondary sexual characteristics in the subsequent statistical analysis.

DNA analyses

Total genomic DNA was extracted from fin tissues using a PureGene DNA isolation kit (Applied Biosystems) following the manufacturer's protocol. The DNA was dissolved in 50 µL TE buffer. I used 13 microsatellite loci (Ssa197, Sle6, Sco200, SsosL456, Otsg83b, Smm21, Sco211, Sco216, Otsg253b, Sco220, MST-85, Sfo12, and Omm1105) for parentage analysis. The loci Sfo12, Ssa197, and MST-85 were known to be diagnostic markers to identify BT, WSC, and HYB (Kitano et al., 2014). Furthermore, allele sizes of SsosL456, Smm21 Otsg83b Otsg253b did not overlap between BT and WSC (Table S1), when I used individuals which have ≥ 99.9% proportion WSC ancestry and ≥ 99.9% proportion BT ancestry subsequently calculated in STRUCTURE (Pritchard et al., 2000). Therefore, seven of the 13 loci had non-overlapping (diagnostic) sets of alleles in BT and WSC. The PCR reactions were performed in 10-µL volumes and the reaction mixtures contained 0.5 U Master Mix (GoTaq, Promega), 0.2 uM of each primer, 0.2 mM dNTP, 50 mM KCL, 15 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, and about 50-100 ng/µL of genomic DNA as a template. The PCR themocycling protocol was as follows: 2 min at 95 ºC, followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 56ºC for 30 s, and extension at 72 ºC for 30 s. The amplified products were analyzed on the genetic analyzer

ABI 3130 (Applied BioSystems) and allele sizes were scored using GeneMapper (GeneMapper v.4.0; Applied BioSystems).

Data analysis

Parental species and hybrids were genetically determined using the software STRUCTURE (Pritchard et al., 2000). When all samples (i.e., BT, WSC, and HYB) were analyzed, the most likely number of putative populations (*K*) was two, representing WSC and BT. Based on $K = 2$, I calculated a hybrid index as the proportion WSC ancestry: this ranges from 0.001 for pure BT (no WSC allele) to 0.999 for pure WSC (no BT allele). First generation hybrids between BT and WSC have a hybrid index of 0.5 with the 13 microsatellite loci and heterozygous for alleles from each of the parental taxa at all the seven diagnostic loci (Table S1). I assumed that fish with a hybrid index of 0.5 but not heterozygous at all the diagnostic loci were post-F_1 hybrids, although it was difficult to distinguish backcrossing hybrids from other post-F1 hybrids.

I performed parentage analysis using CERVUS v3.0.7 (Kalinowski et al., 2007) to identify parentage among candidate parents (the adults captured in October and December of 2013) and offspring (the 0+ fry sampled in May, June, August, October of 2014, and 1+ parr sampled in September 2015). The CERVUS analysis uses a maximum-likelihood approach to predict parent-offspring relationships. For each offspring, a metric of the likelihood that each potential father is the actual father is first calculated as a log-likelihood (LOD) score. Then, CERVUS uses the range of LOD score distributions to calculate a critical LOD score against which LOD scores from the actual population can be evaluated. The LOD scores are then computed for each potential father using their actual

genotype for each individual offspring. The critical LOD score calculated from the simulation step is used to assign fathers to each offspring (Kalinowski et al., 2007). The same procedure was also applied to mother-offspring relationships. Allele frequencies using all genotyped individuals, the expected frequency of heterozygotes (H_e), and the observed frequency of heterozygotes (H_o) were also calculated using CERVUS.

I defined reproductive success as the number of offspring produced by each male or female. To investigate how body size, proportion WSC ancestry, and secondary sexual characteristics influence reproductive success, I used zero-inflated models with negative binomial distribution (ZIB) to model counts of offspring (Zuur et al., 2009), including zeros for males that may have mated, but their offspring was not sampled, because the reproductive success, is a count variable with many zeros (Fig. S2). The statistical analysis with ZIB were only conducted for BT and HYB because the distributions of reproductive success vs. proportion WSC ancestry did not fit to the ZIB for WSC, likely attributable to the low sample size of pure WSC. The number of offspring was used as a response variable, whereas FL (i.e. body size: both males and females), PC1 (i.e., secondary sexual characters: males only), and the height of the caudal peduncle (females only) were used as independent variables. Because body size in salmonid fishes is important not only for male-male interactions during mating but also for the establishment of territories in non-breeding season (e.g. Fleming, 1996), I used both FL and PC1 as the predictor variables to separate these effects. The height of the caudal peduncle in females is associated with the depth of eggs buried and, thus, has a significant effect on reproductive success (Fleming & Gross, 1994). Since a significant correlation was observed between FL and PC1 (Spearman's $r = 0.646$, $P < 0.001$), PC1 was expressed as the residuals from its liner regression against FL. The transformation of PC1 value individuals with remarkable

secondary sexual characteristics, such as higher hump height than average for their size, had a large positive residual value. All statistical analyses were performed using the software R version 3.2.4 (R Development Core Team, 2011).

Results

In total, 666 offspring and 268 potential parents (149 females, 119 males) were collected in October and December 2013, May, June, July and October 2014 and July 2015. Because the total of 934 samples included individuals with multiple captures, data on 61 recaptured individuals were removed from subsequent analysis. Of the 873 samples, 637 offspring and 236 mature individuals (124 females, 112 males) were successfully genotyped at a minimum of seven loci (i.e., 94.9% of possible genotypes). Of the 236 successfully genotyped mature individuals (i.e., the adults), 176 were pure BT (102 females, 74 males), 22 were pure WSC (6 females, 16 males), and 38 were putative hybrids (16 females, 22 males). The number of alleles per locus ranged from two to 24 (mean = 12.2), and expected heterozygosity ranged from 0.15 to 0.76 (mean = 0.41). This led to high combined exclusion probability for the whole set of loci in the parentage analysis ($Pe > 0.85$ for first parent and > 0.99 for identity, Table S1). There were a number of loci that were not in Hardy-Weinberg equilibrium. This is likely because random mating did not occur among BT, WSC, and HYB.

I analyzed morphological variation in males and I confirmed differences of secondary sexual characters between BT and WSC males. 109 of the 112 males were successfully measured the morphological characters. Analysis of variance (ANOVA)

indicated that these groups differed significantly from each other along the first principal component (PC1, $F_{2,107}$ = 27.951, P<0.0001). There was no significant difference among male types in PC2. The BT and WSC males were clearly distinguished by the secondary sexual characteristics (Fig. 1, 2, Table 1). Tukey's pairwise comparisons indicated that BT and WSC males significantly differed from one another in PC1 (P<0.0001), and that HYB and WSC males significantly differed from one another in PC1 (P<0.0001), but that BT and HYB groups were not significant distinct from one another (P=0.4322). The PC1 is based primarily on variation in secondary sexual characters such as hump height and lower jaw length (Fig. 1, Table 1) and explained 40.4% of the variation and PC2 explained an additional 19.6% of the variation.

Of the 637 offspring genotyped, 326 individuals (51%) were assigned to a sampled father, and 367 (58%) were assigned to a sampled mother by CERVUS. Of the 112 mature males, 59 (53%) appeared to produce at least one offspring (maximum 82 offspring) with the median of 1. Of the 124 mature females, 89 (72%) appeared to produce at least one offspring (maximum 84 offspring) with the median of 2. The reproductive success was a count variable with many zeros as expected (Fig. S2).

The ZIB showed that the proportion of WSC ancestry among mature males and females had a strong negative effect on reproductive success (Table 2). Hybrids had lower reproductive success than that of both parental species in males and females (Fig. 3a). Quadratic equations fitted to the median number of offspring per parent among males and females (males, γ^2 =0.8673, y =3.3482x^2 −3.7054x +0.875; females, γ^2 =0.9496, y =12.5x^2 −11.071x +2.2857) estimated that reproductive success decreased by 30.6% and 25.0% for males and females, respectively, in BT with 20 percent proportion WSC ancestry (Fig. 3b). For male BT and HYB, FL had only a small, but statistically significant

positive effect on reproductive success (Table 2, Fig. 4), but variation along PC1 (i.e., prominence of secondary sexual characters) had a 20x stronger positive effect on reproductive success (Table 2, Fig. 4). For female BT and HYB, FL had a significant and positive effect on reproductive success, but caudal peduncle depth had no detectable effect on reproductive success (Table 2, Fig. 4).

Discussion

While past studies have shown the reduction of fitness or reproductive success in hybrids (e.g. Muhlfeld et al., 2009; Lancaster et al., 2007), underling mechanisms have been rarely clarified (but see Svedin et al., 2008; Lemmon & Lemmon, 2010; While et al., 2015). I hypothesized that intermediate morphology is maladaptive especially the one related to sexual selection. My results suggested that PC1 of morphological traits, which was associated with male secondary sexual characteristics, significantly affect the reproductive success of non-native salmonid and its hybrids with native species. Importantly, hybrid index was also selected as a significant factor affecting male reproductive success, suggesting that not only maladaptive morphology but also some other undetermined factor(s) reduce the fitness of hybrids. Hybrid index was also negatively associated with female reproductive success. Although the sample size and number of examined loci were limited, my results were largely consistent with the predictions derived from the general patterns of salmonids' mating systems. In addition, more prominent secondary sexual characteristics of non-native species might result in asymmetric introgression from non-native to native species, which is a serious concern from conservation perspective.

Roles of morphology on reproductive success of salmonids

Because of remarkable morphological variations during breeding seasons, many studies have attempted to clarify the factors affecting reproductive success in salmonids (e.g. Fleming & Gross, 1994; Tentelier et al. 2016). Among them, body size appeared to be the most consistent determinant as the variation could range from < 10 cm to > 100 cm, even within populations (L'abée-Lund, 1989). My population was less variable but still had more than two-fold difference in fork length (129-323 mm). In males, large individuals become dominant and outcompete small males for access to females (Quinn & Foote, 1994; Fleming, 1996, 1998; Blanchfield et al., 2003; Dickerson et al., 2005). In females, fecundity increases exponentially with body size and, thus, large females have much greater advantages (van den Berghe & Gross, 1989). While I found a significant effect of body size in both sexes, the size advantage was not very strong especially for males (Table 2; Fig. 4). It is likely that small males use alternative tactics, such as sneaking, in the presence of bigger males to increase their reproductive success (Theriault et al., 2007). Alternatively, I might have missed some potential parents, which may weaken the pattern (see Limitations of the study section). Importantly, however, although the body size is considered as the most promising factor, field studies under natural conditions often found little or no relationship between male size and reproductive success in salmonids (Garant et al., 2001; Jones & Hutchings, 2002; Seamons et al., 2004; Dickerson et al., 2005).

