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Genomic and phenotypic effects of inbreeding across two different hatchery management regimes in Chinook salmon

Short title: Multigenerational inbreeding in salmon

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Abstract

Genomic approaches permit direct estimation of inbreeding and its effect on fitness. We used genomic-based estimates of inbreeding to investigate their relationship with eight adult traits in a

captive-reared Pacific salmonid that is released into the wild. Estimates were also used to determine whether alternative broodstock management approaches reduced risks of inbreeding. Specifically, 1,100 unlinked restriction-site associated (RAD) loci were used to compare pairwise relatedness, derived from a relationship matrix, and individual inbreeding, estimated by comparing observed and expected homozygosity, across four generations in two hatchery lines of Chinook salmon that were derived from the same source. The lines are managed as "integrated" with the founding wild stock, with ongoing gene flow, and as "segregated" with no gene flow. While relatedness and inbreeding increased in the first generation of both lines, possibly due to population subdivision caused by hatchery initiation, the integrated line had significantly lower levels in some subsequent generations (relatedness: F_2-F_4; inbreeding F_2). Generally, inbreeding was similar between the lines despite large differences in effective numbers of breeders. Inbreeding did not affect fecundity, reproductive effort, return timing, fork length, weight, condition factor, and daily growth coefficient. However, it delayed spawn timing by 1.75 days per one standard deviation increase in F (~0.16). The results indicate that integrated management may reduce inbreeding but also suggest that it is relatively low in a small, segregated hatchery population that maximized number of breeders. Our findings demonstrate the utility of genomics to monitor inbreeding under alternative management strategies in captive breeding programs.

Keywords: inbreeding, relatedness, integrated management, captive breeding, restriction-site associated DNA sequencing, genomics

Introduction

The use of captive breeding as a tool for population and species recovery remains controversial (Bowkett, 2009; Snyder et al., 1996), in part due to accompanying genetic and ecological risks. One genetic risk, inbreeding, occurs when related individuals interbreed (Frankham, Ballou, & Briscoe, 2002; Wright, 1921, 1922). Offspring of such matings may have loci where both alleles are identical by descent (IBD), or derived from a single, ancestral copy. An individual's inbreeding coefficient, introduced as a measure of correlation by Wright (1921), is now commonly considered to be the probability that two alleles at a locus are IBD (Malécot, 1948). Inbreeding can

occur under a variety of circumstances (Jacquard, 1975) and is more likely in small populations (Frankham et al., 2002; Keller & Waller, 2002).

Inbreeding may lead to inbreeding depression, which is a reduction in fitness caused by an increased expression of deleterious recessive alleles or the loss of heterozygosity at loci with a heterozygote advantage (Charlesworth & Willis, 2009). Inbreeding depression has been documented across an array of plants and animals (Brekke, Bennett, Wang, Pettorelli, & Ewen, 2010; Elias, Shipley, McCusker, Sayler, & Jobnson, 2013; Hammerly, Morrow, & Johnson, 2013; Hedrick & Garcia-Dorado, 2016; Hoffman et al., 2014; Huisman, Kruuk, Ellis, Clutton-Brock, & Pemberton, 2016; Keller & Waller, 2002; Woodworth, Montgomery, Briscoe, & Frankham, 2002) and, in addition to affecting individual fitness, can reduce population productivity and increase the risk of extinction (Frankham, 2005; O'Grady et al., 2006; Saccheri et al., 1998). Inbreeding and its potential effects on fitness depend on a variety of factors, including environmental conditions (Armbruster & Reed, 2005; Joron & Brakefield, 2003), the extent to which inbred individuals are purged from the population (Crnokrak & Barrett, 2002; Hedrick, 1994), and sex (Ebel & Phillips, 2016). Further, numerous factors in captive breeding programs can reduce census and genetic population sizes and thus increase the risk of inbreeding, including limited rearing capacity and numbers of breeders, variance in family sizes, and unequal sex ratios (Allendorf, 1993; Frankham et al., 2002; Naish et al., 2008).

Despite their widespread recognition and importance, the genetic, phenotypic, and demographic effects of inbreeding remain unclear (Kardos, Taylor, Ellegren, Luikart, & Allendorf, 2016). Traditionally, estimating inbreeding coefficients and detecting inbred individuals relied on pedigrees spanning at least three generations (i.e. pedigree inbreeding; Malécot, 1948; Wright, 1921, 1922). Yet, such pedigrees are intractable in wild, numerically-abundant, and long-lived populations. Further, inbreeding coefficients derived from pedigrees may lack precision if the pedigree comprises few generations or is incomplete (Reid et al., 2014; Taylor, Kardos, Ramstad, & Allendorf, 2015), and individuals with the same pedigree can have different inbreeding coefficients due to physical linkage and recombination (Kardos, Luikart, & Allendorf, 2015). Genetic markers have also been used to estimate inbreeding by various methods (e.g. Wang, 2011), but these estimates may be imprecise if obtained using small numbers of loci (Hoffman et al., 2014; Taylor, 2015). However, current

approaches provide thousands of loci across the genome and permit direct and accurate estimation of individual inbreeding coefficients (Kardos et al., 2015). One common method for estimating inbreeding from genomic data is to quantify the deviation of individual homozygosity compared to Hardy-Weinberg expectations (this study; Purcell et al., 2007; Yang, Lee, Goddard, & Visscher, 2011). These and other genomic-based estimates have revealed that the negative fitness effects of inbreeding may be more severe than previously thought (Hedrick & Garcia-Dorado, 2016; Hoffman et al., 2014; Huisman et al., 2016). Therefore, there is a need to quantify the effects of inbreeding using genomic approaches in a wide range of populations, especially those of conservation concern.

Pacific salmon may be particularly vulnerable to inbreeding. Wild populations have declined over the last century due to anthropogenic disturbances (Gustafson et al., 2007) and may have an increased risk of inbreeding due to small population sizes and their precise homing tendencies, which promote reproductive isolation between populations (Dittman & Quinn, 1996). Hatchery populations, which are intended to supplement depressed wild stocks and provide fish for commercial and recreational harvest, may inadvertently have higher levels of inbreeding due to management practices (e.g. limited broodstock collection and spawning procedures) and limited rearing capacity (Naish et al., 2008). Numerous studies have documented adverse fitness effects in captive and wild salmonids that were due to inbreeding (Calaprice, 1969; Hard, Connell, Hershberger, & Harrell, 2000; Kincaid, 1976; Naish, Seamons, Dauer, Hauser, & Quinn, 2013; Ryman, 1970; Thrower & Hard, 2009; Wang, Hard, & Utter, 2002). However, the potential consequences of inbreeding over multiple generations can now be more precisely estimated in salmonids with genomic approaches.

