Genetica (2014) 142:281–293
DOI 10.1007/s10709-014-9774-5

Multigenerational outbreeding effects in Chinook salmon (*Oncorhynchus tshawytscha*)

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Received: 27 January 2014 / Accepted: 12 June 2014 / Published online: 22 June 2014

Abstract Outbreeding, mating between genetically divergent individuals, may result in negative fitness consequences for offspring via outbreeding depression. Outbreeding effects are of notable concern in salmonid research as outbreeding can have major implications for salmon aquaculture and conservation management. We therefore quantified outbreeding effects in two generations (F_1 hybrids and F_2 backcrossed hybrids) of Chinook salmon (*Oncorhynchus tshawytscha*) derived from captively-reared purebred lines that had been selectively bred for differential performance based on disease resistance and growth rate. Parental lines were crossed in 2009 to create purebred and reciprocal hybrid crosses (n = 53 families), and in 2010 parental and hybrid crosses were crossed to create purebred and backcrossed hybrid crosses (n = 66 families). Although we found significant genetic divergence between the parental lines ($F_{ST} = 0.130$), reciprocal F_1 hybrids showed no evidence of outbreeding depression (hybrid breakdown) or favorable heterosis for weight, length, condition or survival. The F_2 backcrossed hybrids showed no outbreeding depression for a suite of fitness related traits measured from egg to sexually mature adult life stages. Our study contributes to the current knowledge of outbreeding effects in salmonids and supports the need for more research to better comprehend the mechanisms driving outbreeding depression.

Keywords Outbreeding depression · Heterosis · Aquaculture · Hybrid · Backcrossing

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Introduction

Conservation managers are often confronted with the challenge of whether to inbreed or outbreed populations to either maintain local adaptation or increase genetic diversity, respectively (Fraser 2008; Neff et al. 2011). The outbreeding of populations for conservation purposes is a relatively recent strategy that is predicated on the supposition that imperiled populations could be "genetically rescued" by the infusion of new alleles into the population (Tallmon et al. 2004). The theory of genetic rescue is based on the idea that small populations may suffer from inbreeding effects and that the introgression of novel genotypes could add diversity to that population, thus increasing fitness and "rescuing" the population from extirpation (Tallmon et al. 2004; Edmands 2007). The infusion of novel alleles resulting in superior offspring fitness is known as heterosis (Whitlock et al. 2000), where heterozygosity at a locus provides greater fitness relative to either homozygous genotype (Edmands 2007). Heterosis may also occur through dominance, where the dominant allele from one parent masks the recessive deleterious allele from the other parent (Lynch 1991). Alternatively, depending on the nature of the hybridizing stocks/

populations, outbreeding could result in outbreeding depression where offspring experience reduced fitness relative to their parents (Lynch 1991; Edmands 2007). Outbreeding is of particular concern for salmon populations as they are generally thought to be locally-adapted to their natal streams (Taylor 1991), and thus outbreeding could disrupt gene interactions contributing to local adaptation (Fraser 2008; Neff et al. 2011).

Outbreeding depression can result from additive genetic effects, and these effects are often observed in the first generation hybrid when offspring display a phenotype intermediate to both parents, leading to a reduction in fitness in either parental environment (Lynch 1991; Edmands 2007). Outbreeding depression can also occur through non-additive effects which may be expressed as maladaptive dominance effects leading to "hybrid breakdown" in the offspring of first generation crosses (e.g., Aykanat et al. 2011). We may expect that outbreeding depression in first generation hybrids would occur via *trans*-acting effects, whereby allele interactions between chromosomes result in negative fitness consequences. Additionally, outbreeding depression may result from the disruption of coadapted gene complexes through the introgression of novel alleles and genotypes, and may not be apparent until the second or later generations when divergent parental genomes undergo recombination (Lynch 1991). In this case outbreeding depression may occur in second generation hybrids through *cis*-acting effects, where recombination of parental alleles within a chromosome results in negative fitness consequences. Ideally, outbreeding studies should be multigenerational, given that F_1 hybrids may experience heterosis, and subsequently exhibit outbreeding depression in later generations, as previously documented in copepods (*Tigriopus californicus*; Edmands 1999) and birds (*Melospiza melodia*; Marr et al. 2002).

In fish, outbreeding depression has been detected in survival (Gharrett et al. 1999; Gilk et al. 2004; Tymchuk et al. 2007) and fitness-related traits such as gill morphology (Gharrett and Smoker 1991) and growth rate (Huff et al. 2011). For example, Gilk et al. (2004) observed that hybridization reduced survival in F_2 offspring of pink salmon (*Oncorhynchus gorbuscha*), indicative of outbreeding depression via non-additive genetic effects. Likewise, McClelland et al. (2005) found that F_1 and F_2 hybrid coho salmon (*O. kisutch*) exhibited intermediate weights and lengths relative to parental strains, mostly due to additive and dominance effects and not likely a result of epistatic interactions. Although many other studies have found that outbreeding does not always have negative effects on various physical performance traits, such as body size (Sheffer et al. 1999; Fraser et al. 2008; Houde et al. 2011b), outbreeding depression may be more pronounced in physiological traits since they form the mechanistic basis for a number of complex and inter-linked phenotypic performance responses. Hybridization (F_1) negatively affected cardiovascular performance (Cooke and Phillip 2005) and swimming performance (Cooke et al. 2001) in largemouth bass (*Micropterus salmoides*). Furthermore, Crespel et al. (2011) found outbreeding depression for secondary stress response in F_1 hybrids of domestic brook charr (*Salvelinus fontinalis*), where one hybrid group exhibited an increase in plasma glucose relative to parental strains. Additionally, outbreeding between dwarf and normal whitefish (*Coregonus clupeaformis*) affected male reproductive physiology, as F_2 backcrosses displayed lower sperm velocity compared to parental strains (Whiteley et al. 2009). Negative effects on physiological characteristics can equate to potential fitness reduction, as many physiological responses are important for long-term survival and local adaptation.