The second well-known morphological trait under selection is exaggerating secondary sexual characteristics of male salmonids, such as elongation of the jaw, development of an angular tip or kype, or dorsal hump, which is referred even in *the*

Descent of Man (Darwin 1871). Despite the wide acceptance in salmonids' researchers, surprisingly few studies have actually demonstrated the roles of the sexual traits on reproductive success (Fleming & Gross, 1994). These exaggerated traits are usually observed in large sea-run species or individuals, but even relatively small stream-resident brook trout show such traits (Kazyak et al., 2013), providing a good opportunity to assess their roles. In fact, PC1 clearly distinguished between brook trout and white-spotted charr based on secondary sexual characteristics. Thus, the significant positive effect of PC1 represents one of the rare field evidence on the roles of male secondary sexual characteristic have on reproductive success.

The caudal peduncle area is also reported as an important factor for females in digging during nest construction and appears to influence the depth of egg burial (Fleming & Gross, 1994). By contrast, I did not find a relationship between caudal peduncle depth and reproductive success. This may be because my study occurred in an area where the substrate had been modified by humans and consisted of a concrete base with a shallow layer of pebbles, which likely reduced the available habitat variation in terms of potential nest depth. Based on a questionnaire to farmers, the channel modification occurred about 2002 (S. Fukui, personal communication), in which the time scale is too short for the trait evolution.

Reproductive success of hybrids

My results clearly showed that both male and female hybrids have lower reproductive success compared to the parent species (Fig. 3). This should be partly due to the maladaptive morphology as discussed above, but other undermined factors also reduce the

reproductive success of hybrids because hybrid index had significant effects in both sexes. Unfortunately, the processes underlying the reduced hybrid reproductive success in my study are unknown, because my estimate of reproductive success represents a function of selection at different life-stages, including spawning, eyed egg, egg-to-fry emergence and juvenile survival: selection could have acted at any or all of these stages. Previous laboratory studies have documented lower fitness of hybrids between BT and WSC at multiple stages (Suzuki & Fukuda, 1971; Suzuki & Fukuda, 1973; Suzuki & Fukuda, 1974). First, gonadal development of F1 hybrid males was reduced compared to the parental species (with lower quantity of milt) in both reciprocal crosses. Second, survival and growth rates at the early life stages (i.e., hatching and eyed eggs) of both F2 and some backcrossed hybrids were lower than those of parental species, although some hybrid vigor was also observed in F1 hybrids. Future studies should determine the relative reduction in hybrid fitness at each of the stages.

Conservation implications

I showed that reproductive success of male BT (and its hybrid) was reduced by the maladaptive phenotypes derived from WSC. This, in turn, suggests that native WSC may suffer from asymmetric introgression from non-native BT, which have more remarkable secondary sexual characteristics. That is, hybrid WSC with more pronounced secondary sexual characteristics (via introgression with BT) might enjoy higher reproductive success compared to pure WSC. If this is the case, some genes of the non-native species will persist and spread even when pure BT disappear (Chapter 2), which potentially reduces the likelihood of persistence of pure WSC. Although I could not directly assess the effects of

introgression on the reproductive success of WSC due to insufficient sample size, my previous broader-scale survey showed that most hybrids were derived from backcross with white-spotted charr (Chapter 2). Future study should focus on the direction of hybridization and its mechanisms.

Reduced hybrid reproductive success may inhibit the spread of non-native alleles and the development of hybrid swarms. I used, however, only 13 microsatellite markers to assess the levels of introgression, and therefore non-native (BT) alleles at other loci, particularly those that might be advantageous, may have spread more widely across the WSC genome. For instance, Fitzpatrick et al. (2010) documented the spread of a small portion of introduced alleles up to 90 km into native populations (the California Tiger Salamander: *Ambystoma californiense*) that have no record of non-native species within 60 years, although a majority of single-nucleotide polymorphism markers (65 of 68) showed no evidence of the spread beyond the region where non-native species had been introduced. Further analysis using additional neutral and adaptive loci could improve my understanding of the risk in the spread of non-native genes into WSC populations.

Limitations of the study

While genomic data from next-generation sequencing (NGS) has recently been accumulating, I used only 13 microsatellite loci in the present study. Hybrid index should be less accurate compared to NGS data. Also, I cannot know how much introgression has been occurring in the native species, as well as the loci under selection. In addition, parents were not determined for 40-50% of juveniles, indicating that I should have missed some mature adults. I tried to collect most of the adults in the study reaches by 2-4 passes of

electrofishing sweep until few fish were captured in the last pass. However, since breeding dispersal is common in stream salmonids (Northcote 1997; Hutchings & Gerber 2002), some individuals might have moved to and reproduced in the study tributary between my sampling periods. Importantly, mainstem migratory fish often grow bigger (Koizumi et al. 2006) and, therefore, may attain higher reproductive success compared to smaller residents. I might have failed to collect such large migratory adults, which could explain the low parentage assignment. However, this is a nature of field studies in salmonids: similar attempts for estimating reproductive success showed the equivalent levels of parentage assignments (32-61%: Theriault et al., 2007; Muhlfeld et al., 2009; Serbezov et al. 2010). Most importantly, even with the limited sampling, the results showed consistent patterns predicted from previous studies. Another important aspect I missed is annual variation. More detailed picture will be clarified with NGS approach and additional survey.

Conclusion

This study showed the relative reproductive success of hybrids and their parental species in the wild, despite that there are few field studies on directly quantifying relative fitness of hybrids and their parental species in human-mediated hybrid zones. The reproductive success of hybrids was obviously lower than that of the two parental species. My results supported that both of natural selection derived from outbreeding depression and sexual selection can affect the decision of hybrid fitness, and suggests that the more adaptive male traits derived from non-native species may facilitate the genetic extinction of native population via asymmetric introgression. As this study could not completely separate effects of sexual selection from other factors which can influence hybrid fitness, to quantify

other fitness components, such as survival or growth, would appear to be effective approach for unraveling further causes reducing the hybrid fitness.

Table 1. Eigenvector of morphological variables and the first two components (PC1 and PC2) obtained in the principal component analysis (PC1 explains 40.4% of the variation and PC2 an additional 19.6%).

measurement	variable	PC1	PC2
1-2	snout length	0.34444	0.36449
15-16	under-jaw length	0.42814	0.26132
13-14	pectoral fin length	0.22244	0.09270
3-5	dorsal fin height	-0.08630	0.24612
3-4	hump height 1	0.46144	0.01037
6-12	hump height 2	0.46271	0.02523
8-10	caudal peduncle	0.39417	-0.13014
7-9	caudal length 1	0.14854	-0.61562
9-11	caudal length 2	0.19153	-0.57708

Table 2. Zero-inflated count model with negative binomial distribution of male and female reproductive success. The statistical analysis was conducted separately for males and females.

factor	coefficient	s.e.	*z*-value	*p*-value
Male model				
intercept	-1.5238	0.8689	-1.7540	0.0795
fork length	0.0147	0.0042	3.4600	0.0005
proportion white-spotted charr ancestry	-13.6403	5.1715	-2.6380	0.0084
PC1 (prominence of secondary sexual characters)	0.3073	0.1561	1.9680	0.0490
Female model				
intercept	-10.6836	3.6995	-2.8880	0.0039
fork length	5.4987	1.6464	3.3400	0.0008
proportion white-spotted charr ancestry	-9.8868	3.4380	-2.8760	0.0040
caudal peduncle	-5.1735	6.9735	-0.7420	0.4582

Figure Legends

Figure 1. Representative males of introduced brook trout (above photo), hybrid (middle photo), and native white-spotted charr (below photo). Photographs have been taken in the present study area. Bottom drawing picture represents locations for 16 landmarks identified on each male charr photograph : snout length (1-2), under-jaw length (15-16), pectoral fin (13-14), dorsal fin height (3-5), hump height 1 (3-4), hump height 2 (6-12), caudal peduncle (8-10), caudal length 1 (7-9), caudal length 2 (9-11). Theses nine measurements are used in the principal component analysis. For females, only caudal peduncle (8-10) was used for subsequent statistical analysis with zero-inflated model.

Figure 2. Principal component analysis incorporating nine morphological characters (snout length, under-jaw length, pectoral fin, dorsal fin height, hump height 1, hump height 2, caudal peduncle, caudal length 1, caudal length 2) of the three male groups (native white-spotted charr males, solid circles; hybrids males, grey triangles; introduced brook trout, open circles).

Figure 3. (a) Number of offspring per male (upper left) and female (upper right) versus the proportion native white-spotted charr ancestry (i.e., “0” side is pure BT and “1” is pure WSC). The left plot includes 112 fathers and 327 juvenile assignments, and the right plot includes 124 mothers and 367 juvenile assignments using parentage analysis. Each point represents an estimate for an individual fish from a spawn year. Circles represent brook trout, white-spotted charr, and their hybrids. (b) Plot of the median number of offspring per male (below: solid circles) and female (below: solid triangles) plotted against the proportion white-spotted charr ancestry.

Figure 4. Reproductive success (number of offspring assigned per individual) as a function of male body size (a) and PC1 (b), and female body size (c) for brook trout and hybrid spawners. The *p*-values of above predictor variables which were calculated in the zero-inflated models (see Table 2.) are represented in the top of each plot figure. Open circles are individuals with more than 97% proportion brook trout ancestry, and graduated grey colors are indicate proportion white-spotted charr ancestry.

Figure 1.

