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Significant differences in maternal carotenoid provisioning and effects on offspring fitness in Chinook salmon colour morphs

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Abstract

In oviparous species, maternal carotenoid provisioning can deliver diverse fitness benefits to offspring via increased survival, growth and immune function. Despite demonstrated advantages of carotenoids, large intra- and interspecific variation in carotenoid utilization exists, suggesting trade-offs associated with carotenoids. In Chinook salmon (*Oncorhynchus tshawytscha*), extreme variation in carotenoid utilization delineates two colour morphs (red and white) that differ genetically in their ability to deposit carotenoids into tissues. Here, we take advantage of this natural variation to examine how large differences in maternal carotenoid provisioning influence offspring fitness. Using a full factorial breeding design crossing morphs and common-garden rearing, we measured differences in a suite of fitness-related traits, including survival, growth, viral susceptibility and host response, in offspring of red (carotenoid-rich eggs) and white (carotenoid-poor eggs) females. Eggs of red females had significantly higher carotenoid content than those of white females (6× more); however, this did not translate into measurable differences in offspring fitness. Given that white Chinook salmon may have evolved to counteract their maternal carotenoid deficiency, we also examined the relationship between egg carotenoid content and offspring fitness within each morph separately. Egg carotenoids only had a positive effect within the red morph on survival to eyed-egg (earliest measured trait), but not within the white morph. Although previous work shows that white females benefit from reduced egg predation, our study also supports a hypothesis that white Chinook salmon have evolved additional mechanisms to improve egg survival despite low carotenoids, providing novel insight into evolutionary mechanisms that maintain this stable polymorphism.

Introduction

During early life, maternal effects play an important role in determining offspring phenotype, where both the genotype of the mother and environment that she experiences can have substantial impacts on offspring fitness (Mousseau & Fox, 1998). In oviparous species, an important maternal effect is egg quality, which can be determined by egg size as well as the provisioning of maternally derived compounds such as lipids, antioxidants, antibodies and hormones (Williams, 1994; Hasselquist & Nilsson, 2009; Deeming & Pike, 2013). One group of antioxidants that contributes to egg quality are carotenoid pigments, which have been widely studied

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in the context of maternal effects in birds (Blount *et al.*, 2002; Biard *et al.*, 2005; McGraw *et al.*, 2005; Ewen *et al.*, 2009; Marri & Richner, 2014) and fishes (Svensson *et al.*, 2006; Tyndale *et al.*, 2008; Bazyar Lakeh *et al.*, 2010; Brown *et al.*, 2014; Lehnert *et al.*, 2017a).

Carotenoids are produced by photosynthetic organisms and micro-organisms; therefore, animals cannot synthesize carotenoids *de novo* but must acquire these pigments through their diet (Goodwin, 1986). The maternal provisioning of these antioxidants to eggs can provide physiological benefits (Krinsky, 2001) as carotenoids can help to shield the offspring from oxidative stress that occurs when reactive oxygen species (ROS) accumulate, leading to damage of proteins, lipids and DNA. Carotenoids neutralize some of these ROS that are produced during normal cell metabolism (Chen *et al.*, 2003), and, during embryonic development, egg carotenoids may be especially important for quenching the high levels of ROS produced during this period of rapid growth (Deeming & Pike, 2013). ROS are also generated during the immune response (Nathan & Cunningham-Bussel, 2013), and thus, carotenoids may also have indirect effects on several components of the immune system (reviewed in Chew & Park, 2004). Egg carotenoids can therefore influence offspring fitness in diverse ways, where, across taxa, higher maternal provisioning of carotenoids can increase egg and early life survival (McGraw *et al.*, 2005; Tyndale *et al.*, 2008), enhance immune function (Saino *et al.*, 2003; Biard *et al.*, 2005; Ewen *et al.*, 2009), increase size (Marri & Richner, 2014) and growth rate (Bazyar Lakeh *et al.*, 2010), and improve antioxidant status (McGraw *et al.*, 2005).

Despite demonstrated benefits of maternal carotenoids, large variation still exists within and among species in egg carotenoid content (Withler, 1986; Svensson *et al.*, 2006; Deeming & Pike, 2013). Chinook salmon (*Oncorhynchus tshawytscha*) exhibit such variation, where large differences in egg carotenoid content result from genetic polymorphisms that affect carotenoid deposition into the eggs, skin and flesh (Withler, 1986; Tyndale *et al.*, 2008), consequentially producing two natural colour morphs: red and white (see Fig. 1). Temporal and spatial distributions of red vs. white morphs are not well-characterized within and among populations; however, frequencies can vary from 0 to 100% throughout the western North American range (Hard *et al.*, 1989) and it is estimated that the white morph occurs at a frequency of approximately 10% over the entire range. Unlike the more abundant and widespread red Chinook salmon, white Chinook salmon have lower carotenoid content that translates into white (or pale red) eggs and flesh, and they appear grey in external spawning coloration (Fig. 1). Both morphs consume, absorb and metabolize carotenoids from their environment (Ando *et al.*, 1994); however, genetic differences between morphs result in differences in carotenoid accumulation. One major genomic region appears to be responsible for this polymorphism (Lehnert, 2016). The higher carotenoid concentration found in the red morph is primarily due to differences in the carotenoid astaxanthin, which represents 85–95% of the carotenoids found in Chinook salmon eggs (Tyndale *et al.*, 2008; Garner *et al.*, 2010). Experimental evidence suggests that morphs are not reproductively isolated (Lehnert *et al.*, 2016), and depending on the genotypes of parents, mating between morphs can produce different ratios of red:white offspring (Withler, 1986). Although carotenoid content at the egg stage is entirely dependent on the maternal phenotype (i.e. red females produce only red eggs), offspring from red eggs (maternally determined) may be later categorized as red or white. This offspring phenotype (determined by genotype) will not be expressed until the ocean phase when carotenoids are deposited into the flesh (Withler, 1986; Lehnert, 2016).

In nature, colour polymorphisms can be maintained through various mechanisms, where selection can vary spatially and temporally, and be frequency-dependent thus driving differential trade-offs between morphs (Svensson, 2017). Studies have demonstrated many benefits of carotenoids to salmonids, including egg survival (Tyndale *et al.*, 2008), immune function (Amar *et al.*, 2012), growth rate (Bazyar Lakeh *et al.*, 2010), mate choice (Fleming & Gross, 1994; Craig & Foote, 2001) and sperm quality (Lehnert *et al.*, 2017b). The persistence of both the red and white morph suggests that there are trade-offs associated with carotenoids within Chinook salmon populations. The white morph may have evolved because they benefit from reduced egg predation (Lehnert *et al.*, 2017a) and additional mechanisms may have subsequently evolved to compensate for any additional handicap of limited carotenoids. For example, previous work has demonstrated that, for the Quesnel River population, the white morph of Chinook salmon is significantly divergent from the red morph at two major histocompatibility genes (MHC I and II), with one gene having greater diversity in the white morph consistent with a mechanism to deal with a wider range of pathogens when the immune benefits of carotenoids are limited (Lehnert *et al.*, 2016). To date, the only clearly demonstrated benefit of reduced carotenoids in the white morph is a decrease in egg predation (Lehnert *et al.*, 2017a), whereas costs have been found for egg survival, immune function (Tyndale *et al.*, 2008) and marginally lower sperm velocity (Lehnert *et al.*, 2017b). However, no studies have examined differences in early life survival, growth and immune function within a sympatric population of red and white Chinook salmon.

In our study, we investigate a well-studied mixed population of red and white Chinook salmon from the

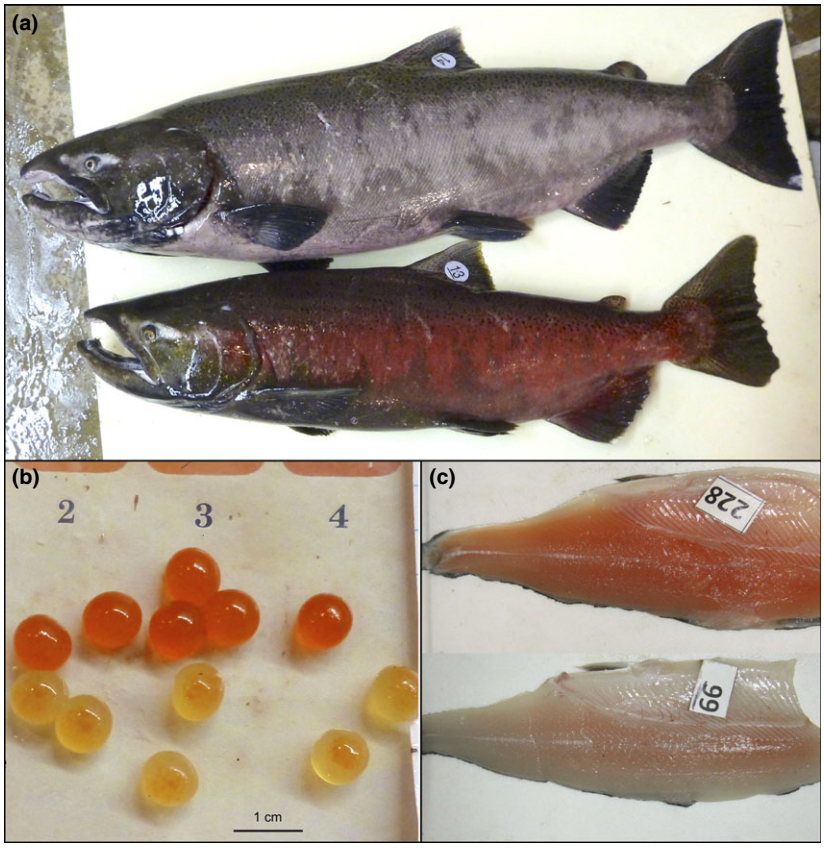

Fig. 1 Photographs showing red and white colour morphs of Chinook salmon (*Oncorhynchus tshawytscha*), where (a) shows external spawning colour of a white (top) and red (bottom) male, (b) shows eggs from red (top) and white (bottom) females and (c) shows differences in flesh pigmentation between red (top) and white (bottom) salmon during the ocean phase.