In our study, we assess multiple generations of outbreeding and test for *trans*- and *cis*-acting outbreeding effects on fitness-related traits in Chinook salmon under a common environment. We investigate F_1 hybrid and F_2 backcrossed hybrid Chinook salmon using a multigenerational approach in order to properly quantify the effects of outbreeding purebred lines. We test for *trans*-acting outbreeding effects on fitness-related traits and survival in F_1 hybrids, and we test for *cis*-acting outbreeding depression in F_2 backcrossed hybrids through survival and a suite of fitness-related traits from egg to mature adult life stages. Although various studies report outbreeding effects in salmonids, no studies have investigated outbreeding in Chinook salmon (*O. tshawytscha*), which is important since it is unlikely that outbreeding effects can be generalized across species (Edmands 2007; McClelland and Naish 2007). Understanding potential outbreeding effects is valuable for both applied hatchery, conservation and aquaculture breeding programs as well as theoretically, since outbreeding depression is a form of reinforcement that can contribute to genetic divergence, local adaptation, and ultimately, speciation (Rundle and Whitlock 2001; Nosil et al. 2005).

Materials and methods

Fish origin

Yellow Island Aquaculture Ltd. (YIAL), an organic Chinook salmon farm located on Quadra Island, British Columbia, Canada, reared the Chinook salmon used in this study. Two specific inbred lines have been held at YIAL since 1997, the originating fish were selected for high performance (HH) and low performance (LL) using growth and survival related gene markers (Docker and Heath

2002). The high performance line was derived from YIAL's domestic stock, which was originally created in 1985 with gametes from Robertson Creek and Big Qualicum hatcheries on Vancouver Island, BC. The high performance line was created after four generations of domestication at YIAL. The low performance line was derived from the wild Big Qualicum river stock in 1997. The lines each began with 26 families, and the lines displayed differential performance for disease resistance during a *Vibrio* outbreak. *Vibrio* is a bacteria that is a common source of salmon mortality in aquaculture during saltwater rearing (Aykanat et al. 2012). Therefore lines were termed high and low performance as a result of the significant difference in their survival during the outbreak (see Bryden et al. 2004). Individual parental fish were also selected based on growth rate (i.e., body size at age) within the two survival groups, where small fish were selected in the LL line and large fish were selected in the HH line. Although the low-performance line often experienced high mortalities, numbers were sufficient to maintain the lines through within-line breeding for 4 generations, generally using less than 20 fish per year to propagate the lines.

First generation (F_1) hybrid mating design and rearing

In November 2009, sexually mature fish from parental HH and LL performance lines were seined from saltwater net pens, transferred to fresh water, and fish were artificially spawned to create two 6 × 6 full factorial crosses. Each 6 × 6 cross included 3 HH and 3 LL females mated to 3 HH and 3 LL males, resulting in 36 families per 6 × 6 cross. Fertilized eggs were incubated in vertical stack incubation trays until hatch then transferred to 200-L tanks (one tank per family). In June 2010, there were 53 families remaining of the total 72 families. Missing families were randomly distributed throughout crosses, resulting in 14 LL × LL (purebred), 14 HH × LL (outbred), 14 LL × HH (outbred) and 11 HH × HH (purebred) families in total, where the first letters denote the dam (female) and the second letters denote the sire (male). Loss of families may have occurred due to variability in fertilization success. Fertilization success was assessed for the original 72 families, where the 52 families that contributed offspring to freshwater tanks were scored as successful (coded as 1) and families that had limited or no offspring remaining after incubation were scored as unsuccessful (coded as 0). Approximately 50 individuals per family were weighed and measured in June 2010 (n = 2516). These fish were subsequently injected with a passive integrated transponder (PIT) tag, which allowed for individual identification of each fish, as each tag has a unique 16 digit numeric code. Tagged fish were transferred to saltwater net pens, where fish were randomly distributed between two net pens with 1,318 and 1,198 fish being allocated to each pen. All fish were weighed and measured in April 2011 (n = 2,162) and October 2011 (n = 684), and saltwater survival was recorded.

Second generation (F_2) backcrossed hybrid mating design and rearing

In November 2010, sexually mature fish (10 males and 10 females) were seined from saltwater net pens and artificially spawned in a full factorial breeding design resulting in 100 crosses (families). All females in the breeding design were purebred HH. Males in the study included outbred (hybrids: HL and LH), as well as purebred (HH). The breeding design thus resulted in 60 outbred families of backcrossed hybrids (30 HH × HL and 30 HH × LH) and 40 purebred (HH × HH) families, although some individual crosses were lost during the study.

Eggs were incubated in vertical stack incubation trays, and dead eggs were counted and removed to determine egg survival. Eggs of each family were split in half, and incubated in two separate cells of the incubation trays (i.e., two cells per family). Cell (egg) position was randomized across stacks, trays and cell position within tray. Eggs were counted between December 17, 2010 and March 2, 2011 on 14 occasions at intervals of less than 2 weeks. Fertilization success was estimated for the 100 families, where families that contributed offspring to freshwater tanks were scored as successful (coded as 1) and those that had limited or no offspring remaining after incubation were scored as unsuccessful (coded as 0). Three females produced non-viable eggs that resulted in the number of families being reduced to 70 immediately after incubation began; however this did not affect relative proportions of cross types since all 10 females were purebred HH. Eggs produced by those three females were excluded from egg survival analysis and remaining experiments. In March 2011, offspring had reached the swim-up fry stage where the yolk sac had been absorbed and feeding began to occur exogenously. At this time, approximately 70–100 fish from each surviving cross (66 families) were transferred to individual 200-L rearing tanks (i.e., one tank per family), and fish were fed to satiation. Of the surviving 66 families, there were 25 HH × HH, 20 HH × HL and 21 HH × LH families. On March 24–25, 2011, a subsample of 20 fish per family were weighed and measured. On June 14–15, 2011, a subsample of 20 fish per family were injected with a PIT tag (see above), then weighed and measured. On July 14, 2011, all tagged fish were transferred to a single saltwater net pen. Tagging of fish allowed for accurate survival and growth records on each individual, and all fish were weighed and measured on three additional occasions including October 29, 2011 (n = 1,261), April 18, 2012 (n = 1,109), and November 2, 2012 (n = 593). From weight and length data

we also determined Fulton's condition factor, calculated as $K = (W \cdot L^{-3}) \times 100$, where W is weight (g) and L is fork length (cm). Specific growth rate (SGR) was determined for the course of the experiment (508 days), using the equation $SGR = (100)\ (\ln W_1 - \ln W_0)\ t^{-1}$, where W_1 is the final weight and W_0 is the initial weight, and t is the number of days in the growth period. Saltwater survival data were coded by individual fish as a binominal data point of "0" for mortality or "1" for survival, and all mortalities were identified as a loss of a PIT tag code and recorded over the course of the experiment from entry into saltwater July 14, 2011 to November 2, 2012.

