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Red and white Chinook salmon: genetic divergence and mate choice

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Abstract

Chinook salmon (*Oncorhynchus tshawytscha*) exhibit extreme differences in coloration of skin, eggs and flesh due to genetic polymorphisms affecting carotenoid deposition, where colour can range from white to bright red. A sympatric population of red and white Chinook salmon occurs in the Quesnel River, British Columbia, where frequencies of each phenotype are relatively equal. In our study, we examined evolutionary mechanisms responsible for the maintenance of the morphs, where we first tested whether morphs were reproductively isolated using microsatellite genotyping, and second, using breeding trials in seminatural spawning channels, we tested whether colour assortative mate choice could be operating to maintain the polymorphism in nature. Next, given extreme difference in carotenoid assimilation and the importance of carotenoids to immune function, we examined mate choice and selection between colour morphs at immune genes (major histocompatibility complex genes: MHC I-A1 and MHC II-B1). In our study, red and white individuals were found to interbreed, and under seminatural conditions, some degree of colour assortative mate choice (71% of matings) was observed. We found significant genetic differences at both MHC genes between morphs, but no evidence of MHC II-B1-based mate choice. White individuals were more heterozygous at MHC II-B1 compared with red individuals, and morphs showed significant allele frequency differences at MHC I-A1. Although colour assortative mate choice is likely not a primary mechanism maintaining the polymorphisms in the population, our results suggest that selection is operating differentially at immune genes in red and white Chinook salmon, possibly due to differences in carotenoid utilization.

Keywords: assortative mate choice, colour polymorphisms, major histocompatibility complex, microsatellites

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Introduction

Understanding the mechanisms promoting the maintenance of variation within and among natural populations is a major goal in the field of evolutionary biology. Specifically, colour polymorphisms are widespread in many taxa and have garnered considerable research interest, as colour traits are often heritable, easily discernable by humans and subject to forces of both sexual and natural selection (Maan & Seehausen 2011; Wellenreuther *et al.* 2014). Colour polymorphisms within species can be observed as discrete colour morphs as well as colour variation that exist across a continuous spectrum. Although many studies have focused on the evolution of colour polymorphisms (see Gray & McKinnon 2007), there is limited empirical data related to the processes that maintain colour polymorphisms in wild populations.

Within populations, the persistence of colour polymorphisms may result from various mechanisms including disruptive selection, frequency-dependent

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selection and/or mate choice (Greene *et al.* 2000; Galeotti *et al.* 2003; Gray & McKinnon 2007; Wellenreuther *et al.* 2014). Disruptive selection may occur if different colour morphs occupy different niches within which fitness can be maximized (Greene *et al.* 2000; Galeotti *et al.* 2003; Munday *et al.* 2003; Hugall & Stuart-Fox 2012). Negative frequency-dependent selection (NFDS) has also long been hypothesized as a mechanism maintaining polymorphisms in nature (Clarke 1962), whereby rare morphs experience a fitness advantage (Sinervo & Lively 1996; Olendorf *et al.* 2006; Takahashi & Kawata 2013). Maintenance of colour polymorphisms may also occur through nonrandom mate choice (Roulin & Bize 2007; Wellenreuther *et al.* 2014), where sexual selection, colour assortative or colour disassortative mating can maintain colour variation within a population (Tuttle 2003; Pryke & Griffith 2007; Elmer *et al.* 2009). For example, Pryke & Griffith (2007) found that in the Gouldian finch (*Erythrura gouldiae*), mates paired assortatively based on head coloration in both wild and captive populations. Assortative mate choice may not by itself maintain polymorphisms; nevertheless, it can act in combination with other selective pressures to retain polymorphisms (Wellenreuther *et al.* 2014).

In nature, Chinook salmon (*Oncorhynchus tshawytscha*) exhibit extreme variation in flesh coloration, where red-coloured individuals coexist with white-coloured individuals with intermediate variants existing across the spectrum. The difference in coloration does not only appear in flesh colour, but also translates into differences in egg and external spawning coloration (Withler 1986). White Chinook salmon do not occur in all rivers, and the percentage of white Chinook salmon within a river can range from 0 to 100% (Hard *et al.* 1989). The difference between red and white Chinook salmon does not appear to reflect diet (Lehnert *et al.* unpublished stable isotope data), but instead results from genetic polymorphisms (Withler 1986). White Chinook salmon have reduced ability to deposit carotenoids into the flesh, eggs and skin, and thus, white individuals have white (or pale) eggs and appear uncharacteristically grey in colour during spawning (see Fig. 1A; Withler 1986; Rajasingh *et al.* 2007). Currently, little is known about the evolutionary mechanisms that maintain the red and white phenotypes in nature.

The maintenance of the colour morphs could be explained if morphs are genetically isolated and undergoing ecological speciation. Reproductive isolation between morphs can be tested using neutral markers (i.e. microsatellites), as genetic divergence between individuals at such loci is expected to occur through reduced gene flow and neutral processes, such as genetic drift, rather than selection (Selkoe & Toonen 2006). In a previous study, genetic isolation of sympatric gold and dark colour morphs of Midas cichlid (*Amphilophus citrinellus*) was revealed by significant genetic divergence between morphs using microsatellite markers (Elmer *et al.* 2009). Additionally, colour-based assortative mate choice could provide another evolutionary mechanism responsible for the persistence of the morphs. Controlled mating experiments are often employed as a first step in testing for mate choice in colour polymorphic species. For example, in cichlid species, laboratory studies have demonstrated that female choice for male coloration plays an important role in speciation (Knight & Turner 2004; Maan & Sefc 2013; Selz *et al.* 2014). In a colour polymorphic population of poison dart frogs (*Dendrobates pumilio*), experiments have shown that red and yellow female frogs show a

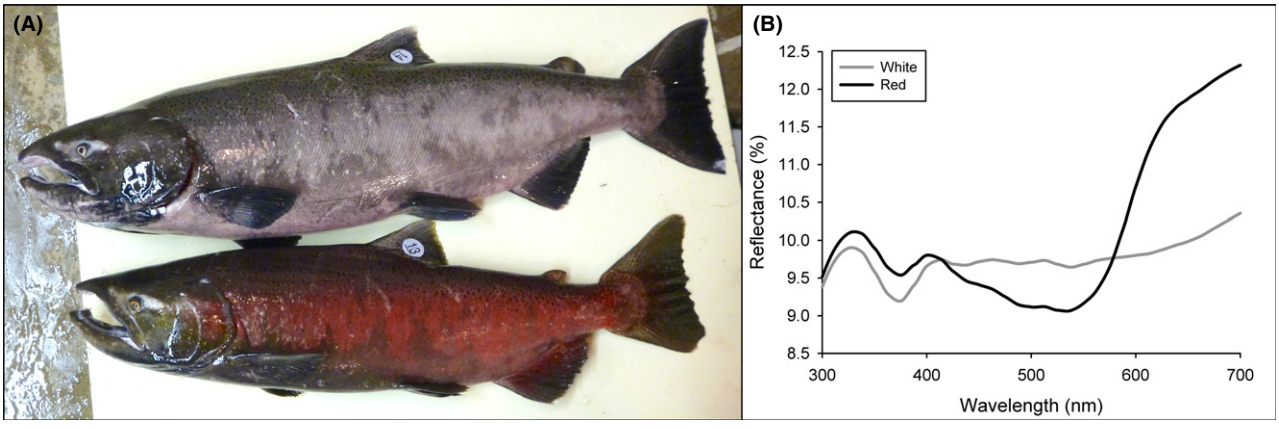

Fig. 1 Photograph (A) of white (top) and red (bottom) Chinook salmon (*Oncorhynchus tshawytscha*) males from the Quesnel River, British Columbia, in the fall of 2013, and mean reflectance spectra (B) for spawning red (n = 19) and white (n = 18) Chinook salmon, including both males and females, captured from the Quesnel River in the fall of 2014.

strong preference for males with the same colour phenotype as their own (Richards-Zawacki & Cummings 2011).