Quesnel River, British Columbia where the frequency of each morph is approximately equal and appears to have remained stable over time (Withler, 1986; Lehnert *et al.*, 2016, 2017a,b). We mated red and white Chinook salmon using a full factorial breeding design and reared the resultant offspring using a common-garden approach to assess the effect of maternal carotenoids on fitness-related traits during early life. We hypothesize that if maternal carotenoids positively influence early life fitness in Chinook salmon, eggs from red morph females (carotenoid-rich eggs) will produce offspring with higher fitness relative to the white morph (carotenoid-poor eggs). If fitness differences are not detected between female morphs, given demonstrated positive effects in many other studies, our results may provide further support of the hypothesis that white Chinook salmon have evolved to cope with their presumed carotenoid handicap (see Lehnert *et al.*, 2016, 2017a). In this case, the relationship between carotenoid content and fitness may differ between colour morphs, where within the red morph, a positive relationship may exist between carotenoids and offspring fitness, but, within the white morph, there may be no effect if white Chinook salmon have evolved carotenoid-independent mechanisms to compensate for the expected reduction in fitness. To test the hypothesis of compensation, we examined the relationship between egg carotenoid content and offspring fitness within each female colour morph separately. Our design also allows us to test the effect of male morph and the interactive effect of male and female morph on offspring performance. In fishes, carotenoid content of males has been demonstrated to have a positive effect on offspring antipredator behaviour (Evans *et al.*, 2004) and disease resistance (Barber *et al.*, 2001); thus, offspring of red males may display higher fitness relative to offspring of white males. In addition, the interaction effect of male and female morphs genomes may also influence offspring fitness, where if genetic incompatibilities exist between morphs, then such crosses may experience fitness reductions relative to crosses within morphs, and disruptive selection may support persistence of each morph. The co-occurrence of red and white Chinook salmon in the Quesnel River system presents a unique opportunity to examine the effect of maternal and paternal carotenoids in a controlled and quantitative way. Our results provide insight into the trade-offs that exist between red and white morphs and potential coadaptation that has evolved in the system; therefore, our work contributes to our understanding of the evolutionary mechanisms operating to maintain polymorphisms in nature.

Materials and methods

Fish and gamete collection

From 18 September 2015 to 30 September 2015, spawning Chinook salmon were collected by seine net from the Quesnel River, Likely, BC, Canada. Salmon were captured by net and transported 5 km to the Quesnel River Research Center (QRRC) where fish were held in seminatural spawning channels at 10 °C until sampling. All fish were characterized as 'red' (pigmented) or 'white' (nonpigmented) based on external spawning coloration (Withler, 1986; Lehnert *et al.*, 2016). Red fish were evident by red skin pigmentation, whereas white fish were grey in colour (Fig. 1a) (see Lehnert *et al.*, 2016). Colour assignment of all females was later confirmed and quantified by egg colour and egg carotenoid content (see below). Following colour morph assignment, fish were fin-clipped for genetic analysis and gametes were collected. Ejaculate (milt) was collected through either live spawning or after euthanization: where males were wiped dry and gentle pressure was applied to the abdomen to express milt for collection. Milt was collected into a plastic bag, sealed and kept cool at approximately 4 °C. For females, fish were euthanized and stripped of eggs. Eggs were placed in plastic bags and stored in the dark at approximately 4 °C until fertilization, with subsets of each sample immediately frozen for later carotenoid analyses.

Breeding design and rearing

The breeding design included four 4 × 4 full factorial crosses, where each 4 × 4 cell included two red and two white males separately crossed with two red and two white females. The replicated full factorial design resulted in 64 families which allowed us to determine the main effect of female colour on offspring performance while also examining the effect of male colour and accounting for variation caused by individual female, male and their interaction. Gamete collection and fertilizations of crosses occurred on September 21, 26 and 29, and all fertilizations were performed within 24 h of gamete collection. Fertilized eggs were moved into a vertical stack incubation tray system with eggs from each family split into replicate cells and subjected to a 100 ppm free iodine disinfectant solution (Ovadine; DynamicAqua Supply, Canada). Eggs were incubated in hatchery (well) water at 10 °C until the eyed-egg stage (250–500 accumulated thermal units, ATU).

Carotenoid quantification

Carotenoid extractions were performed in triplicate, each with ~1 g of unfertilized Chinook salmon eggs (4–5 eggs), under dim light and on ice to prevent degradation. Methods followed those outlined in Li *et al.* (2005) with minor modifications from Farwell *et al.* (2013) (see Appendix S1 for details). Following extractions, total carotenoid content was determined using an Ocean Optics JAZ spectrometer. Extracted carotenoid samples were re-suspended in 1 mL of ethanol and absorbance was measured at 472 nm (λ_{max} for astaxanthin in ethanol) (Withers *et al.*, 1977). Although we did not test for specific carotenoid types, given that astaxanthin represents the primary carotenoid (85–95%) found in Chinook salmon eggs, we assume that carotenoid content is directly related to astaxanthin concentration here (Tyndale *et al.*, 2008; Garner *et al.*, 2010). Extractions were done in triplicate (i.e. three egg batches per female) to test efficiency of extractions and readings of each sample were replicated five times. For red Chinook salmon eggs, samples were diluted as needed by 0.5, 0.25 or 0.125. To determine total carotenoid content (μg carotenoid per 1 g of eggs) of each sample, the per cent extinction coefficient of astaxanthin in ethanol ($E\frac{1\%}{1\,\mathrm{cm}} = 2100$) and absorbance at 472 nm (A) were used in the following equation from McGraw *et al.* (2001):

$$\text{Total carotenoids } (\mu\text{g g}^{-1}) = (\text{A} \times 1\text{mL of voume extract})/(2100 \times 1\text{ g of sample}). \quad (1)$$

Red Chinook salmon egg concentration values were multiplied by their dilution factor to determine total carotenoid concentrations. The total carotenoid concentrations detected here reflect the egg content of each female at the time of fertilization.

Fitness trait measurements

Egg survival

Egg survival to the eyed-egg stage was determined by counting all live and dead eggs. The mean number of total eggs per family per replicate was 368 (range 202–556 eggs). We could not discriminate between dead fertilized and dead unfertilized eggs; thus, eyed-egg survival may be an underestimate of true survival.

On 4 November 2015, live eyed-eggs were transported approximately 8 h from QRRC to Fisheries and Oceans Canada in West Vancouver, Canada. During transport, eggs were kept cool and moist in insulated boxes. After transport, approximately 200 eggs (based on weight) from each cross (i.e. family) were randomly sampled and placed in Whitlock–Vibert boxes in a stacked incubation tray system in 10 °C well water. At this stage, there were 56 (of the 64) families remaining (some lost due to low viability), and 10 families had fewer than 200 eggs (range 87–177 eggs). Eyed-egg survival was recorded as survival between the eyed-egg stage and the fry stage when offspring reached the exogenous feeding stage and the yolk sac was fully absorbed (1000 ATU).

Fry growth and survival

At the exogenous feeding stage (fry), 120 fish from each remaining family (n = 56) were moved into individual 19-L tanks on either 22 December 2015 or 29 December 2015 depending on days post-fertilization. Five families had fewer than 120 fish remaining (range 73–106 fish). On the day following transfer, a total of 15 fish per family were weighed and measured for fork length. Fish were fed daily a diet of low-pigment feed (Taplow Ventures Ltd. Chilliwack, BC, Canada), which contained no added carotenoids and only natural pigments derived from meal made from white-fleshed fish. To determine fry growth, weight and fork length of 15 fish per family were recorded approximately every 8–10 weeks at three more sampling dates including 17 February, 25 April and 7 July. Prior to February measurement, fish from some families were sampled and transported for a viral challenge (see below), and thus, fish were culled across families in February to equalize densities. At the February sampling, mean fish numbers were 34 fish/family with 54 families remaining. No fish were culled after this date; therefore, we had two estimates of fry survival: (1) early fry survival (approximately 4 weeks from freshwater entry to late January) and (2) late fry survival (approximately 20 weeks from February to July).

Susceptibility to viral challenge

Viral challenge conditions

On 27 January 2016, a subsample of fry (n = 1952) from 32 families (two full 4 × 4 crosses) was moved to Fisheries and Oceans Canada Pacific Biological Station (PBS) in Nanaimo, BC for an immune challenge involving live infectious hematopoietic necrosis virus (IHNV). IHNV is a negative-sense single-stranded RNA virus belonging to the family *Rhabdoviridae* (Lapatra, 1998; Bootland & Leong, 2011). This ecologically relevant virus infects both farmed and wild salmonids globally that can lead to significant mortalities (Lapatra, 1998; Bootland & Leong, 2011).