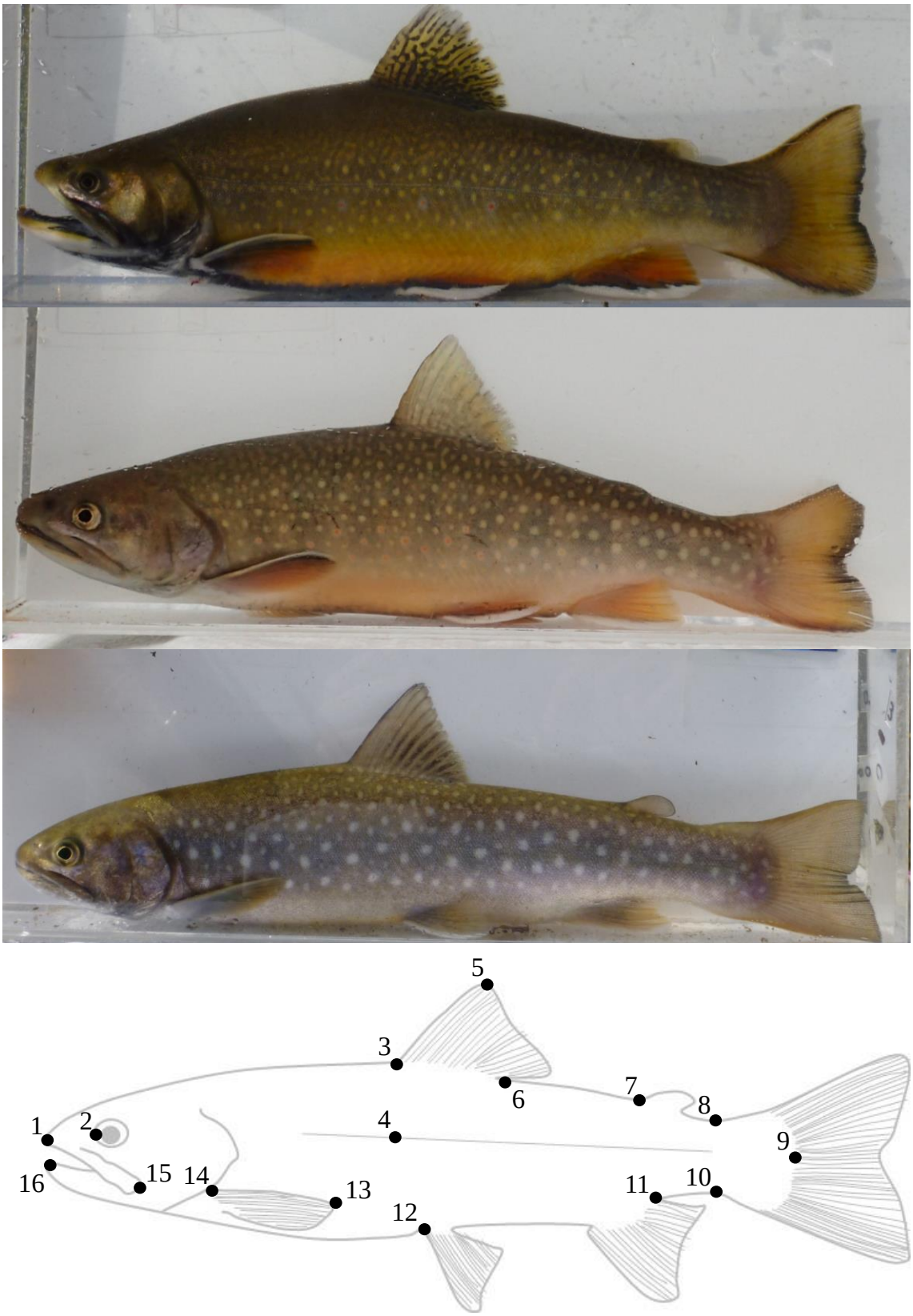


Figure 2.

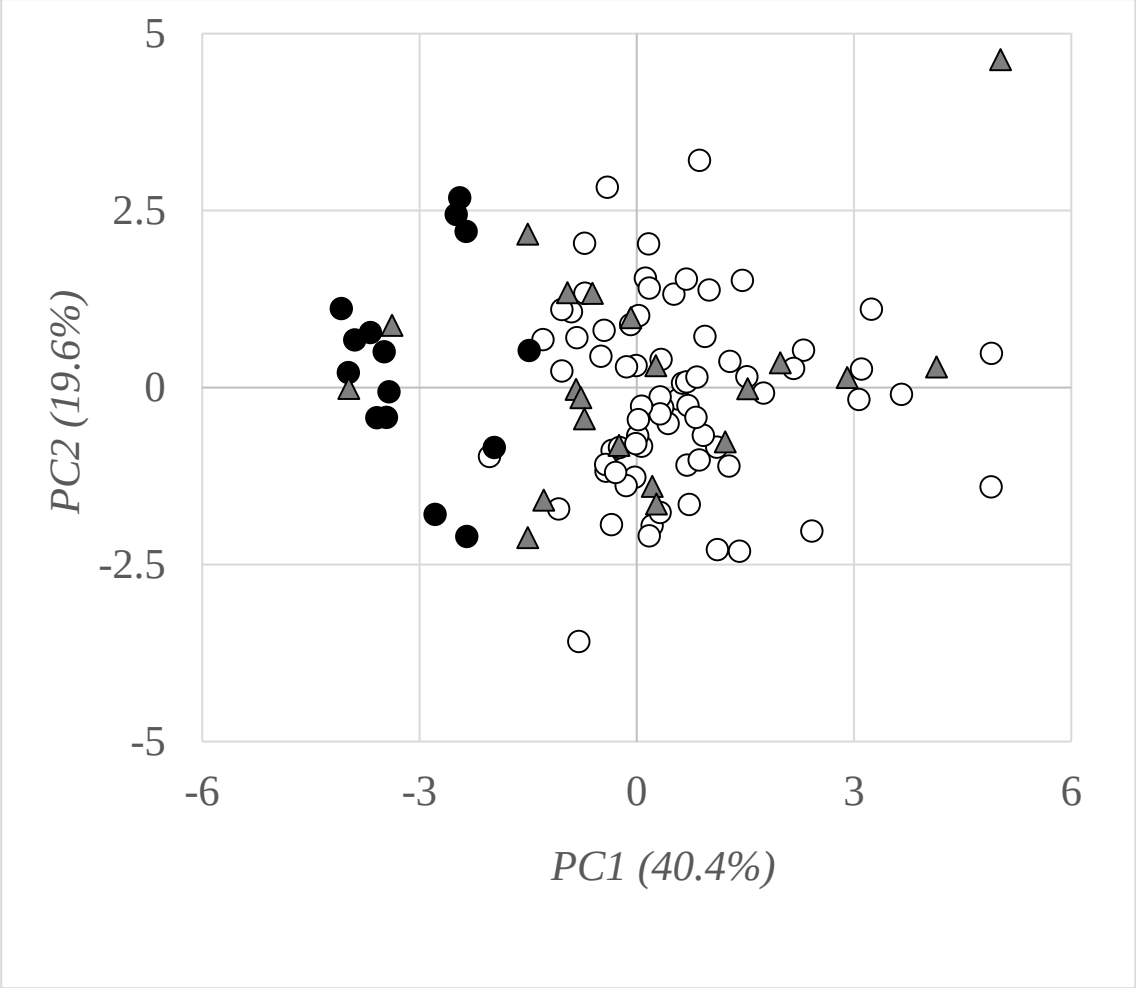


Figure 3.

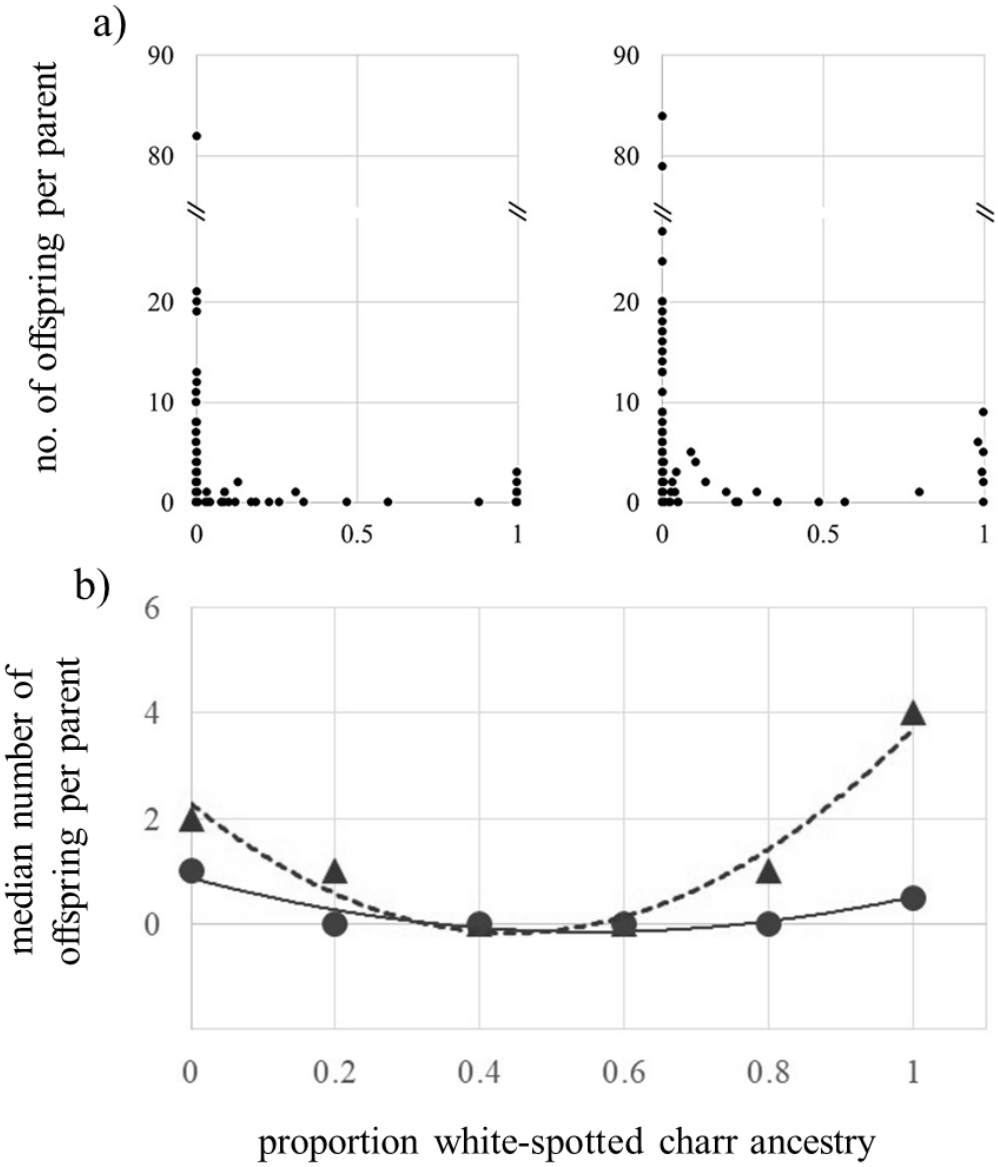
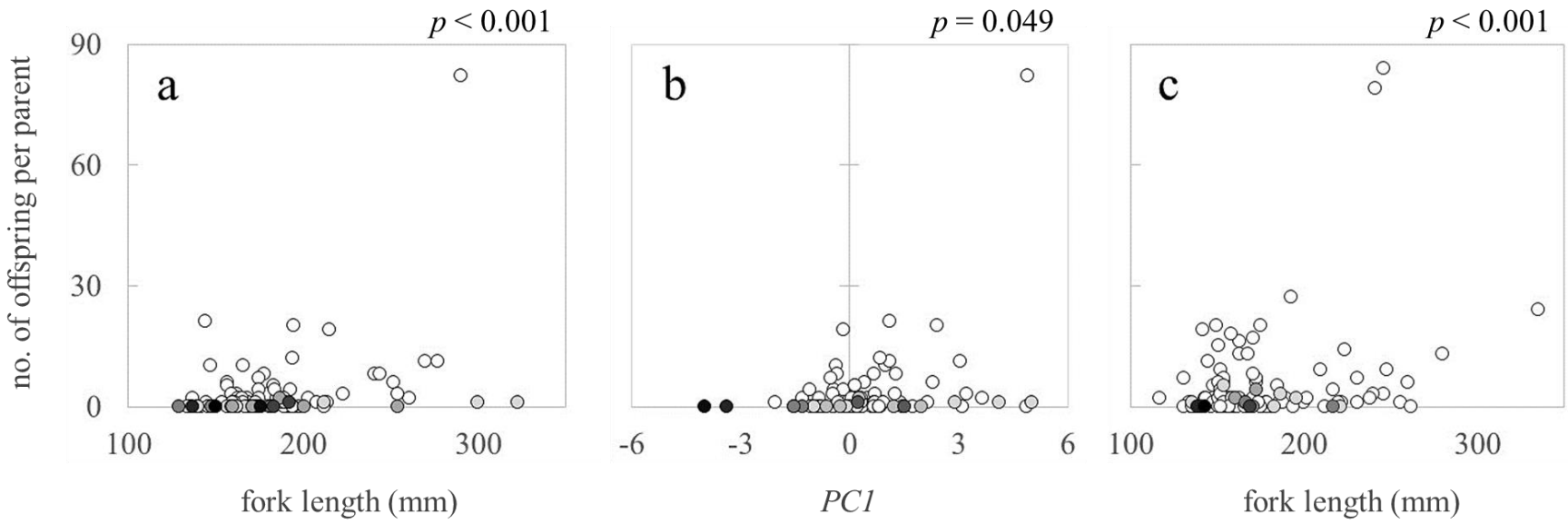