F_2 adrenocortical stress response

To measure the stress response to handling, 36 families with 3–6 individuals per family were chosen to collect baseline and 1-h post-stress plasma cortisol concentration data. Experimental design included the families of 6 females × 6 males, which equated to 12 purebred families and 24 outbred (backcrossed hybrid) families. Fish from those families were randomly selected during sampling on April 18, 2012, and 195 fish were transferred to a 4.5 × 4.5 m net pen to acclimate for at least 40 h. On April 20 between the hours of 9:00–18:00, fish were netted and anesthetized in a clove oil bath, and blood was collected from the caudal vein of fish by ventral insertion of a 1-cc heparinized syringe with a 22-gauge needle. Fish were sampled in groups of 10–15 individuals to ensure that sampling occurred within a short time frame, less than 6 min after capture. Fish recovered in 1,000-L tanks for 1-h, and then blood was taken again to obtain the stress-induced sample. Time of day and time from capture to blood sampling were recorded for all fish. Syringes were kept cool after sampling, transferred to heparinized microcentrifuge tubes on ice and subsequently stored at 4 °C for up to 12 h. Microcentrifuge tubes were centrifuged at 13,000 rpm for 2 min to separate red blood cells and plasma. Plasma was transferred to 1.5 mL tubes and frozen for later laboratory analysis. After the trial, we monitored survival of the sampled (stressed) individuals for 3-weeks post-treatment.

Plasma levels of cortisol from F_2 offspring were measured using a commercial enzyme immunoassay (EIA; Enzo Life Sciences, Inc.) following the supplied kit protocol. Optimization of plasma pooled from several individuals was used to determine optimal plasma dilution prior to assays. Optimal plasma dilution for baseline and stress-induced samples was 1:100, and triplicates for each sample were used in the assay. Samples were analyzed over 19 plates, resulting in an intra-assay variation of 6.7 % and an inter-assay variation of 8.2 %.

F_2 sperm characteristics

On November 2, 2012, F_2 offspring were approximately 2-years of age and many males had attained precocious sexual maturation, which in Chinook salmon are known as "jacks". Jacking rate was recorded, where fish were coded as a binominal data point of "0" for non-jack or "1" for jack. Milt was collected from jacks by applying gentle pressure to the abdomen and milt was stored in whirl pack bags at approximately 4 °C until sperm analysis. Sperm samples were collected from males of all cross types (n = 54), which included 21 purebred HH × HH, 17 outbred HH × HL and 16 outbred HH × LH males. Sperm traits examined included sperm motility, velocity (VAP, average velocity along a smoothed cell path), longevity, and density. Sperm was activated in freshwater and sperm traits were analyzed from video recordings using computer assisted sperm analysis (CASA) following the same protocol described in Lehnert et al. (2012).

Genetic differentiation and diversity

DNA was extracted from fin tissue of 32 individuals from each parental line (HH and LL) using an automated plate-based extraction protocol (Elphinstone et al. 2003). Individuals were genotyped at 10 previously described microsatellite loci: Ots107 (Nelsen and Beacham 1999), RT212, RT191 (Spies et al. 2005), Ots209, Ots211, Ots204, Ots213 (Greig et al. 2003), Omy325 (O'Connell et al. 1997), OtsG67, and OtsG432 (Williamson et al. 2002). Polymerase chain reaction (PCR) was used to amplify DNA at the microsatellite loci with fluorescent dye-labeled forward primers and fragment sizes were visualized using a LiCor 4300 DNA analyzer (LiCor Biosciences, Inc.). Individual genotypes were generated based on fragment sizes scored using GENE IMAGIR 4.05 software (Scanalytics Inc.). Individuals that were missing alleles at more than 4 loci were excluded from the analysis. Genetic differentiation between the two purebred lines was estimated by calculating pair-wise F_{ST} values (Weir and Cockerham 1984) between HH and LL lines using ARLEQUIN version 3.5 at 10,000 permutations (Excoffier and Lischer 2010). Genetic diversity estimates including number of alleles, number of private alleles and expected and observed heterozygosity were calculated using GenAlEx version 6.5 software (Peakall and Smouse 2012) and allelic richness was calculated using FSTAT version 2.9.3 (Goudet 2001).

Statistical analysis

Data were tested for normality using Shapiro Wilks test. Proportional data for F_2 egg survival and sperm motility (at 5, 10 and 15 s post-activation) were arcsine and arcsine

square root transformed, respectively, to successfully achieve normality ($p = 0.19$ and $p > 0.07$). Pearson correlations were used to analyze the relationship between egg survival and female weight as well as egg incubation density. Eggs were randomly spatially distributed during incubation across stacks, trays and cells, and any significant effects of spatial position on egg survival were included as random factors in the models described below.

Data were analyzed in R software (R Development Core Team 2011) using the lme4 package (Bates et al. 2009) with restricted maximum likelihood (REML) methods. First, we tested whether dam, sire and their interaction significantly contributed to the phenotypic variance for the trait of interest. Following the Lynch and Walsh (1998) model, we used the full model to partition phenotypic variance:

$$\text{Full model}: z_{aijk} = \mu + T_a + d_i + s_j + I_{ij} + e_{aijk}$$

where z_{aijk} is the phenotypic value of the kth offspring from the ith dam, jth sire and ath cross type, and μ is the mean phenotypic value of the sample. Dam (d), sire (s) and their interaction (I) were treated as random effects in the model and e represents the residual error. Cross type (T) was included in all models as a fixed effect. To determine the contribution of dam (d), sire (s) and their interaction (I) to the phenotypic variance observed for a specific trait, we compared the fit of various models in a stepwise manner by removing the term and refitting the model. Log-likelihood tests were used to compare the fit of the models where the log-likelihood ratio statistic has a Chi square distribution and the degrees of freedom is equivalent to the number of factors excluded in the model. A significant effect of dam (d) would indicate that maternal and additive genetic effects significantly contribute to the phenotypic variance of a trait (Lynch and Walsh 1998). A significant sire (s) effect would indicate additive genetic effects and a significant interaction (I) effect would indicate significant non-additive genetic effects attributable to the combination of genes from the ith dam and jth sire (Lynch and Walsh 1998). Next we examined the effect of cross type (T) in the model. To determine the effect of cross type on the trait, we compared the fit of the model with and without cross type (T). In this case, the parameters of both models were estimated using maximum likelihood (ML), and a log-likelihood test was used to compare between the two models (with and without the cross type fixed effect). A significant difference between the two models ($p < 0.05$) would indicate a significant effect of cross type. Where significant effects were detected, post hoc pairwise t tests were used for multiple comparisons between cross types using an adjusted alpha level of 0.01. Models were used to compare fork length, weight, and condition factor between the four cross types in the F_1 generation. In the F_2 generation, performance traits (egg survival, fork length, weight, condition factor, growth, sperm and stress metrics) were compared between the three cross types. Significant differences between purebred and outbred (F_1 hybrids or F_2 backcross hybrids) cross types would be indicative of outbreeding effects. Where appropriate, environmental effects were included as random effects in the models. During the saltwater phase of the F_1 generation, net pen effects were included in the models and for the F_2 generation spatial incubation effects were included in the models for egg survival.

Fertilization success and saltwater survival data were compared between F_1 cross types using generalized linear mixed effect model for binomial data with a fixed factor of cross type and the random effects of dam, sire and their interaction. Data were analyzed in R with the "glmer" function, and models were fitted and compared in the same way as described above. The same generalized linear mixed effect model was used to compare F_2 fertilization success, saltwater survival and jacking rate between all three cross types.

Results

First generation F_1 hybrids

Fertilization success did not differ between cross types ($p = 0.61$). The random effects of dam and sire did not contribute to the variance observed for fertilization success. Fork length, weight and condition factor did not differ significantly among F_1 cross types for all sampling times (Table 1; all $p > 0.05$). The random effects of dam, sire and their interaction significantly contributed to phenotypic variance of fork length and weight at all sampling times (Table 1; all $p < 0.03$) and to the phenotypic variance of condition factor in June 2010 (Table 1, $p < 0.001$). Saltwater survival did not differ significantly among F_1 cross types (Table 1; $p = 0.68$), and dam and sire effects significantly contributed to the variance observed in saltwater survival (Table 1; $p = 0.02$). Net pen effects significantly contributed to the phenotypic variance observed for fork length, weight, condition and survival during saltwater rearing (all $p < 0.001$), with the exception of condition in October 2011.

Second generation (F_2) backcrossed hybrids

Egg survival

Fertilization success did not differ among cross types ($p = 0.99$). Dam effects significantly contributed to variance observed for fertilization success ($p < 0.001$) as the

Table 1 Means (±SE) of F_1 cross types of Chinook salmon (*Oncorhynchus tshawytscha*) with the significance (p) of cross type ("Type") and the significant contribution of dam, sire and their interaction to the phenotypic variance of the respective trait

Trait (U)	Purebred	Outbred		Purebred	Significance (p)			
	LL × LL	LL × HH	HH × LL	HH × HH	Dam	Sire	Interaction	Type
Fork length (cm)								
June 2010	8.43 ± 0.03	8.41 ± 0.03	8.03 ± 0.02	8.13 ± 0.03	**<0.001**	**<0.001**	**<0.001**	0.23
April 2011	22.5 ± 0.10	22.3 ± 0.11	21.8 ± 0.10	21.9 ± 0.11	**<0.001**	**<0.001**	**0.006**	0.53
October 2011	34.1 ± 0.25	33.2 ± 0.25	33.5 ± 0.21	33.7 ± 0.26	**0.03**	**<0.001**	**0.001**	0.17
Weight (g)								
June 2010	6.72 ± 0.07	6.83 ± 0.07	5.86 ± 0.05	5.79 ± 0.05	**<0.001**	**<0.001**	**<0.001**	0.06
April 2011	144.2 ± 1.90	139.4 ± 1.99	129.0 ± 1.80	130.0 ± 1.96	**<0.001**	**<0.001**	**0.005**	0.37
October 2011	506.6 ± 10.0	473.2 ± 9.81	471.3 ± 9.07	480.2 ± 10.2	**0.006**	**<0.001**	**0.003**	0.20
Condition (g/cm^3)								
June 2010	1.10 ± 0.005	1.13 ± 0.005	1.12 ± 0.004	1.07 ± 0.005	**<0.001**	**<0.001**	**<0.001**	0.54
April 2011	1.22 ± 0.004	1.21 ± 0.005	1.20 ± 0.004	1.20 ± 0.004	**<0.001**	**0.04**	ns	0.05
October 2011	1.24 ± 0.006	1.26 ± 0.009	1.22 ± 0.008	1.23 ± 0.007	ns	0.90	0.53	0.08
Saltwater survival (%)	27.5 ± 1.76	29.1 ± 1.80	28.8 ± 1.73	22.6 ± 1.78	**0.02**	**0.02**	ns	0.68

ns: variance associated with the parameter is 0, thus the parameter does not contribute to the variance structure of the respective trait. Significant random effects ($p < 0.05$) are indicated in bold

eggs of three females were non-viable. For successful families, egg survival was not influenced by egg incubation density ($r = -0.002$, $p = 0.98$). Mean egg survival and female weight were positively but not significantly correlated ($r = 0.69$, $p = 0.09$). Tray position effects significantly contributed to the variance observed for egg survival ($p = 0.008$), but stack and cell position did not ($p = 0.99$); therefore, tray position was included as a random factor in the mixed models. Mean egg survival did not differ significantly among cross types (Table 2; $p = 0.33$). Dam, sire and interaction effects significantly contributed to variance observed for egg survival (Table 2; $p < 0.001$).

Performance traits and survival

Fork length, weight and specific growth rate did not differ among F_2 cross types for all sampling times (Table 2; all $p > 0.13$). Condition factor significantly differed among F_2 cross types at one sampling time in November 2012 (Table 2; $p = 0.03$)—where backcrossed hybrids (HH × HL and HH × LH) significantly differed from one another ($p = 0.005$)—however hybrids did not differ from the purebred HH cross type. Significant effects of dam and sire were detected for fork length, weight, condition factor and specific growth rate at almost all sampling periods, and significant interaction effects were observed during earlier sampling dates (see Table 2). Saltwater survival was not significantly different among cross types (Table 2; $p = 0.31$), and dam and sire effects significantly contributed to the variance observed for saltwater survival (Table 2; $p < 0.01$).