Fish were approximately 1 g and were chosen as the earliest life stage to likely retain maternal carotenoids in the body, yet were immunocompetent and of suitable size to allow sufficient RNA isolation from tissues. Additionally, an early life stage was chosen as younger fish are more susceptible to IHNV infection (Lapatra, 1998). The IHNV isolate (15–168) used in our study was an endemic strain (U genogroup) obtained from Chinook salmon. To achieve a sufficient virus stock of a known titre for the exposure study, isolate 15–168 was amplified and quantified using cell culture as previously described (Garver *et al.*, 2013). To evaluate IHNV susceptibility and associated immune response differences between the 32 families, two separate virus exposure experiments were conducted: viral challenge I (survival over 35-days) and viral challenge II (gene transcription response).

Viral challenge I: Survival over 35 days after viral exposure

In the first challenge, family susceptibilities to IHNV were measured by exposing duplicate tanks each with 480 Chinook fry (15 fish/family) to IHNV via 1-h immersion in an aerated static bath containing 1.1×10^5 pfu/mL. As a negative control, an identical tank was exposed to Hank's balanced saline solution (HBSS) rather than virus. After the 1-h exposure, water flow was resumed in each tank and fish were monitored three times per day for 35 days. Mortalities were recorded and a fin clip from each dead fry was preserved in high-salt buffer (3.5 M ammonium sulphate; 15 mM EDTA; 15 mM sodium citrate; pH 5.2) for subsequent genetic analyses to identify family following the same methods described for viral challenge II (see below). The remaining carcass was analysed for the presence of IHNV using cell culture as described in Garver *et al.*, (2013).

Viral challenge II: Gene transcription response after viral exposure

For the second challenge, to evaluate potential differences in host response to IHNV between the families, duplicate tanks, each with 128 fish (4 fish/family), were immersion exposed to 1.4×10^4 pfu/mL IHNV, whereas two tanks (mock controls) received HBSS in lieu of virus. After the 1-h exposure, water flow was resumed and fish were maintained for 72 h at which time fish were captured and euthanized in MS-222. Fish were then weighed with a fin clip retained for family identification, and remaining carcass cut on the ventral side to open the abdominal cavity and submersed in a high-salt preservative buffer for later RNA extraction and gene transcription analyses (see below).

Parentage assignment for family identification

Given that fry from 32 families were combined into tanks during experiments, individuals were genotyped at six microsatellite markers to assign fish to their individual family (see Appendix S1 for full genotyping details). For viral challenge I (survival), only mortalities from the experiment were genotyped, whereas for viral challenge II (gene expression), all fish were genotyped. CERVUS version 3.0 (Kalinowski *et al.* 2007) was used to assign parentage to all mortalities using a 1% genotyping error rate and a strict 95% confidence level.

RNA extraction and cDNA synthesis

For viral challenge II (gene expression), total RNA was extracted using mechanical homogenization of gill

tissue in 0.5 mL of TRIzol (Invitrogen, Carlsbad, California, USA). We chose to extract RNA from the gill tissue, as the gills are one site of viral entry for IHNV (Bootland & Leong, 2011), and given the small size of fry, gill tissue was large enough to acquire sufficient RNA. Isolation of total RNA followed the manufacturer's protocol (http://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf), and only samples that were high quality and quantity were used for subsequent analyses (see Appendix S1 for details). Total RNA was diluted to a concentration of 125 ng μL^{-1}, and diluted RNA (0.5 μg) was then treated with DNase I (Promega Corp, Madison, Wisconsin, USA) to remove any genomic DNA contamination. Next, total RNA was converted to complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), with an amount of 0.5 μg of total RNA per reaction.

Quantitative real-time PCR

Primers and TaqMan MGB probes of selected genes (see Table 1 and Table S1) were synthesized and spotted onto OpenArray chips prepared by Applied Biosystems. All primers and TaqMan MGB probes were previously designed in other salmonid studies (Table S1), and selection of candidate genes involved in immune, stress and oxidative stress response is fully described in the Appendix S1 along with methods for OpenArray quantitative real-time PCR (qRT-PCR) using the QuantStudio 12K Flex Real-Time PCR System.

Normalizing gene expression and calculating ΔCt

Relative cycle threshold (C_{RT}) values were obtained for each reaction using ExpressionSuite Software version 1.0 (Applied Biosystems, Foster City, California, USA). Reactions were filtered to remove those that showed no amplification, had an undetermined C_{RT} value or those where the C_{RT} value was >32 cycles. Next, mean C_{RT} was calculated for each sample using technical replicates. In some cases, only one technical replicate was usable. Next, using mean C_{RT} values, we calculated the theoretical starting concentration (N_0) of cDNA in each sample. Starting concentration (N_0) was calculated following Ramakers *et al.* (2003), where mean threshold (N_{CRT}) was divided by mean PCR efficiency (E) to the power of C_{RT} (i.e. $N_0 = N_{CRT}/E^{CRT}$). Mean PCR efficiency and threshold for each gene were determined using LinRegPCR (Ramakers *et al.*, 2003). Starting concentration (N_0) was also calculated for three endogenous controls (EF1A, GAPDH, ARP), and the mean of the three N_0 values was calculated to determine a reference starting concentration. Relative expression was then calculated as N_0 target divided by N_0 reference (Relative expression = N_0 target/N_0 reference) for each sample. Relative expression values were used for subsequent analyses under control (mock) conditions. For fish exposed to live IHNV, relative change in gene expression following IHNV exposure was calculated for each individual based on their family mean expression in the control treatment.

Table 1 List of genes used to measure gene expression in red and white Chinook salmon (*Oncorhynchus tshawytscha*) fry. Gene types included reference genes used as endogenous controls, immune genes and stress/oxidative stress genes. Viral IHNV-N protein gene was also included to score the presence/absence of virus in the sample. Primer and probe sequences along with the original reference for each gene are provided in the Table S1.

Gene type	Primer	Gene	Accession no.
Reference	GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	NM_001124246.1
	ARP	Acidic ribosomal phosphoprotein P0	AY685220
	EF1A	Elongation factor 1-alpha	AF498320.1
Immune	IL1B	Interleukin 1 beta	DQ778946.1
	IL8	Interleukin 8	DQ778949.1
	TNFA	Tumour necrosis factor alpha	DQ778945.1
	IFNA1	Interferron alpha 1	AY788890
	MX1	Mx protein	GT897808
	VIG1	VHSV-induced gene 1	AF076620/CA058263
	IFNG	Interferon gamma	GT897806
	IFR3	Interferon regulatory factor 3	CB515644
	MHCIIB	Major histocompatibility complex class 2	U34718.1
	MHCI	Major histocompatibility complex class 1	AY523661
	CD8	CD8 alpha chain	AF178053
	IGM	Immunoglobulin Mu membrane form heavy chain	X65263/CB506793
	IGT	Immunoglobulin Tau heavy chain	AY870265
	TGFB1	Transforming growth factor beta 1	X99303
	TCRB	T-cell receptor beta chain	AF329700/CB498619
	SAA	Serum amyloid A	NM_001124436.1
Stress/Oxidative stress	GR2	Glucocorticoid receptor 2	AY495372.1
	HSP70	Heat shock protein 70	U35064.1
	HSP90A	Heat shock protein 90a	U89945.1
	META	Metallothionein A	DQ139342.1
	SOD	Superoxide dismutase	AF469663.1
	GPX	Glutathione peroxidase	AF281338.1
	GST	Glutathione *S*-transferase	NM_0011605591
	TRDNX	Thioredoxin reductase	CA057296
Viral protein	IHNV N	IHNV nucleocapsid (N) gene	FJ265710/FJ265715

Table 2 Generalized linear and linear mixed effect models (GLMM and LMM, respectively) examining fitness-related traits (survival, weight and gene expression) in Chinook salmon (*Oncorhynchus tshawytscha*) offspring derived from red and white morphs. All fixed and random terms included in the models for each trait are provided. Density represents rearing density at the life stage tested, date represents date of fertilization for egg survival measures or date of freshwater entry for fry survival and weight measures.

	Fitness traits	Fixed terms	Random terms
Models across female colour morphs	Survival (GLMM)		
	Fertilization to eyed-egg, Eyed-egg to fry	Female colour*Male colour + Female colour + Male colour + Density + Egg mass	Female ID + Male ID + Female ID*Male ID + Date + Tray + Cell
	Early fry, Late fry	Female colour*Male colour + Female colour + Male colour + Density	Female ID + Male ID + Female ID*Male ID + Date
	Weight (LMM)		
	December, February, April, July	Female colour*Male colour + Female colour + Male colour + Density	Female ID + Male ID + Female ID*Male ID + Date
	Viral challenge II: Gene expression (LMM)		
	For each gene in mock and IHNV challenge	Female colour*Male colour + Female colour + Male colour + Weight†	Female ID + Male ID + Female ID*Male ID + Chip + Tank
Models within female colour morphs	Survival (GLMM)		
	Fertilization to eyed-egg, Eyed-egg to fry	Egg carotenoid content + Male colour + Density + Egg mass	Female ID + Male ID + Female ID*Male ID + Date + Tray + Cell
	Early fry, Late fry	Egg carotenoid content + Male colour + Density	Female ID + Male ID + Female ID*Male ID + Date
	Weight (LMM)		
	December, February, April, July	Egg carotenoid content + Male colour + Density	Female ID + Male ID + Female ID*Male ID + Date
	Viral challenge II: Gene expression (LMM)		
	For each gene in mock and IHNV challenge	Egg carotenoid content + Male colour + Weight†	Female ID + Male ID + Female ID*Male ID + Chip + Tank

Interaction of Female colour*Male colour was dropped from models when nonsignificant.
†Weight only included in model if significant correlation between gene expression measure and weight.