The Cle Elum Supplementation and Research Facility (CESRF) in Cle Elum, Washington, U.S.A. provides an unparalleled opportunity to evaluate the effects of inbreeding in captive-reared Pacific salmon, and the management steps that may be taken to reduce these effects. CESRF was initiated in 1997 in response to declining annual returns of anadromous wild spring Chinook salmon (*Oncorhynchus tshawytscha*) to the upper Yakima River, a tributary of the Columbia River. The program was designed to test whether supportive breeding could increase harvest and production in the upper Yakima River Chinook population while minimizing ecological and genetic risks associated with captive rearing (RASP, 1992). To achieve this goal, the hatchery population was divided into a "segregated" (SEG) line, which is not allowed to interbreed with the wild source population, and an

"integrated" (INT) line, which has unrestricted gene flow with the wild stock (Fast et al., 2015). Notably, tissue samples for DNA and extensive phenotypic data, including fork length, weight, and return timing, have been collected from every adult fish spawned in the hatchery since its inception, forming one of the most comprehensive collections available for any Pacific salmon population, wild or hatchery.

The propagation of the integrated and segregated hatchery lines at CESRF also permits evaluation of managed gene flow, or the integrated management approach, to reduce the risks of inbreeding in captive-reared populations. Managed gene flow in the form of integrated hatchery management has been widely implemented throughout the Pacific Northwest (Mobrand et al., 2005; Paquet et al., 2011), because theoretical studies suggest that it can mitigate the risks of domestication selection, inbreeding, and genetic drift associated with captive rearing (Baskett & Waples, 2013; Duchesne & Bernatchez, 2002; Ford, 2002; Lynch & O'Hely, 2001; Mobrand et al., 2005; Paquet et al., 2011). We recently provided the first empirical evidence that managed gene flow successfully reduces genome-wide differentiation (Waters et al., 2015; Waters et al., 2017) and divergence at trait-associated loci (Waters et al., 2018) when compared to a segregated management approach using four generations of data from CESRF. Here, we determine the extent to which managed gene flow may reduce inbreeding and its potential adverse effects on fitness.

We estimated pairwise relatedness (R_{xy}), a measure that may impact inbreeding in subsequent generations, and individual inbreeding coefficients (F) across four generations of the integrated and segregated Chinook salmon hatchery lines at CESRF using 1,100 unlinked restriction site-associated (RAD) loci. Multigenerational observations are advantageous because processes occurring in the wild, including natural selection, could mitigate or exacerbate the effects of inbreeding over time. Further, assessing multiple generations enables the accumulated effects of inbreeding since the initiation of the hatchery to be quantified. We hypothesized that hatchery rearing may inadvertently cause an increase in R_{xy} and F within each hatchery line over time due to factors such as space limitations, broodstock collection practices, and spawning procedures. We then determined if managed gene flow successfully reduced risks by comparing R_{xy} and F between the integrated and segregated hatchery lines over time. Levels of R_{xy} and F were hypothesized to be higher in the segregated line than in the integrated line, since each generation of the segregated line has 3- to 27-

fold fewer effective numbers of breeders (Table S7; Waters et al., 2015; Waters et al., 2017). Last, the relationships between inbreeding coefficient and eight adult fitness-related traits – date of return to freshwater spawning grounds (return timing), fork length, weight, and condition factor at return, fecundity, reproductive effort, daily growth coefficient, and spawn timing – were quantified using linear models. The results will provide a more comprehensive understanding of genetic and phenotypic change that may occur in captive-reared populations and will further inform "best" practices to minimize risks in conservation-focused captive breeding programs.

Methods

Population description

The initial hatchery population at CESRF was founded from 1997–2002 using returning wild adults from the anadromous upper Yakima River Spring Chinook salmon population. Wild adults were collected at the Roza Dam Adult Monitoring Facility (RAMF), located 90 river kilometers south of CESRF, as they returned from the ocean to their freshwater spawning grounds. Adults for broodstock were collected randomly and proportionately (based on average migration timing patterns) over the duration of the run to minimize potential biases and held at CESRF until maturation (Knudsen et al., 2006), at which point they were spawned following a 3x3 factorial mating design (when possible) to increase the effective number of breeders compared to a single pair design (Busack & Knudsen, 2007).

In 2002, the integrated and segregated hatchery lines were initiated by spawning wild (F_1 Wild) and first generation hatchery-origin (F_1 Hatchery) adults, respectively (Figure S1). Broodstock for the integrated line comprise only fish born in the wild, and all returning adults from this line are allowed to spawn in the river. The segregated line, in contrast, uses only returning hatchery-origin fish as broodstock, and no adults from this line are allowed to reproduce naturally; fish from the two lines are differentially marked using visible implant elastomer tags for external identification, so there is minimal risk of misidentification between hatchery lines. The effects of stray fish from other systems are also likely minimal, with the stray rate estimated to be less than 0.06% (Fast et al., 2015). With these practices, fish from the integrated line are exposed to hatchery conditions for just one generation while individuals in the segregated line are exposed every generation. Broodstock collection,

spawning, and rearing procedures for subsequent generations of both lines are conducted in the same manner as the founding generation. Notably, the numbers of adults spawned in the segregated line are typically one quarter of those spawned in the integrated line (Waters et al., 2015), an outcome of the fact that CESRF serves broader restoration goals. Additional details regarding the background of CESRF and the initiation of the integrated and segregated hatchery lines have been described elsewhere (Fast et al., 2015; Knudsen et al., 2006; Waters et al., 2018; Waters et al., 2015).

Sample collection

Broodstock were collected randomly and proportionately across the duration of the run, and tissue samples for DNA were collected during spawning at CESRF and stored in 100% ethanol. Adults from the following years were randomly sub-sampled for this study: 1998 (second year of wild fish used to initiate the hatchery; P_1 Founders), 2002 (F_1 Wild and F_1 Hatchery), 2006 (F_2 INT and F_2 SEG), 2010 (F_3 INT and F_3 SEG), and 2014 (F_4 INT and F_4 SEG; Table S1). A majority (>75%, Table S3; Knudsen et al., 2006) of Chinook salmon from this system spend two years in the ocean and return at age four to reproduce, so these samples represent five generations.

Phenotypic data from returning adults were collected annually upon arrival at RAMF and, for fish used as broodstock, during spawning at CESRF. Eight fitness-related traits were examined for changes due to inbreeding (Table S3a). Return timing, a seasonal phenological trait, corresponds to day of arrival at RAMF. Spawn, or maturation, timing – a related phenology – of all broodstock was estimated weekly at CESRF by manually checking for gonadal ripeness. Gravimetric estimates of fecundity and reproductive effort (proportion of body mass allocated to gamete production) were calculated for females following Knudsen et al. (2008). Fecundity estimates were reduced by 5.5% to correct for bias from residual ovarian fluid remaining within the egg mass (Knudsen et al., 2008). Fork length and weight were measured at both RAMF and upon spawning at CESRF. However, only measurements from RAMF were analyzed here to minimize possible influences from time spent in the hatchery after collection at RAMF and from the development of secondary sex traits. Fulton's condition factor *K* (Ricker, 1975), a body mass index or measure of quality routinely used for fishes, was calculated using fork length and weight measurements at RAMF:

$$K = 100 * \frac{W}{L^3} \quad (1)$$

where W was weight at RAMF in grams and L was length in centimeters. Daily growth coefficient (DGC), which has been shown to be more independent of initial weight than other measures, was calculated according to Cho (1992) and Dupont-Nivet et al. (2010):

$$DGC = 100 \times [((\text{final weight})^{1/3} - (\text{initial weight})^{1/3})/\text{days}] \quad (2)$$

where initial weight was the weight at RAMF, final weight was weight at spawning, and days was the number of days between arrival at RAMF and spawning at CESRF. Here, DGC provided a measure related to weight lost during maturation and holding in the tanks at CESRF.