a)
no. of offspring per parent
90
80
20
10
0
0
0.5
1
b)
median number of offspring per parent
6
4
2
0
0
0.2
0.4
0.6
0.8
1
proportion white-spotted charr ancestry

Figure 4.


p < 0.001
p = 0.049
p < 0.001
a
b
c
no. of offspring per parent
90
60
30
0
100
200
300
-6
-3
0
3
6
100
200
300
fork length (mm)
PC1
fork length (mm)

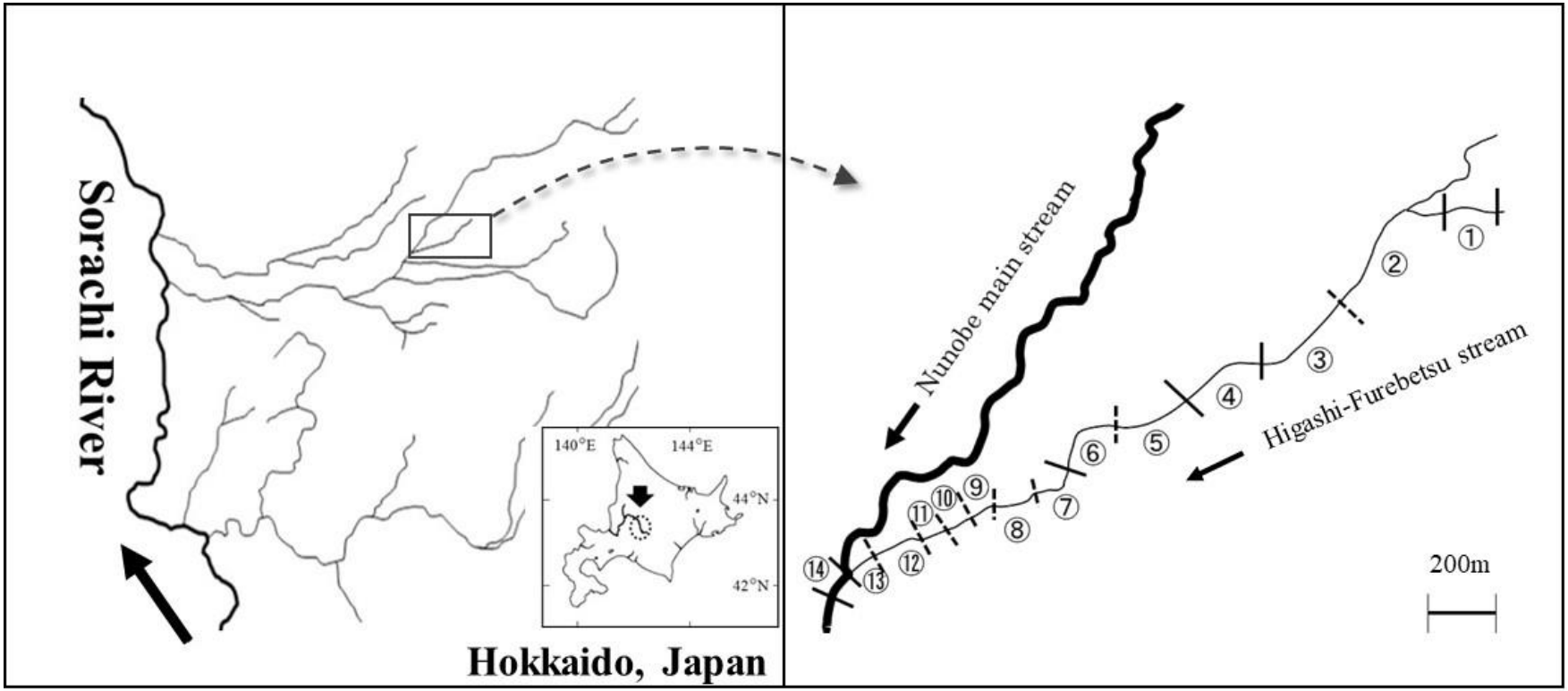


Figure S1. Location of study stream (Higashi-Furebetsu stream) in central Hokkaido, Japan. We established 14 study sections in the study stream. Dashed lines represent dams which have approximately 1m height, respectively, in the study area. Solid lines indicate the boundaries without dam among the study sections.

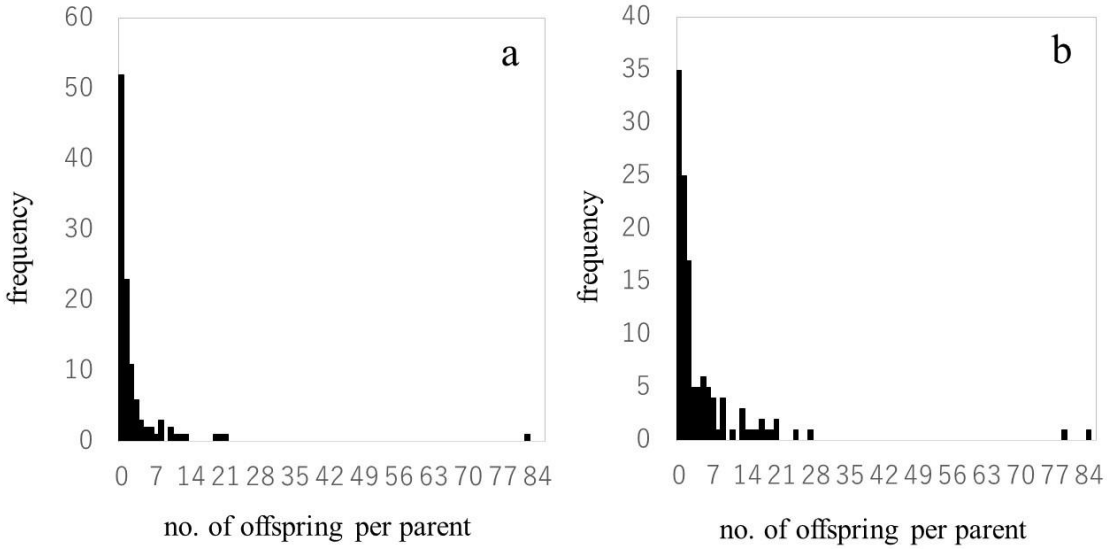


Figure S2. Frequency of assigned offspring in Higashi-Furebetsu stream for male (a) and female (b) brook trout, white-spotted charr, and their hybrids.

Table S1. Characteristics of the microsatellite loci used for parentage analysis of introduced brook trout, native white-spotted charr, and their hybrids in the Higashi-Furebetsu stream.

Locus	*Reference*	*Conc. (µM)*	*T_A (ºC)*	*Alleles*	*He*	*Pe (first parent)*	*Pe (identity)*	*BT size range*	*WSC size range*
Multiplex 1									
Sco200	Dehaan and Ardren 2005	0.17	56.0	24	0.4920	0.1283	0.6878	112-158	140-236
Sco211	Dehaan and Ardren 2005	0.17	56.0	19	0.5830	0.1838	0.7608	228-272	256-300
Sle6	Yamaguchi et al. 2008	0.33	56.0	8	0.5240	0.1388	0.6790	280-282	282-290
Ssa197**	O'Reilly et al. 1996	0.17	56.0	5	0.1520	0.0117	0.2756	148	115-120
SsosL456*	Slettan et al. 1997	0.08	56.0	2	0.1210	0.0073	0.2201	156	160
Multiplex 2									
Smm21*	Crane et al. 2004	0.44	56.0	4	0.1540	0.0119	0.2752	144	114
Otsg83b*	Williamson et al. 2002	0.22	56.0	16	0.2970	0.0448	0.2458	75-79	160-226
Multiplex 3									
Mst85**	Presa and Guyomard 1996	0.07	56.0	5	0.5040	0.1274	0.6807	154-166	120
Sfo12**	Angers et al. 1995	0.29	56.0	11	0.2900	0.0438	0.4810	270-272	208-240
Otsg253b*	Williamson et al. 2002	0.07	56.0	10	0.6140	0.2058	0.7870	136-160	84-88
Sco216	Dehaan and Ardren 2005	0.21	56.0	3	0.1510	0.0113	0.2673	141-153	141
Sco220	Dehaan and Ardren 2005	0.29	56.0	28	0.7630	0.3867	0.3867	264-312	280-380
Omm1105	Rexroad et al. 2002	0.14	56.0	23	0.7470	0.3636	0.9048	122-152	134-206

Conc., final PCR concentration of forward and reverse primers; T_A, annealing temperature; Alleles, total number of observed alleles; H_E, expected heterozygosity; P_E (first parent), exclusion probability for the first parent; P_E (identity), average probability that the set of loci will fail to differentiate between two randomly-selected individuals; Combined exclusion probabilities were 0.8549 and >0.9999 for P_E (first parent) and P_E (identity), respectively. Calculations were made using CERVUS 3.0.7. The size range and of alleles observed at each locus for each species which has >99.9% of own genetic admixture is given. ** Loci are diagnostic between white-spotted charr and brook trout described by previous studies, and * loci shows diagnostic between white-spotted charr and brook trout indicated by the present study.