Statistical analyses

All statistical analyses were carried out in R statistical software v3.3.1 (R Core Development Team, 2016). All generalized linear and linear mixed models used in our study are described below, and all fixed and random terms included in each model are provided in Table 2.

Maternal carotenoids and early life survival measures
First, differences in egg carotenoid content between red and white females were compared using a Mann–Whitney test. Next, we tested for differences in egg survival. Prior to the survival analyses, we removed crosses ($n = 8$ families) that had very low egg survival, and four of these families were sired by a single white male, which likely indicates a sperm viability issue. The removal of these eight families could bias the results for early egg survival measures; therefore, any significant effects of female colour or egg carotenoid content on early survival were re-analysed with all data except families sired by the single nonviable male. The total number of families included in our main analyses was thus 56 families. For all our measures of egg and fry survival, dead eggs/offspring were coded as 0 and live eggs/offspring were coded as 1. Survival was analysed using generalized linear mixed models (GLMMs) with logit link function for binary data run using the *glmer* function in *lme4* package (Bates *et al.*, 2014) and model terms are provided in Table 2. We note that for all models, the interaction of female colour and male colour was tested, and if the interaction was significant, it was included in the models. Random effects incorporated in all models (here and below) include female ID, male ID and their interaction, with additional random terms included if contributing to variance in the observed trait (see Table 2). The interaction of male ID × female ID is equivalent to variance associated with family (or cross), representing the interaction of the parental genomes. We acknowledge that in cases where each family was reared separately (e.g. individual tanks; see below), the effect of male ID × female ID cannot be separated from the effect of environment (tank). Log-likelihood ratio tests were used to determine the significance of each factor in the model, where models were compared with and without each factor. GLMMs were tested for overdispersion, where overdispersion was determined by dividing residual deviation (rdev) by residual degrees of freedom (rdf) for the model, and if the ratio value was <1, then we concluded that the model was not overdispersed.

GLMM for eyed-egg survival was slightly overdispersed (rdev/rdf = 1.12); however, other models did not violate this assumption (rdev/rdf < 1).

We also tested the same models (above) within each female colour morph with the fixed effect of female colour replaced with egg carotenoid content (see Table 2 for model terms). GLMMs for eyed-egg survival were slightly overdispersed within each colour morph (rdev/rdf = 1.16 and 1.07 for red and white morph, respectively); however, other models did not violate this assumption (rdev/rdf < 1). All *P*-values for all survival analyses were corrected for false discovery rate (FDR), where FDR adjustment was performed separately for *P*-values from between morphs and within morphs analyses.

Offspring body size

Fry wet weight and fork length were measured at four sampling points, which included December 2015, February, April and July 2016. Fry weight and length were highly correlated at all time points (all *P*-values < 0.001; all $r > 0.64$); thus, we present only the results of wet weight. Weight was used as response variables in linear mixed models (LMMs) run using the *lmer* function in *lme4* package (Bates *et al.*, 2014), and model terms are provided in Table 2. Model residuals were inspected for conformation to normality. Data were transformed as needed, and generally, model residuals followed a normal distribution, but if transformation (i.e. log, inverse, square root) could not improve normality, analyses were run with nontransformed data. As with the survival measures (above), the same models were run within each female colour morphs with female colour replaced by egg carotenoid content (see Table 2). All *P*-values for all weight analyses were corrected for FDR, where overall samples and within red females and within white females were all adjusted separately.

Viral susceptibility challenges

Viral challenge I: Survival over 35 days after viral exposure. First, we examined whether there was a significant effect of treatment (mock challenge vs. IHNV challenge) on post-challenge survival using GLMMs with logit link function for binary data. Any offspring that died prechallenge was removed from the analysis. GLMMs included treatment (challenge vs. control), female colour and male colour as fixed effects. Random effects in the model included female, male, female × male interaction and tank. If treatment had no effect in the model, then we determined viral exposure had no influence on mortality over the 35-day period.

Viral challenge II: Gene transcription response after viral exposure. LMMs with relative gene expression (mock control) or relative change in gene expression (IHNV challenge) as the response variables were performed for control and IHNV challenge data sets separately. All terms included in LMMs are provided in Table 2. Briefly, LMMs included fixed effects of female colour, male colour and their interaction. Their interaction was only significant within one gene under one treatment condition (Interferon gamma in IHNV challenge, $P = 0.03$) and thus not included in the analyses and not presented here. Fish weight was included as a fixed effect if it was significantly correlated with gene expression ($P < 0.05$). Random effects in the model included female, male, female × male interaction, OpenArray chip and tank. Models were compared in the same way as described above with log-likelihood ratio tests. Residuals of models were inspected for deviations from normality, and data were transformed (natural log-transformed, negative inverse or square root-transformed) if necessary. All *P*-values for all the above gene expression analyses were adjusted for FDR where control and challenge *P*-values were analysed separately.

The same models implemented above were run for offspring of red and white females separately where the fixed effect of female colour was replaced with egg carotenoid content. We primarily tested the effect of carotenoid content on gene expression within each colour morph and each treatment, and other factors in models were only further explored if the carotenoids effect was significant (FDR adjusted $P < 0.05$). Transcriptional profiles among colour morphs were further visualized with a heatmap generated from normalized values (*Z*-scores) of family means for relative expression at each gene within each treatment using the heatmap.2 function in the R package *gplots* (Warnes *et al.*, 2016) with default methods for clustering.

Results

Maternal carotenoids and early life survival measures

Red Chinook salmon females had significantly greater egg carotenoid content than white females ($W = 64$, $P = 0.0002$), and morphs did not overlap in their measures of egg carotenoid content (Fig. 2). Mean (± standard error) carotenoid content of red and white females was 7.56 (± 0.93) and 1.27 (± 0.17) μg g^{-1}, respectively.

There was no significant difference in survival from fertilization to the eyed-egg stage or survival from the eyed-egg to fry stage between red and white females (see Fig. 3 and Table 3; $P > 0.20$). Male colour and egg size also had no effect on either measure of egg survival (all $P > 0.79$); however, egg incubation density had a significant negative effect on survival to the eyed-egg stage ($P < 0.001$), but not eyed-egg to fry survival

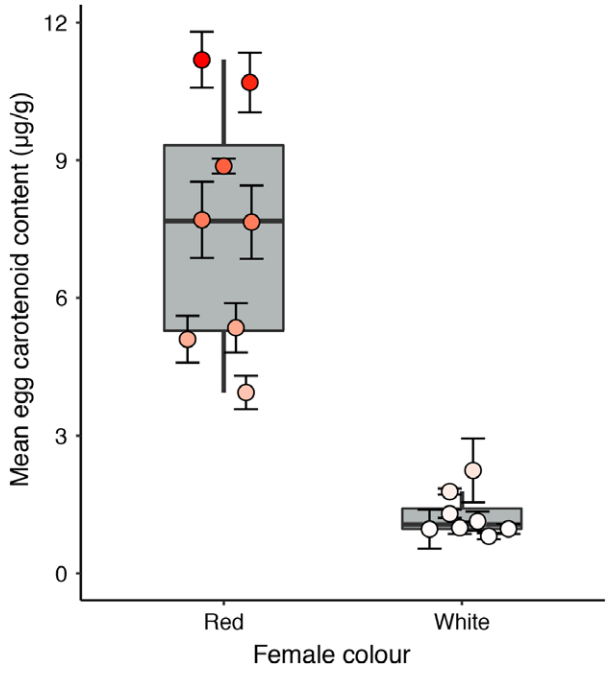

Fig. 2 Boxplot of egg carotenoid content for all red and white Chinook salmon (*Oncorhynchus tshawytscha*) females ($N = 16$), where points are jittered horizontally to show mean values (with standard error) for each female and coloured relative to their carotenoid content (colours do not represent actual colour of egg, but reflect a colour scale to visualize differences). Red and white female groupings were assigned based on external and egg coloration prior to carotenoid extraction.

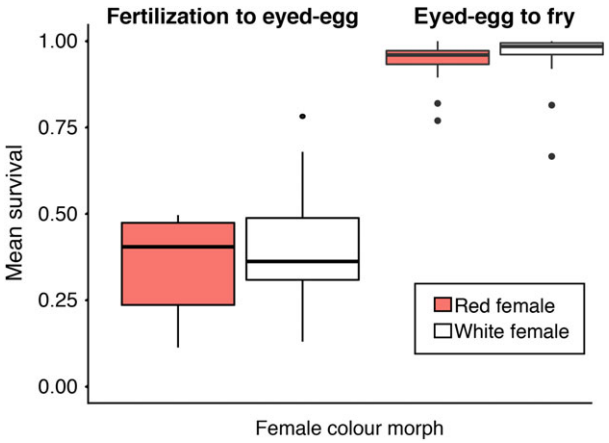

Fig. 3 Boxplot of survival (proportion) derived from red and white Chinook salmon (*Oncorhynchus tshawytscha*) females for two time periods: fertilization to the eyed-egg stage and eyed-egg to fry stage. Each family ($n = 54$) is represented by a single data point where for survival to eyed-egg stage, each data point represents mean family survival (based on family replicates during incubation), whereas survival to the fry stage, each data point represents survival for each family without replication.

($P = 0.99$) (Table 3). Random effects including male x female interaction significantly explained variation in both survival measures, and cell position significantly explained variation in survival to the eyed-egg stage (Table 3). For early and late fry survival, we found no significant effect of female colour, male colour or rearing density (all $P > 0.22$; Table 3). Random effects in the models did not explain any of the variance in early or late fry survival (Table 3). The interaction of male morph x female morph was not significant in any survival model (all $P > 0.05$) and thus was not included in our analyses here.