DNA sequencing and genotyping

DNA from all tissue samples was previously sequenced to investigate population divergence since founding (Waters et al., 2015; Waters et al., 2017) and the effects of domestication selection on genetic diversity underlying fitness-related traits (Waters et al., 2018). Therefore, DNA extraction, RAD library preparation, and bioinformatic processing of sequence data followed the methods reported in Waters et al. (2018) with two exceptions. First, loci were removed if they did not have a minor allele frequency ≥0.05 in the P_1 Founders, rather than ≥0.05 in at least one population, because unbiased estimates of inbreeding rely on polymorphic loci with allele frequencies estimated from a wild base population (Kardos et al., 2015). Second, all loci potentially under selection (i.e. outlier loci) identified from these samples in a previous study (Waters et al., 2017) were removed, because non-neutral loci may also bias inbreeding estimates.

Relatedness and inbreeding

Pairwise relatedness and individual inbreeding were estimated using loci that remained after pruning for linkage disequilibrium (LD) in PLINK (v1.9; Chang et al., 2015; Purcell et al., 2007), because correlations between loci may influence estimates (Blouin, 2003; Kardos et al., 2015; Santure et al., 2010; Wang, 2007). PLINK requires base pair-specific information for each locus in order to prune marker sets for LD. Therefore, all study loci were aligned to the Chinook salmon genome (*Oncorhynchus tshawytscha;* Accession PRJNA432585 released Jan. 16, 2018 on NCBI by Fisheries and Oceans Canada) using *Bowtie2* (v. 2.2.9, Langmead & Salzberg, 2012) with default parameters. Loci that aligned to the Chinook genome with a mapping quality ≥10 were assigned positions and

imported into PLINK. LD pruning was then conducted using conservative parameters (window size = 50, step size = 5, variance inflation factor = 1).

Genomic measures of pairwise relatedness, R_{xy}, and individual inbreeding coefficient, F, within each generation of each hatchery line were then estimated in PLINK (v1.9; Chang et al., 2015; Purcell et al., 2007) with the *make-rel* and *het* functions, respectively, using allele frequencies from the P_1 Founders, the wild base population which we assumed comprised largely unrelated individuals. The *make-rel* function estimates pairwise relatedness from a genomic relationship matrix (Yang et al., 2011), while the *het* measure of F compares the observed number of homozygous genotypes to the expected mean number under random mating (Purcell et al., 2007). Unlike probabilistic estimators such as those implemented in COANCESTRY (Wang, 2011), the PLINK measures of R_{xy} and F are calculated directly from genomic data and can include biologically-relevant negative values. In addition, the *het* measure of F was chosen over an alternative measure, runs of homozygosity (ROH; Broman & Weber, 1999; Curik, Ferencakovic, & Solkner, 2014), for two reasons. First, the marker density (~1 mapped SNP per 0.5 Mb) was too low to accurately detect runs of homozygosity; typical studies that use ROH regularly employ >50,000 loci (e.g. Bosse et al., 2015; Ferenčaković, Solkner, & Curik, 2013; Zhang, Calus, Guldbrandtsen, Lund, & Sahana, 2015). Second, simulations suggest that F based on homozygosity has equal or higher precision than other measures, including ROH, when 5,000 – 10,000 markers are used, as well as low bias when allele frequencies are derived from a wild base population (Kardos et al., 2015). Therefore, we considered the *het* measure of F to be the most appropriate given our data.

Differences in the distributions of R_{xy} and F between successive generations within each hatchery line, as well as differences between the two lines within each generation, were tested using Kolmogorov-Smirnov tests conducted in R (R Core Team 2017), as the data were non-normally distributed.

Effects of inbreeding on traits influencing fitness

The effects of inbreeding coefficient on eight fitness-related traits were quantified using linear models in R (R Core Team 2017). Only four-year-old fish were analyzed because they represented 90% of all adults sampled for this study (Table S3), and sample sizes for three- and five-year-old fish

were too small to accurately estimate age effects. Samples from the P_1 Founders were also excluded to explicitly compare the integrated and segregated hatchery lines in a paired design. Thus, analyses spanned the F_1, F_2, F_3, and F_4 hatchery generations.

Each phenotypic trait was modeled as a function of inbreeding coefficient, hatchery line, generation, and sex. We considered both linear and non-linear effects of inbreeding on fitness, the latter by including a quadratic term (i.e. F^2) in each model. We also included first order interactions of covariates with F to determine if the effects of inbreeding varied by hatchery line, generation, and sex. Continuous variables, specifically trait values and inbreeding coefficients, were standardized (mean centered and divided by the standard deviation) to aid interpretations between variables. All explanatory variables, including generation, were treated as fixed instead of random effects because we were interested in their specific influence on the phenotype. Each trait was modeled with a Gaussian distribution and an identity link function. The general form of each full model was:

$$y_{ijkl} = \beta_0 + \beta_1 F_i + \beta_2 F^2_i + \beta_3 \text{Line}_j + \beta_4 \text{Gen}_k + \beta_5 \text{Sex}_l + \beta_6 F_i * \text{Line}_j \quad (3)$$

$$+ \beta_7 F_i * \text{Gen}_k + \beta_8 F_i * \text{Sex}_l + e_{ijkl}$$

where y_{ijkl} is the standardized trait measurement, F_i is the standardized inbreeding coefficient (continuous variable), Line_j is the hatchery line (categorical), Gen_k is the generation (categorical), and Sex_l is the sex (categorical) of individual i, respectively. Models for gravimetric fecundity and reproductive effort did not include sex, as these traits were only measured in females. Backward model selection was then performed on the full model with the *stepAIC* function of the *mass* package (Venables & Ripley, 2002) in R (R Core Team 2017), using Akaike information criterion (AIC) scores to identify the preferred model for each trait. The main effect of inbreeding coefficient was retained in the preferred models, even if non-influential, to explicitly quantify its effect on the phenotype. Multicollinearity between explanatory variables (i.e. sex, hatchery line, generation, and inbreeding coefficient) included in the preferred models was quantified using the *vif* function of the *car* package in R (Fox & Weisberg, 2011) to ensure that regression results were reliable.

As model selection may downwardly bias *p*-values of explanatory variables in the preferred models (Heinze & Dunkler, 2017), we placed emphasis on the relative magnitudes of regression coefficients when interpreting the effects of inbreeding on trait values. We also estimated post-hoc

statistical power associated with regression coefficients using the *pwr.f2.test* function of the *pwr* package in R (Champely, 2018). A relatively large effect of *F* on a phenotype, along with high statistical power, was considered as evidence that inbreeding affected a fitness-related trait.

Results

Sample collection

Tissues of 753 adult Chinook salmon were sub-sampled for DNA from the P_1 Founders and four generations of the integrated and segregated hatchery lines (Table S1). Phenotypic data were also recorded for these individuals upon their arrival at RAMF and again upon spawning at CESRF (Table S3).