If morphs are not reproductively isolated and mating occurs randomly with respect to colour, mate choice and/or natural selection acting on functional genes could be responsible for the coexistence of red and white individuals. Provided that carotenoids act as immunostimulants and antioxidants (Nakano *et al.* 1995; Blount *et al.* 2003; Faivre *et al.* 2003; Amar *et al.* 2004; but see Costantini & Møller 2008), natural selection may operate differentially on immune genes in red and white individuals. For example, in rainbow trout (*Oncorhynchus mykiss*), circulating levels of carotenoids can be correlated to immunoenhancement (Amar *et al.* 2004, 2012) and protection from oxidative damage (Nakano *et al.* 1995). Similarly, carotenoid supplementation reduces production of endogenous antioxidant enzymes in fishes including olive flounder (*Paralichthys olivaceus*; Pham *et al.* 2014), characins (*Hyphessobrycon callistus*; Wang *et al.* 2006) and yellow croaker (*Pseudosciaena crocea*; Li *et al.* 2014). In Chinook salmon, reduced carotenoid storage in muscle tissue prior to maturation limits the ability of females to move pigments into plasma, eggs and skin (Garner *et al.* 2010b). Thus, white Chinook salmon may experience greater oxidative stress and be immune compromised relative to red conspecifics, unless white individuals have evolved means to counteract these handicaps. One possibility is that white Chinook salmon may benefit from differences at major histocompatibility complex (MHC) immune loci. Two distinct classes (class I and class II) of MHC genes are found in salmon (Miller *et al.* 1997). Class I molecules are typically associated with response to viruses, as the molecules bind peptides derived from intracellular antigens, whereas class II molecules bind peptides derived from extracellular antigens and thus are more commonly associated with bacterial response (Klein 1986). It is possible that mate choice and/or selection operates to increase heterozygosity or to favour specific MHC alleles, both mechanisms having been demonstrated to confer fitness advantages (McClelland *et al.* 2003; Kjøglum *et al.* 2006; Evans & Neff 2009; Evans *et al.* 2010). Given that salmon mate nonrandomly relative to MHC genotype (Landry *et al.* 2001; Neff *et al.* 2008; Yeates *et al.* 2009), we test the hypotheses that mate choice and natural selection contribute to the persistence of the colour phenotypes.

In this study, we investigate a population of colour polymorphic Chinook salmon in the Quesnel River, within which red and white individuals coexist in relatively equal proportions (Withler 1986). First, we test for genetic divergence between red and white individuals at microsatellite marker loci to determine whether reproductive isolation exists between the morphs. Next, we examine mate choice in the system to test whether colour assortative mate choice could be a mechanism acting to maintain the polymorphism in nature. Mate choice is examined under experimental conditions in seminatural spawning channels and determined using behavioural analyses as well as genetic parentage assignment of offspring. Subsequently, genetic differences between morphs at two functional immune genes (MHC class I and class II) were assessed to evaluate the possibility of differential selection pressure operating on immune genes between morphs. Finally, given the importance of MHC genes to mate choice in salmonids (Landry *et al.* 2001; Neff *et al.* 2008; Yeates *et al.* 2009), we test whether nonrandom mate choice at the MHC class II gene can explain the persistence of the two morphs. The coexistence of red and white Chinook salmon in the Quesnel River system provides a unique opportunity to examine the fitness consequences of carotenoids in a controlled and quantitative fashion, thus allowing us to test the long-standing hypotheses about why salmon are red.

Methods

Field collection

Adult Chinook salmon were netted from the Quesnel River in Likely, British Columbia, Canada, during two spawning seasons. Fish were collected from September 13 to 30 in 2013 and September 18 to October 1 in 2014. Fish that were in good condition and that did not exhibit signs of having already spawned were transported in holding tanks with aerated river water approximately 15 min (5 km) to the Quesnel River Research Center (QRRC). Fish were then held in 3000-L freshwater tanks until sampling. Fish were sampled for weight, length and colour, and a fin clip was collected and placed in a high salt preservative buffer (3.5 M ammonium sulphate, 15 mM EDTA, 15 mM sodium citrate, pH 5.2). Colour was assessed visually where individuals were characterized as 'red' or 'white' based on external spawning coloration (Fig. 1A; Withler 1986). Individuals were characterized as red when they showed external red pigmentation, and individuals were characterized as white when they exhibited no external red pigmentation and were grey in coloration. In fall of 2014, spawning colour was also assessed postmortem using a Jaz spectrophotometer (Ocean Optics) with triplicate readings obtained at three locations along the lateral body following a similar protocol to Pitcher *et al.* (2009). The nine spectral readings were averaged and smoothed per individual

using the R package pavo (Maia *et al.* 2013) to generate the average reflectance spectra for red ($n = 19$) and white ($n = 18$) salmon (Fig. 1B). In addition to live adults captured, fin clips for genetic analyses were also collected from carcasses along the shore of the Quesnel River at QRRC. All carcasses were assessed for red or white phenotype by dissection and visual identification.

Neutral genetic divergence and diversity

DNA extraction. Fin clips from adult fish collected in the fall of both 2013 ($n = 73$) and 2014 ($n = 51$) were used to extract DNA following either an automated plate-based extraction protocol (Elphinstone *et al.* 2003) or the Wizard genomic DNA purification kit (Promega Corp.).

Microsatellite genotyping. Spawning salmon from the fall of 2013 and 2014 were genotyped at 14 previously described microsatellite loci, specifically OtsG68, OtsG78b, OtsG432 (Williamson *et al.* 2002); RT212, RT36 (Spies *et al.* 2005); Ots204, Ots209, Ots211, Ots213 (Greig *et al.* 2003); Omm1053 (Rexroad *et al.* 2002); Ots1 (Banks *et al.* 1999); Ots107 (Nelson & Beacham 1999); Omy325 (O'Connell *et al.* 1997); and Oneμ13 (Scribner *et al.* 1999). Polymerase chain reaction (PCR) conditions included: a 5-min denaturation step (94 °C), followed by 33–38 cycles of a 20-s denaturation step (94 °C), a 20-s annealing step (51 °C – Ots213; 52.5 °C – OtsG68, Ots1, OtsG78b; 54 °C – Omy325, Omm1053, Ots209; 56 °C – OtsG432, RT212, RT36; 58 °C – Oneμ13, Ots107; 60 °C – Ots211; and 62 °C – Ots204) and a 30-s extension step (72 °C), followed by a final extension step of 3 min. Amplicons were run on LiCor 4300 DNA analyser (LiCor Biosciences, Inc.), and fragment sizes were scored using GENE IMAGIR 4.05 software (Scanalytics Inc.). DNA extraction and PCR amplification were performed for all fin clips, but only the final numbers of individuals included in the analyses during each year are presented in the results.