Within colour morphs, we found a significant positive effect of carotenoid content on survival to the eyed-egg stage in red females ($P = 0.006$) (Fig. 4; see Table 4 for full model results). The effect of carotenoids remained significant when low survival families (three families from red females) were included in the analysis ($P = 0.004$), as these low survival families (excluded from our main analyses) were derived from two females with the lowest carotenoid content within the morph. There was no relationship between eyed-egg survival and carotenoids in white females ($P = 0.99$) (Fig. 4; see Table 4 for full model results), although there was a significant effect of egg rearing density on eyed-egg survival ($P < 0.001$), which did not influence survival of red female eggs ($P = 0.99$) (Table 4 and Fig. S1). For additional survival measures, we found no significant effect of carotenoid content on survival within either colour morph (see Tables S2 and S3 for full model results).

Offspring body size

Fry sampling was conducted at four time points, and maternal colour had no significant effect on weight across all points (Table 3; $P > 0.38$), nor did the other fixed effects in the model (see Table 3; $P > 0.06$). Further, the interaction of male colour morph x female colour morph was not significant in fry weight models (all $P > 0.05$) and thus was not included in our analyses here. Female ID (random effect) significantly explained variation in weight at several time points representing 10–76% of the observed variation (Table 3), and these effects were strongest at the first sampling time period. Male effects were present (2–13% of the variation) but did not explain significant variation in weight except at the last sampling date (Table 3). The interaction of female × male significantly influenced weight at all sampling points, and accounted for 7–15% of the variation (Table 3).

Within each female colour morph, we found no significant effect of egg carotenoid content on weight at any time (all $P > 0.29$) (see Tables S4 and S5 for full model results).

Table 3 Results of generalized linear and linear mixed effect models (GLMM and LMM, respectively) examining fitness-related traits (survival and weight) in Chinook salmon (*Oncorhynchus tshawytscha*) offspring derived from red and white morphs.

Fixed effects	Intercept		Female colour(W)			Male colour(W)			Density			Egg mass		
Trait (sample size)	Est	SE	Est	SE	*P*	Est	SE	*P*	Est	SE	*P*	Est	SE	*P*
Survival (GLMM)														
Fertilization to eyed-egg (n = 41 244)	6.511	0.630	0.168	0.193	0.71	0.181	0.268	0.80	**−1.268**	**0.091**	**<0.001***	3.263	0.808	0.71
Eyed-egg to fry (n = 10 549)	3.784	4.528	0.596	0.288	0.2	0.128	0.486	0.92	0.035	0.840	0.99	−2.175	4.665	0.91
Early fry (n = 6546)	−4.827	4.436	0.147	0.274	0.86	−0.191	0.261	0.79	1.760	0.930	0.22			
Late fry (n = 1836)	1.055	4.601	0.104	0.456	0.92	−0.197	0.551	0.92	0.962	1.277	0.79			
Wet weight (LMM)														
December (n = 840)	0.457	0.111	0.009	0.020	0.80	0.001	0.005	0.93	−0.011	0.023	0.81			
February (n = 804)	0.926	0.458	−0.092	0.077	0.38	−0.181	0.067	0.06	0.203	0.126	0.26			
April (n = 804)	4.855	1.156	−0.195	0.265	0.63	−0.284	0.166	0.18	0.019	0.322	0.93			
July (n = 803)	5.322	4.325	−0.345	0.719	0.79	−0.228	0.741	0.81	2.042	1.210	0.18			

Random effects	Female ID	Male ID	Variance ± sd (% var) F × M	Date	Tray	Cell
Survival (GLMM)						
Eyed-egg	0.071 ± 0.27 (1.4)	0.140 ± 0.37 (1.4)	**0.222 ± 0.47 (4.2*)**	0.407 ± 0.64 (7.7)	0.862 ± 0.93 (16.3)	**0.280 ± 0.53 (5.3*)**
Eyed-egg to fry	0.016 ± 0.12 (0.3)	0.525 ± 0.72 (11.1)	**0.800 ± 0.89 (17.0*)**	0.087 ± 0.30 (1.9)	ns	ns
Early fry	0.159 ± 0.40 (4.4)	0.116 ± 0.34 (3.1)	0.162 ± 0.40 (4.3)	0.019 ± 0.14 (0.5)		
Late fry	0 ± 0 (0)	0.278 ± 0.53 (6.2)	0.740 ± 0.86 (16.4)	0.196 ± 0.44 (4.3)		
Wet weight (LMM)						
December	**1.5E-03 ± 0.04 (75.6*)**	3.8E-05 ± 0.01 (1.9)	**2.0E-04 ± 0.01 (10.3*)**	ns		
February	0.016 ± 0.13 (10.4)	0.009 ± 0.10 (6.2)	**0.018 ± 0.13 (11.7*)**	0.012 ± 0.11 (7.9)		
April	**0.235 ± 0.48 (18.8*)**	0.057 ± 0.24 (4.6)	**0.084 ± 0.29 (6.7*)**	ns		
July	1.384 ± 1.18 (12.6)	**1.394 ± 1.18 (12.7*)**	**1.655 ± 1.29 (15.1*)**	ns		

ns: indicates the random effect accounted for no variance and was removed from the model.
Significance (*P*-values) of fixed factors are provided along with model estimates and standard error (SE) of each fixed effect. Estimates for colour morphs are for the white morph relative to the red morph. For random effects, the variance with standard deviation (SD) and per cent of total variance (% var) are provided and significant effects are indicated in bold with asterisk. Abbreviated random effect of F × M indicates female ID x male ID interaction. All *P*-values were corrected for false discovery rate (see Materials and methods).

Viral susceptibility challenges

Viral challenge I: Survival over 35 days after viral exposure

All survival challenge tanks started with 480 individuals. Mortalities that occurred before and after the challenge (mock HBSS or IHNV exposure) were recorded, genotyped and assigned to parents. Three post-challenge mortalities (one from control tank and two from a challenge tank) could not be assigned to parents, and therefore, they were excluded from the analyses. Mortalities occurring prior to the challenge (n = 5–9 fish per tank) were removed from the analysis. Following the challenge, mortality remained low (<2.7%) over the 35-day study period regardless of treatment group (mock vs. IHNV). Of the 23 mortalities that occurred in the IHNV-exposed tanks, 20 (87%) tested positive for IHNV, whereas no virus was detected in any of the 10 mortalities that occurred in the mock tank. Using GLMM, we found that none of the variance could be explained by the random effects in the model; therefore, we used generalized linear model (GLM) to test the main effects in the model. Using GLM, we found no significant effect of treatment (P = 0.69). Offspring of red females had higher survival than white females, where mean (± SE) family survival was 98.6 (± 0.25) and 97.2 (± 0.39) %, respectively, but this effect was not significant (P = 0.08). However, male colour did have a significant effect on survival (P = 0.014), where mean family survival of red male offspring was 2% higher than that of white males overall (98.9 ± 0.26 and 96.9 ± 0.37, respectively). We further investigated the effect of male colour on survival within each tank. We found that the difference in survival between offspring of red and white males was statistically significant in the control tank (P = 0.049), where mean family survival of red sired and white sired offspring was 98.9 (± 0.46) and 96.9 (± 0.65) %, respectively.

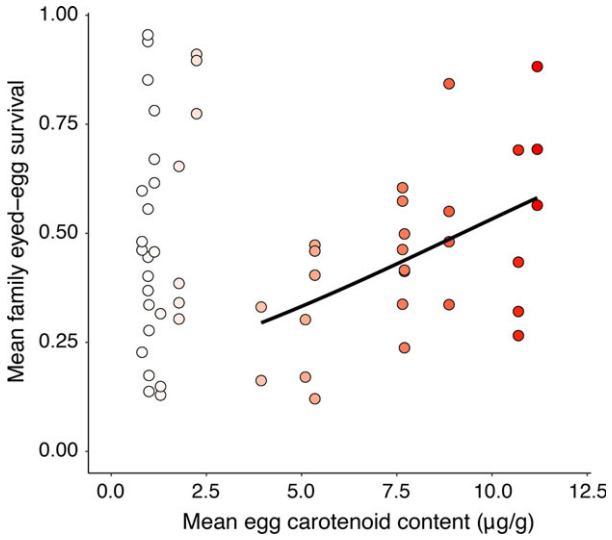

Fig. 4 Mean egg carotenoid content and mean family survival (proportion) to the eyed-egg stage derived from red and white Chinook salmon (*Oncorhynchus tshawytscha*) females. The relationship between carotenoids and survival was only significant within red female morph (shown by the solid line; $P = 0.006$) and not within white females ($P = 0.99$). See Table 4 for full results of generalized linear mixed effect models within each colour morph. Data points are coloured by relative carotenoid content of eggs as in Fig. 2 and jittered horizontally to show all variation.

No difference in survival of red and white sired offspring was found in either of the challenge tanks ($P > 0.18$); therefore, the small difference in survival between offspring of red and white males is not the result of differences in viral susceptibility.