DNA sequencing and genotyping

DNA extraction and RAD sequencing was successful for 696 of the 753 tissue samples, and bioinformatic processing using *Stacks* identified 11,809 biallelic loci. Subsequent filtering of loci and individuals for >50% missing genotypes (following the steps of Waters *et al*. 2018) and a minor allele frequency ≥0.05 in the P_1 Founders reduced the data set to 452 individuals and 7,002 loci. Then, outlier loci that had been previously identified from these samples using two independent methods (Waters et al., 2017) were excluded. The final data set comprised 452 individuals genotyped at 6,805 loci (Tables S2). Specifically, the final data set included 55 fish from the P_1 Founders, 58 and 52 fish from the F_1 Wild and F_1 Hatchery groups, 57 and 53 fish from the F_2 INT and F_2 SEG groups, 69 and 59 fish from the F_3 INT and F_3 SEG groups, and 24 and 25 fish from the F_4 INT and F_4 SEG groups (Table S1).

Relatedness and inbreeding

All 6,805 loci were aligned to the Chinook salmon genome to identify base pair positions for LD pruning in PLINK (Table S4a). The 5,600 loci that aligned to the Chinook genome with mapping quality ≥10 were assigned positions, and LD pruning in PLINK identified 1,100 putatively unlinked loci from which genomic estimates of R_{xy} and F were calculated (Table S4b). However, genomic

estimates of R_{xy} and F obtained from all 6,805 loci and from the 1,100 unlinked loci were comparable (Pearson's $r = 0.76$ and 0.98 for R_{xy} and F, respectively; Table S5).

a. Relatedness

Pairwise relatedness in the P_1 Founders was centered near zero (Figure 1, Table S5a). Slight but significant increases were then observed in the F_1 Wild and F_1 Hatchery groups following initiation of the hatchery (Figure 1, Table 1). Comparisons between subsequent generations revealed a temporally consistent, significant increase in R_{xy} in the segregated line, while R_{xy} did not increase in the integrated line from the F_1-F_4 generations (Table 1). For example, the proportion of R_{xy} values $\geq$ 0.1 steadily increased in the F_2 (0.11), F_3 (0.17), and F_4 (0.21) generations of the segregated line but remained consistent in the integrated line (proportions of 0.06, 0.04, and 0.05 for the F_2, F_3, and F_4 generations, respectively; Figure 1, Table S5a).

Significant differences in the distributions of R_{xy} between the integrated and segregated hatchery lines were observed in the F_2, F_3, and F_4 generations. Specifically, R_{xy} for the segregated line was higher than the integrated line in each generation (Figure 1, Table 1). Despite the differences, median values of R_{xy} remained close to zero (0.02-0.04) in both hatchery lines across all generations.

b. Inbreeding

The distribution of inbreeding coefficients in the P_1 Founders was centered near zero (median = 0.05), although individuals with relatively large F values (positive and negative) were also observed (Figure 2, Table S5b). Levels of F then significantly increased in the F_1 Wild and F_1 Hatchery groups following initiation of the hatchery (Figure 2, Table 1). Distributions of inbreeding values did not significantly change in the F_2 and F_3 generations of either hatchery line (Table 1). Distributions of F in both hatchery lines then significantly decreased in the F_4 generation. Levels of F were not significantly different between the hatchery lines in the F_1, F_3, and F_4 generations (Figure 2, Table 1). The distribution of F values, however, was significantly higher in the segregated line in the F_2 generation.

Effects of inbreeding on traits influencing fitness

The preferred model to quantify the effect of individual inbreeding coefficient on the fitness of four-year-old adults varied for each phenotypic trait (Figure 3, Table 2). Models for fecundity, reproductive effort, return timing, fork length, weight, and condition factor comprised only main effects while those for spawn timing and daily growth coefficient included both main effects and interactions. There was little multicollinearity between explanatory variables (VIFs <2), and post-hoc power associated with regression coefficients was relatively high (>0.8) with a few exceptions (Table 2).

There was a comparatively large, statistically significant relationship between *F* and spawn timing (Figure 3, Table 2). However, this relationship was observed in the integrated line but not in the segregated line, as indicated by the significant interaction between *F* and hatchery line (Figure 3, Table 2). Specifically, spawn timing of adults in the integrated line was delayed by 1.75 days per one standard deviation increase in *F* (~0.16). Notably, the effect of generation (i.e. year) on spawn timing, particularly in the F_3 and F_4 generations, was much larger than any observed inbreeding effects.

There was little evidence for effects of *F* on the other traits. Daily growth coefficient during holding in the hatchery, a trait that is unrelated to growth (e.g. weight) during the oceanic portion of the life cycle, was not affected by inbreeding coefficient in females. However, the inclusion of the interaction term between *F* and sex in the preferred model, although marginally non-significant, suggests that *F* may affect DGC of males. Specifically, inbred males may have a higher rate of weight loss during holding in the hatchery compared to non-inbred males (Figure 3, Table 2). Inbreeding coefficient was not associated with fecundity, reproductive effort, return timing, fork length, weight, and condition factor (Figure 3, Table 2).

Discussion

Effective management of both captive and wild populations requires a comprehensive understanding of demographic, ecological, and genetic risks. Inbreeding is one genetic risk that can affect individual fitness, population productivity, and probability of extinction (Frankham, 2005; Kardos et al., 2016; Keller & Waller, 2002; O'Grady et al., 2006). Yet, the magnitude of these effects remains unclear, largely due to limitations in obtaining precise estimates of individual inbreeding coefficients. Here, we provided the first genomic investigation of inbreeding and its effects on fitness

in Pacific salmon using data from integrated and segregated hatchery lines of Chinook salmon. The two lines represent demographic extremes that provide insight into a range of possible outcomes relevant to risk assessment of captive breeding programs.

We found that integrated management may reduce levels of relatedness and inbreeding over four generations. However, these risks may not be severe even in small, segregated populations, perhaps due to management practices that maximize effective population size and reduce the potential of inadvertent inbreeding. Notably, the effects of inbreeding on fitness varied by trait, sex, and hatchery line. These findings emphasize the importance for all programs to monitor inbreeding and its associated impacts on fitness across a suite of phenotypes and years, as the effects may be case-specific.

Relatedness and inbreeding over time

a. P_1 and F_1 generations

The distributions of relatedness and inbreeding coefficients for the P_1 Founders were centered near zero (Figures 1-2), as would be expected in a wild, randomly mating population of moderate size (Nb ~ 375, Table S7; Waters et al., 2015; Waters et al., 2017). The small but significant increases in levels of relatedness and inbreeding detected in the F_1 Wild and F_1 Hatchery groups may be due to the initiation of the hatchery program, as these values are largely influenced by spawning numbers in the parental generation. Nearly 800 wild adult salmon returned to the system in 1998 (Waters et al., 2015), the second founding year from which a majority of fish in the F_1 generation was derived. Approximately half of these adults were collected as founding hatchery broodstock (i.e. parents of F_1 Hatchery group) while the other half were allowed to spawn naturally in the river (i.e. parents of F_1 Wild group; Figure S1). The division of the wild population into two groups, and the resultant decrease in population sizes, may have inadvertently increased the rate of inbreeding during spawning in both the wild and hatchery. The division may also explain the observed increases in relatedness in the F_1 generation, as the returning F_1 adults in each line represented offspring from a smaller number of parents, and the probability of sampling related individuals may therefore have increased. However, as the study by design lacks a wild control population to which the hatchery lines can be compared, the effects of hatchery initiation on relatedness and inbreeding for each generation cannot

be explicitly quantified. Nevertheless, population recovery plans should account for these potential demographic and genetic costs of removing individuals from the wild when considering a captive breeding as a tool.

b. F_2-F_4 generations

The detection of significant differences in pairwise relatedness (Figure 1, Table 1) between the hatchery lines in the F_2, F_3, and F_4 generations may be due to large differences in estimates of effective numbers of breeders, N_b, as values were 3- to 27-fold higher in the INT line (Table S7; Waters et al., 2015; Waters et al., 2017). However, differences may also be an outcome of the large sample sizes within each group (Table S5a) and the associated power to detect slight differences. This is suggested by the fact that differences were detected even though the median values of R_{xy} remained close to zero in both hatchery lines across all generations (Figure 1).