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Chapter 4

HYBRIDS AS POTENTIAL MEDIATERS SPREADING THE NON-NATIVE GENES: COMPARISON OF SURVIVAL, GROWTH, AND MOVEMENT AMONG NATIVE, NON-NATIVE, AND HYBRID CHARRS

Abstract

Comparing multiple fitness components and potential movement of the wild hybrids with their parental species is necessary to fully understand the consequences of human-mediated introgression, but studies tracking both parental species and their hybrids at individual-level are limited. Here I compered the growth, survival, and movement of sympatric introduced brook trout (BT: *Salvelinus fontinalis*) and native white-spotted charr (WSC: *S. leucomaenis*) with their hybrids (HYB) in a stream of the northern Japan, using mark-recapture [passive integrated transponder (PIT) tags] data collected over 4 years (1087 marked individuals). The growth data from a single cohort showed that HYB grew faster than BT and WSC. In addition, there is no evidence that the hybrid survival was lower than the both parental species throughout the entire study periods. Furthermore, HYB showed high mobility similar to WSC, while BT showed the lowest mobility. Together, although my previous studies have documented the reductions of BT distribution and HYB reproductive success, non-native genes can pose a threat to native WSC via relatively higher survival, growth, and/or mobility of HYB.

Keywords: introgression, introgressive hybridization, native charr, fitness components, invasive species, growth rate, survival rate, mark-recapture, pit-tag, movement,

Introduction

Hybridization and introgression with non-native species are serious threats to native species worldwide (Rhymer & Simberloff, 1996; Allendorf et al., 2001). The irreversible effects of introgressive hybridization between native and non-native species can cause the breakdown of inherent gene complexes and ecological adaptations in native populations, which can contribute to the extinction of rare or endangered taxa (Rhymer & Simberloff, 1996). The rate of hybridization and introgression can further be exacerbated by increasing land use and global climate change (Allendorf et al., 2001; Muhlfeld et al., 2014; Muhlfeld et al., 2017). Therefore, there is a growing need to better understand the relative difference in fitness between hybrid and parental species under natural conditions because this is the most informative approach for estimating and forecasting the effects of hybridization (Svedin et al., 2008).

Although field studies on quantifying a fitness component of hybrids between non-native and native species are getting increased (e.g. Muhlfeld et al., 2009; Casas et al., 2012), much less have compared multiple fitness components of hybrids with that of the parental species owing to the difficulty of long-term individual tracking. As selection pressure against hybrids should shift throughout their life-stages (Lemmon & Lemmon, 2010), estimating hybrid fitness components in a limited period can mislead the effects of

introgression. Furthermore, consequences of introgression may depend on dispersal ability of hybrids. Kovach et al. (2014) have documented that the continuous immigration of hybrid adults from neighborhood populations is likely to sustain continuous introgressive hybridization, despite strong natural selection against hybrids (Muhlfeld et al., 2009). Nevertheless, dispersal has been frequently ignored (Nathan, 2001; Kovach et al., 2014) because it is generally difficult to measure the dispersal of hybrids simultaneously with their parental species in the wild. Thus, most studies on human-mediated hybridization have poorly understood the potential importance of hybrids as a mediator spreading non-native genes and also the life-stages of hybrids upon which strong selection act. Such information is essential for the effective management and conservation of native biodiversity (Ellstrand et al., 1999; Allendorf et al., 2001; Fitzpatrick et al., 2010).

Fishes are particularly prone to hybridization and introgression because of their external fertilization system and the absence of strong prezygotic reproductive barriers (Hubbs, 1955; Scribner et al., 2001). In addition, introductions of non-native fishes are an issue worldwide (Rahel & Olden, 2008) and hybridization with native species is an increasing concern (Aboim et al., 2010). These characteristics of fishes allow enough samples of wild hybrids for quantifying multiple fitness components. Furthermore, stream-dwelling fishes serves as an excellent model system to measure the dispersal distance of individual (e.g. Pépin et al., 2012; Terui et al., 2017), which should be an indication of gene flow potential. Because stream fishes generally move linearly along the stream by continuously established study sections, I can easily measure the distance each individual moved.

Brook trout (BT: *Salvelinus fontinalis*) is the second most widely introduced salmonid (49 countries; Fausch, 2008), and hybridization with non-native BT threatens the

existence of native charr species in North America and Japan (e.g. Leary et al., 1993: DeHaan et al., 2010: Kitano et al., 2014: Fukui et al., 2016). In North America, a rapid displacement of bull trout (*S. confluentus*) by hybridization with BT was documented (Leary et al., 1993; Leary et al., 1995). In the Far East, introgressive hybridization between introduced BT and native white-spotted charr (WSC: *S. leucomaenis*) have been described in the upper Sorachi River, Hokkaido, Japan (Kitano et al., 2014; Fukui et al., 2016). Although I recently demonstrated the lower reproductive success of hybrids (HYB) compared to the parental species (Fukui et al. 2018), there is no study evaluating other fitness component(s) and movement of hybrids.

The main questions of this study are: 1) whether hybrids have lower survival and growth from juvenile to adult stage compared to parental species, 2) in which life-stage hybrids suffer natural selection, and 3) whether HYB spread non-native genes by dispersal. To answer these questions, I first compared the survival, growth, and body size of HYB with those of BT and WSC using 569 PIT (passive integrated transponder)-tagged individuals that were born in 2013 (a single cohort). Body size and growth rate is closely tied to fitness especially in salmonids, because they determine the establishment of territories (and thus, foraging success and mating success) and also affect age at the first maturation (Shearer & Swanson, 2000). I also compared the dispersal distance of HYB with that of BT and WSC using 1087 PIT-tagged individuals over 4 years. Although dispersal distance is not necessarily equivalent to gene flow, this should be a good indicator for the spread of non-native genes (Kovach et al., 2014).

Materials and methods

This study was conducted in the Higashi-Furebetsu stream, a tributary of the upper Nunobe stream, in central Hokkaido, Japan (Fig. S1). Based on species distribution data from a previous survey (Fukui et al., 2016), I considered that the tributary was suitable to investigate the interactions between WSC and BT because both species and their hybrids were consistently observed and the tributary size was relatively small (2–4 m in stream width). Fourteen contiguous sections from a 2,615 m length stream reach were used as permanent study sites with a length of 100–425 m (mean ± SD = 186.8 ± 71.2 m) in each section. Other fish species collected during the field survey included freshwater sculpin (*Cottus nozawae*) and Siberian stone loach (*Barbatula barbatula*). Annual mean water temperature was 7.1 ºC, ranging from 0.6 to 14.7 ºC during July 2015 – July 2016 (measured at the section 6, Fig. 1a).

The field survey in the Higashi-Furebetsu was conducted eight times, August, October and December 2013, May, August and October 2014, July 2015, and October 2016. Two-four pass backpack electrofishing (200-300 V) was conducted in each section. The captured fish were anesthetized, identified to species based on appearance (Nakabo, 2000), and measured the fork length (nearest mm). Individuals with ambiguous wavy lines on the dorsal fin were also identified as putative hybrids between BT and WSC (Kitano et al., 2014). My visual species identification for BT, WSC, and HYB had 83% accuracy (164 of 204 individuals) based on species determination using 13 microsatellites loci: misclassification generally occurred for later-generation (post-F1) hybrids, whereas pure BT and WSC, as well as F1 hybrids, were identified correctly (S. Fukui, unpublished data). Although I could not determine the details of hybrids genetically for logistical reasons, I

believe that the relatively high classification will not significantly bias the results. Individuals exceeding 47 mm in fork length were implanted with 8 mm PIT tags into the body cavity during the first four field surveys (i.e. from August 2013 to May 2014). After tagging, fish were released back into the sections from which they were captured. Individuals of 2013 cohort, which were used for subsequent growth and survival analysis, were distinguished from other generations based on length-frequency histogram (Fig. 1b).

Given that growth can be age dependent, I used the single cohort born in 2013. I used only the individuals captured on two consecutive sampling occasions for measurements of seasonal growth rate (mm/day). Of the eight sampling occasions, data of the first seven sampling were used because few tagged individuals were collected in October 2016 probably because of longevity. To test the difference in the growth rate among species, one-way ANOVA was conducted for each of the six periods. I also compared body size trajectory among species. I firstly applied the von Bertalanffy growth curve, but the data was not fitted because the body size did not approach plateau (Fig. 1c). Thus, analysis of covariance (ANCOVA) was used to test the differences in growth rate between the hybrids and their parental species using the age (month) as a covariate. The interaction between species and age was also tested. ANCOVA was performed with the software R version 3.2.4 (R Development Core Team, 2011).

Apparent survival, together with capture probability, was estimated using Cormack-Jolly-Seber (CJS) models in the program MARK (White & Burnham, 1999). Survival estimates in CJS models do not distinguish between death and emigration and are therefore minimum estimates of true survival (Lebreton et al., 1992). Before beginning the analysis, I formulated an *a priori* list of candidate models (number of candidate models = 40) that reflected hypotheses concerning the effects of age (i.e. age-0+ or older), species,

hybrid or not, season (i.e. overwinter or not) on survival. I also hypothesized that capture probability would vary by sampling effort (i.e. the number of electrofishing pass), age, and species. Theses hypotheses were included in the candidate models. I used model selection methods based on the Akaike Information Criterion corrected for small sample size and over-dispersion (ΔAICc) to identify the best predicted model and other models with ΔAICc ≤ 2, which are considered to be statistically equivalent (Burnham & Anderson, 2002).

I examined within-study section dispersal of recaptured BT, WSC, and HYB by recording the distance between the initial capture section and recaptured section. Distance was calculated using the center of each section. I only used individuals that were recaptured in consecutive sampling events. Dispersal distance and direction were evaluated during the six intervals between my seven consecutive field-observations. The estimated dispersal distance was log-transformed for GLMM with normal distribution to determine whether the dispersal significantly differed among species. The section ID where individuals were recaptured was used as a random factor in the GLMM. I also used individual ID as a random factor because some individuals were recaptured more than once.

Results

Non-native BT was dominant in the Higashi-Furebetsu stream: of the 1087 pit-tagged fish, 761 (70.0%) were BT, 265 (24.4%) were WSC, and 61 (5.6%) were putative hybrids (Fig. 1b). Of the tagged fish, 597 individuals (54.9 %) were recaptured at least once throughout the field surveys, and 569 (52.3%) were assigned to be the cohort born in 2013. Of the cohort, 368 (64.7%) were classified to BT, 154 (27.1%) to WSC, and 47 (8.3%) to putative

hybrids.