Behavioural mate choice experiment

Spawning channels. Salmon captured through netting in September 2013 (as described above) were sampled and subsequently used for a mate choice experiment. Fish were externally tagged with a white Petersen disc (Floy Tag Inc.) that was 3 cm in diameter and numbered on both sides of the body. Tagged fish were then moved onto a spawning channel, which was 70 m long and was divided into multiple sections that were each 9.5 m long by 2 m wide. Water depth was approximately 0.5–0.75 m, and water velocity was approximately 0.06–0.1 m/s. Gravel depth was 0.4 m and was composed of gravel ranging from 0.02 to 0.1 m in diameter. Six sections of the spawning channel were used where six fish were placed within each section, which included two females (1 red and 1 white) and four males (2 red and 2 white). The section provided 9.5 m^2 of spawning area per female, which is greater than previous successful experiments with Chinook salmon (Neff *et al.* 2008; Lehnert *et al.* 2013). When possible, we attempted to size match red and white males within a section so that males of similar sizes were represented by each colour. However, given that our fish were captured from a wild population, it was difficult to select for matched sizes in all cases. Among all sections, there was no significant difference in fork length between red and white males ($P = 0.56$), where the mean (±SE) fork length for red and white males was 86.8 (±3.32) cm and 89.5 (±3.32) cm, respectively. The range in fork length for red and white males was 70–107 cm and 75–110 cm, respectively. Females were limited in number and thus were not matched for size. Care was taken to utilize only fish that appeared to be in good reproductive condition based on visual inspection, as we chose fish with limited fin deterioration and scale loss and no evidence of prior spawning.

Video recordings and behaviour. Fish were left to spawn and video recordings were taken haphazardly during daylight hours throughout the experimental period (September 19–October 11). GoPro cameras were placed underwater at the downstream end of the spawning channel section, and video recordings occurred over 30- to 98-min intervals between 08:00 and 19:00. The hours of videos recorded varied between channel sections, as this was dependent on the number of days where possible spawning activity (both males and females present) could occur in the channel section, as well as the number of channel sections with possible spawning activity as only two GoPros were used and were thus rotated among spawning sections throughout the day. Approximately 68-h video was recorded and analysed, where the mean (±SE) number of hours per channel section was 11.4 (±0.68) hours. All video recordings were analysed for three aggressive behaviours (similar to Garner *et al.* 2010a), which included biting, chasing (pursuit of another individual) and lunging (rapid increase in swimming speed towards another individual). Two observers recorded behaviours, where each observer recorded behaviour from three of the channel sections. Each time a behaviour was observed in a video, the fish displaying the behaviour and the fish receiving the behaviour were recorded, and the number of each

aggressive behaviour by a female towards a male was calculated for each female and male pair.

Offspring collection. After spawning, fish were removed and sampled postmortem for body weight and gonad weight. Fertilized eggs were left to incubate in the gravel and offspring were collected at two sampling periods. Eggs were collected at the eyed stage (250–500 accumulated thermal units, ATU) by hydraulic sampling (see Lehnert *et al.* 2013) from 11 to 14 November 2013. Sampling was conducted across the entire area of the spawning channel section. Both eyed eggs and newly hatched alevins were collected from the spawning channels with a total of 2039 live offspring (1373 eyed eggs and 666 alevins). Dead eggs were counted and discarded, but we could not differentiate between dead fertilized and unfertilized eggs. Live eyed eggs and alevins were preserved for genetic analysis. Eggs collected were assumed to be a subsample of the total eggs in the substrate; thus, offspring were also collected at the emerging fry stage (approximately 1000 ATU). Fry were captured using a minnow seine net from 11 to 12 January 2014. Total number of fry collected was 2030, where a subsample of 100 fry were kept per spawning section for genetic analysis with the exception of one section that had fewer than 100 fry (n = 13, Section 1 excluded from analyses).

Genotyping and parentage assignment. Parent and offspring DNA was extracted using an automated plate-based extraction protocol (Elphinstone *et al.* 2003). Parents and offspring were PCR-amplified at 6 previously described microsatellite loci: Ots107 (Nelson & Beacham 1999), Ots211 (Greig *et al.* 2003), Omy325 (O'Connell *et al.* 1997), OtsG432, OtsG68 (Williamson *et al.* 2002) and Oneμ13 (Scribner *et al.* 1999). PCR conditions and allele scoring followed the same methods as described for adult spawners (described above). Parentage was assigned using CERVUS version 3.0 (Kalinowski *et al.* 2007) at an 85–95% confidence range with 1% genotyping error. The stated parameters resulted in assignment rates between 95.4 and 100% per spawning section for all eggs and alevins genotyped at 2 or more loci (see Table 1). After initial parentage analysis of fry, it was evident that movement had occurred between channels following fry emergence. To assign parentage to fry, parents that successfully contributed to eggs and alevins in surrounding channels were also included in the assignment analysis when assignment rates were low. Upon inclusion of a greater pool of potential parents, fry assignment rates increased and were between 82 and 98% per spawning section (see Table 1). All offspring (eggs, alevins and fry) were assigned at the strict 95% confidence, with the exception of only 4 fry that were assigned at the relaxed confidence of 85%. For microsatellite loci genotyped, the mean (±SE) probability of exclusion of a parent pair per locus was 0.82 (±0.05) and ranged from 0.68 to 0.95.

MHC genetic divergence and mate choice

MHC genotyping. In addition to microsatellite loci, DNA extracted from spawning salmon from the fall of 2013 (n = 73) was also genotyped at two MHC loci including the class II beta 1 gene (MHC II-B1) and class I alpha 1 gene (MHC I-A1) using primers designed by Miller *et al.* (1997). Both primers amplify the peptide-binding regions of the molecules. We performed the following steps in replicate. PCRs were performed using the following: a 5-min denaturation step (94 °C), followed by 30 cycles of a 20-s denaturation step (94 °C), a 20-s annealing step (52.5 °C – MHC II-B1; 63.5 °C – MHC I-A1) and a 30-s extension step (72 °C), followed by a final extension step of 3 min. PCR products from both loci were pooled by individual (5 μL of each locus/individual), cleaned by precipitation with isopropanol, resuspended in ddH_2O then individually barcoded. Barcoding PCR included: a 2-min denaturation step (94 °C), followed by 7 cycles of a 30-s denaturation step (94 °C), a 30-s annealing step (60 °C) and a 1-min extension step

Table 1 Number of Chinook salmon (*Oncorhynchus tshawytscha*) eggs, alevins and fry assigned parentage from experimental spawning channels where parentage assignment rates were based on the number of offspring genotyped at 2 or more microsatellite loci (see text for details).