Stress response

Baseline plasma cortisol and stress response (change in plasma cortisol) were significantly related to time of day (Pearson correlation, $R^2 = 0.29$ and 0.34, respectively, p values <0.001), but stress-induced cortisol was not significantly related to time ($p = 0.87$). Time of day was thus included as a random factor in the mixed models. Baseline cortisol, stress-induced cortisol and stress response did not differ significantly among F_2 cross types (Table 2; $p > 0.06$, $n = 194$). Dam effects significantly contributed to the variance observed for baseline and stress induced plasma cortisol (Table 2; $p < 0.001$). There was no difference among cross types for survival 3-weeks post-stress experiment (Table 2, $p = 0.10$).

Sperm traits

Jacking rate did not differ significantly among F_2 cross types (Table 2; $p = 0.94$) and sire effects significantly contributed to the variance observed for jacking rate ($p = 0.002$). All sperm metrics were analyzed using mixed models, however we found that dam, sire and interactions effects did not contribute significantly to the variance observed for any sperm traits ($p > 0.08$). Sperm motility was significantly different among cross types across all time points post-activation (Fig. 1a; $p < 0.03$). Post-hoc tests revealed that outbred HH × HL jacks were significantly higher in sperm motility compared to outbred HH × LH jacks at all time points ($p < 0.002$), and purebred HH × HH jacks did not differ from either outbred cross ($p > 0.01$).

Table 2 Means (±SE) of outbred (F_2 backcrossed hybrids) and purebred Chinook salmon (*Oncorhynchus tshawytscha*) with the significance (p) of cross type ("Type") and the significant contribution of dam, sire and interaction to the phenotypic variance of the respective trait

Trait (U)	Outbred		Purebred	Significance (*p*)			
	HH × HL	HH × LH	HH × HH	Dam	Sire	Interaction	Type
Egg survival (%)	69.9 ± 2.2	69.7 ± 2.2	73.6 ± 2.2	**<0.001**	**<0.001**	**<0.001**	0.33
Fork length (cm)							
March 2011	3.6 ± 0.006	3.6 ± 0.006	3.6 ± 0.005	**<0.001**	**<0.001**	**0.003**	0.83
June 2011	7.7 ± 0.02	7.8 ± 0.02	7.8 ± 0.02	**<0.001**	**<0.001**	0.08	0.84
October 2011	15.4 ± 0.05	15.4 ± 0.05	15.5 ± 0.04	**<0.001**	**<0.001**	**0.04**	0.99
April 2012	22.2 ± 0.09	22.2 ± 0.10	22.0 ± 0.08	**<0.001**	**<0.001**	ns	0.81
November 2012	30.1 ± 0.19	29.4 ± 0.22	29.6 ± 0.16	**0.002**	**<0.001**	0.45	0.42
Weight (g)							
March 2011	0.44 ± 0.003	0.45 ± 0.003	0.45 ± 0.003	**<0.001**	**0.02**	**<0.001**	0.13
June 2011	5.26 ± 0.05	5.31 ± 0.04	5.45 ± 0.04	**<0.001**	**<0.001**	**0.002**	0.90
October 2011	41.6 ± 0.5	41.2 ± 0.4	41.8 ± 0.4	**<0.001**	**<0.001**	**0.002**	0.98
April 2012	136.8 ± 1.7	134.0 ± 1.9	131.8 ± 1.5	**<0.001**	**<0.001**	0.58	0.75
November 2012	388.7 ± 7.89	354.8 ± 8.72	365.2 ± 6.38	0.05	**<0.001**	0.94	ns
Condition (g/cm^3)							
March 2011	0.94 ± 0.006	0.96 ± 0.005	0.96 ± 0.005	**<0.001**	**<0.001**	**<0.001**	0.60
June 2011	1.14 ± 0.003	1.13 ± 0.003	1.14 ± 0.003	**<0.001**	**<0.001**	**<0.001**	0.67
October 2011	1.12 ± 0.003	1.11 ± 0.003	1.12 ± 0.003	**<0.001**	**<0.001**	0.14	0.82
April 2012	1.23 ± 0.004	1.21 ± 0.004	1.21 ± 0.004	**<0.001**	**<0.001**	0.55	0.52
November 2012	1.39 ± 0.01^{a}	1.34 ± 0.01^{b}	1.38 ± 0.009^{ab}	**<0.001**	ns	ns	0.03*
Specific growth rate (g/day)	0.84 ± 0.005	0.82 ± 0.005	0.82 ± 0.004	**<0.001**	**<0.001**	0.70	0.50
Baseline cortisol (ng/ml)	192.6 ± 11.2	160.4 ± 10.3	152.5 ± 9.4	**<0.001**	ns	ns	0.38
Stress induced cortisol (ng/ml)	167.0 ± 6.6	178.3 ± 6.7	170.8 ± 6.4	**<0.001**	ns	ns	ns
Stress response (ng/ml)	18.2 ± 8.7	−25.6 ± 9.8	17.9 ± 9.1	0.39	ns	ns	0.06
3-weeks post stress survival (%)	79.1 ± 5.0	87.1 ± 4.3	92.4 ± 3.0	0.67	ns	ns	0.10
Saltwater survival (%)	48.0 ± 2.5	38.8 ± 2.4	47.6 ± 2.2	**0.01**	**<0.001**	ns	0.31
Jacking rate (%)	5.0 ± 1.1	4.6 ± 1.0	5.6 ± 1.0	0.36	**0.002**	0.60	0.94

Different letters (^{a,b}) represent significant differences for pairwise comparisons of cross type ($p < 0.01$)

ns: variance associated with the parameter is 0, thus the parameter does not contribute to the variance structure of the respective trait. Significant random effects ($p < 0.05$) are indicated in bold. Asterisk (*) denotes significant cross type effects ($p < 0.05$)

Sperm velocity (VAP) did not differ significantly among cross types at any time point post-activation (Fig. 1b; $p > 0.13$). Sperm longevity differed significantly among cross types (Fig. 1c; $p = 0.03$) where outbred groups HH × HL and HH × LH differed from each other ($p = 0.005$). Sperm density was not significantly different among cross types (Fig. 1d; $p = 0.064$).