The range of body size was relatively wide (41–387 mm) (Fig. 1b), and individual body size trajectories also showed a rapid growth and high variation (Fig. 1c). Fork lengths of WSC and HYB were longer than that of BT throughout the study period. One-way ANOVA found that HYB growth rate (mm/day) were significantly greater than BT in only a single period, from summer to autumn in 2014 (P = 0.023). ANCOVA revealed that HYB became larger than WSC in later periods as shown by the significant interaction term and initial body size was smallest in BT (Fig. 1c, Table 1). Growth rate was strongly seasonal (Fig. 1d). Growth rates were generally low (< 0.2 mm/day) across the study period except during spring to summer in 2014 (Fig. 1d). During this term (i.e. age-1+ spring-summer), growth rate was high and variable among/within parental species and their hybrids (0.4–0.8 mm/day with 75% interval). A significant difference of growth rate among the hybrids and parental species was found only in a period from summer to fall of 2014 (i.e. age-1+): HYB had higher growth rate ($F_{1, 45}$ = 6.1035; p = 0.017).

My best model for survival was species dependent (i.e. WSC, HYB, or BT) with capture probability varying by “sampling effort (i.e. pass number of electrofishing)”, “age (i.e. 0+age or more than 1+ age)”, and “species” (Table 2). The second model, which included the interaction of “hybrids or parental species” and “age”, showed ΔAICc < 2. The third model, which included the interaction of “winter season” and “age”, also showed ΔAICc < 2. The most important variable was the “species” as it appeared in the top three models. According to the best model, apparent survival was highest in HYB (ϕ > 0.999 ± <0.001; Fig. 2), followed by BT (ϕ = 0.830 ± 0.019), and lowest in WSC (ϕ = 0.458 ± 0.037).

Of the 706 dispersal events detected, 524 (74.2%) were BT, 120 (17.0%) were

WSC, and 62 (8.8%) were HYB. The mean distances moved (± SD) were 98.2 ± 224.8 m, 259.6 ± 441.8 m, and 221.8 ± 322.3 m for BT, WSC, and HYB, respectively. Median distances moved were 0 m for all the species and 75th quantiles were 118.5 m, 385.0 m, and 385.0 m for BT, WSC, and HYB, respectively. Proportion of long-distance (> 100 m) movement was highest in HYB (45.2% of the 28 events), followed by WSC (44.2% of the 53 events), and lowest in BT (28.8% of 151 events) (Fig. 3). GLMM showed that the dispersal of HYB was significantly greater than BT, while there was no statistical difference between WSC and HYB (Table 3).

Discussion

While field studies quantifying a single fitness component of hybrids between non-native and native species are getting increased (e.g. Fitzpatrick & Shaffer, 2007; Muhlfeld et al., 2009), much less studies have compared multiple fitness components. To evaluate hybrid fitness using several fitness components is important because selection pressure against hybrids should shift throughout their life-stages (Lemmon & Lemmon, 2010) and dispersal of hybrids can sustain introgression (Kovach et al., 2014). I firstly tested whether selection occurred against HYB by comparing survival and growth rate with parental species in the system where selections were observed during reproduction and hatching stages (Fukui et al., 2018). I found no evidence of reduced survival or growth in HYB: rather HYB survival and growth were higher than parental species. Secondly, I compared HYB dispersal with BT and WSC to evaluate the potential effect of gene flow or introgression. HYB showed high dispersal tendency comparable to WSC, while BT showed the lowest. Taken all the

results together, despite lower reproductive success of HYB and low mobility of BT, HYB with high growth, survival, and mobility can sustain introgressive hybridization to native WSC. Although I did not determine the degree of genetic admixture for each hybrid (e.g. F1, post-F1), the clear patterns and high visual identification of hybrids (83%, S. Fukui, unpublished) assured the validity of the present study.

Growth

Previous lab experiments using brook trout, white-spotted charr, and their hybrids, demonstrated that F1 hybrids from female BT and male WSC had slower growth compared to parental species, whereas another side combination of F1 hybrids and most hybrid crosses (F2 and backcross) had the same or higher growth rate (Suzuki & Fukuda, 1971; Suzuki & Fukuda, 1974). In my field study, reduced growth was not observed: rather, hybrids grew faster in some period. Body size trajectory also indicates higher growth of HYB, although there is some possibilities that size dependent mortality/dispersal generated this pattern. Hybrid vigor is often suggested as a reason for higher fitness of hybrids (Lynch, 1991; Rieseberg et al., 1999; Fitzpatrick & Shaffer, 2007; Lippman & Zamir, 2007) and is generally found in F1 hybrids because hybrid vigor results from heterozygote advantage. In the Higashi-Furebetsu River, however, most hybrids were of post-F1 (i.e. the later generation hybrids) (Fukui et al. 2018) and, therefore, hybrid vigor is probably unlikely.

Alternatively, hybrids may invest more energy to body growth compared to the parental species because of the lower fertility. Although F1 and post F1 hybrids between BT and WSC have some fertility (Suzuki & Fukuda, 1971; Suzuki & Fukuda, 1973b; Suzuki &

Fukuda, 1974; Fukui et al. 2018), gonads of HYB (F1 and post F1 hybrids) appeared to be not always fully developed (Suzuki & Fukuda, 1993b; Suzuki & Fukuda, 1974; S. Fukui, unpublished data). Energy allocation of HYB to growth is also inferred from the results that HYB grew faster than the parental species at a period from summer to fall of 1+ year age (i.e. 2014). Because male WSC and both sex of BT begin to mature at this period (S. Fukui, unpublished), the parental species have to invest more energy to gonadal development rather than somatic, thereby reducing the growth rate.

Another possible explanation for high growth of HYB is that initial body size (i.e. at August 2013) advantage positively affected later growth rate. For stream-dwelling salmonids, it is well known that resource competition largely depend on individual body size (e.g. Nakano, 1995). Therefore, size advantage in early life stages can last long (Einum & Fleming, 2000). The reason why initial body size of HYB was larger than BT is unknown, but many of the HYB in 2013 cohort might have resulted from the mating between female WSC and male BT. Breeding season and, hence, fly emergence is earlier in WSC compared to BT (S. Fukui, unpublished data), resulting in larger body size of WSC in early life stages (e.g. spring-summer age-0+). Because males generally mature earlier within breeding season than females in salmonids (protandry: Morbey, 2000), protandrous BT males might have mated with WSC females.

Growth rates were highest in spring to summer at age-1+ for all the species with large variations, while these were relatively slow in other periods. Such seasonal growth patterns were also found in other stream-dwelling salmonids, including brook trout at native ranges (Carlson et al., 2007; Utz & Hartman, 2009; Xu et al., 2010; Hoxmeier et al., 2013), and appeared to be reflected increased water flow and food variability during spring. Furthermore, growth in summer is generally limited due to increased water

temperatures (Carlson et al., 2007; Xu et al., 2010; but see Hoxmeier et al., 2013), which is consistent to my results. I consider that growth of age-1+ would be the most important for the decision of individual life-history (i.e. age at maturation) and subsequent fitness across the both parental species and the hybrids.

Survival

I found no evidence for lower survival of HYB. Rather, the best model in the CJS models showed that the apparent survival of HYB were greatest. As discussed above hybrid vigor might contribute to the high HYB fitness (Suzuki & Fukuda, 1971), but F1 hybrids were rare in this study stream (Fukui et al., 2018). Alternatively, large body size of HYB at age-0+ winter (mean fork length at December 2013: HYB = 113.3 mm, BT = 84.3 mm, WSC = 110.7 mm) might have reduced HYB mortality, because age-0+ winter can be a critical period for salmonids (Brown et al., 2011) and the body size at this period is a key factor for winter survival (Quinn & Peterson, 1996). Furthermore, because of the poor gonad development at fall of 1+ year-old for HYB (S. Fukui, unpublished), hybrids may able to invest more energy to body growth at prior to the severe winter season. HYB can also store more energy compared to parental species if imperfectly matured HYB exhibit less active reproductive behavior because reproduction is very costly for salmonids (Hutchings, 1994). Such lower reproductive activity could significantly increase the winter survival of HYB.

The estimated survival rates of HYB was close to 1 with <0.0001 confidence interval. This seems not realistic because the model predicts that the hybrid individuals do not die. The reason why the model estimated such an unusually high survival remains unknown because of the complexity of the models (i.e. 40 candidate models with 5 possible

explanatory variables). This might be due to low capture probability of HYB or the inability to separate mortality and emigration. Because the apparent survival includes the emigration to other river stretches, this is probably underestimated (Kéry et al., 2012). Because mobility was highest in WSC (see the next section), the lowest apparent survival might result from emigration. Future study is needed to assess the emigration probably using stationary PIT-tag detector. On the other hand, since BT was more sedentary than HYB, survival of HYB should be higher than that of BT considering higher chance of emigration in HYB.

Movement

My results showed that dispersal of HYB was intermediate between the parental species. This is consistent with a few previous studies that investigated migration behavior of wild hybrids, i.e. between coastal cutthroat trout (*Oncorhynchus clarki clarki*) and steelhead trout (*O. mykiss*) (Moore et al., 2010), and between Swainson's thrushes (Helbig, 1991; Delmore & Irwin, 2014). Dispersal of hybrids are still poorly understood because dispersal is generally difficult to accurately measure in the field and also is a complex process involving multiple biotic and abiotic factors, such as genotypes, competition, ontogeny, and temporary changing environment (Nathan, 2001; Kovach et al., 2014). The intermediate dispersal tendency of the current studies might suggest some additive genetic control of movement behavior.

Although it has been considered that intermediate migration behaviors may reduce hybrid fitness relative to pure species (Helbig, 1991; Delmore & Irwin, 2014), I found higher survival of HYB. For migration (i.e. seasonal return movement between

habitats), the place to move and timing is critical for survival and, therefore, intermediate behavior should be maladaptive. For non-migrating species, dispersal may be advantageous especially in spatially or temporally variable environment. Since streams are dynamics system, favorable habitats, such as foraging and refuge areas, change by space, time and ontogeny for stream fishes (Gowan & Fausch, 2002; Petty & Grossman, 2010; Koizumi et al., 2013). As a result, movements are potentially important for HYB individuals to exploit heterogeneous habitats and maximize fitness.

Hybrid fitness components and management implication

To date, most studies on ecological isolation have evaluated hybrid fitness during a single or limited portion of the life cycle (e.g., Fitzpatrick & Shaffer, 2007; Lancaster et al., 2007). Such studies can mislead the consequences of introgression. In my case, for example, as HYB showed lower reproductive success than parental species (Fukui et al., 2018), one might think that non-native BT genes will be excluded in the near future. On the other hands, my view including the survival and growth results indicates that the HYB which successfully developed until juvenile can contribute to sustain the introgression for long-term. Whether introgression persists/spreads depends on the relative strengths of selection and dispersal at different life stages (e.g. Kovach et al., 2014). Similar results have been also found by Lemmon & Lemmon (2010): although hybrids were disfavored in mate choice and fertilization, hybrid viability was greater than parental species in chorus frogs (*Pseudacris* spp.). From a conservation perspective, empirical studies evaluating multiple fitness components of hybrids would provide not only accurate basic knowledges on the effects of introgression but also important knowledges for the management of non-native

genes, such as “how to remove the hybrids”.