Levels of *F* differed between the two lines only in the F_2 generation, where the segregated line had higher levels of inbreeding. The lack of temporal trends within hatchery lines and additional differences between lines, despite the large differences in N_b (Table S7), may be due to the "best-practice" management principles employed CESRF, which aim to reduce negative ecological and genetic effects of hatchery rearing (Fast et al., 2015). Broodstock are collected randomly and proportionately over the entire return of adults, which reduces inbreeding (Kincaid, 1983; Wang et al., 2002) and other genetic risks associated with captive breeding. The facility also employs 3x3 factorial matings during spawning, which equalize sex ratios, reduce variation in family sizes (at least at early life stages), and increase the effective population size (Busack & Knudsen, 2007). As a comparison, Naish et al. (2013) – the only other study to quantify rates of inbreeding in a salmonid population that spends a portion of its life in the wild – documented an accumulation of inbreeding over four generations in an adult hatchery steelhead population using reconstructed pedigree data. However, in contrast to the factorial matings at CESRF, where each group is fertilized separately, spawning in the early generations of the hatchery steelhead population relied on pooling of eggs and sperm from multiple adults, which increased the likelihood of sperm competition, variance in reproductive success, and inbreeding. Modifications to mating strategies may therefore reduce inbreeding and other

risks (e.g. genetic drift) in captive breeding programs (Busack & Knudsen, 2007; Willoughby et al., 2015)

An alternative but not mutually exclusive explanation for the observed levels of *F* is that inbreeding may be occurring at CESRF, particularly in the small segregated line, but inbred individuals may be purged from the population after they are released into the wild (Crnokrak & Roff, 1999; Thrower & Hard, 2009) and thus are not detected in adult samples. Returning adults are the small fraction of fish that survived the selective pressures imposed by the freshwater and marine environments and may represent the "most-fit" individuals from the population. Estimates of inbreeding, along with fitness effects, may therefore be downwardly biased in one or both hatchery lines if inbred fish experienced a disproportionately high level of mortality prior to adulthood. Reduced survival of inbred individuals has been observed in many species, including salmonids (e.g. Brekke et al., 2010; Huisman et al., 2016; Wang et al., 2002). Notably, Thrower and Hard (2009) showed that inbred rainbow trout and steelhead experienced substantially higher (~80% greater) mortality than non-inbred fish when released into the wild, marine environment (survival rates were not significantly different during captive rearing in freshwater). Reduced survival of inbred families may also upwardly bias estimates of relatedness in the returning adults, as these fish may represent a smaller number of families. From a management perspective, the purging of inbred individuals could be beneficial, because it mitigates unintended fitness consequences to the wild population (Baskett & Waples, 2013). However, the loss of fish also reduces the effectiveness of captive breeding programs to supplement wild populations. Since the effects of inbreeding can vary by life stage (Grueber, Laws, Nakagawa, & Jamieson, 2010; O'Grady et al., 2006), it is important for CESRF and other programs to explicitly test for inbreeding across the entire life cycle in order to fully quantify the risks.

Reduced levels of inbreeding in the F_4 generation

The distributions of individual *F* decreased in the F_4 generation for both hatchery lines. The inclusion of paralogous loci or contamination between samples, both of which would cause an increase in individual heterozygosity and a concomitant decrease in *F*, are unlikely explanations for the observed decrease (Supplemental Information, Figures S2-S3). Similarly, the reduced sample sizes in the F_4 generation, which were approximately half of those from previous generations, do not

explain the reduced levels of inbreeding (Supplemental Information, Figure S4). Rather, it was likely due to environmental effects, especially since the change was observed in both hatchery lines. As inbreeding depression can be exacerbated under stressful conditions (Armbruster & Reed, 2005), it is possible that inbred fish in the F_4 generation experienced unusually high mortality after their release from the hatchery, perhaps due to the anomalous warm-water mass (i.e. "the Blob") that first appeared in the northeastern Pacific Ocean in 2013 (the last year at sea for fish from the F_4 generation) and had negative ecological impacts throughout the region (Cavole et al., 2016). However, while estimates of smolt-to-adult returns for CESRF Chinook salmon indicate a low survival rate of the F_4 generation, the P_1 and F_2 generations had similarly low survivals (Sampson, Fast, & Bosch, 2016).

Effects of inbreeding on traits influencing fitness

The use of genomic approaches has revealed that the fitness consequences of inbreeding are more severe than previously estimated in some organisms (e.g. harbor seals and red deer; Hoffman et al., 2014; Huisman et al., 2016). Here, one of the eight traits analyzed was significantly affected by inbreeding, although only returning adults were sampled. The results support evidence from simulations that inbreeding may not substantially contribute to the reduced fitness observed in some populations of hatchery-reared salmonids (Christie, French, Marine, & Blouin, 2013).

Inbreeding did not detectably affect the fecundity and reproductive effort of females. Similarly, Naish et al. (2013) did not detect any effects of inbreeding on fecundity, gonad weight (similar to reproductive effort), and reproductive success in adult steelhead that spend a portion of their lives in the wild. However, reduced fecundity due to inbreeding was observed in an experimental line of rainbow trout (Su, Liljedahl, & Gall, 1996). These differences may be due to the environments in which the studies were conducted (captive or wild; Armbruster & Reed, 2005), levels of variation in trait or *F* values, species and population-level differences, or other factors. For example, the fitness effects of inbreeding may be undetectable if the rate of inbreeding is slow (Wang et al., 2002).

Trait values for return timing, fork length, weight, condition factor, and daily growth coefficient were also not significantly associated with inbreeding, which differs from the results of other studies. Naish et al. (2013) detected that inbred steelhead returned later than non-inbred fish, and multiple studies documented reduced weights of inbred fish in both experimental (Kincaid, 1983;

Su et al., 1996; Wang et al., 2002) and wild environments (Naish et al., 2013). The results observed here may be due to the sampling of returning adults rather than fish at earlier life stages or any of the other factors previously described.