Spawning channel section	Number assigned		% Assignment (Total number assigned)	Number untested (Genotyped at <2 loci)	
	Eggs	Alevins		Eggs	Alevins
Section 1	5	76	100 (81)	12	5
Section 2	483	116	95.4 (599)	5	0
Section 3	98	58	95.7 (156)	29	1
Section 4	85	176	96.3 (261)	0	2
Section 5	290	141	100 (431)	10	4
Section 6	276	81	98.3 (357)	20	11

	Fry	% Assignment	Fry
Section 1	–	–	–
Section 2	98	98	0
Section 3	92	92.9	1
Section 4	95	95	0
Section 5	91	97.8	7
Section 6	82	82	0

(72 °C), followed by a final extension of 5 min. Next, Agencourt AMPure XP (Beckman Coulter, Inc.) was used to purify barcoded amplicons, and amplicons were pooled and then gel-extracted using GenCatch Gel Extraction Kit (Epoch Life Science Inc.). The library was run on a 2100 Bioanalyzer (Agilent Technologies) to assess size distribution and concentration of DNA fragments in preparation for dilution. Replicate libraries were combined, and then emulsion PCR was performed using an Ion OneTouch System (Life Technologies) with an Ion OneTouch 400 bp template kit. An Ion Torrent Personalized Genome Machine (Life Technologies) was used to sequence the library with a 318™ chip (Life Technologies).

MHC genotypes were determined using JMHC software (Stuglik *et al.* 2011) and followed a similar protocol to that described by Lighten *et al.* (2014). Briefly, JMHC was used to identify all sequence variants (i.e. potential alleles) for each gene. Given that PCRs were performed in duplicate for each locus, each individual was genotyped twice and thus assigned two different barcodes. Therefore, for the remaining analyses, we separated barcodes from the two replicate PCRs and analysed the replicates independently to generate replicate genotypes for each individual. For all individuals within a replicate, sequence variants occurring at high frequency (these comprised approximately 26–30 sequence variants containing >65% of the total read number once singletons were removed) were compared using a neighbour-joining tree in CodonCode Aligner (CodonCode, Dedham, MA, USA). Based on the neighbour-joining tree, we identified putative true alleles versus alleles that were likely sequencing errors/artefacts (mismatched at 1–3 bp). Sequence reads from a putative true allele and its artefacts were combined to increase sequencing depth (Lighten *et al.* 2014). Next, following Lighten *et al.* (2014), and assuming that an individual could have either one or two alleles, individual genotypes were estimated by model fitting. Model fitting is described in Lighten *et al.* (2014) and uses two approaches: (i) copy number variation (CNV) model and (ii) degree of change (DOC) model. Briefly, the CNV approach considers the number of reads for a maximum of 10 sequence variants ('alleles') per individuals (Lighten *et al.* 2014). Assuming a one-locus model, approximately equal number of reads should be observed for both alleles if an individual is considered heterozygous (Lighten *et al.* 2014). An excel macro fits the data to possible genotype models (in our case heterozygous or homozygous) based on the observed number of reads for the potential alleles and then compares the fit of the possible models (Lighten *et al.* 2014). The two models that fit the data best are compared, and if there is a significant difference between the two models, then the best-fit model is used to assign the genotype (Lighten *et al.* 2014). In cases, where there was no significant difference between the two models, we also considered the second approach (DOC) offered by Lighten *et al.* (2014), where the model considers the change in number of reads between each allele from the most abundant to the least abundant allele. In this approach, the allele with the greatest DOC value is considered the last true allele in the genotype (Lighten *et al.* 2014). We considered the genotype to be true if the DOC and best-fit CNV model agreed. Generally, genotypes were easily assigned using the CNV approach. A few individuals had ambiguous genotypes, and therefore, individuals were compared with their replicate genotype to ensure accurate genotyping.

Statistical analyses

Neutral genetic divergence and diversity. First, microsatellite loci were tested for linkage disequilibrium (LD) to assess suitability of makers for further analyses. Loci were tested for conformity to Hardy–Weinberg equilibrium (HWE) using GENEPOP version 4.2 (Rousset 2008). ARLEQUIN version 3.5 was used to determine genetic divergence (F_{ST}) between red and white individuals in the population (Excoffier & Lischer 2010). Population differentiation based on allele frequency distributions was also tested using Fisher's exact test in GENEPOP version 4.2 (Rousset 2008). Genetic diversity estimates, including heterozygosity observed (H_O) and expected (H_E), were calculated using GENALEX version 6.5 (Peakall & Smouse 2012), and FSTAT was used to estimate allelic richness (Goudet 2001). Estimates of genetic diversity were compared between red and white groups using a Mann–Whitney U-test.

Female aggression towards males. Aggressive behaviour by females towards males was compared between colour-matched and colour-mismatched pairs. For all females that performed at least one aggressive act, we summed the number of each behaviour (lunging, biting and chasing) for each male–female pair in the channel. Next, given that the three behaviours were significantly correlated (all $r > 0.70$, $P < 0.01$), we ran a principle component analysis (PCA) using the three aggressive behaviours using JMP version 12 (SAS Institute Inc.). Any informative PC axis (eigenvalue >1) was used as a response variable in our model. We used a linear mixed model in the lme4 package (Bates *et al.* 2009) with R software (R Core Team, 2014). The model included a fixed factor as pair type (colour-matched versus colour-mismatched), with random effect of channel section, and the model was weighted by the amount of time a female was observed on video. Two observers recorded

behaviours for different channel sections (G.F. observed sections 1, 2 and 5; M.L. observed sections 3, 4 and 6). To compare scoring between observers, both observers recorded behaviours for the same video. We calculated two measures of reliability in scoring, including Cohen's kappa for interrater reliability (IRR; Cohen 1960) and intraclass correlation (ICC, Shrout & Fleiss 1979). IRR was 0.88, suggesting that behaviours were interpreted in the same way by each observer and ICC was 0.82, indicating consistency in scoring between observers.

Colour assortative mate choice. All analyses were conducted in R software (R Core Team, 2014), unless otherwise stated. Parentage of eyed eggs and alevins was considered a quantification of mate choice. First, a chi-square test was used to compare the number of offspring (eggs and alevins) produced by colour assortative and disassortative mating. The difference in number of offspring produced by colour assortative and disassortative mating between red and white females was also tested with a chi-square test, as well as differences between individual females. Next, number of colour assortative and disassortative mating events was compared using a chi-square test for both the number of primary mating events (i.e. based on a female's primary mate) and for all mating events combined (i.e. all mating pairs detected). Using Fisher's exact test, we also compared the number of colour assortative and disassortative mating events between red and white females to determine whether female preference for colour differed between female phenotypes.

Offspring survival (fry). In addition to parentage assignment of eggs and alevins, fry were also parentally assigned, and the parentage of fry was considered as a measure of offspring survival. Because fry appeared to have moved between sections of the spawning channel (described above), channels could no longer be considered as independent replicates. However, we chose to consider the overall proportions of each mating pair within the fry sample and compare those to the proportions for the same mating pair at the egg/alevin stage. The proportions at both early life stages were calculated as the proportion of offspring from the total genotyped offspring population (egg/alevins $n = 1885$, fry $n = 458$). To test whether offspring from assortative and disassortative mating events experience differences in survival between the egg and fry stages, we calculated the change in proportion from the egg stage to the fry stage. Change in offspring proportion between assortative and disassortative mating pairs was compared using a Mann–Whitney U-test, as sample sizes were not equal between groups.