Genetic differentiation and diversity

Genetic differentiation between parental HH and LL purebred lines was highly significant ($p < 0.001$), with a pair-wise F_{ST} of 0.130 between purebred lines. Estimates of genetic diversity including number of alleles, allelic richness, number of private alleles and observed and expected heterozygosity are presented in Table 3. Genetic diversity estimates varied between the two parental lines, with the LL line generally showing reduced genetic diversity relative to the HH line.

Discussion

The potential for outbreeding depression, either in the first generation hybrids, or the second generation backcrosses, is an important consideration when developing effective management protocols for salmonid conservation. Furthermore, a better understanding of the role of outbreeding depression in limiting gene flow among locally-adapted salmon populations is critical for determining the

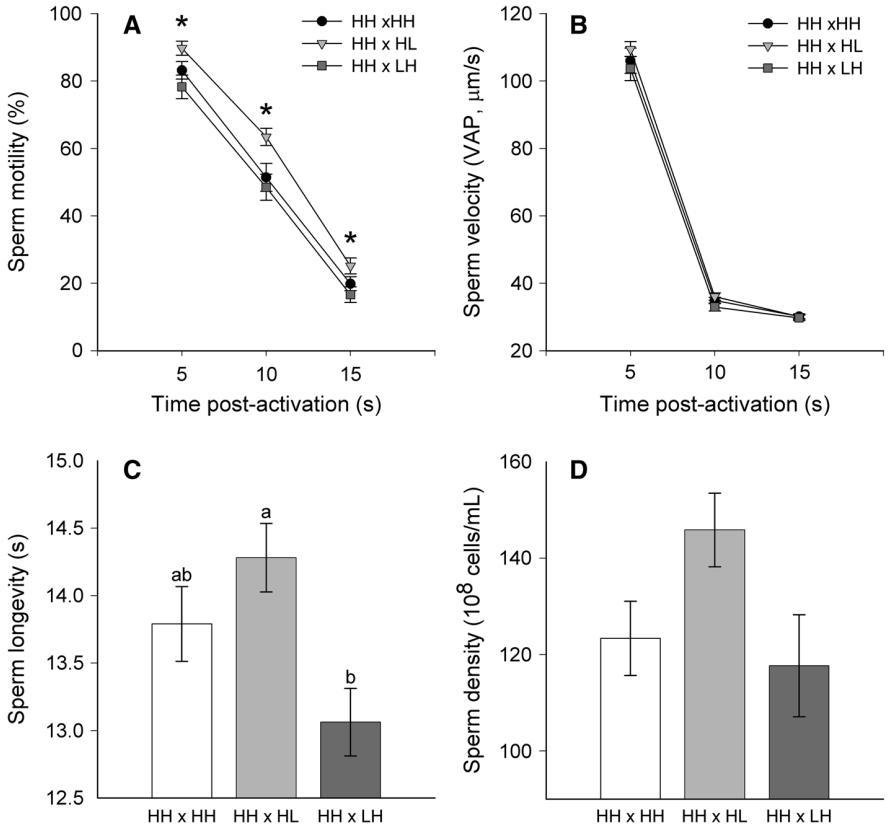

Fig. 1 Means (±SE) of purebred and F_2 backcrossed hybrid Chinook salmon (*Oncorhynchus tshawytscha*) males for sperm traits: **a** percent sperm motility, **b** sperm velocity (average path velocity, VAP), **c** sperm longevity and **d** sperm density. *Asterisks* over time periods and *different letters over bars* indicate significant differences between male types ($p < 0.05$)

Table 3 Genetic information for 10 microsatellite loci for two parental strains of Chinook salmon (*Oncorhynchus tshawytscha*) including sample size (N), mean number of alleles (A_N), mean allelic richness (A_r), total number of private alleles (A_P), and mean observed (H_o) and expected heterozygosity (H_e)

Parental line	N	A_N	A_r	A_P	H_o	H_e
HH	27	9.10	8.49	44	0.81	0.83
LL	25	7.30	6.94	26	0.79	0.74

evolutionary significance of isolated populations of salmonids. We found no significant differences between F_1 hybrids and purebred cross types for fork length, weight, condition factor and saltwater survival. The F_1 hybrid performance was neither intermediate (which, in nature, could result in an inappropriate phenotype in either parent environment), nor indicative of outbreeding depression. Furthermore, no hybrid cross performed significantly better than the best parental line, therefore indicating no favorable heterosis in F_1 Chinook salmon, similar to what has been seen in wild-farmed F_1 hybrid Chinook salmon (Bryden et al. 2004).

In the second generation, outbred backcrosses (both HH × HL and HH × LH) did not differ significantly from the purebred (HH) cross type for egg survival, fork length, weight, condition, growth, stress metrics and sperm metrics, indicative of no evidence for outbreeding depression acting on these performance traits. Furthermore, F_2 cross types did not differ in saltwater survival. While we did not find differences between outbred and purebred cross types, there were significant differences between backcrossed hybrids (HH × HL and HH × LH) for some performance traits (including condition factor, sperm longevity and sperm motility), perhaps due to trans-generational epigenetic effects (Bossdorf et al. 2008). Although poorly understood, epigenetic effects (e.g., genetic imprinting) have been previously suggested for differences in gene transcription between reciprocal hybrids in trout (Aykanat

et al. 2011). Though we found no evidence of outbreeding depression, consistent with some previous research (Sheffer et al. 1999; Fraser et al. 2008; Dann et al. 2010; Houde et al. 2011a, b), other studies have reported outbreeding depression in fishes (Cooke et al. 2001; Cooke and Phillip 2005; Huff et al. 2011), including salmonids (Gharrett and Smoker 1991; Gilk et al. 2004; Tymchuk et al. 2007). The disparity in results between studies and species highlights the need to conduct situation specific studies for proper quantification of outbreeding effects to develop suitable management and conservation strategies (Houde et al. 2011b).