Spawn timing was the only trait that exhibited a significant association with inbreeding. Interestingly, this trait may also be responding to domestication selection, as multiple loci associated with this trait overlapped with signatures of adaptive divergence in the hatchery (Waters et al., 2018). Here, we observed that inbred fish of the integrated line matured later than non-inbred fish, with a 1.75 day delay in spawn timing for every one standard deviation increase in F (~0.16; Figure 3, Table 2). The biological impacts of later spawn timing are unclear. Later spawn timing in salmonids can confer higher fitness due to reduced risks of redd superimposition (Essington, Sorensen, & Paron, 1998; Hendry, Morbey, Berg, & Wenburg, 2004) and predation on their offspring (Brännäs, 1995). Later spawn timing may also negatively affect fitness because individuals face increased competition for mates and territories (Foote, 1990), and their offspring may suffer higher mortality due to factors associated with their comparatively smaller size (Einum & Fleming, 2000). Numerous studies have documented correlations between a similar trait, arrival date on the spawning grounds, and fitness (Anderson, Faulds, Atlas, Pess, & Quinn, 2010; Anderson, Faulds, Atlas, & Quinn, 2013; Ford, Hard, Boelts, Lahood, & Miller, 2008; Kodama, Hard, & Naish, 2012; Lin et al., 2016; Sard et al., 2015; Williamson, Murdoch, Pearsons, Ward, & Ford, 2010), though the strength and direction of these correlations varied between years and systems, in part due to stochastic environmental factors. Yet, the studies of Chinook salmon suggest that later arrival date of adults frequently reduces their reproductive success (Anderson et al., 2013; Sard et al., 2015; Williamson et al., 2010).

Importantly, the large generational effects on trait values support the concept that environmental conditions may exacerbate or mitigate the fitness consequences of inbreeding (Armbruster & Reed, 2005; Joron & Brakefield, 2003). Likewise, the interaction between F and hatchery line for spawn timing suggests that the effects of inbreeding may vary between populations. Here, the different effects of inbreeding on spawn timing in the integrated and segregated hatchery lines may be due to other genetic differences (i.e. genome-wide divergence and domestication selection) that have been previously identified (Waters et al., 2015; Waters et al., 2017).

Management implications

Wild and hatchery-reared salmon provide millions of dollars to local economies and are important to the ecology and culture of the Pacific Northwest. However, numerous wild salmon populations across the region are declining (Gustafson et al., 2007), and many are listed under the Endangered Species Act. Supportive breeding programs in the form of hatcheries exist to supplement these declining populations, and much effort has been aimed at understanding the long term impacts of these programs. However, research has largely focused on the impacts of domestication selection to the fitness of hatchery fish. In contrast, genetic effective population sizes in hatcheries are typically much smaller than census sizes (Busack & Currens, 1995; Fraser, 2008; Naish et al., 2013; Naish et al., 2008) and are likely unrecognized causes of reduced fitness. It is important to fully understand all potential risks in order to develop comprehensive benefit-risk assessments for supplementation programs and to maximize their effectiveness to rebuild natural populations.

This study is the first to assess the multigenerational risks of inbreeding in Pacific salmon using genomic approaches. We document an influence of inbreeding on the phenology of adult spawners, which could have biological implications for individual fitness and population productivity. The varied results highlight the importance of broad-scale, long-term monitoring in captive breeding programs. Further, we provide the first empirical evidence that integrated management, or managed gene flow, may reduce levels of relatedness and the rate of inbreeding. Yet, the results also indicate that these risks may not be severe in segregated hatchery populations with appropriate broodstock management approaches. Until earlier life stages can be investigated, we recommend that hatcheries adopt conservative protocols to minimize the risks of inbreeding, such as those already employed at CESRF, including random and proportionate broodstock collection and factorial matings. These findings provide a better understanding of genetic and phenotypic change that may occur in captive-reared populations and will further inform research on "best" practices to minimize risks in conservation-focused captive breeding programs.

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Data accessibility: Raw sequence data that support the findings of this study are openly available in the NCBI Sequence Read Archive at https://www.ncbi.nlm.nih.gov/sra, accession SRP127673. Sequencing barcodes for each individual in the raw data are provided in Table S11 of Waters et al. (2018). The R script to quantify the effects of inbreeding on phenotypes will be deposited in the Dryad Digital Repository.

Author contributions: C.D.W. and K.A.N. designed the study; D.E.F., C.M.K., and W.J.B. provided critical data and insight on the study system; C.D.W., J.J.H., and K.A.N. analyzed the data, C.D.W. drafted the paper; J.J.H., D.E.F., C.M.K., W.J.B, and K.A.N. contributed to the writing and revision of the manuscript and gave final approval for publication.

Tables (moved to Excel file that will be uploaded separately)

Figure Legends

Figure 1. Split violin plots of pairwise relatedness, R_{xy}, for all pairs of individuals within the P_1 founders (grey) and the F_1, F_2, F_3, and F_4 generations of the integrated (INT, in blue) and segregated (SEG, in red) hatchery lines estimated from 1,100 unlinked loci. The median values for each

distribution are denoted by solid horizontal lines while the vertical black bars span the interquartile ranges.

Figure 2. Split violin plots of individual inbreeding coefficients, *F*, for the P_1 founders (grey) and the F_1, F_2, F_3, and F_4 generations of the integrated (INT, in blue) and segregated (SEG, in red) hatchery lines estimated from 1,100 unlinked loci. The median values for each distribution are denoted by solid horizontal lines while the vertical black bars span the interquartile ranges.