MHC genetic divergence and mate choice. First, genetic divergence and diversity at MHC genes between red and white spawning salmon in the population were analysed in the same way as neutral loci (described above). Next, we tested whether mate choice occurred randomly based on MHC II-B1 divergence (genotyping analyses described above). MHC II-B1 alleles were compared using MEGA version 5 (Tamura *et al.* 2011) to determine the number of amino acid (AA) differences between male and female genotypes. The average divergence between potential mates was calculated similar to Landry *et al.* (2001), where the number of AA differences between each male and female allele combination was calculated to determine the average number of AA differences between mates. For each mating pair, the expected proportion of heterozygote offspring based on parental genotypes was calculated to determine an index of mate choice based on heterozygosity. Following Neff *et al.* (2008), we used a Monte Carlo simulation in Excel (Microsoft Corporation) to randomly assign males to female mates, and we included all males, however, we used only females that were observed to have successfully reproduced based on genetic data. In our simulation, females were randomly assigned to males in their spawning section based on their number of observed mates in the experiment, and the MHC divergence and heterozygosity value for each female were calculated as a weighted average based on the per cent offspring sired by different males. We generated a distribution for red and white females separately based on 5000 simulations of random mating. Observed MHC divergence and heterozygosity indices among mates were compared against the estimated distributions to determine P values (one-tailed).

Results

Neutral genetic divergence and diversity

For fall of 2013, we included only individuals that were genotyped at 7 or more of the 14 microsatellite loci in our analyses. Three loci (Ots209, OtsG78 and Ots204) showed highly significant deviations from HWE in both red and white groups; thus, these loci were removed from further analyses and were not genotyped for fall of 2014 samples. Next, linkage disequilibrium (LD) was tested and an adjusted P value of 0.0045 (0.05/11) was used to account for multiple comparisons among the 11 loci. No pairs of loci showed evidence of significant LD in either sampling year (all P values >0.005) or overall (all P values >0.02); thus, all further analysis included

11 loci with 69 individuals in 2013 (n = 33 red; 36 white) and 45 individuals in 2014 (n = 22 red; 23 white). Genetic divergence was not significant between red and white groupings in either sampling year (2013: F_{ST} = 0.002, P = 0.16; 2014: F_{ST} = −0.0003, P = 0.37; see Table 2). Fisher's exact tests also showed no significant population differentiation based on allele frequencies over all loci between red and white groups in both 2013 (χ^2 = 21.2; P = 0.51) and 2014 (χ^2 = 23.8; P = 0.36). In both sampling years, genetic diversity estimates did not differ significantly between red and white, including heterozygosity observed (P values >0.90) and expected (P values >0.74) (see Table 2). Estimates of allelic richness were also similar between red and white individuals in both 2013 (P = 0.84) and 2014 (P = 0.95) (Table 2). Fisher's exact tests were used to assess genetic differentiation between the sampling years, and we found only 1 of 11 loci showed a significant difference in allele frequencies between 2013 and 2014 samples. Therefore, samples were combined to assess genetic divergence and differentiation between red and white over both sampling years, and we found no significant genetic divergence between red and white (F_{ST} = 0.0005, P = 0.25) and no significant population differentiation between red and white based on allele frequencies (χ^2 = 25.91, P = 0.26).

Behavioural mate choice experiment

Female aggression towards males. A total of 275 aggressive acts by females towards males were observed in the videos. Both reproductively successful and unsuccessful females exhibited aggressive acts. Females in four of the six channel sections (no female aggression towards males was observed in sections 3 and 6) exhibited aggression towards at least one male within her section. Thus, our analyses included eight females and 16 males from four channel sections, which resulted in 32 pair combinations (18 colour-matched and 18 colour-mismatched). Of the 32 pair combinations, at least 1 aggressive act was observed for 25 pairs, and nine of these 25 pairs had reproductive events (i.e. produced offspring). PCA revealed that one PC axis (PC1 herein referred to as aggression score) could explain 85.4% of the variation (eigenvalue = 2.56). Aggression scores were log-transformed for linear mixed modelling to meet assumption of normality for model residuals (Shapiro–Wilks test, P = 0.08). We found no significant difference in female aggression towards males when

Table 3 Number of offspring (eggs and alevins) produced by red and white Chinook salmon (*Oncorhynchus tshawytscha*) females through colour assortative and disassortative mating in experimental spawning channel sections. Significant differences in number of offspring produced by colour assortative and disassortative mating are indicated in footnote.

		Number of offspring	
Female ID	Section	Assortative	Disassortative
Red 1	1	81	0
Red 12	2	424	0
Red 26	3	21	135
Red 27	4	42	219
Red 28	5	391	0
Red 36	6	154	0
Total red		1113	354
White 11	2	0	175
White 33	5	28	12
White 48	6	203	0
Total white		231	187
Total overall		1344	541

Between individual females: χ^2 = 1582.9, P < 0.001.
Between red and white females: χ^2 = 66.50, P < 0.001.
Overall: χ^2 = 342.07, P < 0.001.

Table 2 Estimates of neutral (microsatellites) and functional (major histocompatibility complex genes) genetic diversity and genetic divergence (F_{ST}) for red and white Chinook salmon (*Oncorhynchus tshawytscha*) from the Quesnel River over multiple sampling years. Genetic diversity estimates include mean allelic richness (A_R) for microsatellites or number of alleles (A_N) for functional genes, and heterozygosity (observed H_O and expected H_E).

Loci/Genes	Year	Colour	No. fish	A_R (or A_N)	H_O	H_E	Divergence
Microsatellites	2013	Red	33	13.24	0.805	0.824	F_{ST} = 0.002
		White	36	13.82	0.832	0.824	
	2014	Red	22	11.20	0.819	0.812	F_{ST} = −0.0003
		White	23	11.44	0.843	0.823	
MHC II-B1	2013	Red	30	4	0.467	0.517	F_{ST} = 0.0346*
		White	31	5	0.774	0.673	
MHC I-A1	2013	Red	26	8	0.346	0.791	F_{ST} = 0.0495*
		White	30	8	0.433	0.788	

*Significant genetic divergence between red and white groups (P < 0.05).

comparing between colour-matched and colour-mismatched males (LMM, $\chi^2 = 0.039$; $P = 0.84$).

Colour assortative mate choice. In the spawning channels, three of the 12 females failed to reproduce, and 50% of the males ($n = 12$) did not successfully contribute to the parentage of eyed eggs and alevins (hereafter referred to as offspring). All three females that did not spawn were white females, and males that did not spawn included five red and seven white males. Successful males and females showed variation in mating success, as the number of offspring assigned to females ranged from 40 to 424 offspring, whereas the variation in males ranged from 19 to 599 offspring.

Based on parentage assignment of offspring, there was a significant difference in the number offspring produced by colour assortative and disassortative mating ($\chi^2 = 342.07$, $P < 0.001$; Table 3). Furthermore, there was a significant difference between red and white females in the number of offspring produced by assortative and disassortative mating ($\chi^2 = 66.50$, $P < 0.001$; Table 3), where approximately 75.9% and 55.3% of offspring from red and white females, respectively, were produced through assortative mating (Fig. 2). When comparing individual females, we also found a significant difference between females in the number of offspring produced by colour assortative and disassortative mating ($\chi^2 = 1582.9$, $P < 0.001$; Table 3). The number of primary mating pairs (i.e. mate that sired the majority of a female's eggs, which was >70% of a female's offspring in our study) that were colour assortative was greater than, but not significantly different from, the number of mating pairs that occurred through colour disassortative mating ($\chi^2 = 1.00$, $P = 0.32$; Table 4), and qualitatively similar results were found when considering all mating events ($\chi^2 = 2.57$, $P = 0.11$; Table 4). Furthermore, the number of colour assortative versus disassortative primary mating events was not significantly different between red and white females (Fisher's exact test, $P = 0.99$; Table 4), and the same was true when considering all mating events (i.e. primary and secondary mates) (Fisher's exact test, $P = 0.58$; Table 4).