Viral challenge II: Gene transcription response after viral exposure

In total, 493 offspring were sampled for RNA; however, samples were excluded if RNA quality/quantity was low ($n = 31$ of 493) and samples were excluded if parentage could not be assigned with 95% confidence ($n = 26$ of 493). A total of 436 individuals were analysed for transcription at all genes being assayed; however, as described in the methods, only individuals that generated usable transcription data were included in the final analyses (i.e. C_{RT} values were filtered and screened). Four genes, including CD8, IFNA, SAA and IHNV-N, were not further analysed because they showed limited or no amplification in our qRT-PCR reactions. IHNV-N gene represented the gene used to detect viral RNA in our sample. The lack of amplification of IHNV-N likely indicates that the virus was absent in the sample (or levels were too low to detect) as there did not appear to be any amplification across all samples.

We analysed relative gene expression for 21 genes, where 15 genes showed high amplification and the remaining six genes showed more variable amplification where individuals were excluded due to lack of or low amplification (see Tables 5 and 6 for sample size). Under control (mock) conditions, results of LMMs showed no significant effect of female colour on relative expression of immune, stress and oxidative stress genes (all $P > 0.16$; see Table 5 and Fig. S2). Following exposure to IHNV, offspring of red and white females also showed no difference (all $P > 0.15$; Table 6) and generally limited changes in relative gene expression for immune genes (Fig. 5) and stress and oxidative

Table 4 Results of generalized linear mixed effect models examining survival to the eyed-egg stage in Chinook salmon (*Oncorhynchus tshawytscha*) within red and white female colour morphs.

	Fixed effects						Random effects				
Survival to eyed-egg		Est	SE	χ^2	d.f.	*P*		% var	χ^2	d.f.	*P*
White females	Intercept	8.93	1.38				Cell	**9.8**	**727.17**	**1**	**<0.001**
	Carotenoids	−0.03	0.36	0.01	1	0.999	Tray	3.1	0.59	1	0.99
	Male colour(W)	0.30	0.25	1.05	1	0.863	Female × Male	**4.4**	**209.13**	**1**	**<0.001**
	Egg density	**−1.38**	**0.19**	**40.53**	**1**	**<0.001**	Female	1.4	0.43	1	0.99
	Egg mass	−0.95	1.60	0.03	1	0.999	Male	0.3	0.02	1	0.99
							Date	27.8	6.48	1	0.07
							Residual	53.2			
Red females	Intercept	0.89	1.28				Cell	**10.8**	**813.13**	**1**	**<0.001**
	Carotenoids	**0.27**	**0.05**	**11.36**	**1**	**0.006**	Tray	0.0	0.00	1	0.99
	Male colour(W)	0.44	0.39	1.16	1	0.734	Female × Male	**7.3**	**255.14**	**1**	**<0.001**
	Egg density	−0.01	0.19	0.00	1	0.999	Female	0.0	0.00	1	0.99
	Egg mass	−12.15	2.15	2.49	1	0.557	Male	8.5	3.00	1	0.47
							Date	0.0	0.00	1	0.99
							Residual	73.4			

Model estimates (Est) and standard error (SE) and results of fixed and random factors are provided. The percentage of the observed variation (% var) accounted for by each random factor is provided, and significant effects are indicated in bold. All *P*-values were corrected for false discovery rate (FDR).

stress genes (Fig. 6). Additionally, we found no difference in gene expression between the offspring of red and white males under control or challenge conditions (all $P > 0.16$; Tables 5 and 6). The effect of weight and some random effects explained expression of some genes (see Tables 5 and 6). Overall, female colour morph or male colour morph had no influence on gene expression results under mock or challenge conditions, nor was there a consistent pattern in the direction of expression differences when comparing morph types for both sexes (Tables 5 and 6).

Within each female morph, we found no significant effect of egg carotenoid content on gene expression in the fry under challenge or control conditions (all $P > 0.10$; see Table S6), and these limited differences were further visualized by transcriptional profiles (Fig. S3).

Discussion

Maternal carotenoids have been shown to provide important benefits to offspring in oviparous species, where carotenoids can impact egg and early life survival (McGraw *et al.*, 2005; Tyndale *et al.*, 2008), immunity (Saino *et al.*, 2003), body size (Marri & Richner, 2014), growth rate (Bazyar Lakeh *et al.*, 2010) and antioxidant status (McGraw *et al.*, 2005). In our study, we examined a wide range of fitness-related measures in the offspring derived from the eggs of carotenoid-rich (red) and carotenoid-poor (white) morphs of Chinook salmon. We found that the red female morph had significantly higher egg carotenoid content relative to the white female morph (~6-fold more); however, this did not translate into fitness-related differences that we assessed, including survival, growth or viral susceptibility, between progeny from the two female colour morphs. However, within female colour morphs, we found a significant positive effect of egg carotenoid content on survival to the eyed-egg stage (the earliest fitness measure) within the red female morph but not within the white female morph. White females gain benefits from reduced egg predation relative to red females (Lehnert *et al.*, 2017a); however, in the absence of predation, our results show that eggs of white females have similar survival to those of red females, and unlike red females, carotenoids in white eggs have no effect on egg survival. These results may support the hypothesis that white females benefit from additional

Table 5 Results of linear mixed effect models examining relative gene expression of immune stress and oxidative stress genes in Chinook salmon (*Oncorhynchus tshawytscha*) fry after exposure to control conditions.

		Fixed effects															
		Intercept		Female colour(W)			Male colour(W)			Weight			Random effects				
Gene	*N*	Est	SE	Est	SE	*P*	Est	SE	*P*	Est	SE	*P*	*f*	*m*	*I*	*C*	*T*
GPX	229	0.209	0.016	−0.002	0.016	0.99	0.010	0.011	0.90							*	
GR2	229	−0.346	0.044	0.035	0.048	0.99	0.007	0.038	0.99								
GST	230	0.701	0.059	−0.039	0.026	0.53	0.041	0.046	0.90					*		*	
HSP90A	229	−4.429	0.096	−0.098	0.086	0.73	0.003	0.077	0.99								
HSP70	230	−1.715	0.126	−0.060	0.077	0.98	0.021	0.091	0.99	−0.044	0.110	0.99	*	*		*	
IFNG	179	−8.729	0.096	0.055	0.066	0.97	−0.004	0.082	0.99							*	
IFR3	227	−5.631	0.155	0.085	0.056	0.52	−0.003	0.101	0.99	**0.699**	**0.174**	**0.003**					
IGM	142	−4.022	0.130	0.035	0.096	0.99	0.144	0.075	0.30							*	
IGT	187	−4.764	0.157	−0.055	0.124	0.99	−0.105	0.077	0.61							*	
IL1B	118	2.448	0.135	−0.044	0.072	0.99	0.102	0.074	0.58							*	
IL8	213	−2.330	0.119	−0.027	0.042	0.99	−0.059	0.053	0.75	0.223	0.131	0.39				*	
META	230	−3.505	0.190	−0.071	0.162	0.99	0.268	0.150	0.33								
MHCI	230	0.843	0.138	0.080	0.088	0.88	−0.027	0.102	0.99	0.314	0.128	0.08	*	*			
MHCIIB	230	0.846	0.095	−0.074	0.035	0.30	−0.060	0.061	0.88							*	
MX1	229	−3.797	0.309	0.284	0.118	0.16	0.017	0.209	0.99	**1.021**	**0.278**	**0.004**		*			*
SOD	228	0.167	0.007	0.002	0.004	0.99	−0.005	0.004	0.65								
TCRB	230	−3.593	0.094	0.069	0.100	0.98	0.055	0.096	0.99								
TGFB1	223	−3.221	0.113	0.009	0.059	0.99	−0.031	0.048	0.99							*	
TNFA	116	−4.615	0.141	−0.103	0.112	0.88	−0.014	0.087	0.99							*	
TRDNX	200	−4.430	0.170	−0.082	0.072	0.75	−0.180	0.108	0.45							*	
VIG1	138	−5.544	0.469	−0.048	0.183	0.99	0.158	0.222	0.99	**1.382**	**0.522**	**0.045**					

We used relative gene expression as the response for the control (mock) challenge. Significance (adjusted *P*-values) of fixed factors in the model are provided and the significance of random effects in the model are denoted by an asterisk (*). Estimates for colour morphs are for the white morph relative to the red morph. Random effects include female ID (*f*), male ID (*m*), their interaction (*I*), OpenArray chip (*C*) and tank (*T*). All *P*-values were adjusted for false discovery rate and significant values are indicated by bold font.

Table 6 Results of linear mixed effect models examining relative gene expression of immune stress and oxidative stress genes in Chinook salmon (*Oncorhynchus tshawytscha*) fry after exposure to challenge (infectious hematopoietic necrosis virus; IHNV) conditions.