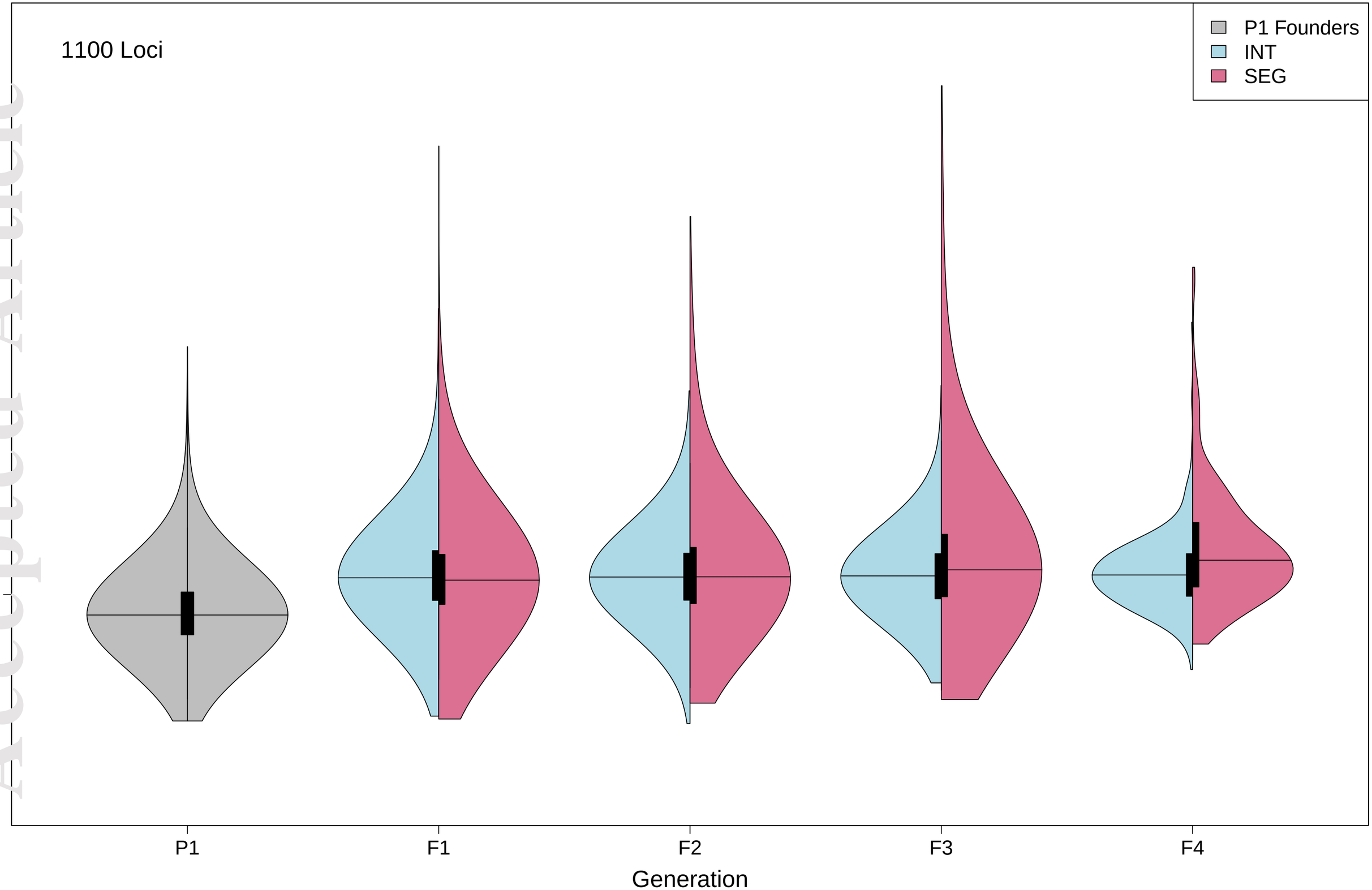
Figure 3. Standardized regression coefficients (points) and their 95% confidence intervals (whiskers) of explanatory variables included in the preferred models for each phenotypic trait. The left panel shows model coefficients for fecundity, reproductive effort, return timing, and spawn timing while the right panel displays results for condition factor, fork length, weight, and daily growth coefficient (note different x-axis scales). The reference levels for all regressions are outbred (F=0) females from the integrated hatchery line in the F_1 generation; all coefficients in the plot therefore refer to the deviation from these reference levels. For example, the effects of inbreeding coefficient (*F*) on trait values are shown in the top row of each panel, the effects of the F_2, F_3, and F_4 generations on trait value (i.e. difference between F_1 and each subsequent generation, independent of inbreeding coefficient) are shown in separate rows, and the effects of sex (i.e. difference of males from females) on trait value are shown in the "sex" row. Inbreeding coefficient significantly affected spawn timing. Note that terms included in the preferred models varied for each trait.

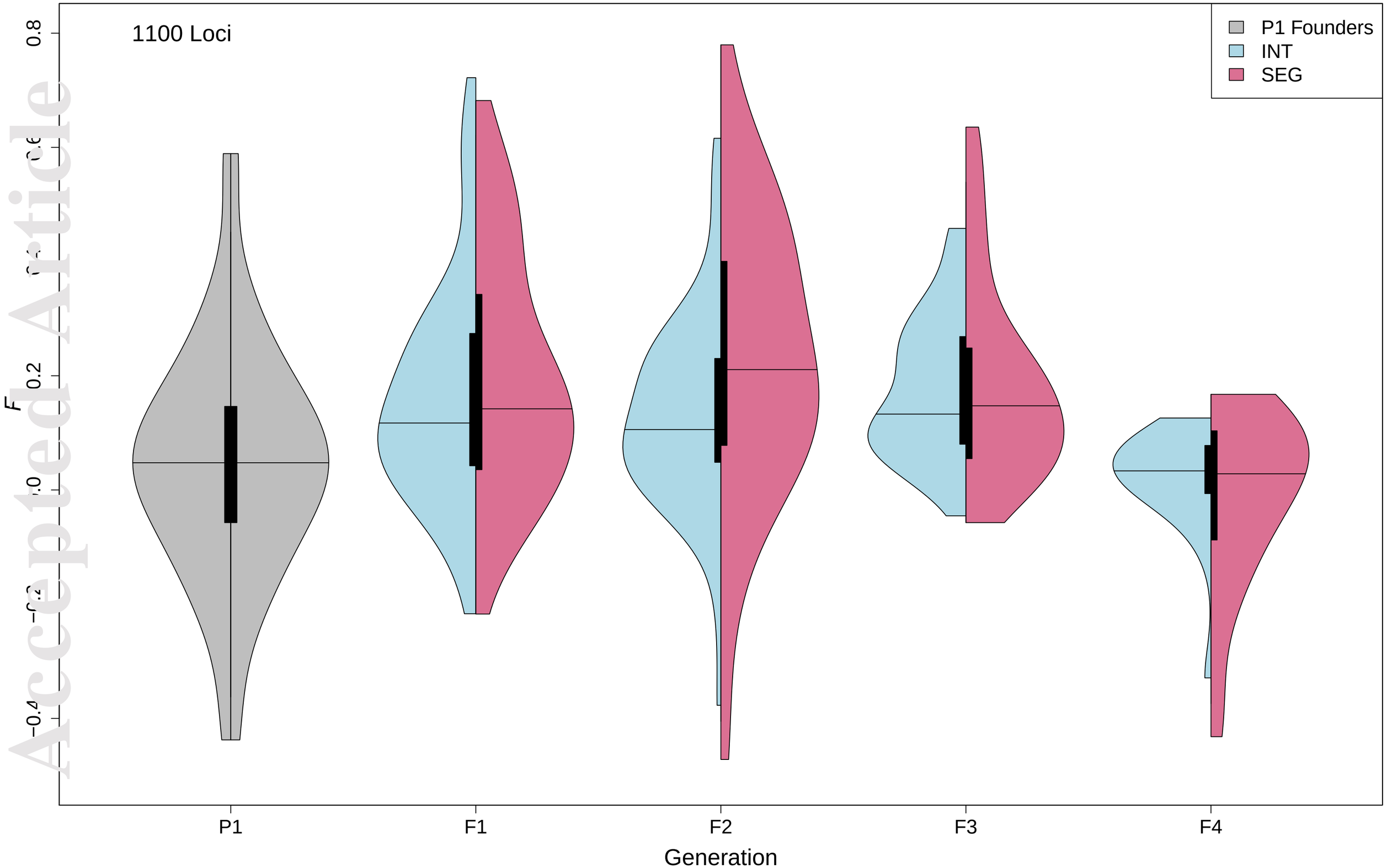
Table 1. Test statistics, *p* values, and the direction of significant differences from Kolmogorov-Smirnov tests conducted between subsequent generations (gens) within the integrated (INT) and segregated (SEG) hatchery lines. Tests were also conducted between the hatchery lines within each generation. Significant differences are in bold, and NA denotes no significant difference.