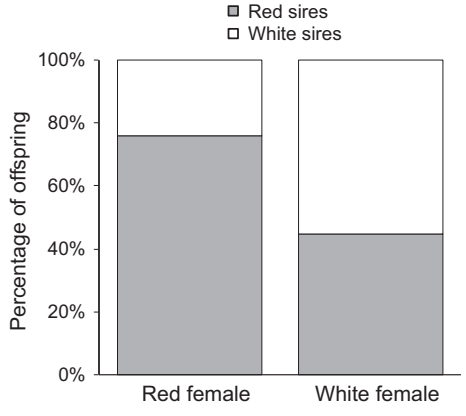

Fig. 2 Percentage of offspring (including eggs and alevins) from red ($n = 1467$ offspring) and white ($n = 418$ offspring) Chinook salmon (*Oncorhynchus tshawytscha*) females that were sired by red and white males in experimental spawning channels.

Offspring survival (fry). There was no significant difference in offspring survival between assortative and disassortative mating pairs (Mann–Whitney U-test, $P = 0.65$). The mean (±SE) change in proportion of offspring between the egg and fry stage for colour assortative and disassortative mating pairs was −0.62 (±2.60) % and 1.38 (±4.22) %, respectively.

MHC genetic divergence and mate choice

Genotypes for four individuals at MHC I-A1 and six individuals at MHC II-B1 were not identical between replicate samples, and thus, genotypes were discarded. For MHC II-B1 analyses, we removed individuals that were not replicated ($n = 7$). Therefore, our analyses included 30 red and 31 white individuals. Because the sample size was lower for MHC I-A1 (due to individuals that did not amplify), all individuals with genotypes were analysed for divergence and diversity estimates including nonreplicated individuals ($n = 26$ red; 30 white).

Analyses of MHC I-A1 and MHC II-B1 genes identified 8 and 6 alleles, respectively, in the Quesnel River population (Fig. 3; Table S1 and Appendix S2, Supporting information). The MHC I-A1 locus exhibited significant deviations from HWE in both red and white groups ($P < 0.001$) due to a significant heterozygote deficiency. MHC II-B1 did not deviate significantly from HWE in either red or white group (P values >0.06). Both MHC I-A1 and MHC II-B1 loci showed significant genetic divergence between red and white individuals in the population, where F_{ST} values were 0.0495 ($P = 0.028$) at MHC I-A1 and 0.0346 ($P = 0.039$) at MHC II-B1 (Table 2). At MHC II-B1, both red and white individuals shared the same three major alleles and there was no significant difference in the frequency of these alleles between red and white fish (Fisher's exact test, $P = 0.06$; Fig. 3B). In contrast, for MHC I-A1, the allele frequency distributions differed significantly between red and white groups (Fisher's exact test, $P = 0.01$; Fig. 3A). Heterozygosity estimates (H_O and H_E) were greater for white than red individuals at both loci (Table 2), where the observed percentage of heterozygotes at MHC II-B1 was 47%

Table 4 Number (and percentage) of mating events for colour assortative and disassortative mating in red and white Chinook salmon (*Oncorhynchus tshawytscha*) females in experimental spawning channels when considering only primary mating events, as well as all mating events.

	Primary mating events		All mating events	
Female colour	Assortative (%)	Disassortative (%)	Assortative (%)	Disassortative (%)
Red	4 (67)	2 (33)	7 (78)	2 (22)
White	2 (67)	1 (33)	3 (60)	2 (40)
Total	6 (67)	3 (33)	10 (71)	4 (29)

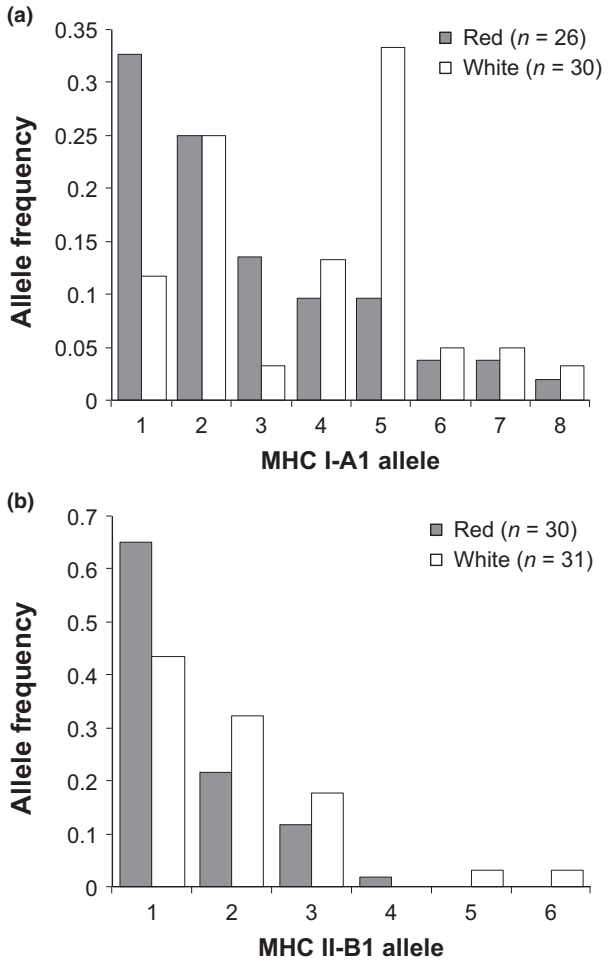

Fig. 3 Allele frequency distributions for spawning red and white Chinook salmon (*Oncorhynchus tshawytscha*) from the Quesnel River, British Columbia, at two major histocompatibility complex (MHC) genes, including (A) MHC class I alpha 1 and (B) MHC class II beta 1. MHC I-A1 alleles are referred to as OTS A-1 to 8, and MHC II-B1 alleles are referred to as OTS B-1 to 6.

and 77% for red and white, respectively, and for MHC I-A1, heterozygosity was 35% and 43% for red and white, respectively.

The estimated distribution of random mating based on average MHC II-B1 divergence and heterozygosity between mates are provided in Fig. 4. Red and white females mated randomly based on MHC II-B1 amino acid divergence (all P values >0.10; Fig. 4A,B). Furthermore, red and white females mated randomly based on heterozygosity at the MHC II-B1 gene (all P values >0.27; Fig. 4C,D).