Many fitness-related traits in our study showed significant effects of dam, sire and interaction on phenotypic variance. Significant interaction effects indicative of non-additive genetic effects (i.e., epistasis or dominance) were found for weight, length and condition during most sampling periods in the F_1 generation and in earlier stages of ontogeny (but not later) in the F_2 generation. However, we acknowledge that the interaction effects detected during the freshwater rearing stage could be reflective of tank effects, as each family was reared in a separate tank. Nevertheless, we still detect significant interaction effects during later saltwater rearing for some traits in both the F_1 and F_2 generation. Additionally, we recognize that outbreeding can influence the variance components associated with the interaction of dam and sire thus effects may vary between cross types (Cavalli 1952). Nevertheless, the presence of non-additive genetic variance in Chinook salmon are consistent with other studies that found non-additive genetic effects on early survivorship and body size (Pitcher and Neff 2006, 2007) and cytokine transcription in response to *Vibrio* (Aykanat et al. 2012). Non-additive genetic effects are common in salmon (Rye and Mao 1998; Gilk et al. 2004; Gallardo et al. 2010; Aykanat et al. 2011) and the fact that many traits show non-additivity further complicates our ability to predict effects of outbreeding on fitness in salmonids.

While other studies have detected outbreeding effects in salmonids, many of these studies neglect to include parental effects in their analysis, which may lead to false detection of outbreeding depression. For example, if we used a one-way ANOVA to examine cross type effects in the F_2 generation, we would have found significant effects ($p < 0.05$) for several traits. For instance, without considering parental effects, weight in June 2011 was significantly different among cross types ($p = 0.004$), where outbred (HH × HL) significantly differed from the purebred (HH) cross type (Tukey post hoc test, $p = 0.005$). However, after including parental effects in the model, we found no difference between cross types (Table 2; $p = 0.90$) as dam, sire and their interaction significantly contributed to the phenotypic variance of weight. In Atlantic salmon (*Salmo salar*), Houde et al. (2011a) found significant maternal and paternal cross type effects, but no significant effects of outbreeding when controlling for those effects. Our F_2 breeding design helped eliminate maternal cross-type effects, but for many traits we still found significant dam and sire effects (i.e., maternal and additive genetic effects) and, in some cases, significant interaction effects (i.e., non-additive genetic effects). We therefore used mixed effect models to control for these parental effects in our analysis of both generations to best detect true outbreeding effects.

Although outbreeding effects are not easily predictable, it is expected that the magnitude of outbreeding effects will be dependent on the genetic differentiation between the parental populations (Edmands and Timmerman 2003; Edmands 2007; but see McClelland and Naish 2007). While the HH and LL performance lines have experienced the same rearing and environmental conditions for a number of generations, we still found substantial genetic differences ($F_{ST} = 0.130$) between the lines likely resulting from artificial selection, inbreeding and subsequent drift. We acknowledge that the origin of the lines may be problematic, as the HH line was derived by crossing Big Qualicum (BQ) and Robertson Creek (RC) salmon; therefore, *cis*- and *trans*-acting outbreeding effects may already be operating due to several rounds of recombination between BQ and RC chromosomes. Regardless, the lines show significant genetic differentiation and we argue that the crossing of HH and LL lines could still act to detect outbreeding effects. Furthermore, although parental lines we used for our study were partly derived from the same original population (i.e., BQ), the lines were artificially selected for differential performance, and fish have been documented to respond and diverge rapidly under strong selective forces (Heath et al. 2003; Hutchings and Fraser 2007). Moreover, the parental lines were propagated from the BQ stock during different brood years as the HH line was derived from BQ and RC salmon in 1985 and the LL line was derived from BQ salmon in 1997. Significant temporal genetic differences have been documented in Chinook salmon at 5- to 12-year intervals within a single population (Walter et al. 2009), thus it is likely that there were genetic differences between the BQ stocks used to generate the original lines and hence may in part explain the substantial genetic differentiation (F_{ST}) found in our study. Differences between the lines were also clear by the large number of private alleles (alleles unique to one group) found in each parental line. The HH line had a greater number of private alleles relative to the LL line, and the number of private alleles in the HH and LL lines were similar to those previously reported for their originating populations of RC ($N = 43$) and BQ ($N = 25$), respectively (Kim et al. 2004). Genetic diversity estimates also

differed between lines, as the HH parental line showed higher allelic richness and heterozygosity relative to the LL line, which may be expected as the HH line was originally derived from two source populations (BQ and RC) whereas the LL line was derived from a single source population (BQ).

Despite the genetic differences between the parental lines, the 2009 cohort (F_1) of performance crosses did not differ significantly for the performance traits described in our study; however, Falica and Higgs (2013) found significant differences between HH and LL crosses in the swim performance for critical speed at 30 s (U-crit30s). Furthermore, the 2008 cohort of HH and LL crosses at YIAL differed significantly in fork length and saltwater survival (Falica 2011). Thus if outbreeding depression occurs in Chinook salmon, we would have expected to detect some level of performance loss given our breeding design and range of phenotypic traits measured. Houde et al. (2011b) found that Atlantic salmon populations ranging in F_{ST} values from 0.0353 to 0.0953 did not experience outbreeding depression in backcrossed hybrids in the wild. Leberg (1993) reported that mosquitofish (*Gambusia holbrooki*) populations with F_{ST} values between 0.016 and 0.032 exhibited no evidence of outbreeding effects, whereas pink salmon with similarly low genetic differentiation exhibited outbreeding depression for survival (Gharrett et al. 1999; Beacham et al. 1988). Clearly, the relationship between F_{ST} and outbreeding depression is not straightforward, making it difficult to predict outbreeding effects based on simple genetic differentiation, likely due to species- and possibly population–specific effects (Edmands 2007; Houde et al. 2011b). Although predictions of outbreeding depression based on measures of neutral genetic differentiation (e.g. microsatellite F_{ST}) may be problematic, divergence based on functional gene markers would be useful for outbreeding studies, as genes acted on by natural selection would provide more accurate and direct information about locally adaptive differences between populations (Heath et al. 2006). Currently many outbreeding studies use G_{ST} or F_{ST} based on neutral markers to describe divergence between lines or populations, but we acknowledge that our high level of genetic divergence based on F_{ST} may not necessarily reflect functional divergence between our parental lines. Given the lack of phenotypic differences between the lines, the lack of detected outbreeding effects in our study may be due to a lack of functional differences between the lines. However, outbreeding effects may still be expressed as anomalous hybrid phenotypes even when the parental stocks do not differ phenotypically (Aykanat et al. 2011). Although our study may not be directly comparable to the crossing of locally-adapted wild salmon populations, our study is appropriate for informing aquaculture breeding programs. Furthermore, we argue that our study represents a strong preliminary examination of outbreeding effects in Chinook salmon that should promote further investigation in this field.