In this study case, selectively removing HYB at the juvenile stage may be more effective to protect pure WSC populations, because there should be a stronger negative influence on WSC in the life stages from juvenile to adult where HYB performance (i.e. survival and growth) is high. Further studies should evaluate resource competition (e.g. food, territory) during these life stages among the native, nonnative and hybrid species.

Table 1. ANCOVA result of for growth (fork length) trajectories of hybrids (HYB), compared with the parental species (brook trout, BT, and white-spotted charr, WSC).

	Estimate	s.e.	*t*-value	*p*–value
Intercept	70.834	3.587	19.75	< 0.001
Monthly age	19.114	0.840	22.758	< 0.001
Brook trout	-20.070	3.842	-5.224	< 0.001
White-spotted charr	5.871	4.270	1.375	0.169
Monthly age × brook trout	-2.218	0.898	-2.469	**0.014**
Monthly age × white-spotted charr	-3.748	1.190	-3.151	**0.002**

Table 2. Ranking of Cormack-Jolly-Seber models estimating survival (*S*), capture probability (*P*) for the 2013 cohort of introduced brook trout, native white-spotted charr, and their hybrids, using program MARK.

Model	AICc	Δ AICc	*Wi*	*K*	Deviance
S(species), *P*(pass + age + species)	2448.72	0.00	0.47	21	315.10
S(age*winter + species), *P*(pass + age + species)	2450.51	1.79	0.19	24	310.62
S(age + species), *P*(pass + age + species)	2453.79	5.06	0.04	24	313.90
S(age + species + winter), *P*(pass + age + species)	2458.53	9.81	0.00	30	306.02
S(age*winter + species), *P*(pass + age)	2462.33	13.60	0.00	12	347.27

Table 3. GLMM results of hybrid mobility, compared with the parental species. Site ID and sample ID were used to the random effect.

factor	coefficient	s.e.	*z*-value	*p*-value
(intercept)	3.0122	0.3382	8.9058	**<0.0001**
Compared with WSC	0.1556	0.3726	0.4174	0.6764
Compared with BT	-0.6911	0.3287	-2.1022	**0.0355**

Figure Legends

Figure 1. Annual water temperature at a mediate section (i.e. section 6) in 2016 (a), length-frequency histograms (b), growth trajectories of individuals captured seven times of introduced brook trout (open circle and dotted black line), native white-spotted charr (solid circle and solid black line), and their hybrids (grey circle and bold grey line) (c), and growth rates (median = bold lines, 25th to 75th quantile = box, 2.5–97.5% quantile = whisker) across sampling occasions (d) for sampled introduced brook trout (white), native white-spotted charr (black), and their hybrids (grey).

Figure 2. Estimates of putative survival rate (mean and 95% confidence interval) of introduced brook trout (BT: a open circle), hybrids (HYB: a grey circle), and native white-spotted charr (WSC: a solid circle), using the best model by the model selection with program MARK.

Figure 3. Distance moved by individual introduced brook trout (a,d; n=524), white-spotted charr(b,e; n=120), and their hybrids (c,f; n=62) recaptured in consecutive sampling events. Left graphs (a-c) are used the absolute value of the distance moved. Negative numbers in d-f are downstream movement.

Figure 1.

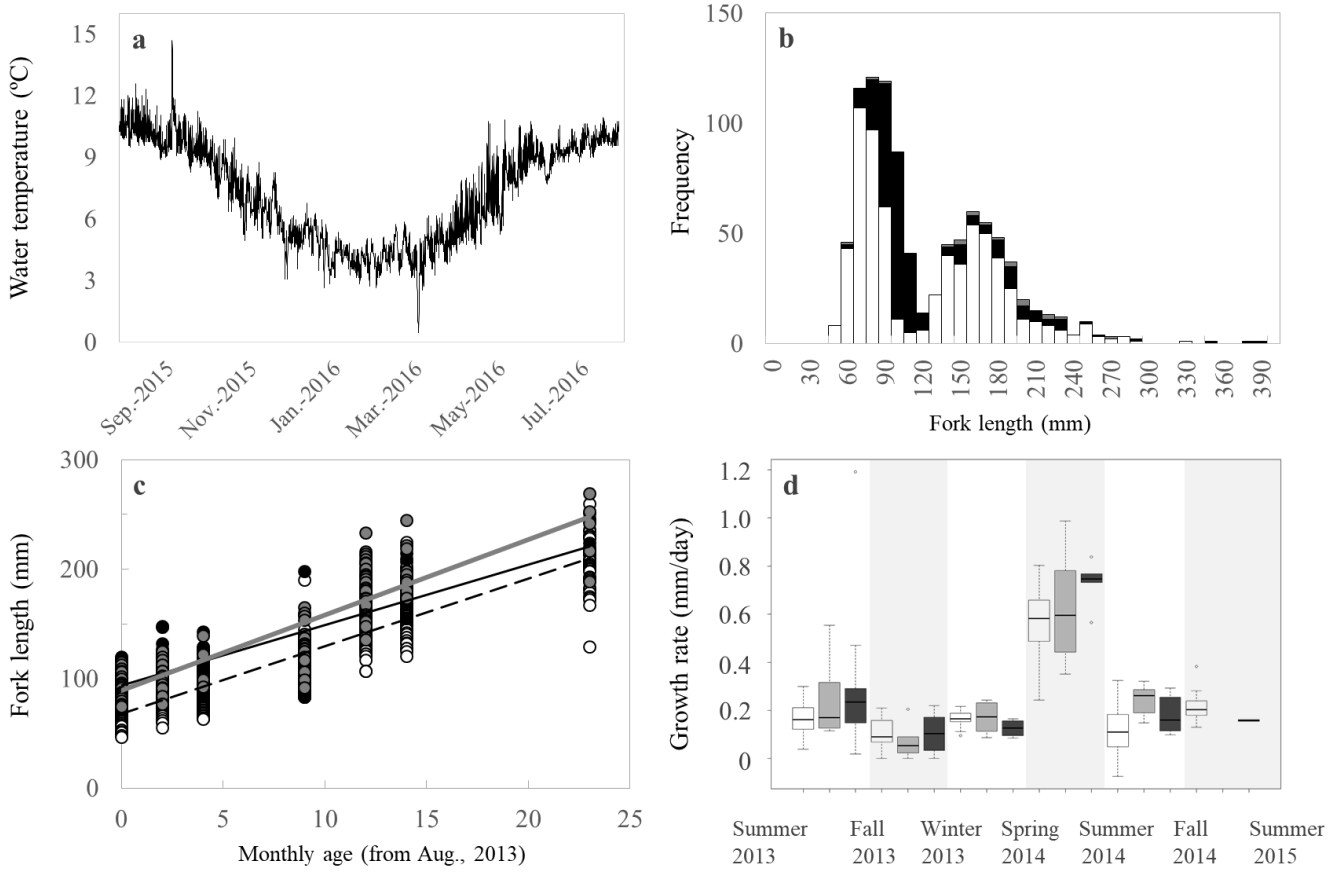

a
Water temperature (ºC)
15
12
9
6
3
0
Sep.-2015
Nov.-2015
Jan.-2016
Mar.-2016
May-2016
Jul.-2016
b
Frequency
150
100
50
0
0
30
60
90
120
150
180
210
240
270
300
330
360
390
Fork length (mm)
c
Fork length (mm)
300
200
100
0
0
5
10
15
20
25
Monthly age (from Aug., 2013)
d
Growth rate (mm/day)
1.2
1.0
0.8
0.6
0.4
0.2
0
Summer 2013
Fall 2013
Winter 2013
Spring 2014
Summer 2014
Fall 2014
Summer 2015

Figure 2.

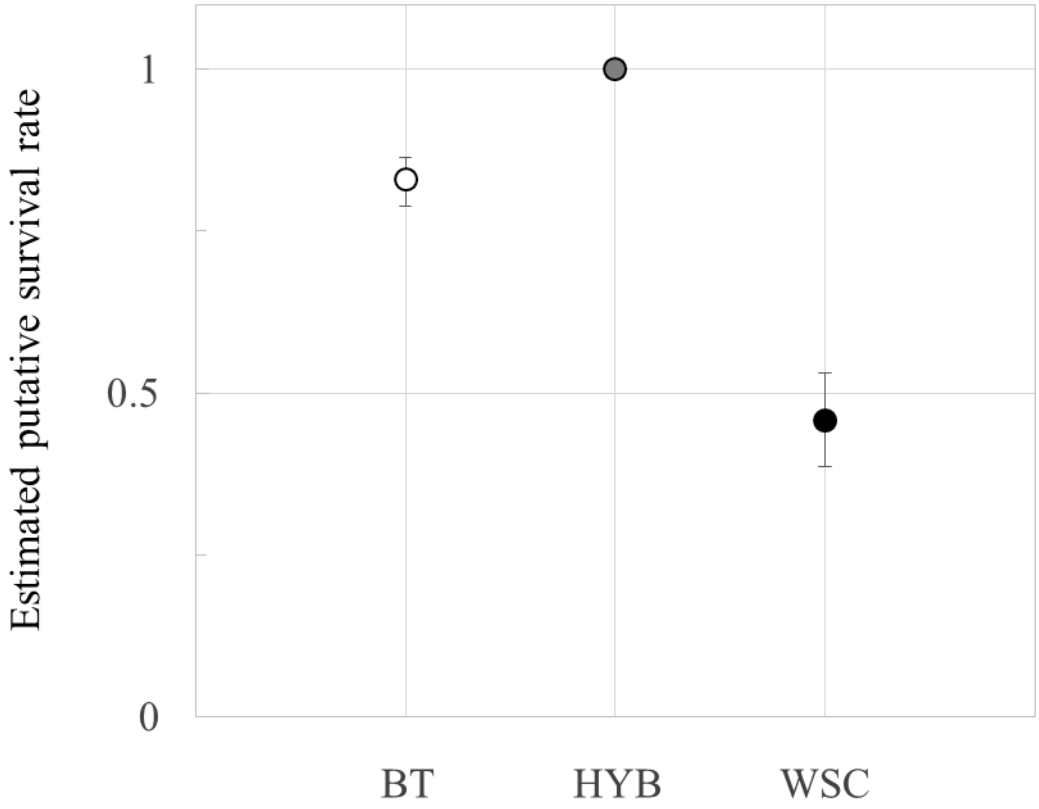
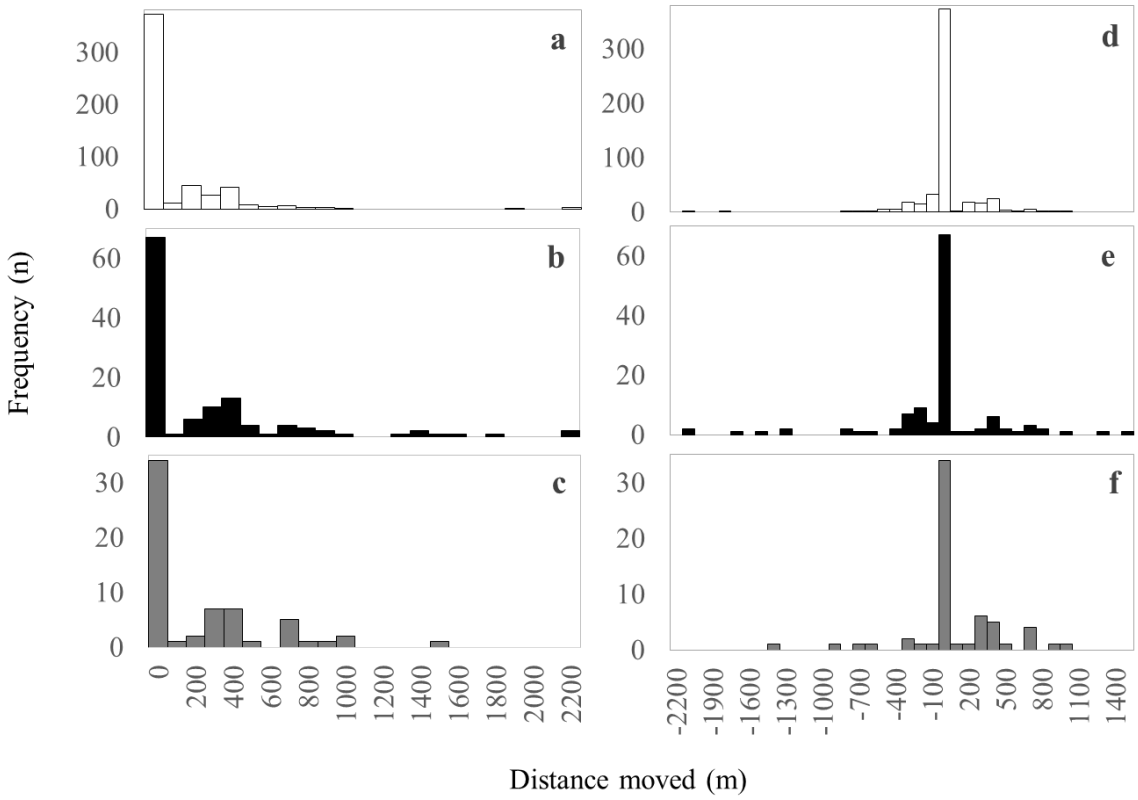