Discussion

In our study, we examined how mate choice as well as neutral and functional genetic processes can shape phenotypic variation in a population of colour polymorphic Chinook salmon. Based on neutral microsatellite markers, we found no genetic differentiation between red and white phenotypes over multiple years (overall F_{ST} = 0.0005), suggesting that there are no barriers to gene flow between the phenotypes. Furthermore, in experimental spawning channels, female aggression towards males was not dependent on the colour of the male relative to the female. Specifically, females displayed the same amount of aggression towards colour-matched and colour-mismatched males, thus indicating no evidence of a behavioural bias by females based on male colour. However, we found significantly more offspring were produced through colour assortative than disassortative mating under experimental conditions, although the difference was primarily driven by red females producing more offspring with red males than white males. Nevertheless, we found no difference in the number of mating pairs occurring through colour assortative and disassortative mating. We also found no difference in offspring survival between colour assortative and disassortative mating, indicative of no negative consequences on early life survival of interbreeding between red and white individuals. Therefore, although we found some degree of assortative mating (71%) in our experiment, substantial gene flow between the morphs makes it unlikely that colour-based assortative mating is a primary mechanism underlying the continued presence of the polymorphism. Furthermore, the

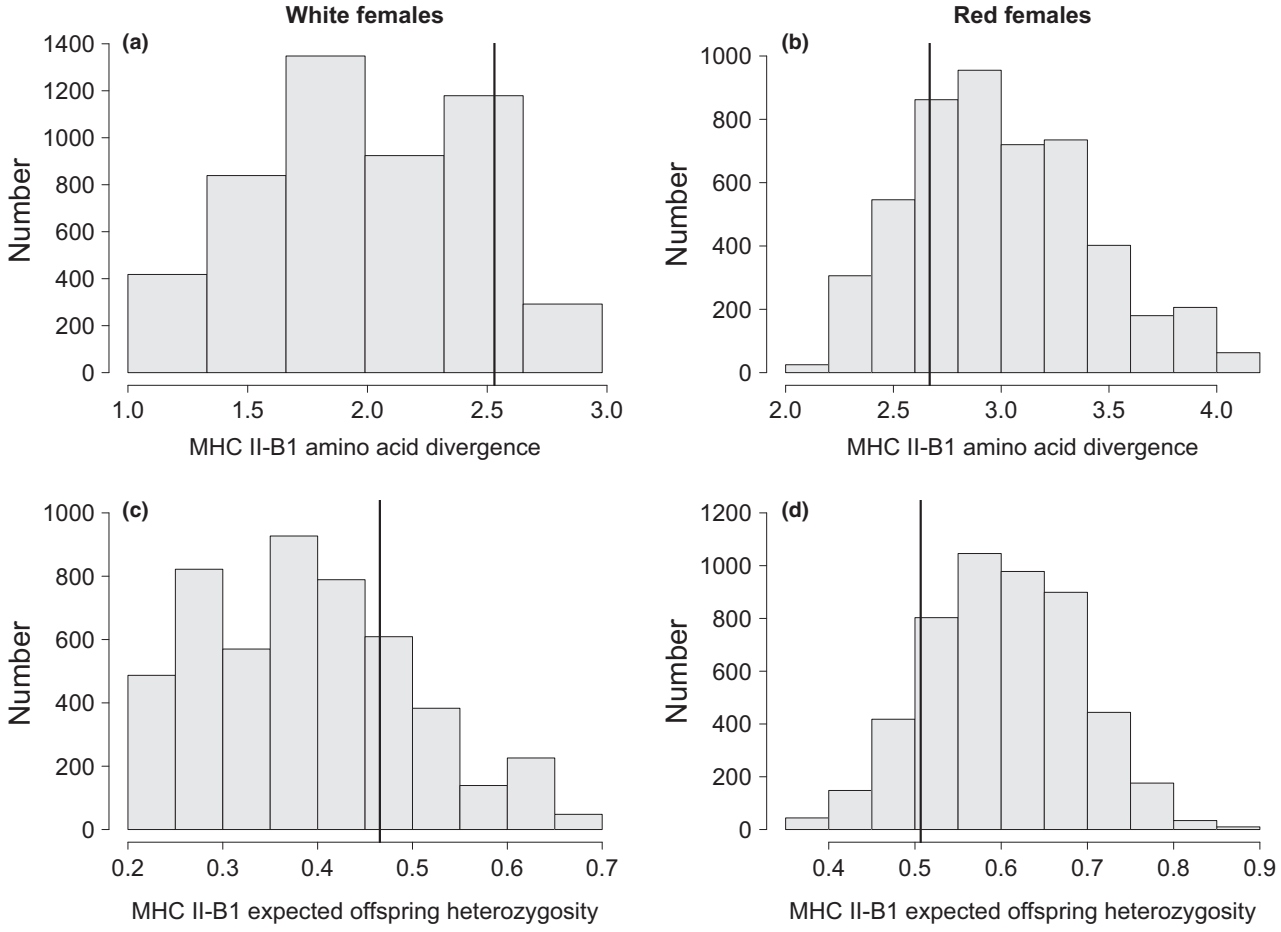

Fig. 4 Distribution of average MHC II-B1 amino acid divergence (A, B) and MHC II-B1 expected offspring heterozygosity (C, D) between Chinook salmon (*Oncorhynchus tshawytscha*) mates based on 5000 simulations of random mating in experimental spawning channels. White (A, C) and red (B, D) female distributions are represented with the average observed value indicated by a solid line.

persistence of white individuals as well as the interbreeding of red and white morphs suggests that red spawning coloration previously shown to be important to sexual selection in salmon (Fleming & Gross 1994; Skarstein & Folstad 1996; Craig & Foote 2001) is not universally so, at least not in populations where red and white individuals exist in sympatry. Mate choice in red and white Chinook salmon in the Quesnel River may be driven by other factors (discussed below).

Previous studies on colour polymorphisms and genetic divergence have found that, similar to our study, sympatric colour morphs exhibited weak or no genetic divergence at neutral markers (Walter *et al.* 2009; Huyghe *et al.* 2010). For example, in Dalmatian wall lizards (*Podarcis melisellensis*), there was evidence of weak genetic divergence between three colour morphs where overall F_{ST} between morphs was −0.001 (Huyghe *et al.* 2010). Although Walter *et al.* (2009) observed significantly more colour assortative than disassortative mating among colour polymorphic sailfin silversides, genetic data revealed no significant differentiation between the blue and yellow colour morphs (F_{ST} = 0.003). Given that the flesh colour phenotype in Chinook salmon is controlled by few loci, it is possible that if these alleles do not affect fitness the polymorphism would be maintained in nature despite interbreeding between red and white individuals. Withler (1986) examined inheritance patterns in offspring from red and white Chinook salmon parents. Based on offspring phenotypes, Withler (1986) proposed that flesh colour may be controlled by two loci, each with two alleles, where one red determining allele (dominant) must be present at both loci to exhibit red coloration. Thus, crosses between red and white parents can produce both red and white offspring, where the ratio of red/white offspring will depend on the genotype of the parents (Withler 1986). In the Quesnel River, assuming the Withler (1986) two-locus model and equal frequencies of red and white alleles in the population, we would expect 56% of the population to have the red

phenotype (9:7 ratio of red/white individuals). The frequency of red and white individuals in the Quesnel River population is estimated to be approximately 50% red and 50% white (Withler 1986). Therefore, even some bias for assortative mating, as we found in our study, could lead to equal proportions of red and white individuals that are found in the Quesnel River population assuming equal fitness among colour genotypes. In this case, an increase in assortative mating by red females (as demonstrated in our study) could consequently result in an increase in the proportion of white individuals in the population due to more opportunities for white–white mating events. Thus, interbreeding between red and white Chinook salmon could occur, yet colour diversity in the population could also be maintained through mechanisms such as balancing selection acting on the red and white alleles.