		Fixed effects															
		Intercept		Female colour(W)			Male colour(W)			Weight			Random effects				
Gene	*N*	Est	SE	Est	SE	*P*	Est	SE	*P*	Est	SE	*P*	*f*	*m*	*I*	*C*	*T*
GPX	203	−0.046	0.020	−0.003	0.013	0.99	−0.001	0.009	0.99	0.034	0.017	0.25			*	*	
GR2	206	−0.019	0.028	−0.044	0.031	0.54	−0.033	0.031	0.87								
GST	206	−0.023	0.038	0.007	0.024	0.99	−0.053	0.024	0.23						*	*	
HSP90A	206	0.000	0.002	0.000	0.002	0.99	−0.001	0.002	0.99						*	*	
HSP70	204	−0.025	0.021	−0.003	0.016	0.99	0.002	0.006	0.99	0.011	0.017	0.99	*			*	
IFNG	143	0.000	0.000	0.000	0.000	0.41	0.000	0.000	0.59								
IFR3	206	−0.001	0.001	0.001	0.001	0.99	0.000	0.001	0.99								
IGM	146	−0.005	0.005	0.001	0.002	0.99	−0.005	0.003	0.23	0.010	0.005	0.38					
IGT	170	0.002	0.002	−0.001	0.002	0.99	−0.001	0.001	0.87								
IL1B	109	−1.750	2.052	2.774	1.528	0.37	−3.170	1.868	0.37								
IL8	185	−0.014	0.008	−0.001	0.007	0.99	0.000	0.009	0.99						*		
META	206	0.003	0.021	0.000	0.025	0.99	−0.031	0.020	0.50						*		
MHCI	206	0.004	0.138	−0.085	0.086	0.91	−0.015	0.086	0.99							*	
MHCIIB	206	−0.249	0.179	−0.092	0.118	0.99	0.111	0.095	0.87						*	*	
MX1	206	−0.016	0.014	−0.001	0.008	0.99	0.010	0.017	0.99							*	
SOD	206	−0.003	0.003	0.000	0.001	0.99	0.003	0.001	0.16							*	
TCRB	206	−0.002	0.004	0.003	0.004	0.99	−0.005	0.004	0.66						*		
TGFB1	201	−0.005	0.003	0.000	0.002	0.99	0.001	0.002	0.99							*	
TNFA	99	−0.001	0.002	0.002	0.002	0.61	−0.001	0.001	0.99						*		
TRDNX	193	−0.001	0.002	0.003	0.001	0.15	0.000	0.001	0.99							*	
VIG1	115	−0.003	0.005	0.004	0.005	0.99	0.002	0.006	0.99								

We used the change in relative gene expression for the IHNV challenge treatment as the response. Model estimates with standard error (SE) and significance (adjusted *P*-values) of fixed factors in the model are provided and the significance of random effects in the model are denoted by an asterisk (*). Random effects include female ID (*f*), male ID (*m*), their interaction (*I*), OpenArray chip (*C*) and tank (*T*). All *P*-values were adjusted for false discovery rate and significant values are indicated by bold font.

mechanisms that have evolved to compensate for lower survival associated with their reduced carotenoid content during early life. Such potential mechanisms are discussed below.

In Chinook salmon, a positive relationship between egg carotenoid content and egg survival has been previously documented across populations with variation in egg carotenoid content (Tyndale *et al.*, 2008). Although some studies have failed to detect a positive association between carotenoids and egg survival in other salmonids (Torrissen, 1984; Christiansen & Torrissen, 1997; Wilkins *et al.*, 2017), Craik (1985) proposed that a critical threshold for egg carotenoid content may exist in salmonids (ranging between 1 and 3 μg g^{-1} for rainbow trout), above which egg survival will be high. This has been supported in Chinook salmon where the carotenoid threshold was estimated to be 2 μg g^{-1} (Tyndale *et al.*, 2008). Our data do not support a threshold effect across all females or within colour morphs, as the relationship between carotenoids and survival does not appear to be asymptotic within the red morph. Interestingly, within the white morph group, carotenoids were not related to eyed-egg survival, although survival was related to incubation density within this morph. Although density effects were not of primary study in our experiment, the stronger effect of density on white salmon eggs warrants further investigation and may suggest that the white morph occupies (and is restricted to) river habitats with cold temperatures and high water flows (like the Quesnel system) that help to maintain high oxygen levels in nests during incubation (Hard *et al.*, 1989). In this case, differences in maternal behaviour during nest building could also contribute to morph differences in sympatric systems.

Maternal carotenoids appear to only have a positive effect on fitness at the earliest measurable stage in our study and only within the red colour morph – we found no effects of carotenoids (between or within female morphs) on survival or size after the eyed-egg stage. Results were also consistent with offspring performance after exposure to infectious hematopoietic necrosis virus (IHNV), a pathogen that has been detected in the Quesnel River. In fact, we did not detect any strong response to the challenge overall, and we acknowledge that several factors may influence viral susceptibility in salmonids including environmental condition, age, life history variation, population and viral strain (Lapatra, 1998; Garver *et al.*, 2006;

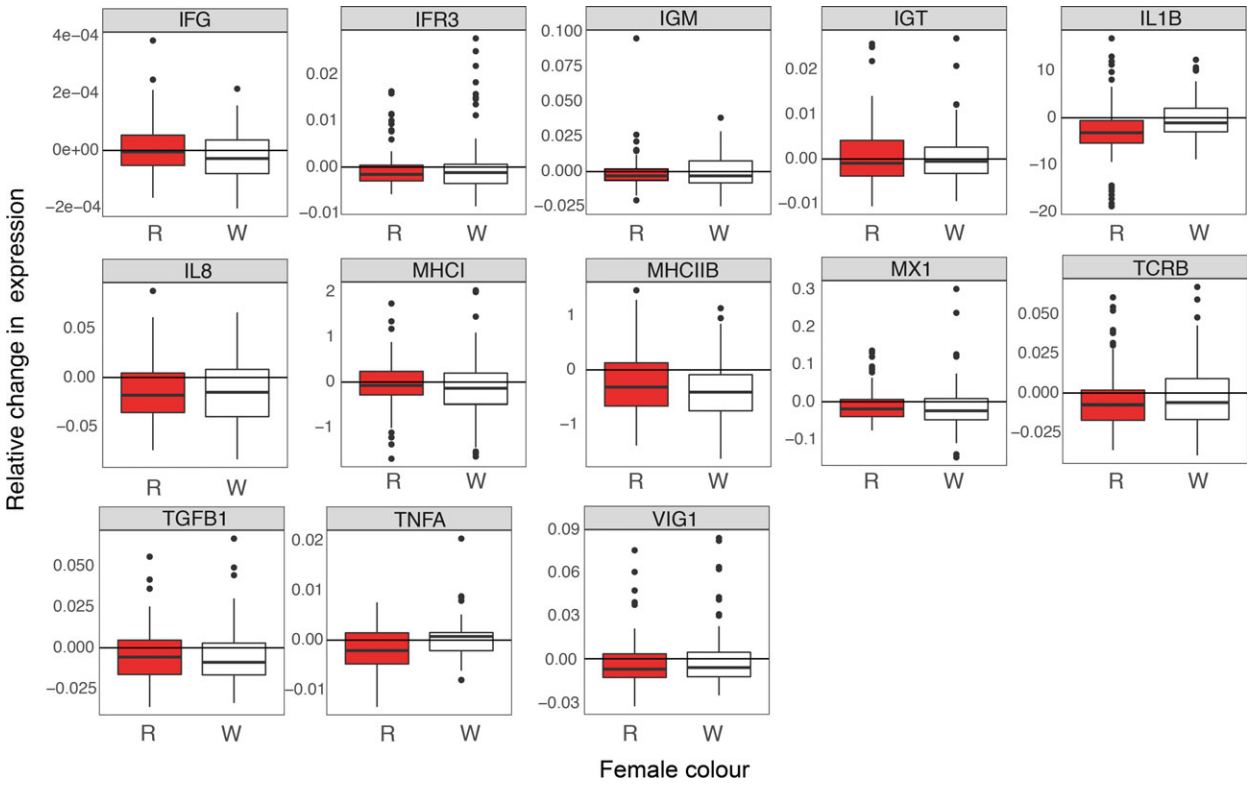

Fig. 5 Boxplots of change in relative gene expression (relative to the family mean under control conditions) of immune genes in Chinook salmon (*Oncorhynchus tshawytscha*) fry from red and white females after exposure to infectious hematopoietic necrosis virus (IHNV). Relative expression was measured in all fish at 72 h post-challenge. Line at zero represents no change in relative expression compared to the control treatment.

Hernandez *et al.*, 2016). It is possible that 72 h post-challenge may not have been the optimal time to detect differential gene expression; however, this seems unlikely as other studies in salmonid fry have demonstrated the responses to viral exposure within 2–3 days (Yamamoto *et al.*, 1990; Ogut & Reno, 2004; Purcell *et al.*, 2004), and even over a 35-day period, we found no effect of the virus on survival. The low mortality and lack of host response to virus likely suggest that Quesnel River Chinook salmon fry were not highly susceptible to the IHNV isolate and dose used here.