Figure 3.


a
b
c
d
e
f
Frequency (n)
Distance moved (m)
300
200
100
0
60
40
20
30
10
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
-2200
-1900
-1600
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500
800
1100
1400

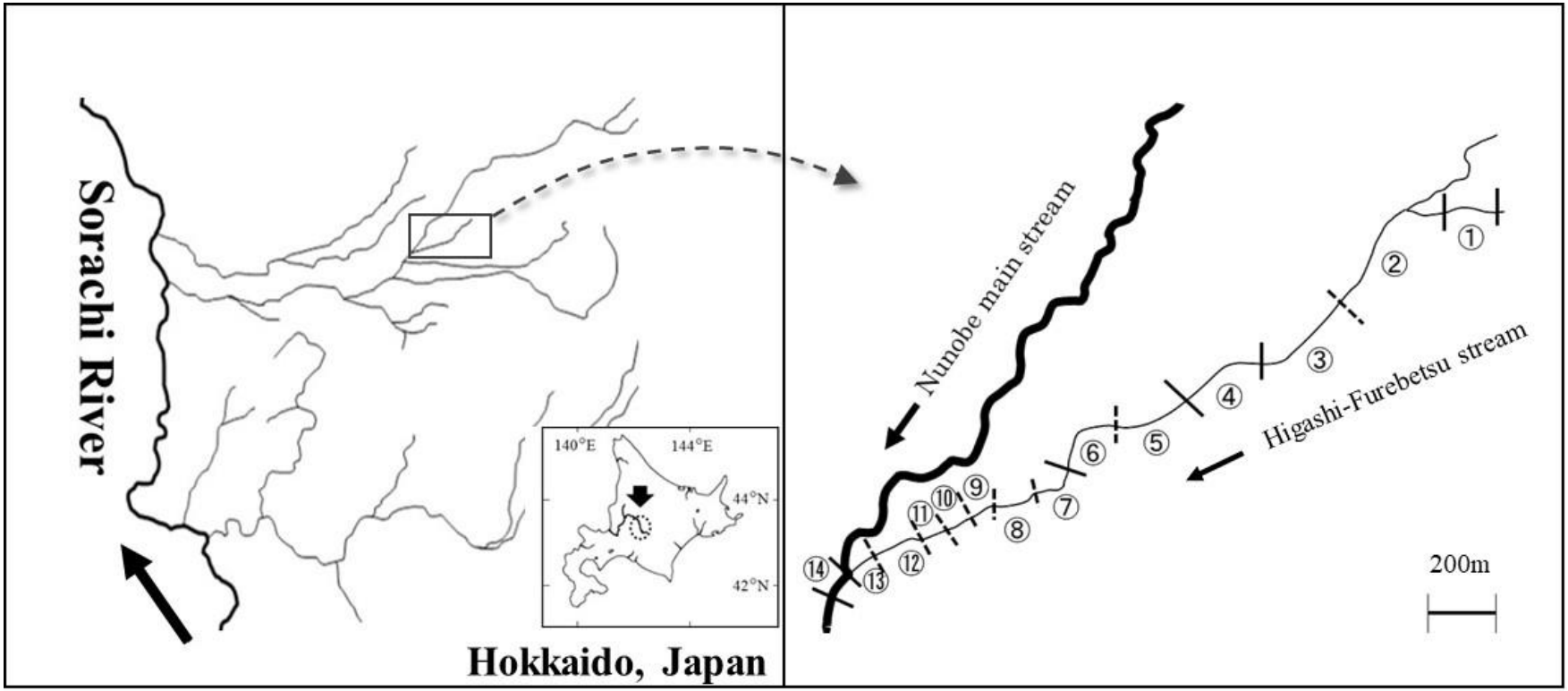

Figure S1. Location of study stream (Higashi-Furebetsu stream) in central Hokkaido, Japan. We established 14 study sections in the study stream. Dashed lines represent dams which have approximately 1m height, respectively, in the study area. Solid lines indicate the boundaries without dam among the study sections.

Chapter 5

CONCLUSIONS AND MANAGEMENT RECOMMENDATIONS

General discussion

The fundamental question of my dissertation was how the introgressive hybridization between non-native BT and native WSC can be sustained in the wild. I found that introgression in the upper Sorachi River has been sustained for more than 10 years due to a combination of three mechanisms: (1) introgression of more adaptive male secondary sexual characteristics derived from non-native BT; (2) relaxed natural selection against HYB from juveniles until maturation; and (3) high mobility of HYB. This thesis underscores the importance for jointly investigating multiple fitness components and dispersal ability of hybrids.

As consequences of the hybridization, a series of my studies showed the different biological features between WSC and HYB. First, male secondary sexual characteristics have a genetic component and introgression altered the reproductive morphology of WSC. As it is considered that such developed secondary sexual characteristics contribute to reproductive success through male-male competition and female choice (Fleming & Gross, 1994; Quinn & Foote, 1994), hybrids would disturb the mating behavior and sexual selection of WSC. Second, I found that even small amounts of introgression markedly reduce individual reproductive success in the wild. Finally, HYB

had some size advantages over the parental species from the initial life stages. Because the competition is generally size-dependent in salmonids and subdominant are often forced to emigration (Nakano, 1995). High mobility of native WSC might be partially due to the competition from HYB. Future studies are needed to investigate resource competition among the species.

My findings have significant implications not only to the protection and restoration of pure WSC populations, but also to guide the fundamental conservation policy and management decisions. To date, “how much hybridization is acceptable to maintain the evolutionary legacy of a species, subspecies or evolutionarily significant unit?” poses difficult conservation questions owing to little empirical information about long-term consequences of introgressive hybridization. Therefore, under the Ministry of the Environment (MOE), the Ministry of Agriculture, Forestry and Fishery (MAFF), and the Ministry of Land, Infrastructure, Transport and Tourism (MLIT) in Japan, there is no reasonable decisions of conservation and management for hybrid individuals between non-native species and native species (Ministry of Environment in Japan, 2015). My empirical data suggest that the level of anthropogenic introgression should be much less than 20% to maintain individual fitness and protect the biological features of the pure native populations, given that 20% of nonnative genes can dramatically decrease reproductive success. Future studies are necessary to assess whether a very low level of introgression (less than ten percent) impacts the fitness and other biological features, and to test how the results of the present study can be changed in other species and/or environmental conditions.

My results provide an insight to how selection against hybrids shifts across life stages: both sexual selection and outbreeding depression acted in the life stages from

maturation to the juveniles of the next generation: then, the successfully developed HYB juveniles received relaxed selection until maturation. These results are consistent with previous studies (Simovich 1985; Simovich et al. 1991; Parris 1999; Parris et al. 1999; Parris 2001a,b,c). To date, however, only a few studies have quantified multiple individual-fitness components in the wild and have focused on changing natural selection against hybrids (Lemmon & Lemmon, 2010; Lackey & Boughman, 2016). This is particularly true in human‐mediated hybrid zones, compared to extensive studies on natural hybrid zones (e.g. Svedin et al. 2008; Lemmon & Lemmon, 2010; Lackey & Boughman, 2016). Therefore, in which life-stage hybrids with non-native genes suffer stronger natural selection, have much less known, despite this question is critical for deciding conservation planning.

I conclude that native WSC populations are placed in a large conservation risk by the long-term sustainable introgression from non-native BT. Given future climate changes, the presence and degree of hybridization may be more proceeded, as suggested in other areas (e.g. Muhlfeld et al. 2009; 2014). For planning of the most effective management to suppress or eliminate the non-native BT genes, some of management schemes that may prove useful in stopping or slowing the progress of hybridization: (1) selectively removing hybrid juvenile; (2) installing barriers to isolate non-hybridized WSC populations, or to preclude access to other non-hybridized WSC populations used by hybrids; and (3) to keep or modify flow and habitat conditions that favor native WSC. My results also suggests that adaptive reproductive traits of male BT (Chapter 3), relaxed selection against HYB which successfully developed until juvenile (Chapter 4), and a high mobility of HYB (Chapter 4) should pose threats to pure native WSC populations, despite a decreasing distributional BT range (Chapter 2), a lower reproductive success of HYB

(Chapter 3), and the lowest mobility of pure BT (Chapter 4). Further information in other hybrid systems of plants and animals are needed for conservation policy and management decision based on scientific knowledges.

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