Comparison		R_{xy}			F		
		D	***p* value**	**Difference**	**D**	***p* value**	**Difference**
between gens	P_1 vs. F_1 Wild	**0.428**	**<2.2e-16**	**$F_1 > P_1$**	**0.267**	**0.036**	**$F_1 > P_1$**
	F_1 Wild vs. F_2 INT	0.025	0.676	NA	0.171	0.305	NA
	F_2 INT vs. F_3 INT	0.024	0.667	NA	0.241	0.053	NA
	F_3 INT vs. F_4 INT	0.044	0.733	NA	**0.614**	**2.94e-6**	**$F_3 > F_4$**
	P_1 vs. F_1 Hatch	**0.393**	**<2.2e-16**	**$F_1 > P_1$**	**0.346**	**0.003**	**$F_1 > P_1$**
	F_1 Hatch vs. F_2 SEG	**0.056**	**0.027**	**$F_2 > F_1$**	0.144	0.570	NA
	F_2 SEG vs. F_3 SEG	**0.082**	**6.48e-5**	**$F_3 > F_2$**	0.231	0.084	NA
	F_3 SEG vs. F_4 SEG	**0.109**	**0.005**	**$F_4 > F_3$**	**0.490**	**2.17e-4**	**$F_3 > F_4$**
between lines	F_1 Wild vs. F_1 Hatch	0.041	0.175	NA	0.173	0.333	NA
	F_2 INT vs. F_2 SEG	**0.062**	**0.007**	**SEG > INT**	**0.329**	**0.004**	**SEG > INT**
	F_3 INT vs. F_3 SEG	**0.136**	**3.33e-16**	**SEG > INT**	0.165	0.352	NA
	F_4 INT vs. F_4 SEG	**0.217**	**2.61e-6**	**SEG > INT**	0.315	0.121	NA

Table 2. Coefficient estimates (standard errors), *p* values, and post-hoc power for terms included in the preferred linear models for eight fitness-related traits. Terms not included in the preferred models are denoted by N/A. Note that all continuous variables were standardized prior to analyses; regression coefficients therefore refer to the number of standard deviations from the reference level. The reference level is females from the integrated hatchery line in the F_1 generation. Models for fecundity and reproductive effort did not include sex as a covariate, as these traits were only measured in females. Significant effects of *F* on trait values are in bold. Additional model information for each trait is also included.

	Fecundity	Repro. Effort	Return Timing	Spawn Timing	Fork Length	Weight	Condition Factor	DGC
Intercept	0.312 (0.146)	-0.164 (0.138)	0.082 (0.114)	0.808 (0.102)	0.186 (0.119)	0.178 (0.109)	0.077 (0.088)	0.039 (0.086)
	p=0.034	p=0.235	p=0.473	p=3.76e^{-14}	p=0.120	p=0.104	p=0.381	p=0.651
F	0.004 (0.069)	0.057 (0.072)	0.006 (0.056)	**0.250 (0.079)**	0.064 (0.053)	0.077 (0.048)	-0.053 (0.066)	-0.013 (0.047)
	p=0.956; pwr=0.15	p=0.426; pwr=0.92	p=0.917; pwr=0.29	**p=0.002; pwr=1.00**	p=0.225; pwr=1.00	p=0.109; pwr=0.72	p=0.427; pwr=0.56	p=0.782; pwr=0.36
F^2	N/A	N/A	N/A	N/A	N/A	N/A	0.094 (0.065)	N/A
							p=0.148; pwr=1.00	
F_2 gen	-0.775 (0.177)	-0.151 (0.188)	0.299 (0.146)	-0.278 (0.127)	-0.618 (0.136)	-0.743 (0.124)	-0.626 (0.110)	0.263 (0.098)
	p=0.00002; pwr=1.00	p=0.422; pwr=0.98	p=0.041; pwr=1.00	p=0.029; pwr=1.00	p=7.27e^{-6}; pwr=1.00	p=5.46e^{-9}; pwr=1.00	p=2.55e^{-8}; pwr=1.00	p=0.007; pwr=1.00
F_3 gen	0.160 (0.170)	0.428 (0.180)	-0.181 (0.141)	-0.542 (0.122)	0.234 (0.131)	0.164 (0.120)	-0.061 (0.106)	0.690 (0.095)
	p=0.348; pwr=0.99	p=0.019; pwr=1.00	p=0.199; pwr=1.00	p=1.30e^{-5}; pwr=1.00	p=0.075; pwr=1.00	p=0.172; pwr=1.00	p=0.564; pwr=0.93	p=2.61e^{-12}; pwr=1.00
F_4 gen	-0.258 (0.239)	0.512 (0.251)	-0.190 (0.191)	-0.743 (0.165)	0.157 (0.178)	0.855 (0.163)	1.672 (0.144)	-1.554 (0.127)
	p=0.281; pwr=0.99	p=0.043; pwr=1.00	p=0.321; pwr=1.00	p=9.21e^{-6}; pwr=1.00	p=0.379; pwr=0.99	p=2.68e^{-7}; pwr=1.00	p<2e^{-16}; pwr=1.00	p<2e^{-16}; pwr=1.00
Hatchery Line	-0.212 (0.132)	N/A	N/A	-0.929 (0.093)	-0.392 (0.102)	-0.437 (0.093)	-0.185 (0.082)	0.212 (0.073)
	p=0.109; pwr=1.00			p<2e^{-16}; pwr=1.00	p=0.0001; pwr=1.00	p=3.86e^{-6}; pwr=1.00	p=0.024; pwr=1.00	p=0.004; pwr=1.00
Sex	N/A	N/A	-0.217 (0.110)	N/A	0.205 (0.104)	0.207 (0.095)	N/A	-0.625 (0.075)
			p=0.049; pwr=1.00		p=0.049; pwr=1.00	p=0.030; pwr=1.00		p=2.05e^{-15}; pwr=1.00
F:Hatchery Line	N/A	N/A	N/A	-0.297 (0.097)	N/A	N/A	N/A	N/A
				p=0.002; pwr=0.77				
F:F_2 gen	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
F:F_3 gen	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
F:F_4 gen	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
F:Sex	N/A	N/A	N/A	N/A	N/A	N/A	N/A	-0.135 (0.074)
								p=0.068; pwr=0.97
Observations	204	201	336	334	335	336	335	328
R^2	0.161	0.074	0.053	0.298	0.183	0.315	0.471	0.586
Adjusted R^2	0.14	0.055	0.039	0.285	0.168	0.303	0.461	0.577
Residual Std. Error	0.927 (df = 198)	0.972 (df = 196)	0.980 (df = 330)	0.846 (df = 327)	0.912 (df = 328)	0.835 (df = 329)	0.734 (df = 328)	0.651 (df = 320)
F Statistic	7.595*** (df = 5; 198)	3.901*** (df = 4; 196)	3.729*** (df = 5; 330)	23.083*** (df = 6; 327)	2.208*** (df = 6; 328)	25.26*** (df = 6; 329)	48.617*** (df = 6; 328)	64.649*** (df = 7; 320)
Note:	*p<0.1; **p<0.05; ***p<0.01							





F
F_2 Gen
F_3 Gen
F_4 Gen
SEG Line
Sex
F:SEG Line
−1.0
−0.5
0.0
0.5
1.0
Coefficient
Fecundity
Repro. Effort
Return Timing
Spawn Timing
F
F^2
F_2 Gen
F_3 Gen
F_4 Gen
SEG Line
Sex
F:Sex
−1.5
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1.0
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Coefficient
Condition Factor
Fork Length
Weight
DGC