While we found no evidence of neutral genetic divergence, we did find significant functional genetic differences between red and white individuals at two MHC genes. Therefore, if mating occurred nonrandomly at the MHC genes in the present study, this would contribute to the maintenance of the polymorphism. However, when we examined mate choice based on MHC II-B1, which is the gene class commonly found to be associated with mate choice in salmon (Landry *et al.* 2001; Skarstein *et al.* 2005; Neff *et al.* 2008), we found no evidence for nonrandom mating. Thus, our results indicate that natural selection, rather than mate choice, is likely shaping the differences between the morphs at MHC II-B1. Mate choice based on MHC I-A1 was not assessed, as not all individuals in the experiment were successfully genotyped. Nonrandom mate choice at MHC I-A1 may explain the significant difference in allele frequencies detected in our study. Although few studies have examined mate choice in relation to MHC class I genes in salmonids, Yeates *et al.* (2009) found that Atlantic salmon (*Salmo salar*) males with more similar MHC class I genotypes to the female had greater competitive fertilization success relative to males with dissimilar genotypes. Therefore, if red and white Chinook salmon are choosing mates with more similar MHC I-A1 alleles, this could provide a mechanism for maintenance of the two colour morphs in the system, a hypothesis that warrants further investigation.

Carotenoids have been found to enhance immune response across a broad range of taxa including salmon (Blount *et al.* 2003; Grether *et al.* 2004; Amar *et al.* 2012; Butler & McGraw 2013); thus, we speculate that white Chinook salmon may be immune compromised relative to red individuals unless they have evolved compensatory mechanisms to deal with this handicap (Tyndale *et al.* 2008). In our study, white individuals were 30% more heterozygous than red individuals at the MHC II-B1 gene. However, given that no evidence for nonrandom mate choice based on MHC II-B1 was identified, diversity at MHC genes may be explained through balancing selection, where individuals that are heterozygous at the MHC gene exhibit a fitness advantage over homozygotes (see Bernatchez & Landry 2001). Therefore, it is possible that white Chinook salmon may enhance immunity through increased heterozygosity at the MHC gene (McClelland *et al.* 2003; Evans & Neff 2009). Indeed, in Chinook salmon, evidence of a heterozygote advantage at MHC II-B1 gene has been demonstrated for resistance to bacterial (Evans & Neff 2009) and viral pathogens (Arkush *et al.* 2002). Although there is some evidence that carotenoids can also improve specific (adaptive) immunity (Grether *et al.* 2004), the majority of research indicates that carotenoids improve nonspecific (innate) immunity (Blount *et al.* 2003; Amar *et al.* 2012; Butler & McGraw 2013). Given that heterozygosity at the MHC II-B1 gene can be beneficial, why do red individuals not increase both their nonspecific immunity (via carotenoids) and specific immunity (via MHC diversity)? One possibility is that excessive MHC diversity within an individual can result in autoimmune issues (Penn & Potts 1999); thus, there may be an optimal number of MHC alleles (Milinski *et al.* 2005), which may vary depending on the level of other immune response capability (e.g. carotenoids present or not).

In addition to differences found at MHC II-B1, we found a significant difference in allele frequencies between red and white groups and a significant heterozygote deficiency overall at the MHC I-A1 gene. Previous studies have also found evidence for heterozygote deficiency at MHC class I genes in Chinook salmon populations (Miller *et al.* 1997; Heath *et al.* 2006). Although we cannot rule out mate choice for shaping differences in MHC I-A1 gene, in salmon, specific alleles can infer fitness advantages as local adaptation may drive directional selection for certain alleles that enhance immunity when encountering common pathogens. In Atlantic salmon, specific alleles at MHC I-A1 have been demonstrated to confer higher fitness when exposed to infectious salmon anaemia (ISA) virus (Kjøglum *et al.* 2006). Although red and white Chinook salmon are thought to utilize the same environments, the extreme difference between the morphs in carotenoid assimilation may result in deviation in susceptibility to different viruses. For example, in rainbow trout, fish fed a carotenoid (astaxanthin) diet had significantly higher survival after exposure to a virus than fish fed a control diet (Amar *et al.* 2012). Moreover, two alleles in white individuals that occur at a high frequency (33.3%

for OTS A-5 and 13.3% for OTS A-4) lacked a two-codon insertion, which were termed *motif 2* by Miller *et al*. (1997) (Fig. 3A; for DNA sequences, see Appendix S2, Supporting information). The two alleles each occurred at <10% frequency in red individuals (Fig. 3A). Interestingly, *motif 2* alleles have been found at high frequency in another white Chinook salmon population (Harrison River), and these alleles could be used to differentiate the Harrison population from the Nechako River population (Miller *et al*. 1997). Thus, the insertion may be selected against in white populations, and alleles lacking the insertion (*motif 2*) at MHC I-A1 locus may infer some fitness advantage in white individuals. Nonetheless, studies designed to test for differences in susceptibility between morphs to both viral and bacterial pathogens are required to further characterize the MHC allelic differences detected in our study.

There is little known about the evolutionary processes maintaining the sympatric red and white Chinook salmon phenotypes, and our study is the first to quantify neutral and functional genetic differences between the phenotypes, as well as examine mate choice in this system. We demonstrate that gene flow is occurring between red and white individuals, although some bias for colour assortative mating was detected which may explain in part the relatively equal proportions of red and white individuals in the Quesnel River population. Nevertheless, colour assortative mating is likely not the primary mechanism operating to maintain the polymorphism. However, our study highlights that natural selection is likely operating differentially on red and white Chinook salmon at two immune genes, as we found that white individuals were more heterozygous at the MHC II-B1 gene and there was a significant difference in allele frequencies at the MHC I-A1 gene between red and white conspecifics. The possibility of nonrandom mating at MHC I-A1 may explain differences in MHC detected here, but no evidence for mate choice based on MHC II-B1 was observed. If the limited ability to store carotenoids in tissues subsequently impacts the immune response in white Chinook salmon, our research provides a possible compensatory genetic mechanism that may allow white Chinook salmon to deal with an immune handicap. No studies to date have specifically tested whether white Chinook salmon are actually immunocompromised relative to the red morph, and thus, the present interpretation is based on current knowledge of carotenoids, MHC and immune function in fishes. Our study contributes to our understanding of the evolutionary factors that maintain the red/white colour polymorphism in Chinook salmon and also adds to our growing understanding of the more general evolutionary question of: why are salmon red?

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All authors contributed to aspects of the study conception and design. S.J.L. collected field data, carried out experiments and molecular laboratory work, performed statistical analyses and drafted the manuscript. All authors edited and approved the final manuscript.

Data accessibility

Mate choice and genotype data are available through Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.sh391.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 MHC II-B1 and MHC I-A1 alleles and their corresponding allele frequencies, GenBank accession number (with original reference in footnote) for red and white Chinook salmon (*Oncorhynchus tshawytscha*) spawners in the Quesnel River (see text for details).

Appendix S2 DNA sequences for MHC I-A1 and MHC II-B1 alleles found in spawning Chinook salmon (*Oncorhynchus tshawytscha*) in the Quesnel River, British Columbia, Canada.