Limited differences in overall fitness between colour morphs are perhaps surprising given the large differences in maternal carotenoid provisioning; however, our results are consistent with Tyndale *et al.* (2008), where egg carotenoids were correlated with egg survival in Chinook salmon but not linked to later fitness measures such as offspring growth or size. These expected differences are based on the assumption that the only difference between red and white eggs is carotenoid (primarily astaxanthin) concentration. Yet, these colour phenotypes have been shaped by natural selection over evolutionary time, and therefore, it seems likely that white Chinook salmon have evolved compensatory mechanisms to counteract the deficiency of carotenoids in their eggs, consistent with our findings. Recent studies have found that white Chinook salmon may have evolved because of benefits from reduced egg predation (Lehnert *et al.*, 2017a) and additional mechanisms may have evolved to offset their presumed handicap such as functional genetic mechanisms (major histocompatability genes; Lehnert *et al.*, 2016). White Chinook salmon may have also evolved to increase the concentration of alternative antioxidants into their eggs, such as vitamin E, vitamin C or retinoids, which are found in salmon eggs (Cowey *et al.*, 1985; Garner *et al.*, 2010) and/or through increased concentration of maternal antibodies in eggs (Blount *et al.*, 2002). If white Chinook salmon have evolved such mechanisms, this may evoke selection on coadapted alleles, and indeed, in other species, tightly linked genes can facilitate the evolution of alternative morphs (McKinnon & Pierotti, 2010; Kupper *et al.*, 2016). For example, in the white-throated sparrow (*Zonotrichia albicollis*), genes modulating colour and behaviour are tightly linked through a chromosomal inversion (Zinzow-Kramer *et al.*, 2015). In this case, we may expect to detect an effect of parental morph on offspring

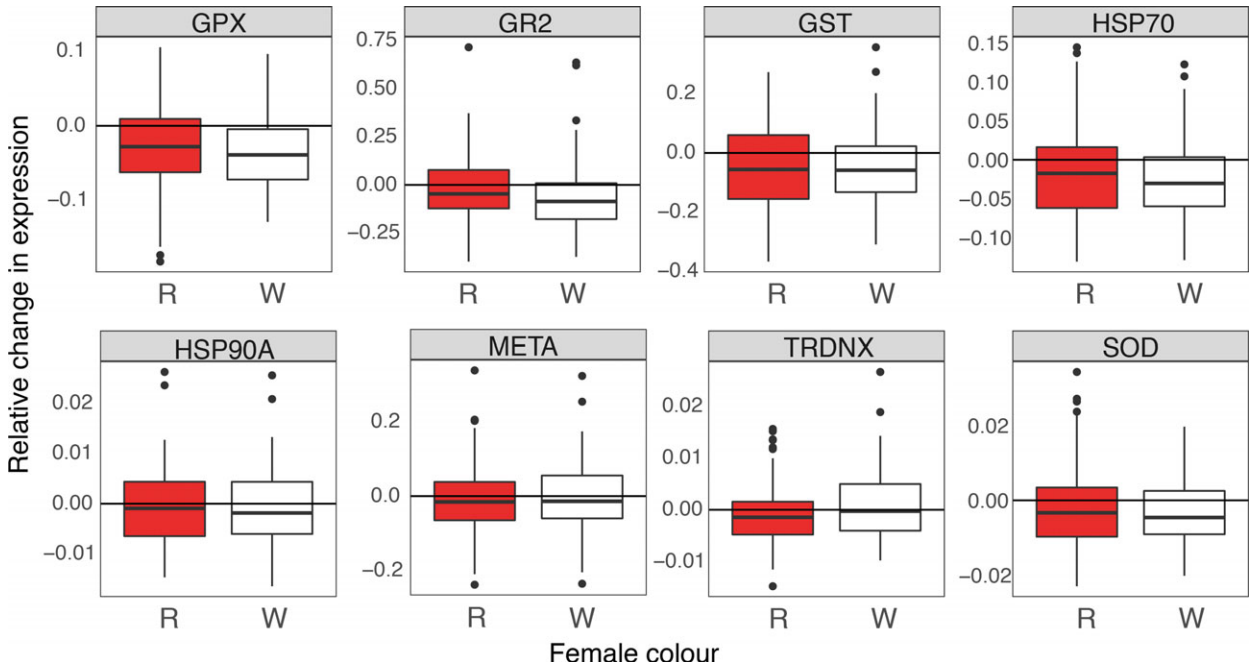

Fig. 6 Boxplots of change in relative gene expression (relative to the family mean under control conditions) of stress and oxidative stress genes in Chinook salmon (*Oncorhynchus tshawytscha*) fry from red and white females after exposure to infectious hematopoietic necrosis virus (IHNV). Relative expression was measured in all fish at 72 h post-challenge. Line at zero represents no change in relative expression compared to the control treatment.

fitness, where, in the salmon scenario for example, crosses between white females and red males may produce offspring with lower fitness relative to crosses between white females and white males. However, we did not detect an interaction effect of male × female morph on fitness-related traits. Recombination can disrupt the linkage of *cis*-acting coadapted alleles along a chromosome, and thus, it may require at least an additional generation of mating (i.e. F_2 generation and later) to detect potential negative consequences of crossing between morphs. Therefore, in our study, coadapted alleles influencing colour and egg survival in white females may contribute to offspring fitness in a dominant way (i.e. sex-dependent dominance) overriding any potential influence of male genotype. Sex-dependent dominance has been documented in other salmonids, such as for a life history trait in Atlantic salmon (*Salmo salar*) (Barson *et al.*, 2015); nonetheless, more work is needed to better understand the genomic architecture of these colour morphs.

We did not assay carotenoid content of males in our study; however, red and white males show clear differences in external carotenoid coloration during spawning in the Quesnel system. Salmon have a nonresource-based mating system where males provide no parental care, and in these systems, male traits may signal genetic quality and females may select males to obtain superior offspring (Neff & Pitcher, 2005). However, evidence that carotenoids signal male genetic quality and contribute to offspring fitness is scarce in nonresource-based mating systems (but see Evans *et al.*, 2004). Here, we found no difference between offspring of red and white sires in terms of egg and offspring survival, offspring size and offspring gene expression. Offspring of red males did show significantly higher survival in the viral challenge I; however, differences found between male morphs were not associated with viral susceptibility as this difference was only found in the mock (no virus exposure) tank. Nonetheless, despite a lack of strong differences, it is worth noting that early life measures such as egg survival and early fry weight were higher in offspring of white males, but at later stages, fry survival and fry weight were higher in offspring of the red morph (see estimates from Table 3). These data are consistent with ontogenetic trade-offs between morphs, and similar shifts can also be noted in the female colour morphs.

Although the limited effects of carotenoids found in our study beyond the egg stage are comparable to some other studies, inconsistencies in the literature also exist and may arise from differences in the context of which variation in maternal carotenoids is studied as follows: experimental manipulation vs. natural variation. In birds and fishes, many diet manipulation studies have found evidence that maternal carotenoid supplementation can influence offspring performance (McGraw

et al., 2005; Ewen *et al.*, 2009; Bazyar Lakeh *et al.*, 2010; Brown *et al.*, 2014), whereas studies using natural variation in egg carotenoid content have found no link between egg carotenoids and several fitness traits such as offspring growth or size in Chinook salmon (Tyndale *et al.*, 2008), embryo survival in brown trout (*Salmo trutta*) (Wilkins *et al.*, 2017) and offspring survival and size in the two-spotted goby (*Gobiusculus flavescens*) (Svensson *et al.*, 2006). We acknowledge that these studies and our study examine the effects of carotenoids under laboratory conditions where selection is relaxed, and thus, fitness effects may differ from natural conditions. Nonetheless, it is also possible that maternal diet quality influences offspring fitness in these studies (Brown *et al.*, 2014). Despite the limited ability of white Chinook salmon females to deposit carotenoids into their tissues, they still consume and metabolize these pigments. Therefore, if maternal dietary carotenoid availability and thus quality of the maternal diet (and not egg carotenoid content itself) influence offspring performance (see Brown *et al.*, 2014), this could also explain why the offspring of red and white Chinook salmon females exhibited similar performance in our study.

In conclusion, we measured a suite of fitness-related traits in offspring derived from red and white Chinook salmon, which differed significantly in egg carotenoid content, yet we found no measurable differences in offspring fitness. The only effect of carotenoids detected in our study was found within the red female morph, where egg carotenoid content was positively related to survival to the eyed-egg stage. Our results highlight the possibility that although white Chinook salmon may have evolved due to the benefit of reduced predation at the egg stage (Lehnert *et al.*, 2017a), additional evolutionary pressure may have shaped the white morph to compensate for any additional costs of limited egg carotenoids such as maternal provisioning of alternative resources or behavioural strategies in spawning habitat preference. In nature, variation in carotenoid utilization exists (Svensson *et al.*, 2006; Lehnert *et al.*, 2017a; Wilkins *et al.*, 2017) and this variation may be shaped by trade-offs where animals such as white Chinook salmon can incur their own benefits by employing different tactics, and thus may explain in part how this unique polymorphism can be maintained through balancing selection.

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Conflict of interest

Authors have not conflict of interest to declare.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1 Methods.
Table S1 Primer and Taqman probe sequences for all genes used in the study.
Table S2 Results of generalized linear mixed effect models examining egg and fry survival in Chinook salmon (*Oncorhynchus tshawytscha*) within the red female morph.
Table S3 Results of generalized linear mixed effect models examining egg and fry survival in Chinook salmon (*Oncorhynchus tshawytscha*) within the white female morph.
Table S4 Results of linear mixed effect models examining wet weight in Chinook salmon (*Oncorhynchus tshawytscha*) fry within the red female morph.
Table S5 Results of linear mixed effect models examining wet weight in Chinook salmon (*Oncorhynchus tshawytscha*) fry within the white female morph.
Table S6 Results of linear mixed effect models examining relative gene expression (and change in relative expression for challenge) of immune genes, stress and oxidative stress genes in fry of red and white female colour morphs of Chinook salmon (*Oncorhynchus tshawytscha*) after exposure to control or challenge (infectious hematopoietic necrosis virus; IHNV) conditions.
Figure S1 Relationship between egg density and eyed-egg survival in A) white and B) red Chinook salmon females.
Figure S2 Boxplots of relative gene expression of immune, stress and oxidative stress genes in Chinook salmon (*Oncorhynchus tshawytscha*) fry from red and white females under control conditions.
Figure S3 Heatmaps showing normalized (*Z*-score) values of mean relative expression of Chinook salmon (*Oncorhynchus tshawytscha*) fry by family ($n = 32$) for 21 genes involved in immune, stress, and oxidative stress response under (A) control (mock) challenge and (B) viral (infectious hematopoietic necrosis virus; IHNV) challenge.

Data deposited at Dryad: https://doi.org/10.5061/dryad.33m546g

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