It is possible that the aquaculture environment used for the current study was not suitable for detecting outbreeding effects, as natural selection pressures are often relaxed and detection of outbreeding depression is dependent on the environment (Tymchuk et al. 2007). Outbreeding depression in pink salmon occurred under natural conditions (Gharrett and Smoker 1991; Gilk et al. 2004), whereas, no evidence of outbreeding depression in Atlantic salmon was detected under experimental conditions (Houde et al. 2011a). Nevertheless, studies have detected outbreeding effects in salmonids in experimental settings (McClelland et al. 2005; Crespel et al. 2011). In nature, we may expect greater differences indicative of outbreeding depression to occur, as heightened detrimental genetic effects have been detected under more stressful conditions, as demonstrated in *Drosophila* (Kristensen et al. 2008). Additionally, outbreeding depression may not be apparent until the F_3 (or later) generation in salmonids (as observed by Tymchuk et al. (2007)), as salmonids are residual tetraploids with low recombination rates (Allendorf and Thorgaard 1984). Edmands and Timmerman (2003) found that the extent of outbreeding effects will be greater in species with higher recombination rates, therefore investigating three generations or more of outbreeding may prove beneficial to studies of outbreeding depression, particularly in species like salmonids.

Furthermore, including F_2 hybrids (F_1 hybrid $\times$ F_1 hybrid) instead of backcrosses would also have been useful to detect *cis*-acting outbreeding effects, as it would provide greater potential for co-adapted gene complex breakdown. Additionally, having both pure parental lines (HH and LL) for F_2 comparisons would also strengthen our ability to detect *cis*-acting outbreeding effects. However, our backcross design still allows for the detection of *cis*-acting outbreeding effects on one chromosome as well as a higher possibility of *trans*-acting incompatibilities, indeed other studies have reported outbreeding depression in backcrosses (McGinnity et al. 2003; Tymchuk et al. 2007; Whiteley et al. 2009; Huff et al. 2011). Additionally, backcrossing may be a more ecologically relevant situation, as in nature backcrossing may be more likely to follow a one-time hybridization event. For example, when farm salmon escape and hybridize with wild stocks future generations are likely to be composed of hybrids backcrossed to that wild population (McGinnity et al. 2003). Therefore we recommend that further outbreeding studies should not only include hybrids but also backcrosses in order to fully explore potential ecological outbreeding consequences.

Although the HH and LL lines showed genetic differences, there were no performance differences detected in our study; however, the lines have shown differential performance for various traits in previous experiments (see Bryden et al. 2004; Falica 2011; Falica and Higgs 2013). Our design may be confounded by the fact that the parental lines have been cultured under the same rearing conditions for several generations, and this may have resulted in similar selection pressures that may reduce the functional differences between the lines over time. Nevertheless, even though the lines have been reared under similar conditions for generations, under many circumstances (i.e., supplementation and enhancement or captive breeding programs) divergent lines will be derived from a single population and adapted to the same environment, and in these cases the possibility of outbreeding effects should not be ignored. In the instance of fish hatchery and enhancement programs, hatchery and wild fish may diverge genetically after multiple generations and interbreeding can nonetheless result in outbreeding depression (Miller et al. 2004; Araki et al. 2007). Similarly, outbreeding depression has been documented in pink salmon where interbreeding between odd- and even-year broodlines occurred, despite the fact that both lines were adapted to identical environments and comprised the same population (Gharrett and Smoker 1991; Gharrett et al. 1999). Furthermore, captive breeding programs may create inbred lines under artificial conditions, which may be crossed in an attempt to exploit heterosis (Falconer and Mackay 1996), however there is also the potential for outbreeding depression in these situations. Finally, these circumstances are not limited to fish populations, as crossing inbred lines created under culture conditions commonly occurs in plants and animals used for agriculture (Lippman and Zamir 2007), and the release of captive bred animals (including plants, birds and mammals) from the same source may be used for reintroduction or species recovery (Snyder et al. 1996). Although our results suggest a lack of outbreeding effects in Chinook salmon under our experimental conditions, we may expect different results in other populations or other species. Therefore, because of the unpredictable nature and lack of mechanistic understanding of outbreeding depression, we suggest that any case of crossing divergent lines should be treated with caution, regardless of local environment and origin, especially when information on the lines is limited.

While the issues addressed above may explain in part why we did not observe outbreeding depression, our study nonetheless provides novel data on outbreeding effects in Chinook salmon. For F_1 hybrids and F_2 backcrossed hybrids, we found no negative fitness consequences of outbreeding when fish are reared under the same environmental conditions, and thus our data imparts valuable information for Chinook salmon aquaculture breeding programs. On the other hand, we found no evidence for favorable heterosis in F_1 hybrids either. Our study provides a good starting point for outbreeding studies in Chinook salmon, and we suggest that future studies should be multigenerational, include data for various life stages and incorporate parental effects into the analysis to accurately quantify outbreeding effects. Finally, we recognize that it is difficult to generalize outbreeding effects for a species on the basis of a single study; however, our study contributes to the current knowledge of outbreeding in salmonids. Although outbreeding effects will increase with genetic distance, outbreeding effects may also be influenced by population size, mutation rate and recombination rate (Edmands and Timmerman 2003), therefore as previously recommended by Houde et al. (2011b) outbreeding effects should be studied at the population level, as the nature of hybridizing stocks and subsequent outbreeding effects will undoubtedly vary from experiment to experiment and from species to species.

Acknowledgements Yellow Island Aquaculture Ltd. provided experimental fish and facilities for the study. We are grateful to B. Coristine, B. Falica, A. Heath, C. Heath, J. Heath, K. Johnson, K. Jones and M. Wiper for assistance in the field and to C. Harris for assistance with hormone assays. This work was supported by the Collaborative Research and Development (CRD) and Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the University of Windsor (DDH). All research followed the University of Windsor and Canadian Council of Animal Care guidelines.

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